Proceedings of 8th International Conference on Mushroom Biology and Mushroom Products

Volume II

World Society of Mushroom Biology and Mushroom Products
ICAR-Directorate of Mushroom Research, Solan
Mushroom Society of India, Solan

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Manjit Singh
Chairman Steering Committee, 8th ICMBMP

World Society of Mushroom Biology and Mushroom Products
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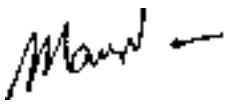
PREFACE

The eighth International Conference on Mushroom Biology and Mushroom Products is a continuum of the conferences organized by World Society of Mushroom Biology and Mushroom Products since 1993 after every three years in one or other part of the world. The seventh conference was held in Arcachon, France in October 2011 where we agreed to hold the eighth conference in India. Considering the changes taking place all over the globe and impetus towards growth of mushroom research and development in India, this was an apt decision and the interactions during the event will help all of us to promote the research in various facets of mushroom biology and mushroom products.

We thank our colleagues from all parts of the world for their overwhelming response to the call for presentations and participation in eighth International Conference on Mushroom Biology and Mushroom Products jointly organized by World Society of Mushroom Biology and Mushroom Products (WSMBMP), ICAR-Directorate of Mushroom Research, Solan (ICAR-DMR) and Mushroom Society of India (MSI) at New Delhi from 19-22 November 2014.

In this conference there were 231 contributions as abstracts that were grouped into 10 sessions. Full papers were received for 90 presentations. The presentations were grouped into 10 sessions that are: (i) Biodiversity and taxonomy, (ii) Genomics, genetics and breeding, (iii) Bioinformatics and nanotechnology, (iv) Biology, biochemistry, physiology and development, (v) Waste conversion & utilization, substrates, casing and crop management, (vi) Myco-molecules, medicinal, nutritional and nutraceutical properties, (vii) Mycorrhizal, entomopathic and other novel mushrooms, (viii) Pests and diseases, (ix) Value addition and mushroom products and (x) Economics, social, IT and marketing issues. Volume II contains the papers included in sessions VI to X.

I would like to thank all members of Scientific Advisory Committee and Organizing Committee for their help and support. I particularly thank my colleagues at ICAR-DMR who have been working for this conference since the inception of the concept to organize the conference in India. We thank Indian Council of Agricultural Research and Department of Agriculture and Cooperation, Government of India for their financial support for this conference. I thank Mrs. Sunila Thakur for her secretarial support.



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POTENTIAL OF MUSHROOM BIOACTIVE MOLECULES TO DEVELOP HEALTHCARE BIOTECH PRODUCTS

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ABSTRACT

Mushrooms are widely appreciated all over the world for their nutritional value and medicinal properties. They have low fat, high protein and vitamins contents. Mushrooms contain several minerals and trace elements, as well as substantial amount of dietary fibers. Basidiomycetes mushrooms (phylum Basidiomycota) including agaric and bracket fungi are also producers of bioactive molecules and valuable enzymes with different therapeutic effects. Therefore, they are considered as perspective organisms to develop different healthcare biotech-product. The main groups of bioactive molecules produced by different mushrooms are polysaccharides, terpenoids, phenolics, lectins etc. More than 126 therapeutic effects (immune-modulating, antimicrobial, antiviral, antioxidant, hypocholesterolemic, ect.) of these molecules were revealed.

Nowadays, interest to biotechnological cultivation of Basidiomycetes mushrooms is related with the growing demand of different mushroom-based biotech-products in pharmaceutical, food and cosmetic industries. The submerged cultivation of mycelium has significant industrial potential to obtain biomass and desired bioactive molecules for further development of consistent and safe healthcare products.

Several pharmaceuticals (krestin, lentinane, coriolan, schyzophillan, etc.) formulated from medicinal mushrooms are already available in the world market. The majority of mushroom products possesses beneficial health effects owing to the synergistic action of present bioactive molecules and can be used on a regular basis without harm. Nutritive, anti-inflammatory, regenerative and antioxidant properties of several mushrooms makes their usage perspective in manufacturing of cosmetic products. Formulation of balanced food for pets is anew area of application of mushroom biotech-products. Establishment and maintenance of culture collections play important role in studies of biodiversity, genetic resources and biotechnological potential of Basidiomycetes mushrooms.

Keywords: basidiomycetes mushrooms, bioactive molecules, biotechnological potential

INTRODUCTION

Since ancient times mushrooms were widely appreciated all over the world, particularly in China, Korea, Japan, Central and North American countries for their nutritional value and medicinal properties. Mushrooms have a low fat and high protein content, a high content of several vitamins (B, C, D, K), minerals (potassium, phosphorus) and trace elements (selenium). Mushrooms contain substantial amount of dietary fibers, as well. Modern scientific data has documented that mushrooms represent an unlimited source of bioactive molecules as an example of molecular diversity with recognized potential in drug discovery and development. Basidiomycetes mushrooms (phylum Basidiomycota) including agaric and bracket fungi are natural source of bioactive molecules and valuable enzymes with around 126 therapeutic effects [1-3]. Therefore, they are considered as perspective organisms to develop different healthcare biotech-product.

BIOACTIVE MOLECULES WITH MUSHROOM ORIGIN

Bioactive molecules produced by mushrooms are mainly belongs to the polysaccharides, glucans, terpenoids, phenolic compounds, lectins, statins, etc. [4-6]. They have immune-modulating, antioxidant, genoprotective, antitumor, hypocholesterinemic, antidiabetic, hepatoprotective and other medicinal effects [2,3,7,8]. Fungal polysaccharides are the most potent mushroom-derived substances with antitumor and immune-modulating properties. They are present in cell wall with different types of glycosidic linkages, such as (1→3) and (1→6)-β-D-glucans. Polysaccharides possess significant immune-stimulating, antitumor, antioxidant, antibacterial and antiviral activities. Fungal terpenoids (tri- and sesquiterpenes)

have cytotoxic, antibacterial, antifungal, hypocholesterolemic, hypoglycemic, hypotensive and antioxidant effects. Chitin and chitosan isolated from fungal cell wall are regulating the functions of liver, gastro-intestinal tract and kidney. Fungal pigment melanin possesses antioxidant, immune-modulating, anti-mutagenic and radioprotective properties.

Wood inhabiting mushrooms from genera *Ganoderma, Fomes, Fomitopsis, Inonotus, Phellinus, Trametes, Schyzophillum* and others are reported as a source of different biomolecules, such as glucans, phenolic compounds and terpenoids with immune-modulating and antimicrobial activities [2]. About 400 bioactive molecules including different triterpenes, polysaccharides, proteins, sterols, and fatty acids have been isolated from *Ganoderma* species *G. lucidum, G. applanatum* and *G. tsugae*. Among them lanostan-type triterpenoids are promising candidates to develop antitumor drugs [9,10]. Two glucans grifon and grifolan isolated from maitake mushroom *Grifola frondosa* possesses immune-modulating and antitumor effects. New glycoprotein complex obtained from maitake helps to maintain healthy cardiovascular function [11]. Medicinal mushrooms *Polyporus (=Grifola umbellata) umbellatus* and *Polyporus alveolaris* contain cytotoxic steroids and polypeptides with immune-stimulating, anticancer, anti-inflammatory, antibacterial, hepatoprotective and antifungal effects [12]. An antitumor product lentinan was developed from water-soluble antitumor polysaccharide isolated from shiitake mushroom *Lentinula edodes*.The antitumor β-D-glucan extracted from cultural broth of *S. commune* was developed to anticancer biotech-product under the name schyzophillan. Polysaccharide based products from *Agaricus brasiliensis (=A. blazei)* are using to combat physical and emotional stress, stimulate immunity, reduce levels of blood cholesterol and sugar, prevent osteoporosis and gastric ulcer. Antitumor protein flammulin was isolated from enokitake mushroom *Flammulina velutipes* [5,13].

Several bioactive molecules, such as sesquiterpenes (cuparane, illudins), quinones (5-methoxy-p-toluquinone, benzoquinone, lagopodins, hydroxylagopodins), polysaccharides, proteins (hydrophobins and galectins), sterols, derivatives of imidazole, indolic (triptamine, serotonin, bufotenin, psilocin, psilocibin, ergothionin) and volatile (skatole) compounds were reported in Coprinoid mushrooms. They possess antifungal, antibacterial, antiviral, fibrino- and thrombolytic, antiprotozoal, neuro- and vasotonic effects [14].The oyster mushrooms (*Pleurotus* spp.) are potential producers of statins, which are inhibitors of HMG-CoA reductase, a key enzyme in cholesterol metabolism in the human body. Significant reduction of blood cholesterol level was observed when biotech-product plovastin obtained from submerged mycelia of *P. ostreatus* and *P. eryngii* var. *ferulae* was used as a dietary supplement [4]. Antimycotic biotech-product mucidermin was developed from antifungal compound mucidin (strobilurin A) isolated from *Oudemansiella mucida*. Antiviral agents against different types of viruses (Papilloma, H5N1, HSV-1 and 2, Hepatitis B,C,D,E, AIDS, etc.) are also actively being searched from different mushroom species (*Piptoporus betulinus, Fomitopsis officinalis, Coprinellus micaceus*, etc) to develop new class of mushroom-based antiviral biotech-products [3,14].

Bioactive proteins lectins and hydrophobins of potential medicinal interest are so far described from mushrooms. Lectins are carbohydrate-binding proteins with agglutinating properties that are widely found in eukaryotes. The hydrophobins are most interesting candidates for various medical applications, such as increasing biocompatibility of medical implants devices, immobilization of antibodies in a biosensor and stabilizing oil vesicles for drug delivery [15].

Polysaccharides, sesquiterpenes, lectins, phenolic compounds and other biomolecules with different therapeutic effects (antibacterial, antifungal, cytotoxic, anti-inflammatory, insecticidal, nematocidal, antioxidant and others) were detected in several edible medicinal ectomycorrhyzal mushrooms [3] (Table 1). Mushrooms are also producers of extracellular proteolytic enzymes with fibrinolytic and thrombolytic activities [16].

Thus, the available information about bioactive molecules and enzymes of medicinal mushrooms suggests that they are promising biological organisms to develop health enhancing biotech-products.

MUSHROOM-BASED BIOTECH-PRODUCTS

Nowadays, different mushroom-based health care commercial biotech-products with preventive and curative effects are available and largely consumable in the world market. They sold in dried forms as healthy food ("nutraceuticals") and as

functional food additives ("pharmaceuticals" or "nutriceuticals"). The majority of mushroom products possesses beneficial health effects owing to the synergistic action of present bioactive molecules and can be used on a regular basis without harm.

Mushroom polysaccharide-based biotech-product krestin (PSK) was produced from *Trametes versicolor*. Preparation befungin obtained from Chaga mushroom (*Inonotus obliquus*) was approved as an antitumor drug in Russia and reportedly successful in treating breast, lung, cervical, and stomach cancers. Healthy food developed from biotechnologically cultivated mycelia of medicinal edible mushrooms *Hericium erinaceus* and *Tremella* spp. in combination with other natural substances (medicinal plants, algae, etc.) possess antioxidant and immune-stimulating activity, regulate the level of blood lipids and sugar [17,18]. Available biotech-products from *H. erinaceus* help to control Alzheimer disease and bleeding. They are used as anti-cancer and sarcoma agents. Some mushroom products are able to decrease high glucose and lipid levels in blood and recommended as neuro- and vasotonics, hepatoprotective and thrombolytic agents [3].

Nutritive, anti-inflammatory, regenerative and antioxidant properties of several mushrooms (*Lentinula edodes, Ganoderma lucidum, Fomes officinalis, Hypsizygus ulmarius,Tremella* spp.) makes their usage perspective in manufacturing of cosmetic products [6]. Mushrooms are currently proposed as highly active ingredients in world production of hair and skin care products. *Tremella* mushrooms contain hydrophilic agent – polysaccharide glucuronoxylomannan (GXM) with anti-inflammatory and wound healing properties largely used in cosmetology [19]. *Tremella* cosmetic products are applicable in treatment of neurodermatitis and sclerodermatitis.They prevent skin pigmentation and stimulate blood circulation. Fungal chitosan is also wildly used in cosmetology as an emulgatory, gel-forming, protective and anti-bacterial agent.

A new area of application of mushroom biotech-products is formulation of balanced food for pets. Except medicinal plants and other ingredients such food additionally contains mushroom dietary fibers and polysaccharides stimulating immune activity in animals. The pet food supplements are obtaining from mycelial biomass of different mushrooms.

Thus, Basidiomycetes mushrooms have significant biotechnological potential. Biological characteristics of mycelia, particularly fast growth and easy reproduction in culture conditions is assisting biotechnological cultivation of medicinal mushrooms to obtain desired bioactive molecules and biotech-products.

BIOTECHNOLOGICAL ROLE OF CULTURE COLLECTIONS

Establishment and maintenance of cultures collections of different group of mushrooms are of valuable importance to study their biodiversity, genetic resources and biotechnological potential. Availability of culture collections is also requested for biotechnological cultivation of mycelia.

Currently, two different approaches in biotechnological cultivation of mushrooms are used. First is fruiting bodies production and second-cultivation of mycelia. The fruiting bodies production is a long-term process and takes 1-2 months, while cultivation of mycelia takes several days. The submerged cultivation of mycelia has significant industrial potential and it is the best technique to obtain biomass and desired bioactive molecules for further development of consistent and safe healthcare mushroom biotech-products.

Study of genetic resources and biotechnological potential of medicinal mushrooms and establishment of their culture collection in Armenia was initiated in 1983. Presently fungal collection at the Yerevan State University (FCC-YSU) consists around 144 species and 500 strains of Basidiomycetes mushrooms [20]. Among them 94 species and 430 strains are Basidiomycetes mushrooms. The medicinal properties (antifungal, antibacterial, antiviral, immune-modulating, antioxidant, cytotoxic, mitogenic/regenerative etc.) of 43 species and 308 strains are reported in our publications [21,22].

Studies of mushroom collections from FCC-YSU were realized in different culture conditions (static, submerged). Favorable growth parameters (temperature, pH, medium) for biotechnological cultivation of mycelia were revealed. Colony and pellet morphology, biomass formation, characteristics of the life cycle, the presence and type(s) of asexual sporulation (anamorph), ability to form teleomorph (fruiting bodies) *in vitro* and other mycelial characters were observed and their

Table 1. Therapeutic effects of bioactive molecules in several mycorrhiza forming Basidiomycetes mushrooms [3]

Species	Bioactive molecule	Therapeutic effect
Boletus (=Xerocomus) badius	Polysaccharides, polyphenolics, N-ethyl-γ-glutamine (L-theanine analog)	Antimitotic, antitumor, immune-modulating, antioxidant, neurotropic
Boletus edulis	Lectin, polysaccharides, polyphenols, ergothioneine	Antitumor, immune-modulating, antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, mitogenic, neurotropic
Cantharellus cibarius	Polysaccharides, cibaric acid, phenolic compounds	Antioxidant, antimicrobial, antifungal, insecticidal, nematicidal
Cantharellus tubaeformis	Polysaccharides, 10-hydroxy-8-decenoic acid	Antioxidant, antimicrobial, antifungal, anti-inflammatory, insecticidal, nematicidal, hypocholesterolemic, hypoglycemic, hypotensive, antitumor
Lactarius deliciosus	Sesquiterpenoids, lectin, phenolic compounds	Antibacterial, antifungal, cytotoxic, anti-inflammatory, insecticidal, nematocidal, antioxidant
Lactarius flavidulus	Polysaccharides, flavidulols A-D	Antitumor, antibacterial, cytotoxic, anti-inflammatory, immune-suppressive
Lactarius necator	Alkaloids necatorin and necotoron	Antibacterial, antifungal
Lactarius volemus	Phenolic acids, lectin	Antioxidant
Lyophillum decastes	(1→3)- and (1→6)-beta-D-glucans, phenolics	Antitumor, anti-bacterial, hypochole-stero-lemic, hypoglycemic, hypotensive, anti-inflammatory, immune-modulating, radio-protective, antioxidant
Morchella esculenta	Galactomannan (α-d-glucan)	Immune-modulating
Russula delica	Lectin	Antiproliferative, antiviral
Russula paludosa	Peptide	Antiviral
Rusulla virescens	Polysaccharides	Antioxidant, hypoglycemic, hypocholesterolemic
Russula xerampelina	Polysaccharides	Antitumor, antiparasitic
Suillus bovines	Suillin	Immune-suppressive, antibacterial
Suillus granulatus	Tetraprenylphenols	Antitumor
Suillusluteus	Phenolics, polysaccharides	Antifungal, antioxidant, immune-modulating
Tricholoma lobayense	Polysaccharides, polysaccharide-protein complex	Immune-modulating, antitumor
Tricholoma giganteum	Polysaccharides	Immune-modulating, antitumor
Tricholoma matsutake	α-d-glucan	Immune-modulating
Tricholoma mongolicum	Lectins, polysaccharide-peptide complex	Immune-modulating, antitumor, hypotensive, vasorelaxing
Tricholoma portentosum	Polysaccharides, phenolic compounds	Antitumor, antibacterial, antifungal, fibrinolytic

taxonomic significance was evaluated [13, 23 and others]. Screening of chemical composition (polysaccharides, phenolics, terpenoids, proteins, sugars, fatty acids, etc.) of mycelial and fruiting bodies' extracts of different collections was realized [3].

Despite of commercial importance, there are gaps to be filled in the current knowledge on taxonomy and biology of medicinal mushrooms. Genetically identified collections of polypore and coprini mushrooms, as well as other agaric and bracket fungi were screened for their taxonomic verification and phylogenetic analysis. Several new species, such as

Ganoderma applanatum, Coprinellus strossmayeri, Coprinellus radians and others were originally described for Armenian mycobiota [24-26]. Studies of genetic resources and biotechnological potential of medicinal mushrooms in Armenia are in progress.

CONCLUSION

Further sustainable research of natural and genetic resources of medicinal mushrooms using improved screening methods of genomics, proteomics and metabolomics will assist further biotechnological cultivation and usage of their bioactive molecules to develop novel healthcare biotech products with a positive global impact on human welfare and environmental conservation.

ACKNOWLEDGEMENTS

The research was supported by SCSRA, joint Armenian-Russian research project 13RF-110 and RFBR grant 13-04-90607_Arm. S.M. Badalyan thanks to DAAD for technical grant (#548.104401.317).

REFERENCES

[1] Wasser SP. (2002). Medicinal mushrooms, as a source of antitumor and immunomodulating polysaccharides. *Appl Microbiol Biotechnol.* 60: 256-274.

[2] Wasser SP. (2010). Medicinal mushroom science: history, current status, future trends, and unsolved problems. *Int. J Med. Mushr* 12: 1-16.

[3] Badalyan SM. (2012). Edible Ectomycorrhizal Mushrooms In: Edible Ectomycorrhizal Mushrooms. Soil Biology series.Vol. 34.Springer-Verlag. A. Zambonelli& G. Bonito, Eds. pp. 317-334.ISBN: 978-3-642-33822-9.

[4] Gunde-Cimerman N and Cimerman A (1995). *Pleurotus* fruiting bodies contain the inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme a reductase-lovastatin. *Exp. Mycol*. 19: 1-6.

[5] Wasser SP and Weis AL. (1999). Medicinal properties of substances occurring in higher Basidiomycetes mushrooms: current perspectives. *Int J Med Mushr* 1: 31-62

[6] Badalyan SM. (2001). The main groups of therapeutic compounds of medicinal mushrooms. *Probl. Med. Mycology*. 3: 16-23.

[7] Badalian S M. (2000). Antitumor and immune-modulating activities of compounds from several basidiomycete mushrooms.*Probl. Med. Mycology.* 2:23-28.

[8] Lindequist U *et al*. (2005). The pharmacological potential of mushrooms. *Ev. Based Comp. Alt. Med.* 2: 285–299.

[9] Fatmawati S *et al*. (2010). Ganoderic acid Df, a new triterpenoid with aldose reductase inhibitory activity from the fruiting body of *Ganoderma lucidum*.*Fitoterapia*. 81: 1033-1036.

[10] Fatmawati S *et al.* (2013). Structure-activity relationships of lanostane-type triterpenoids from *Ganoderma lingzhi* as á-glucosidase inhibitors.Bioorg Med Chem Lett. 1: 5900-5903.

[11] Kodama N *et al*. (2002). Effects of D-fraction, a polysaccharide from *Grifola frondosa* on tumor growth involve activation of NK cells. *Biol. Pharm. Bull*. 25: 1647-1650.

[12] Jiang J and Sliva D. (2010). Novel medicinal mushroom blend suppresses growth and invasiveness of human breast cancer cells. *Int. J Oncology* 37: 1529-1536.

[13] Badalyan SM *et al*. (2006). Morphology, Growth Characteristics and Genetic Variability of The Edible Medicinal Mushroom *Flammulina velutipes* (Curt. : Fr.) Sing. Collections.*I nt. J. Med. Mushr*. 8: 263-278.

[14] Badalyan SM *et al*. Bioactive compounds and enzymes of coprinoid mushrooms of potential medicinal interest.Review, (in preparation).

[15] Walser PJ *et al*. (2004). Structure and functional analysis of the fungal galectin CGL2.Structure, 12: 689-702.

[16] Denisova NP. (2005). Proteolytic enzymes of Basidiomycetes (taxonomical and ecological aspects). In: *Int. J. Med. Mushr*. 7: 395-396.

[17] Khan MA *et al*. (2013). *Hericium erinaceus*: an edible mushroom with medicinal values. *J Complement Integr Med.* 24: 10.

[18] Standish LJ *et al*. (2008). *Trametes versicolor* mushroom immune therapy in breast cancer. *J Soc. Integr.Oncol*. 6: 122–128.

[19] De Baets S and Vandamme EJ. (2001). Extracellular *Tremella* polysaccharides: structure, properties and applications. *Biotech. Letters*. 23: 1361-1366.

[20] Badalyan SM *et al*. (2005). Catalogue of the Fungal Culture Collection at the Yerevan State University, Armenia. Afrikyan E.G. & Abrahamyan J.H. Eds. Yerevan: YSU press. 58 p. ISBN: 5-8084-0642-0.

[21] Badalyan SM *et al*. (2014). Genetic resources and cultural characteristics of several medicinal polypore mushrooms (Basidiomycota, Polyporales). *Int. J Med. Mushrooms*, (in press).

[22] Saltarelli R *et al*.(2014). Biochemical characterization, antioxidant and antiproliferative activities of different *Ganoderma* collections. *J. Microbiol. Biotechnol*., accepted.

[23] Badalyan SM *et al*. (1995). Investigation of fungal metabolots and acute toxisity studies from fruit-bodies of *Hyphaloma* species (Strophariaceae). *Cryptogamie Mycol*. 16: 79-84.

[24] Badalyan SM *et al*. (2011). New Armenian wood-associated Coprinoidspecies: *Coprinopsis strossmayeri* and *Coprinellus* aff. *radians. Diversity*. 3: 136-154.

[25] Badalyan SM *et al*. (2011). Taxonomic significance of anamorphic characteristics in the life cycle of coprinoid mushrooms. In: Proceed. VII Int. Conf. Mushr. Biol. & Mushr. Prod. Savoie J.M., Foulongne-Oriol M., Largeteau M. and Barroso G. Eds. P. 140-154.

[26] Badalyan SM *et al*. (2012). Morphological and genetic characteristics of different collections of *Ganoderma* P. Karst.species. The 18th Congr. ISMS. Zhang J., Wang H. and Chen M. Eds. P. 247-254.

ADVANCES IN CULTIVATION OF MEDICINAL FUNGI BIOMASS AND PHARMACEUTICAL COMPOUNDS IN BIOREACTORS

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ABSTRACT

Original strains of *Ganoderma lucidum* (MZKI G97) and *Grifola frondosa* (GF3) isolated from Slovenian forests were cultivated using solid state cultivation. In *G. lucidum* in 18 days of solid state cultivation to 5.77 mg /g of extracellular and 1.45 mg/g intracellular polysaccharide was produced at the end of the cultivation. In *G. frondosa* solid state cultivation in 38 days of cultivation 3.80 mg/g of extracellular and 0.70 mg/g of intracellular polysaccharide was produced. Polysaccharides were further separated by ion-exchange, gel and affinity chromatography. The isolated polysaccharides were mainly β-D-glucanes. Immunostimulatory effects of isolates were tested on induction of cytokine (TNF-a , IFN-g and IL12) synthesis in primary cultures of human mononuclear cells (PBMC) isolated from a buffy coat.

Keywords: *Ganoderma lucidum*, *Grifola frondosa*, polysaccharides, immunomodulatory effects

INTRODUCTION

Basidiomycets of various species and their wide range of pharmaceutically interesting products are in the last decades one of the most attractive groups of natural products in Asia and North America. Research in physiology, basical and applied studies in fungal metabolism, process engineering aspects and clinical studies last decade represent large cotribution to the development of this potentials that initiates the development of new drugs, specially those on the palette of over counter remedies.

G. frondosa also known as Maiitake and *G. lucidum*, are lignin degrading basidiomycete with excellent nutritional and medicinal properties. Pharmaceutically active compounds from *G. lucidum* include triterpenoids, polysaccharides (1,6-β-D-glucans and 1,3-β-D-glucans), proteins, proteoglycans, steroids, alkaloids, nucleotides, lactones and fatty acids, amino acids, nucleotides, alkaloids, steroids, lactones and enzymes. Over 100 triterpenoids were found in *Ganoderma* spp., such as ganoderic (highly oxygenated C_{30} lanostane-type triterpenoids), lucidenic, ganodermic, ganoderenic and ganolucidic acids, lucidones, ganoderals and ganoderols *G. frondosa* active compounds primarilly belong to the group of polysaccharides (especially 1,6-β-D-glucans and 1,3-β-D-glucans), glycoproteins, and proteins. This products have been used for treatment of a series of diseases, including hepatitis, arthritis, nephritis, bronchitis, asthma, arteriosclerosis, hypertension, cancer and gastric ulcer. Newer investigations report on *G. frondosa* antiallergenic constituents, immunomodulatory action and treatment of HIV infections, antitumor and cardiovascular effects, liver protection and detoxification and effects on nervous system [1].

A large and diverse spectrum of chemical compounds with a pharmacological activity has been isolated from the mycelium, fruiting bodies and sclerotia of *Ganoderma* mushrooms: triterpenoids, polysaccharides, proteins, amino acids, nucleotides, alkaloids, steroids, lactones, fatty acids and enzymes

G. lucidum isolates effects on angiogenesis, reduction of benign prostatic hyperplasia, antibacterial and antiviral effects, effects on lipid metabolism and hypertension, antidiabetic activity, vitality and performance enhancement, antioxidant effects, and beneficial cosmetic effects on skin.

As *G. frondosa* and especially *G. lucidum* are very rare in nature, the amount of wild mushroom is not sufficient for commercial exploitation. Cultivation on solid substrates, exploing various agricultural wastes from food and wood industy,

stationary liquid medium or, in the last time, by submerged cultivation using various complex media, have become essential to meet the increasing demands on the international markets.

G. lucidum and *G. frondosa* polysaccharides (especially β-D-glucanes) have been recognised as effective anti-cancer drugs remarqably improving immunosystem in human, veterinary an fishery use. In human body they induce activity of cytokines IL-1, IL-6, TNF-α and IFN-γ production by human macrophages and T-lymphocytes.

A successful artificial cultivation of *G. lucidum* and *G. frondosa* has been reported on solid substrates, utilising e.g. sawdust and agricultural wastes as the main media components [2], as well as submerged cultivation in liquid media [3]. The quality and content of physiologically active substances vary from strain to strain and also depends on location, culture conditions [4] and growth of the mushroom [5].

The main goals of this research were to test the ability of submerged cultivation for the production of *G. lucidum* biomass, and to evaluate the potential immunostimulatory effects of polysaccharides tested on the induction of cytokine (TNF-α IFN-γ) synthesis in primary cultures of human mononuclear cells.

MATERIALS AND METHODS

Strains

Original strains of *G. lucidum* (MZKI G97) and *G. frondosa* (GF3) isolated from Slovenian forests were cultivated in solid state cultivation where beech saw dust, wheat bran and mineral salts as well as milled whole corn plant (*Zea mays*), olive oil and mineral salts substrates were used. Production of extracellular and intracellular polysaccharides in both cultivation was studied.

Substrates

Solid state cultivation of *G. lucidum* and *G. frondosa* biomass producing 1,6-β-D-glucans and 1,3-β-D-glucans in solid substrates from food and wood industy wastes were applied. Original technology of cultivation of fungal biomass has been developed recently for small and pilot-plant production of intra- and extracellular polysaccharides.

Bioreactor

In solid state cultivation in 30l horizontal stirred tank reactor (HSSR) was used. (Fig. 1). Sterilisation was performed *in-situ*.

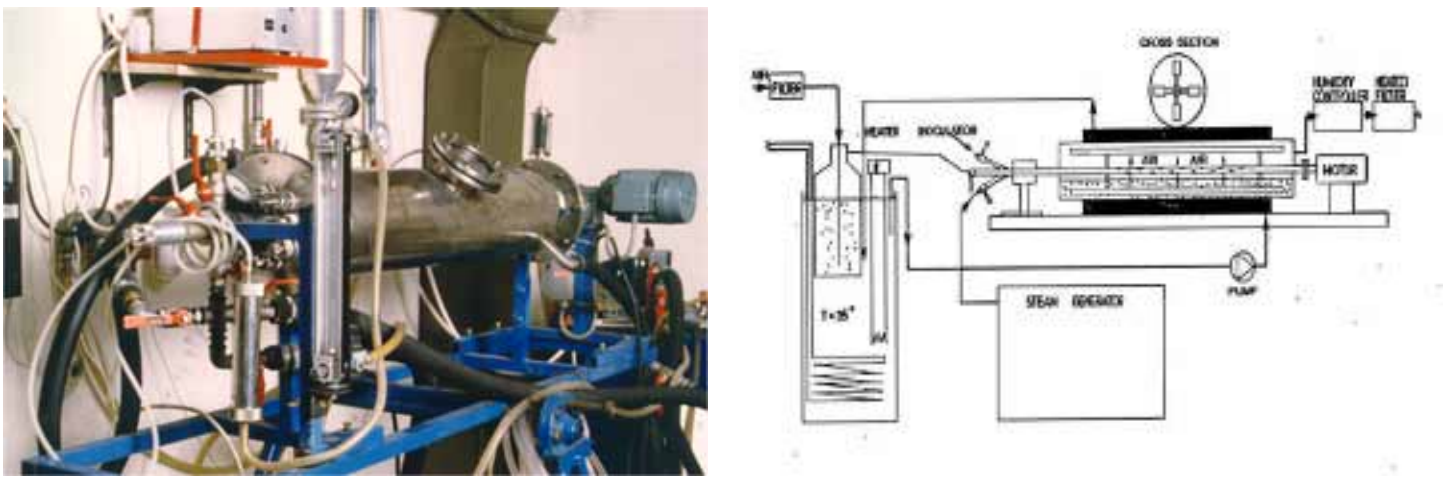


Figure 1. Horizontal stirred tank reactor and equipment

In *G. lucidum* cultivation temperature was 30 °C, while at *G. frondosa* used temperature was 28 °C. Aeration in solid state cultivation aeration was 5 l $min.^{-1}$ and only periodically mixing N = 8 rpm, 5 min/day was used with both fungi in all of the experiments.

Biomass

In solid state cultivation for biomass determination on solid particles determination of glucosamine (chitin) contents and glucosamine assay with 3 methyl-2-benzothiazole hydrazone were used.

Microscopy

Fungal growth in solid state cultivation was monitored by electron microscopy using Field-Emission Scanning Electron Microscope Karl Zeiss Supra 35 VP (Fig. 2).

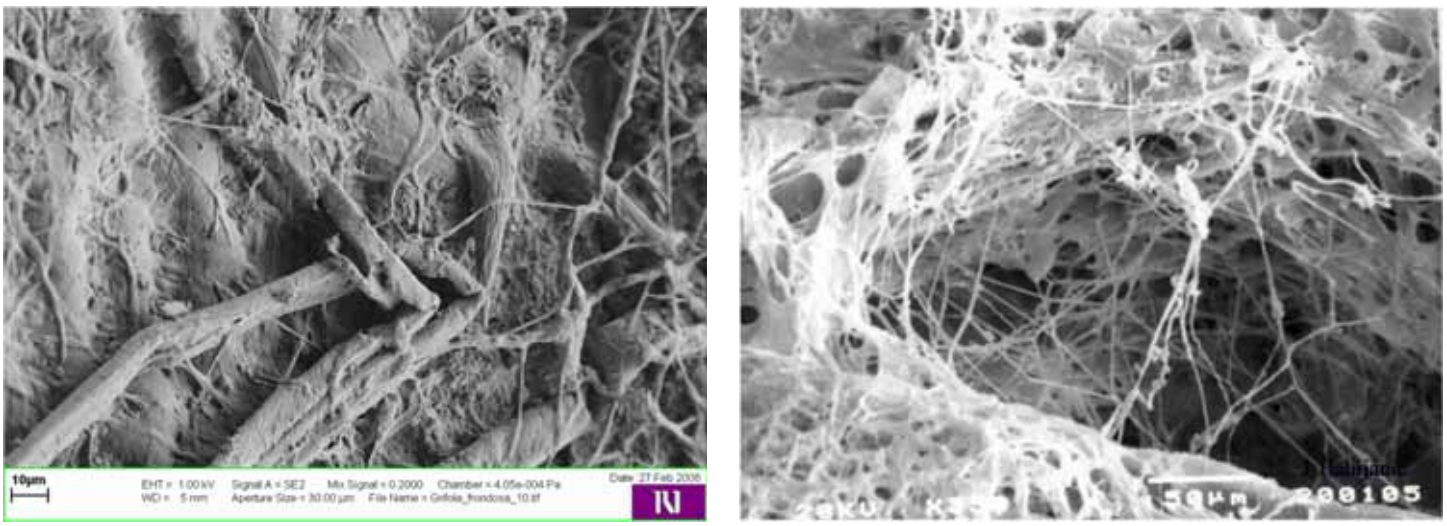


Figure 2. Fungal growth in solid state cultivation a) *G.rifola frondosa*, b) *G.anoderma lucidum* Field-Emission Scanning Electron Microscope Karl Zeiss Supra 35 VP

RESULTS AND DISCUSSION

Cultivation

G. lucidum in 18 days of solid state cultivation produced 5.77 mg /g of extracellular and 1.45 mg /g intracellular polysaccharide at the end of the cultivation

In *G. frondosa* in solid state cultivation in 38 days of cultivation produced 3.80 mg/g of extracellular and 0.70 mg/g of intracellular polysaccharide was produced. Polysaccharides were further separated by ion-exchange, gel and affinity chromatography.

Extraction and fractionation of polysaccharides

Fungal mycelium was separated from the submerged cultivation broth by vacuum filtration. Cultivation medium was concentrated at T = 50 °C and a reduced pressure. Extracellular polysaccharides were precipitated from the concentrate by 96% ethanol, filtered, washed with acetone and dried (fraction A). The mycelium was extracted with 85% ethanol to eliminate low molecular components. Then, the first fraction of intra-cellular polysaccharides was extracted with hot water (T = 100 °C, 3 hours), filtered, concentrated and precipitated by 96% ethanol (fraction B). The mycelium was further extracted with 1% ammonium oxalate solution (T=98 °C, 3 hours) (fraction C), and with 5% sodium hydroxide solution (T= 25 °C, 12 hours), from which polysaccharides were precipitated by acetic acid (fraction D), and from the remaining solution by ethanol (fraction E). Samples of fractions A - E were used in cytokine assays (Table 1).

Table 1. Extraction and fractionation of polysaccharides

Fraction	Properties	Mass (mg)	Yield (%)
A	extracellular polysaccharides, water soluble, precipitated with 96% ethanol	1616	1,37
B	intracellular polysaccharides, hot water extract, precipitated with 96% ethanol	2414	2,04
C	intracellular polysaccharides, 1% ammonium oxalate solution extract, precipitated with 96% ethanol	1183	1,002
D	intracellular polysaccharides, 5% sodium hydroxide solution extract, precipitated with acetic acid	2068	1,75
E	intracellular polysaccharides, 5% sodium hydroxide solution extract, precipitated with 96% ethanol	650	0,55

Polysaccharide fractions A and B were further fractionated and purified by ion-exchange chromatography on DEAE-cellulose (column 20 x 3.0 cm, elution with water, 0.1M $NaHCO_3$, 0.3M $NaHCO_3$, 0.5M $NaHCO_3$ and 0.1M NaOH), gel filtration on Sepharose 4B (column 70 x 1.2 cm, elution with water), and affinity chromatography on Concavalin A-Sepharose 4B (column 20 x 1.2 cm). For β- polysaccharides, the column was eluted with 0.1 M phosphate buffer (pH=7,0) in 1M NaCl, and for α- polysaccharides with 0.1 M glucose in 1M NaCl. In all cases, the absorbance of chromatographic fractions was measured at 480-490 nm by Dubois method.

Evaluation of cytokine inducing capacity

Human peripheral blood mononuclear cells (PBMC) from the buffy coat of a healthy blood donor were isolated by a density gradient centrifugation with Ficoll-Paque (Pharmacia, Sweden). The cells were cultured in a tissue culture medium RPMI 1640 (Sigma, USA) supplemented with 100 U ml^{-1} penicillin (Sigma, USA), 100 mg ml^{-1} streptomycin (Sigma, USA), 20 mM Hepes (Sigma, USA) and 10 % heat-inactivated AB normal human serum (Sigma, USA). The 1x 10^6 cells (final culture volume 1.5 ml) were plated in 24-well culture plates (Nunc, Denmark) with each of five fractions alone in different concentrations (3.25, 12.5, 50, 100, 400 mgm l^{-1}), at T=37 °C in a humidified atmosphere of 5% CO_2 in air. Cultures of untreated cells in RPMI 1640 without active substances were considered as a negative control. To rule out a possible contamination by the endotoxin - a lipopolysaccharide from the Gram negative bacterial cell wall (LPS) of our polysaccharide samples, the samples with polysaccharide concentrations of 12.5, 100, 400 mg ml^{-1} with added polymyxin B (Sigma, USA) in concentration 10 mg ml^{-1}, were tested parallely.

The concentration of cytokines (pg ml^{-1}) in PBMC culture supernatant was measured by commercially available ELISA kits, TNF-α from DPC (USA) and IFN-γ from Endogen (USA), according to the manufacturer instructions. The detection limit for TNF-α was 15.0 pg ml^{-1} and for IFN-γ 1.0 pg ml^{-1}, respectively

Table 2. The concentration of IFN-γ in culture supernatants of human PBMC incubated for 72 hours with two polysaccharide fractions showing the strongest TNF-α inducing capacity (B and C), with polymixin B

Concentration of polysaccharide fraction [mg/ml]	Concentration of IFN-γ pg/ml]	
	Polysaccharide fraction B	Polysaccharide fraction C
12.5	<1	<1
100	1.23	2.15
400	1.39	2.18

Immunomodulatory activity

In vitro testing of immunomodulatory effects of polysaccharide fractions from *G. lucidum* mycelium proved the induction of moderate amounts of TNF-α in the extent of < 3.0 pg ml^{-1} to 630 pg ml^{-1} , while TNF-α induction at *G. frondosa* was

up 322 pg ml^{-1} at a polysaccharide concentration of 200 µg ml^{-1} for the intracellular fraction. of a culture supernatant (Figs.3 and 4 a,b,c).

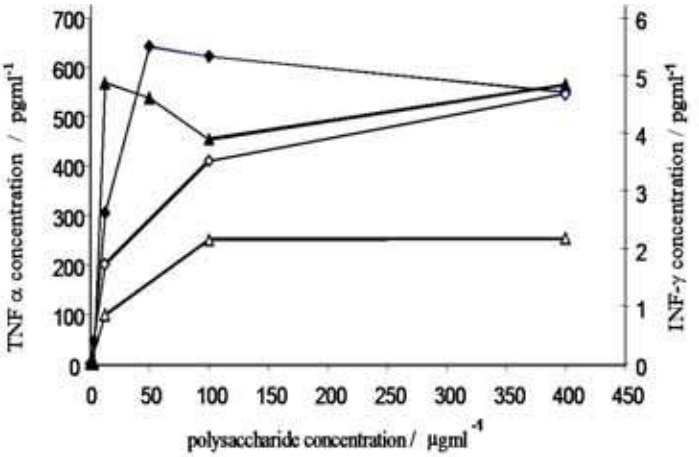


Figure 3. Induction of TNF-α and IFN- γ production by the most active *Ganoderma lucidum* polysaccharides (sample C) from ammonium-oxalate extract, in the presence of polymyxin and without polymyxin

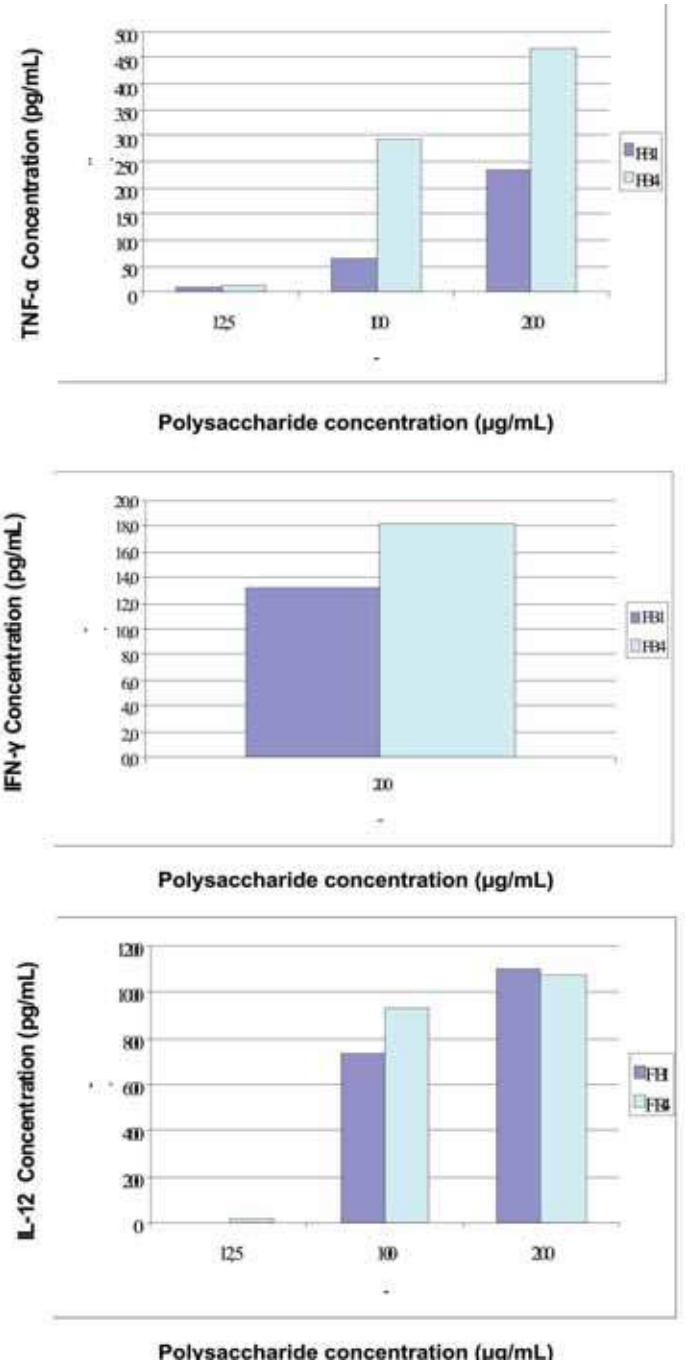
♦ TNF- ; ◊ TNF-α + polymixin ; Ä IFN-γ ; ²⁰% IFN-γ + polymixin

CONCLUSION

Cultivation of fungal biomass of *G. lucidum* and *G. frondosa* isolated from the Slovenian forest by solid state cultivation enables high production of pharmaceutically active fungal biomas that enables the production of fungal polysaccharides mostly 1-3 and 1 – 6 β-D-glucanes, which are known from literature as the main immunomodulatory substances of both fungi.

Polysaccharide fractions from both fungal mycelium proved to be inducers of production of cytokines TNF-α, IFN-γ and at *G. frondosa* also IL-12, that are comparable to the those ammounts of cytokines that are inducing activity of romurtide, which has been used as a supporting therapy in cancer patients treated with radiotherapy and/or chemotherapy.

Consequently, the polysaccharides isolated from the Slovenian *G. lucidum* strain represent a potential and promising natural immunomodulatory substance, which could be efficiently and economically produced by solid state cultivation and production of *G. lucidum* and *G. frondosa* biomass. The reported results represent valuable information on active fungal polysaccharides produced and isolated from the European *Ganoderma* and *Grifola* spp. Solid state



Figures 4 a,b,c. Immunostimulatory effects of *Grifola frondosa* polysaccharides. The comparison of different isolates on induction of cytokines TNF α , IFN- γ and IL-12

cultivation are enabling large scale production of fungal polysaccharides - particulary suitable as feed grade imunostimulatory compounds for veterinary use.

ACKNOWLEDGEMENTS

The project was co-financed by the Ministry of Science and Technology of the Republic of Slovenia.

REFERENCES

[1] Hirose K *et al.* (1988). Antiviral agent. Japanese patent JP 63316734. 1988 December 26.

[2] Triratana S *et al.* (1991). Cultivation of *Ganoderma lucidum* in sawdust bags. In: Science and Cultivation of Edible Fungi, Proceedings of the 13th International Congress on the Science Cultivation of Edible Fungi, Dublin, 1-6 September 1991 (ed. M.J. Maher). pp. 567-572, Rotterdam: AA Balkema.

[3] Kohlmünzer S *et al.* (1989). Polysaccharides in mycelial culture of *Ganoderma applanatum* (Pers.) *Pat. Herba Hungarica*. 28: 87-94.

[4] Habjanic J and Berovic M. (2002). Process of cultivation of fungus *Ganoderma lucidum* on a solid cultivation substrate. Patent SI 20923.

[5] Miyahara R *et al.* (1987). Chemical structures and changes of extracts during growth of reishi (*Ganoderma lucidum*). *Mokuzai Gakkaishi*. 33: 416-422.

ANTIPROLIFERATIVE ACTIVITY OF BIOACTIVE COMPOUNDS FROM MUSHROOMS OF INDIAN ISOLATES

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ABSTRACT

Biodiversity study on mushrooms of basidiomycetes was initiated and more than seven hundred species were described by Natarajan and associates during 1975-2008 from South India. But the bio-documentation of many medicinal mushrooms were initiated a decade ago by Vaidya and associates from Western India, Janardhanan and associates from Kerala and Kaviyarasan and associates from South India. *Lentinus tuberregium, Neolentinus kauffmanii* and *Agaricus heterocystis,* were studied for their medicinal properties such as antitumor, antiviral, antimicrobial and antioxidant activities. These indigenous mushrooms are effective against many cancer lines and induce apoptosis and results in tumor cell death. Antiangiogenesis effect of *Trametes hirsuta* extract was well established with fertilized hen eggs. These results, clearly established their candidature for drug formulations. Two novel anticancer compounds extracted from *Lentinus tuberregium* were filed for patent for their anticancer properties. Polysaccharides from *Tramates hirusuta,* an indigenous isolate was also very effective against many cancer lines. Currently few more edible mushrooms are being studied for their medicinal properties.

INTRODUCTION

Many clinically important drugs, such as aspirin, digitoxin, progesterone, cortison and morphine, have been derived directly or indirectly from higher plants. Less well-recognized but of great clinical importance are the widely used drugs from fungi such as the antibiotics, penicillin and griseofulvin, the ergot alkaloids and cyclosporine [1]. Mushrooms are the macrofungi with a distinctive fruiting body, which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand [2]. Mushrooms constitute at least 14,000 and perhaps as many as 22,000 known species. The number of mushroom species on the earth is estimated to be 140,000, suggesting that only 10% are known [3].

For millennia, mushrooms have been valued by mankind as an edible and medical resource. A number of bio- active molecules, including antitumor substances, have been identified in many mushroom species. During the last two decades there has been an increasing recognition of the role of the human immune system for maintaining good health. Mushrooms such *as Ganoderma lucidum* (reishi), *Lentinus edodes* (shiitake), *Inonotus obliquus* (chaga) and many others have been collected and used for hundreds of years in Korea, China, Japan, and eastern Russia. It is notable and remarkable how reliable the facts collected by traditional eastern medicine are in the study of medicinal mushrooms [4,5]. In India, the knowledge of indigenous mushroom consumption as food and medicine prevails from time immemorial. But there is no authentic record of our own. But two authentic reports on medicinal uses were recorded by Petch, [6] on the uses of *Termitomyces* mycelial mass near Thanjavur, Tamilnadu, India and Gordon Wasson of Germany. A good book on Soma drink referred in Rig Vedas stating that *Amanita muscaria* extract was the Soma drink by comparing the descriptions in the Vedas with the structural description of *Amanita* has been written. Use of compounds from *Phellinus* sp. as preservative was recorded by Sharifi *et al.* [7] and their antitumor activity was studied by Meera and Janardhanan [8]. Natarajan and associates during 1978-2008 has studied biodiversity of Agarics diversity of South India. Recently many indigenous edible and medicinal mushrooms were studied by our group for their antioxidant and antitumor activities using cell lines and presented in this paper [9-14].

CURRENT STATUS OF RESEARCH

The fruiting body and the mycelium of mushrooms contain compounds with a wide range of medicinal properties. Currently a lots of research is being carried out to prove the medicinal properties such as antitumor properties, antiviral, antibacterial

and immunomodulatory properties of the bioactive metabolites at both national and international level. Mushrooms are rich sources of β-glucan, proteoglycan, lectin, phenolic compounds, flavonoids, polysaccharides, triterpenoids, diatery fibre, lentinan, schizophyllan, lovastatin, pleuran, steroids, glycopeptides, terpenes, saponins, xanthones, coumarins, alkaloid, kinon, fenil propanoid, kalvasin, porisin, AHCC, maitake D-fraction, ribonucleases, eryngeolysin, and also have been used extensively in traditional medicine for curing various types of diseases such as antimicrobial, antiviral, anticancer, antitumor, antiinflamatory, cardiovascular diseases, immunomodulating, central activities etc. [15-17, 4]. Medicinal mushroom research has focused on discovery of compounds that can modulate positively or negatively the biologic response of immune cells. Those compounds, which appear to stimulate the human immune response, are being sought for the treatment of cancer, immunodeficiency disease or for generalized immunosuppression following drug treatment. They are also sought for combination therapy with antibiotics and as adjuncts for vaccines [18]. Wasser [4] reported that mushroom polysaccharides are regarded as biological response modifiers (BRM). This basically means that they cause no harm and place no additional stress on the body, but help the body to adapt to various environmental and biological stresses. Mushroom polysaccharides support some or all of the major systems of the body, including nervous, hormonal and immune systems as well as regulatory functions. The polysaccharides from mushrooms do not attack cancer cells directly, but produce their anti-tumour effects by activating different immune response in the host [4].

Polysaccharides are a structurally diverse class of macromolecules able to offer the highest capacity for carrying biological information due to a high potential for structural variability [4]. Whereas the nucleotides and amino acids in nucleic acids and proteins effectively, interconnect in only one way, the monosaccharide units in polysaccharides can interconnect at several points to form a wide variety of branched or linear structures [19]. This high potential for structural variability polysaccharides gives the necessary flexibility to the precise regulatory mechanisms of various cell-cell interactions in higher organisms. The polysaccharides of mushrooms occur mostly as glucans. Some of which are linked by β-(1-3), (1-6) glycosidic bonds and α-(1-3) glycosidic bonds but many are true heteroglycans.

BIOACTIVE POLYSACCHARIDES

Polysaccharides are the best known and most potent mushroom- derived substances with antitumor and immunomodulating properties [20-24]. Historically, hot-water-soluble fractions (decoctions and essences) from medicinal mushrooms, i.e., mostly polysaccharides, were used as medicine in the Far East, where knowledge and practice of mushroom use primarily originated [5]. Ikekawa *et al*. [25] published one of the first scientific reports on antitumor activities of essences obtained from fruiting bodies of mushrooms belonging to the family Polyporaceae (Aphyllophoromycetideae) and a few other families, manifested as host-mediated activity against grafted cancer – such as Sarcoma 180 – in animals [26,27]. Soon thereafter the first three major drugs were developed from medicinal mushrooms. All three were polysaccharides, specifically β-glucans: krestin from cultured mycelial biomass of *Trametes versicolor* (Turkwey Tail), lentinan from fruiting bodies of *L. edodes*, and schizophyllan from the liquid cultured broth product of *Schizophyllum commune*.

Hobbs [5] reported that *L. edodes* produces two bioactive preparations, which are efficient immune modulators, mycelium extract and lentinan. These two bioactive polymers appear to act as host defense potentiators restoring and enhancing the responsiveness of host cells to lymphocytokines, hormone and other biologically active substances. The immunopotentiation has been shown to occur by stimulating the maturation, differentiation or proliferation of cells involved in host defense mechanism. Many interesting biological activities of lentinan including increase in the activation of non- specific inflammatory response such as acute phase protein production; vascular dilation and haemorrhage-inducing factor in vivo [28], activation and generation of helper and cytotoxic T cells [15].

Chihara *et al*. [15] reported that lentinan increase host's resistance against various kinds of cancer and has the potential to restore the immune function of affected subjects. The interaction of lentinan with many kinds of immune cells was not known until recently. Ross *et al*. [29] provided an insight into receptor binding in immune cells by β-glucan from fungi and further showed that β-glucan from yeast bind to iC3b- receptors (CR3, CD11b/CD18) of phagocytic and natural killer (NK) cells. When this happens, it will stimulate phagocytosis and/or cytotoxic degranulation. Lentinan has also been shown to stimulate peripheral blood lymphocytes in vitro to increase interleukin-2-mediated LAK cell (lymphokine-activated

killer cell) and NK cell activity at levels achievable in vivo by administration of clinical doses of lentinan. This observation was made using the blood of healthy donors and cancer patients. Lentinan has also been shown to inhibit suppressor T cells activity in vivo and to increase the ratio of activated T cells and cytotoxic T cells in the spleen when administered to gastric cancer patients undergoing chemotherapy.

BIOACTIVE SMALL MOLECULES

Apart from Polysaccharides mushrooms also contains valuable bioactive small molecule that have antitumor, antimicrobial and antiviral properties. The small molecule which possess anti-microbial activity includes Applanoxidic acid A, isolated from *Ganoderma annulare* (Fr.) Gilbn., shows weak antifungal activity against *Trichophyton mentagrophytes* [30]. Steroids like 5a-ergosta- 7,22-dien-3b-ol or 5,8-epidioxy-5a,8a-ergosta-6,22- dien-3b-ol, isolated from *Ganoderma applanatum* (Pers.) Pat., proved to be weakly active against a number of gram- positive and gram-negative microorganisms. Oxalic acid is one agent responsible for the antimicrobial effect of *Lentinula edodes* (Berk.) Pegler against *S. aureus* and other bacteria [31]. Ethanolic mycelial extracts from *L. edodes* possess antiprotozoal activity against *Paramecium caudatum* [32]. The antimicrobial activity of *Podaxis pistillaris* (L.: Pers.) Morse, used in some parts of Yemen for the treatment of 'nappy rash' of babies and in South Africa against sun burn, is caused by epicorazins [33]. These substances belong to the group of epipolythiopiperazine-2,5- diones, an important class of biologically active fungal metabolites. Other antimicrobial compounds from the Aphyllophorales were summarized by Zjawiony [34].

In contrast to bacterial infectious diseases, viral diseases cannot be treated by common antibiotics and specific drugs are urgently needed. Antiviral effects are described not only for whole extracts of mushrooms but also for isolated compounds. They could be caused directly by inhibition of viral enzymes, synthesis of viral nucleic acids or adsorption and uptake of viruses into mammalian cells. These direct antiviral effects are exhibited especially by smaller molecules. Indirect anti-viral effects are the result of the immunostimulating activity of polysaccharides or other complex molecules.

Several triterpenes from *Ganoderma lucidum* (M. A. Curtis: Fr.) P. Karst. [i.e. ganoderiol F , ganodermanontriol, ganoderic acid B are active as antiviral agents against human immunodeficiency virus type 1 (HIV-1). The minimum concentration of ganoderiol F and gano- dermanontriol for complete inhibition of HIV-1 induced cytopathic effect in MT-4 cells is 7.8 mg/ml. Ganoderic acid B inhibits HIV-1 protease [35]. Ganodermadiol , lucidadiol and applanoxidic acid G , isolated from *G. pfeifferi*, but also known from other *Ganoderma* species, possess *in vitro* antiviral activity against influenza virus type A. Further, ganodermadiol is active against herpes simplex virus type 1, causing lip exanthema and other symptoms [36]. *In vitro* antiviral activity against influenza viruses type A and B was demonstrated for mycelial extracts of *Kuehneromyces mutabilis* (Schaeff.: Fr.) Singer & A. H. Sm. [37], extracts and two isolated phenolic compounds from *Inonotus hispidus* (Bull.: Fr.) P. Karst [38] and ergosterol peroxide, present in several mushrooms. The antiviral activity of *Collybia maculata* (Alb.&Schwein.: Fr.) P.Kumm. (vesicular stomatitis viruses in BHK cells) is caused by purine derivatives [39]. Thus many drugs are formulated not only to treat against diseases but to stimulate the immune system to resist the pathogens using biomolecules from mushrooms.

CURRENT STATUS OF RESEARCH IN OUR LABORATORY

The major objective of research is to study the biodiversity of basidiomycetes of both Eastern Ghats (Thirumala hills, Kolli hills and Javvadi hills etc) and Western Ghats besides the plains of Tamil Nadu. Besides the biodiversity study bio-documentation of these organisms is the need of the hour. Medicinal properties of few South Indian mushroom species were charcterised by isolating few bio active molecules from indigenous mushroom species. An intracellular fibrinolytic protease from *Ganoderma lucidium* isolate VK12 (Fig 1) was isolated and purified [10], which has the potential to be used as an alternative to the commercially available Urokinases having many side effects on the patient for treatment of cardio vascular diseases. The enzyme was purified to homogeneity and molecular mass of was determined as 33.2 kDa. By enzyme kinetic studies the enzyme was characterized as metalloprotease. The purified fibrinolytic protease showed anticoagulant activity with human plasma. Moreover the purified protease protected pulmonary mice thromboemolism to the extent of 70%. The survival rate of mice treated with purified protease were 36, 72 and 81% at doses 20, 40 and 60µg/kg respectively, compared to 9% in the control [40].

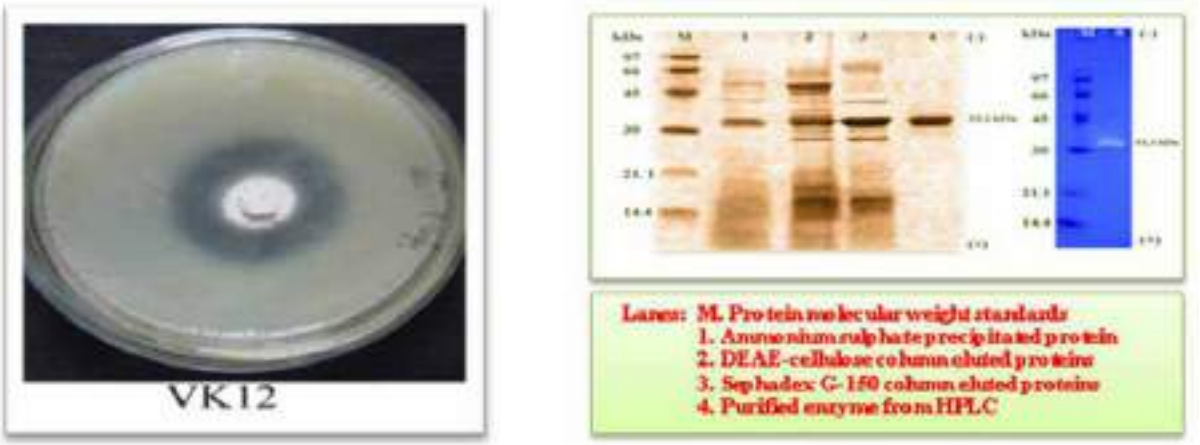

Figure 1. Extracellular fibrinolytic protease from *Ganoderma lucidum* strain VK-12 1a) Mycelium showing fibrinolytic activity on agar plate 1b) Purification of fibrinolytic protease from *Ganoderma lucidum* strain VK-12.

Figure 2. Fruiting body of Indigenous agaric *Agaricus heterocystis* Heinem and Gooss – *VKJ 17*

Though some of the agaric species are growing wildly in our tropical envirnment, no studies were carried out to cultivate them. *Agaricus heterocystis* had formed fruit bodies in the agar medium itself [41]. Cultivation of the indigenous wild edible variety *A. heterocytis strain VKJ17* (Fig. 2) was later standardized by Jagadish *et al.* [42]. In addition to the study on the nutritive value, their edibility and the medicinal properties such as antioxidant capacity and antitumor activity were evaluated [42]. Moreover the ethanolic extract of this mushroom induces apoptotic mode of cell death in HL-60 cell line. Two more terpenoid compounds were isolated namely C1-AGH and C2-AGH were shown in the Fig 3 which were active against human viruses such as HSV type1, type 2 and Influenza viruses A and B.

Lentinus tuberregium, an edible mushroom consumed by Kaani tribes of Paechi parai forest of Western Ghats (Fig. 4) are known to have antitumor, antioxidant and antimicrobial diterpene compounds. They were isolated from the *Lentinus*

Figure 3. Two antiviral compounds active against influenza virus A and B and HSV Type I and Type II viruses obtained from *Agaricus heterocystis* strain VKJ-17 3a) C1-AGH 3b) C2-AGH

Figure 4. Fruiting body of *Lentinus tuberregium* (Fr.) Fr strain VKJM 24

tuberregium VKJM 24 in another biodocumentation study [11]. Two compounds namely LT-1 and LT-2 were isolated, purified and their structure has been elucidated using various techniques and shown in the Fig. 5. They were tested against various cell lines *viz.*, SK-OV-03 (ovarian cancer), A673 (Rhabdomyosarcoma), HCT-116 (Colorectal Carcinoma) and MCF-7 (Breast Cancer) and the viability of cells was determined by the MTT assay. Of the four cancer lines tested, both LT1 and LT2 exerted maximal growth inhibition in SK-OV-03, followed by A673. On the other hand, HCT-116 showed moderate growth inhibition.

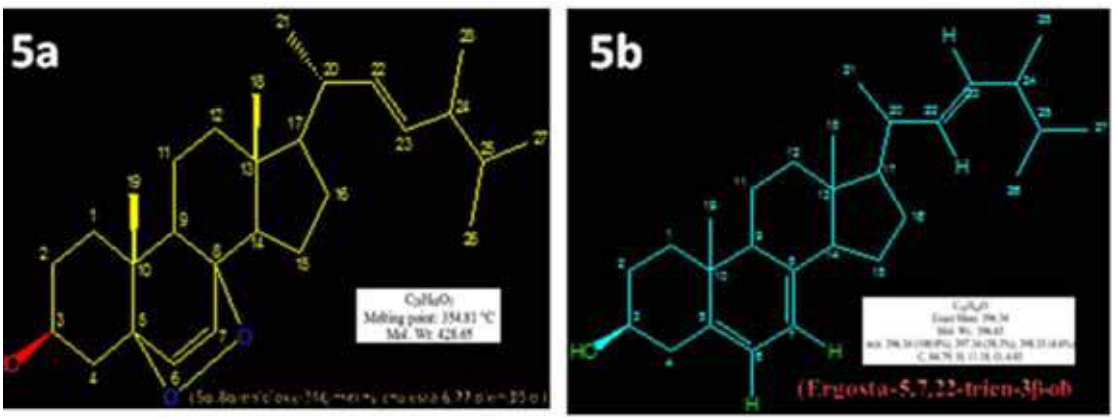

Figure 5. Two compounds namely LT-1 and LT-2 isolated from *Lentinus tuberregium* VKJM

Neolentinus kauffmanii (Fig. 6) strain *VKGJ01* was isolated during biodiversity study on Western Ghats [43] which is being consumed by the Kanni tribes of Kanyakumari forests of Western ghats as food additive and they claim many medicinal properties. Its cultivation was standardised for mass production. Both fruit body and mycelium were screened for bioactive compounds for antimicrobial and antitumor activity. A compound of steroid nature namely β-sitosterol with a molecular formula of $C_{28}H_{50}O_3$ and molecular weight 414.71 g/mol^{-1} was isolated from *N. kauffmanii* and studied for anticancer activity (Fig. 7). The study clearly showed that the compound exerted significant inhibitory effect on the HepG2 lung cancer cell lines. Both crude extract of mycelium and fruit body exhibited similar activities [14]. A Polysaccharide of glucan nature with antiangiogenic property was isolated from *Pleurotus eryngii* [12].

Figure 6. Fruiting body of *Neolentinus kauffmanii* Smith A.H. strain VKGJ01

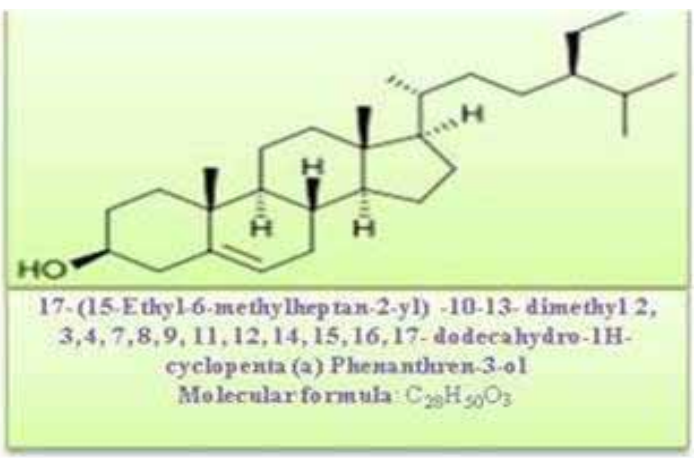

Figure 7. Structure of purified anticancer compound NK-1 active against liver cancer cell lines obtained from *Neolentinus kauffmanii* strain VKGJ01

Besides these agaric members a polypore namely *Trametes hirusuta* was isolated from suburb of Chennai (Fig 8) and its cultivation was standardized for the fruit body production. Though extensive studies on *Trametes versicolor* were carried out and the extracellular polysaccaride was shown to have antitumor activity and marketed globally as Krestin an anticancer drug [44]. Since, no such studies were carried out on indigenous *Trametes hirusuta* a study was carried out for biodocumetation of this mushroom [45]. An extracellular β-glucan was isolated from the *Trametes hirsuta strain VKESR* culture filtrate (Fig. 9) with moderate in *vitro* antioxidant and immunomodulatory potentials and showed good antiproliferative activity in colon, liver and leukemic cells lines. Further, it induced apoptosis through intrinsic mitochondrial mediated pathway in the cell lines. Moreover, it possesses good *ex vivo* antiangiogenic and anticancer potentials against DEN induced hepatocellular carcinoma in rats [45].

Figure 8. Fruiting body of *Trametes hirsuta* (Wulf.) Pil. strain VKESR

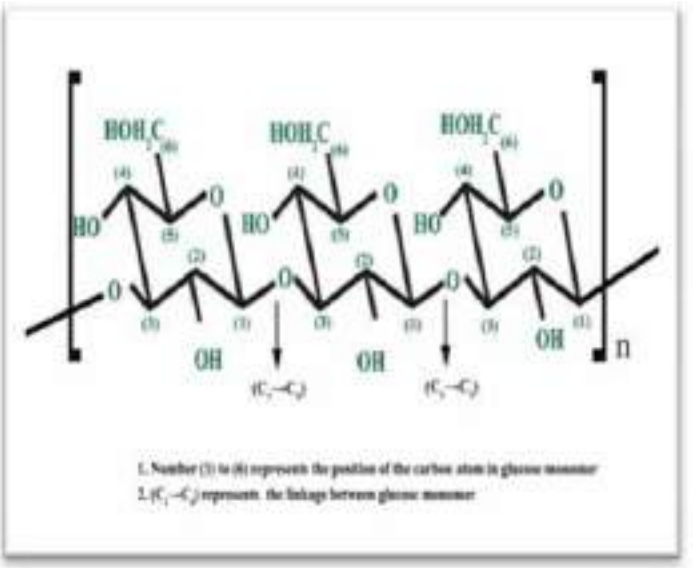

Figure 9. Structure of antitumor and immunomodulatory extracellular β– Glucan obtained from *Trametes hirsuta* (Wulf.) Pil. strain VKESR

COMMERCIAL STATUS OF BIOMOLECULES

At present, between 80 and 85% of all medicinal mushroom products are derived from the fruiting bodies, which have been either commercially farmed or collected from the wild mushrooms. Only15% of all products are based on extracts from mycelia. Examples are PSK and PSP from *T. versicolor* and Tremellastin from *Tremella mesenterica*. (Retzius): Fr. A small percentage of mushroom products are obtained from culture filtrates, e.g. Schizophyllan from *S. commune* and protein-bound polysaccharide complex from *Macrocybe lobayensis* (R. Heim) Pegler & Lodge [syn. *Tricholoma lobayense* R. Heim] [46]. After production, suitable galenic formulations like capsules, tablets or teas have to be developed, dependent on the material. Mixtures of several mushrooms or of mushroom and substrate become more and more common [47].

Lentinan from *L. edodes* fruit-bodies, Schizophyllan from *S. commune* mycelial broth, PSK and PSP, from mycelial cultures of *Trametes versicolor* and Grifron-D from fruit-bodies of *G. frondosa* were clinically tested commercial anticancer and immunomodulating drugs. All have been gone through Phase I, II and III clinical trials mainly in Japan and China but now in US. However, in many cases the standards of these trials may not meet current Western regulatory requirements. In many cases there have been significant improvements in quality of life and survival. Increasingly, several of these compounds are now used extensively in Japan, Korea and China, as adjuncts to standard radio and chemotherapy. While most of these clinical studies have used extracts from individual medicinal mushrooms, some recent studies from Japan have shown that mixtures of extracts from several known medicinal mushrooms, when taken as a supplement, have shown beneficial effects on the quality of life for some advanced cancer patients.

FUTURE FOCUS IN THE RELEVANT FIELD

The above studies clearly show that mushrooms, similar to plants, have a great potential for the production of useful bioactive metabolites and they are a prolific resource for drugs. The responsible bioactive compounds belong to several chemical groups, very often they are polysaccharides or triterpenes. One species can possess a variety of bioactive compounds, and therefore, has probabibility of having higher pharmacological effects. The best example is *G. lucidum*, which not only contains >120 different triterpenes but also polysaccharides, proteins and other bioactive compounds [48,49]. However, one main pre-requisition to use as drug, nutraceutical or other purpose is the continuous production of mushrooms (fruiting bodies or mycelium) in high amounts and in a standardized quality. In the opinion of Chang [46], mycelial products are the 'wave of the future' because they ensure standardized quality and year around production. A further necessity is the establishment of suitable quality parameters and of analytical methods to control these parameters. Nevertheless, the legal regulations for authorization as drug or as dietary supplements or as food should get more attention [44]. Control of possible side effects (i.e. allergies) during broad use is necessary. Finally, also the nutritional value of mushrooms should be taken into account.

Currently, biodocumentation of few more indigenous mushrooms from Indian forest ecosystem are being carried out in our group. Further, focusing on standardization of mass production of fruit bodies and mycelium for more compound production to develop novel anticancer drugs from the indigenous mushrooms in collaboration with various organizations. Thus many more miles to go before we get a fruitful drug with following vision and concepts. More studies are needed to demonstrate anti-viral, anti-tumor and anti-cholesterol process with high-quality long term double-blinded placebo-controlled studies with large trial populations to ensure safety and efficacy of medicinal mushrooms with statistical power.

ACKNOWLEDGEMENTS

I thank the former Directors of C.A.S in Botany Prof. N. Anand, and the present Director Prof. R. Rengasamy for the help and guidance.

REFERENCES

[1] A Ajith T and K Janardhanan K. (2007). Indian medicinal mushrooms as a source of antioxidant and antitumor agents. *J. Clin. Biochem Nutr*. 40(3): 157-162.

[2] Chang ST and Miles PG. (1989). Edible mushrooms and their cultivation, CRC Press.

[3] Hawksworth DL. (2001). The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycological Research*. 105(12): 1422-1432.

[4] Wasser SP and Weis AL. (1999). Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. *Crit Rev Immunol*. 19(1): 65-96.

[5] Hobbs C. (2000). Medicinal value of *Lentinus edodes* (Berk.) Sing. (Agaricomycetideae). A literature review. *International Journal of Medicinal Mushrooms*. 2(4): 287-302.

[6] Petch T. (1906). *The Fungi of Certain Termite Nests*.

[7] Sharif A *et al.* (2005) Evaluation of crude sesquiterpenoid extract of *Phellinus fastuosus* as a natural preservative. *Hindustan Antibiot. Bull.* 47-48(1-4): 20-23.

[8] Meera CR and Janardhanan KK. (2012). .Antitumor Activity of a Polysaccharide-Protein Complex Isolated from a Wood-Rotting Polypore Macro Fungus *Phellinus rimosus* (Berk) Pilat. *J. Environ. Pathol. Toxicol. Oncol*. 31(3): 223-232.

[9] Jagadish KL *et al.* (2009). Comparitive study on the antioxidant, anticancer and antimicrobial property of *Agaricus bisporus* (J E Lange) Imbach before and after boiling. *African Journal of Biotechnology*. 8(4). 654-661.

[10] Kumaran S *et al.* (2011). Studies on screening, isolation and purification of a fibrinolytic protease from an isolate (VK12) of *Ganoderma lucidum* and evaluation of its antithrombotic activity. *Med. Mycol. J*. 52(2): 153-162.

[11] Manjunathan J *et al.* (2011). Taxonomic Studies on *Lentinus tuberregium* (GQ292711) Tamil Nadu, India. *Research J. Pharm. and Tech*. 4(2)

[12] Shenbhagaraman R *et al*. (2012). Optimization of extracellular glucan production from *Pleurotus eryngii* and its impact on angiogenesis. *Intl. J. Biol. Macromol*. 50(4): 957-964.

[13] Johnsy G and Kaviyarasan V. (2012). Nutritive Value of Edible Wild Mushrooms Collected from the Western Ghats of Kanyakumari District. *World journal of Dairy and Food Sciences*. 11(1).

[14] Johnsy G. (2012). Studies on nutritional and medicinal value of an indigenous isolate of *Neolentinus kauffmanii* Smith A.H. (VKGJ-01). Ph.D., Thesis submitted to University of Madras, Chennai, India.

[15] Chihara G. (1992). Immunopharmacology of Lentinan, a polysaccharide isolated from *Lentinus edodes*: its applications as a host defence potentiator. *Intl. J. Oriental Medicine* 17: 57-77.

[16] Wang H *et al*. (2000). A new lectin with highly potent antihepatoma and antisarcoma activities from the oyster mushroom *Pleurotus ostreatus*. *Biochem. Biophys. Res. Commun.* 275(3): 810-816.

[17] Bobek P *et al*. (2001). Effect of pleuran (beta-glucan from *Pleurotus ostreatus*) in diet or drinking fluid on colitis in rats. *Nahrung* 45(5): 360-363.

[18] Jang M *et al*. (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 275(5297): 218-220.

[19] Sharon N and Lis H. (1993). Carbohydrates in cell recognition. *Sci Am*. 268(1): 82-89.

[20] Mizuno T *et al*. (1996) .Antitumor activity and chemical modification of polysaccharides from niohshimeji mushroom, *Tricholma giganteum*. *Biosci Biotechnol Biochem*. 60(1): 30-33.

[21] Lorenzen K and Anke T. (1998). Basidiomycetes as a source for new bioactive natural products. *Current Organic Chemistry*. 2(4): 329-364.

[22] Borchers AT *et al*. (1999). Mushrooms, tumors, and immunity. *Proc Soc Exp Biol Med*. 221(4): 281-293.

[23] Mizuno M *et al*. (1999). Anti-tumor polysaccharide from the mycelium of liquid-cultured *Agaricus blazei* mill. *Biochemistry and Molecular Biology International*. 47(4): 707-714.

[24] Reshetnikov SV *et al*. (2001). Higher basidiomycota as a source of antitumor and immunostimulating polysaccharides (Review). *Intl. J. Med. Mushr*. 3(4): 361-394.

[25] Ikekawa T *et al*. (1969). Antitumor Activity of Aqueous Extracts of Edible Mushrooms. *Cancer Research* 29(3): 734-735.

[26] Ikekawa T *et al*. (1982). Studies on antitumor polysaccharides of *Flammulina velutipes* (Curt. ex Fr.) Sing.II. The structure of EA3 and further purification of EA5. *J. Pharmacobiodyn* 5(8): 576-581.

[27] Ikekawa T *et al*. (1992). Antitumor activity of *Hypsizigus marmoreus*. I. Antitumor activity of extracts and polysaccharides. *Chem. Pharm. Bull. (Tokyo)*. 40(7): 1954-1957.

[28] Maeda YY *et al*. (1991) Genetic control of the expression of two biological activities of an antitumor polysaccharide, lentinan. *Intl. J. Immunopharmacology* 13(7): 977-986.

[29] Ross GD *et al*. (1999) .Therapeutic intervention with complement and beta-glucan in cancer. *Immunopharmacology* 42(1-3): 61-74.

[30] Smania EFA *et al*. (2003) .Antifungal activity of sterols and triterpenes isolated from *Ganoderma annulare*. *Fitoterapia*. 74(4): 375-377.

[31] Bender S *et al*. (2003). A Case for Caution in Assessing the Antibiotic Activity of Extracts of Culinary-Medicinal Shiitake Mushroom *Lentinus edodes* (Berk.) Singer] (Agaricomycetideae). 5(1): 6.

[32] Badalyan S. (2004). Antiprotozoal activity and mitogenic effect of mycelium of culinary-medicinal shiitake mushroom *Lentinus edodes* (Berk.) Singer (Agaricomycetideae). 6(2): 8.

[33] Lindequist U. (1998). *Schizophyllum* In: Schneider G, Hänsel R, Blaschek W, editors. HAGERs Handbuch der Pharmazeutischen Praxis. Berlin, Heidelberg, New York: Springer-Verlag;. pp. 528–34.

[34] Zjawiony JK. (2004). Biologically active compounds from aphyllophorales (polypore) fungi. *J. Natural Products*. 67(2): 300-310.

[35] El-Mekkawy S *et al*. (1998). Anti-HIV-1 and anti-HIV-1-protease substances from *Ganoderma lucidum. Phytochemistry* 49(6): 1651-1657.

[36] Mothana RAA *et al*. (2003). Antiviral lanostanoid triterpenes from the fungus *Ganoderma pfeifferi. Fitoterapia* 74(1–2): 177-180.

[37] Mentel R *et al*. (1994). *In vitro* antiviral effect of extracts of *Kuehneromyces mutabilis* on influenza virus. *Pharmazie*. 49(11): 859-860.

[38] Awadh Ali *et al*. (2003). Antiviral activity of *Inonotus hispidus*. *Fitoterapia*. 74(5): 483-485.

[39] Leonhardt K *et al*. (1987). 6-Methylpurine, 6-methyl-9-beta-D-ribofuranosylpurine, and 6-hydroxymethyl-9-beta-D-ribofuranosylpurine as antiviral metabolites of *Collybia maculata* (Basidiomycetes). *Zeitschrift fur Naturforschung. C. J. Biosciences* 42(4): 420-424.

[40] Kumaran S. (2008). Studies on production, purification and characterisation of fibrinolytic and antithrombotic protease from a South Indian isolate of *Ganoderma lucidum* (FR.) Karst. Ph.D., Thesis submitted to University of Madras, Chennai, India.

[41] Natarajan K *et al.* (2002). Basidiocarp production in *Agaricus heterocystis* Heinem. and Gooss. in nutrient agar media. *Current Science*. 82: 9-10

[42] Jagadish L. (2010). Cultivation and medicinal properties of Indigenous Agaric. *Agaricus heterocystis* Heinem and Gooss – *VKJ 17*. Ph.D., Thesis submitted to University of Madras, Chennai, India.

[43] Davidson Sargunam S *et al.* (2012). Mushrooms in the food culture of the Kaani tribe of Kanyakumari district. *Indian Knowledge of traditional knowledge* 11(1): 150-153.

[44] Wasser SP. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology* 60(3): 258-274.

[45] Shenbhagaraman R. (2012). Therapeutic potential of extracellular â- glucan from *Trametes hirsuta* (Wulf.)Pil. strain VKESR against malignant neoplasm. Ph.D., Thesis submitted to University of Madras, Chennai, India.

[46] Chang ST. (2001). A 40-Year Journey Through Bioconversion of Lignocellulosic Wastes to Mushrooms and Dietary Supplements. 3(2-3): 1.

[47] Wasser SP *et al.* (2000). Dietary Supplements from Medicinal Mushrooms: *Diversity of Types and Variety of Regulations* 2(1): 19.

[48] Kim HS *et al.* (1999). *In vitro* chemopreventive effects of plant polysaccharides (*Aloe barbadensis Miller*, *Lentinus edodes*, *Ganoderma lucidum* and *Coriolus versicolor*). *Carcinogenesis* 20(8): 1637-1640.

[49] Gao Y *et al.* (2004). Immunomodulating activities of *Ganoderma*, a mushroom with medicinal properties. *Food Reviews International* 20(2): 123-161.

[50] Hobbs C. (1995). Medicinal Mushrooms: An Exploration of Tradition, Healing & Culture, Botanica Press.

[51] Ikekawa T. (2001). Beneficial Effects of Edible and Medicinal Mushrooms on Health Care. 3(4): 8.

[52] Kumar M. (2009). Taxanomic studies on Agaric diversity of Kolli hills, Eastern ghats, India. Ph.D., Thesis submitted to University of Madras, Chennai, India.

[53] Manjunathan J. (2011). Isolation, Purification, Characterization and Evaluation of Antimicrobial and Anticancer Compounds from Indigenous Isolate of *Lentinus tuberregium* (Fr.) Fr. Ph.D., Thesis submitted to University of Madras, Chennai, India.

[54] Sekar Kumaran *et al.* (2011). Screening of Fibrinolytic protease from south Indian isolates of *Ganoderma lucidum*. *International J. Pharma. and Bioscience.* 2(1): 419-431.

CALOCYBE INDICA POLYSACCHARIDES ALLEVIATES COGNITIVE IMPAIRMENT, MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS INDUCED BY D-GALACTOSE IN MICE

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ABSTRACT

The aim of this study was to investigate the protective effect of *Calocybe indica* crude polysaccharides (CICP) against D-galactose induced cognitive dysfunction, oxidative damage and mitochondrial dysfunction in mice. Mice were subcutaneously injected with D-galactose (150 mg/kg per day) for 6 weeks and were administered CICP simultaneously. Aged mice receiving vitamin E (100 mg/kg) served as positive control. Chronic administration of D-galactose significantly impaired cognitive performance oxidative defense and mitochondrial enzymes activities as compared to control group. The results showed that CICP (200 and 400 mg/kg) treatment significantly improved the learning and memory ability in Morris water maze test. Biochemical examination revealed that CICP significantly increased the decreased activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), mitochondrial enzymes-NADH dehydrogenase (ND), malate dehydrogenase (MDH), isocitrate dehydrogenase (ICDH), Na^+K^+, Ca^{2+}, Mg^{2+}ATPase activities, elevated the lowered total anti-oxidation capability (TAOC); glutathione (GSH) and vitamin C decreased the raised acetylcholinesterase (AChE) activities; malondialdehyde (MDA), hydroperoxide (HPO), protein carbonyls (PCO), advanced oxidation protein products (AOPP) levels in brain of aging mice induced by D-gal in a dose-dependent manner. In conclusion, present study highlights the potential role of CICP against D-galactose induced cognitive impairment, biochemical and mitochondrial dysfunction in mice.

Keywords: *Calocybe indica*, oxidative stress, antioxidants, aging, neuro degenerative disease

INTRODUCTION

Ageing is a complex natural phenomenon that is frequently accompanied by the occurrence of several diseases, such as schizophrenia, cognitive impairment, Alzheimer's and Parkinson's diseases and others. Anti-aging has already become a major public issue with the increasing elderly population in the world. Memory decline is characteristic of aging and age-related neurodegenerative disorders, which result in a progressive loss of cognitive function, specifically in spatial memory [1]. Abundant evidence has pointed to an important role of oxidative stress during the pathogenesis of brain aging, age-associated or neurodegenerative diseases [2–4]. Indeed, with increasing age, accumulation of oxidant damaged cellular macromolecules, such as DNA, proteins and lipids of cell membranes takes place [5,6]. Brain, with high oxygen demand, high level of unsaturated lipids, and relatively deficient in anti-oxidative defense mechanism, is the most susceptible organ to oxidative damage. Thus, antioxidant therapy may be an important avenue for managing neurodegenerative diseases.

Further, evidence suggests that mitochondria are both producers as well as targets of reactive oxygen species, which increases oxidative damage [7]. As a consequence, damaged mitochondria progressively become less efficient, losing their functional integrity and release more reactive oxygen molecules [8]. Increasing oxidative burden deteriorates functional mitochondria during aging. Mitochondria are the major source of energy or adenosine triphosphate (ATP) for the normal functioning of eukaryotic cells. Dysfunction of mitochondria is well known to generate reactive oxygen species (ROS), reduce mitochondrial ATP production, increased mitochondrial deoxyribonucleic acid (DNA) mutations, increase in abnormal mitochondrial criste structures and impairs intracellular calcium level [9]. Increased ROS generation with compromised mitochondrial function ultimately affects neurons and accelerates neurodegenerative process [10].

D-galactose (D-gal) is a physiological nutrient and can be metabolized at normal concentration. In animals, galactose is normally metabolized by D-galactokinase and galactose-1-phosphate uridyltransferase but over-supply of D-galactose results its abnormal metabolism [11].D-galactose converts into galactitol, which does not metabolize by above enzymes but accumulate in the cell, that leads to osmotic stress and ROS production [12]. D-galactose is a reducing sugar that reacts with free amines of amino acids in proteins and peptides to from advanced glycation end products (AGE), which in turn causes activation of receptor for advanced glycation end products. These sequences of events cause oxidative stress and cellular damage [13,14]. AGE increases with age and has been linked pathologically in many age related pathologies such as diabetes, arteriosclerosis, nephropathy and Alzheimer's disease.The D-gal-lesioned rodents have been used for brain aging studies, as D-gal induced behavioral and neurochemical changes can mimic many characters of the natural brain aging process [15-18].

Administration of antioxidants via oral gavage is thought to be an effective approach to prevent free radicals from oxidizing sensitive biological molecules, thus slowing aging process and preventing diseases. Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products. Nowadays more and more attention was cast on mushroom polysaccharide by biochemical and nutritional researchers due to their various biological activities used in health care food or medicine, especially antioxidant, immunostimulatory and antitumor effects (19-22]. However, studies of the antiaging effects of *C. indica* crude polysaccharides (CICP) in animal models are scarce. Therefore, it is necessary to investigate the effect of CICP in an animal model to develop a neuroprotective drug. The aim of present study has been designed to explore the possible effect of CICP against D-galactose induced cognitive dysfunction oxidative damage and mitochondrial dysfunction in mice.

MATERIALS AND METHODS

Animals

Female Balb/C mice (20-25 g) were used. The mice were housed in the temperature and humidity controlled room (temperature 23 ± 2 °C and humidity 50 ± 10%) with a 12 hr light-dark cycle with and water *ad libitum*. Experimental animals were handled according to the University and Institutional Legislation, regulated by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Preparation of the extract

Fruiting bodies of *Calocybe indica* were collected from a local mushroom farm. The fruiting bodies of the mushroom were cut into small pieces, dried at 40-50 °C for 48 h and powdered.The water soluble polysaccharides was prepared following the procedure described by Cheng *et al.* [23] with some modifications. Polysaccharides were extracted from dried mushrooms (~1.5 g) with boiling water (50 ml) for 4 h under agitation. The residue was then extracted with two further portions of boiling water over a total 6 h extraction. The resulting suspension was then centrifuged (4000 r/min for 10 min). It was then concentrated in a rotary evaporator under reduced pressure at 50 °C. The concentrated supernatants were then precipitated with three volumes of absolute ethanol (95%) and polysaccharides were precipitated overnight at 4 °C. The precipitated polysaccharides were collected after centrifugation at 3000 g for 40 min followed by filtration, before being lyophilised, resulting in a crude polysaccharide (CICP).

Experimental design

The mice were randomly divided into five groups, each consisting of 10 animals. The five groups were designated as follows: group I (normal control group); group II (D-gal model group); group III (CICP + D-gal); group IV (CICP + D-gal); group V (Vit E + D-gal). Mice in group II to group V were subcutaneously intraperitoneally (i.p.) injected with D-gal (in normal saline) once daily at a dose of 150 mg/kg per day for 6 weeks, while mice in group I were treated with the same volume of physiological saline. From the first week, after the injection of D-gal, group III and group IV mice were orally administrated with crude mushroom polysaccharide fraction (CICP) at the doses of 200

and 400 mg/kg per day and group V mice were treated with Vit E by intragastric administration at the dose of 100 mg/kg per day, respectively. At the same time, the normal control group (group I) and the D-gal model group (group II) mice were given the same volume of physiological saline for 6 weeks by intragastric administration.

Behavioral assessments

Assessment of cognitive performance: Learning and memory ability was detected by Morris water maze test [24]. In this test, mice were trained to find a platform (6 cm in diameter) hidden 1 cm below the water surface in a circular water tank (100 cm in diameter, 45 cm in height). Each mouse received four training sessions per day for 4 consecutive days. One day before the first trial, each mouse received 4 times of pre-training: mouse was put on the platform for 20s, then given a 30s free swim and placed on the platform, where it was allowed to rest for another 20s rest. For each trial, the mouse was placed in the water facing the pool wall at one of four starting quadrant point, and the time required for the mouse to find the hidden platform was recorded. A mouse that found the platform was allowed to stay on it for 20s and then returned to its cage for 40s inter-trial interval. If the mouse did not find the platform within 60s, it would be placed on it for 20s, and the escape latency (finding the submerged platform) was recorded as 60s. After the 4th training trial, the platform was removed and each mouse was allowed to swim freely for 60s as the probe test. The time that mice spent in the target quadrant (where the platform was once hidden) was measured.

Preparation of brain tissue homogenate: According the method of [24], all mice were deeplyanesthetized and sacrificed by decapitation after behavioral testing. Brains were promptly dissected and the tissues were minced and homogenized (10% w/v) in Tris-HCl buffer (0.1 M; pH 7.4) and centrifuged at 3000 g for 20 min at 4 °C.

Preparation of mitochondrial fraction: About 10% of the brain tissue homogenate of rats was prepared in 50 mM phosphate buffer (pH 7.0) containing 0.25 mol/l (w/v) sucrose. Homogenate was centrifuged initially at 3,000 g for 10 min and the supernatant was subjected to 11,000 g for 10 min at 4 °C in a cooling centrifuge. The mitochondrial pellets were washed twice with phosphate buffer to removethe sucrose and suspended in phosphate buffer.

Biochemical assessments: The brain homogenate were assayed for AChE, SOD, CAT, GPx, GR, GST, GSH, Vit C, LPO and HPO. The mitochondrial fraction were assayed for ND, MDH, ICDH, AOPP, PCO and TAOC.

Determination of Acetylcholine esteraseactivity: Acetylcholine esterase activity was estimated according to the method of Shinomol and Muralalidana [25, 26]. Acetythiocholine is hydrolysed to thiocholine by acetylcholine esterase, which reacts with DTNB and give yellow colour chromophore 5-thio-2-nitrobenzoic acid (TNB), which is measured at 412 nm. The enzyme activity is expressed as µM of substrate hydrolyzed/min/mg protein.

Determination of NADH dehydrogenase activity: NADH dehydrogenase activity was determined by the method of King and Howard [27]. The method involves catalytic oxidation of NADH to NAD^+ with subsequent reduction of cytochrome c and is assayed by measuring the increase in absorbance at 550 nm against reagent blank.

Determination of Malate dehydrogenase activity: Malate dehydrogenase activity was estimated by the method of Mehler*et al.* [28]. It catalyses the reversible conversion of oxaloacetic acid to malic acid. The activity was expressed as µmoles of NADH oxidized/ min/mg protein using the extinction coefficient of NADH 6.22$mM^{-1}cm^{-1}$.

Determination of Isocitrate dehydrogenase activity: Isocitrate dehydrogenase activity was estimated according to the method of Varley [29]. ICDH catalyses the decarboxylative oxidation of trisodium-isocitrate by $NADP^+$ yielding α-ketoglutarate, CO_2 and NADPH. The increase in absorbance associated with the reduction of NADP to NADPH is measured spectrophotometrically at 340 nm. The activity was expressed as micromoles of NAD^+reduced/min/mg protein using extinction coefûcient 6.22 $mM^{-1}cm^{-1}$.

Determination of total antioxidant capacity: The total antioxidant capacity (TAOC) of the sample was assayed according to the method of Benzie and Strain [30] using ferric reducing ability as a measure of antioxidant power. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue coloured Fe II-

tripyridyltriazine compound from colourless oxidized Fe III form by the action of electron donating antioxidants.The results are expressed as µM/mg protein.

Determination of Superoxide dismutase activity: Superoxide dismutase activity was assayed by the method of Das [31]. The method involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound, which subsequently reacts with naphthylamine to produce a red azo compound whose absorbance is measured at 543 nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

Determination of Catalase activity: Catalase activity was assayed by the method of Sinha [32]. The enzyme assay was based on the utilization of H_2O_2 by the enzyme. Dichromate in acetic acid was converted to perchloric acid and then to chromic acetate when heated in presence of H_2O_2. The chromic acetate thus produced is measured spectrophotometrically at 610 nm. The activity of catalase was expressed as µM of H_2O_2 consumed/min/mg protein.

Determination of Glutathione peroxidase activity: Glutathione peroxidase activity was measured by Ellman [33]. The assay measures the enzymatic reduction of H_2O_2 by GPx through consumption of reduced glutathione (GSH). Glutathione was measured by its reaction with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to give a yellow derivative that absorbs at 412 nm. The activity was expressed in term of µg of glutathione oxidized/min/mg protein or ml of serum.

Determination of Glutathione reductase activity: Glutathione reductase activity assay was based on the method of Beutlur [34]. The enzymatic activity was assayed photometrically by measuring NADPH consumption. In the presence of GSSG and NADPH, GR reduces GSSG and oxidizes NADPH, resulting in a decrease of absorbance at 340 nm.The activity was expressed as µmoles of glutathione utilised/ min/mg protein.

Determination of Glutathione-S-transferase activity: Glutathione-S-transferase activity was measured by the method of Habig [35]. Glutathione-S-transferase catalyses the reaction of 1-chloro 2, 4 dinitrobenzene (CDNB) with the sulphydryl group of glutathione. The CDNB- Glutathione conjugate absorbs light at 340 nm and the activity of the enzyme can therefore be estimated by measuring the increase in absorbance. The results were expressed as nmoles of CDNB conjugated/min/mg of protein.

Determination of ATPase activity: Na^+K^+ ATPase was assayed by the method of Bonting [36], Ca^{2+}ATPase [37], Mg^{2+}ATPase [38] based on the inorganic phosphorus liberated, which is estimated by Fiske and Subbarow method.

Determination of reduced glutathione: Reduced glutathione level was measured according to the method of Moron [39] based on the formation of a yellow colored complex with Ellman's reagent measured at 412 nm. The amount of glutathione was expressed as µg/mg protein.

Determination of vitamin C: The level of vitamin C was determined by the method described by Omaye [40]. The dehydro-ascorbic acid formed from the oxidation of vitamin C by copper, formed a colored product on treatment with 2,4-dinitrophenylhydrazine, whose absorbance was measured at 520 nm.The results were expressed as µg/mg protein.

Determination of protein carbonyl: Protein carbonyl was estimated by the method of Levine *et al.* [41]. Protein carbonyl utilizes the DNPH reaction and the amount of protein hydrozone produced is quantified spectrophotometrically at an absorbance of 370 nm. The results were expressed as µM of carbonyl/ mg protein.

Determination of advanced oxidation protein product: Advanced oxidation protein products (AOPP) level was determined according to the method described of Kayali *et al.* [42].The concentration of AOPP was calculated using the extinction coefficient 26 $mM^{-1}cm^{-1}$ and expressed as µM/mg protein.

Determination of Lipid peroxidation: The extent of lipid peroxidation in the brain was determined quantitatively by performing the method as described by Niehius [43]. In this method malondialdehyde and other TBARS were estimated

by their reactivity with thiobarbituric acid (TBA) in acidic condition to generate a pink coloured chromophore, which were read at 535 nm. MDA content was expressed as nmol per g tissue.

Determination of Hydroperoxides: The hydroperoxide was determined by the method of Jiang *et al* [44].In this method, oxidation of ferrous ion (Fe^{2+}) under acidic condition, in the presence of xylenol orange leads to the formation of a chromophore with an absorbance maximum at 560 nm.Hydroperoxides were expressed as mM/g tissue.

Determination of Protein: The protein content was estimated by Lowry's method [45] using bovine serum albumin as a standard.

Statistical analysis

Values are expressed as mean ± SEM.The data are reported as the mean ± standard deviation and were analysed by SPSS (version 20.0 SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant differences between the means were determined by Duncan's Multiple Range tests. $p<0.05$ was considered significant.

RESULTS

Effect of CICP on memory performance in Morris water mazetask in D-galactose treated mice

One of the age related declines of brain function is the cognitive behavioral deficit. It has been well established that water maze performance declines with aging of the animals and it is a sensitive method for revealing the impairment of spatial learning and memory [46]. As indicated in Fig. 1 the D-gal induced group took longer time to find the hidden platform as compared with the normal control group, suggesting that the D-gal treated mice had significant cognitive impairment. The prolonged escape latency in the D-gal model group was shortened by administration of CICP (200, 400 mg/kg per day) and Vitamin E (100 mg/kg day).

The time the mice spent swimming in the target quardant on day 5th are shown in Fig. 2. The D-gal mice crossed the former location of the platform less frequently ($p<0.05$) than normal mice, but CICP (200, 400 mg/kg b.wt) and vitamin E (100 mg/kg) treatment could improve the impaired performance. In addition, mice in the CICP treated groups reached the target quadrant more rapidly and the time they spent swimming in the target quadrant were longer ($p<0.05$) than that of the D-gal mice. The above results indicated that CICP prevented D-gal induced spatial learning and memory dysfunction in mice.

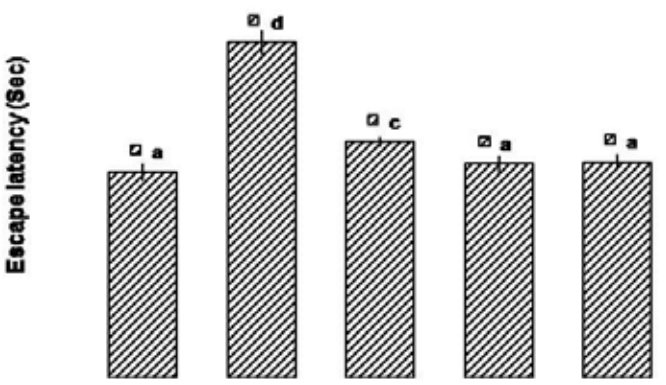

Figure1. Effect of CICPon the escape latency of mice in the water maze test on 5th day. Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT

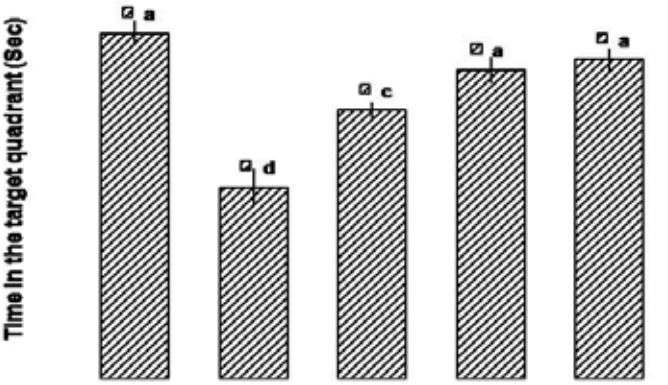

Figure 2. Effect of CICPon the time that mice spent in swimming in the target quadrant on 5th day Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT

Our present study demonstrated that long term administration of D-gal induced impairment of learning and memory in water maze tests. However, water maze tests suggested that CICP significantly reversed the behavioral retrogression, such as learning and cognition indicating that CICP had potential to preventlearning and memory deficits.

Effect of CICP on the activities of acetyl cholinesterase in D-gal induced mice

Cholinergic system is of predominant importance in learning and memory processes [47]. Acetyl cholinesterase, one of the specific cholinergic marker proteins, is responsible for degradation of acetyl cholinesterase to acetate and choline in the synaptic cleft. Therefore, we analyzed the acetyl cholinesterase activity in the brain of each group of mice. Figure 3 described the acetyl cholinesterase activity in the brain of all groups of mice. D-gal induced mice displayed a remarkable increase in AChE activity ($p<0.05$) compared with the normal control. The increase in AChE activity was restored to near normal in CICPand vitamin E treated mice. CICPat the dose of 400 mg/kg b.wt exhibited more significant effect than the lower dosage (200 mg/kg b.wt) and vitamin E at a dose of 100 mg/kg b.wt showed comparable effect to that of CICP (400 mg/kg b.wt).

Effect of CICPon the activities of brain mitochondrial NADH dehydrogenase, MDH and ICDH in D-gal induced mice

Administration of D-Gal for 6 weeks caused significant alterations in mitochondrial enzyme activities. D-galactose treatment significantly decreased NADH dehydrogenase, MDH, ICDH activities as compared to control mice ($p<0.05$). However, CICP (200 and 400 mg/kg b.wt) and Vit E (100 mg/kg b.wt) treatment significantly restored the mitochondrial enzyme activities to near normal (Table 1).

Table 1. Effect of polysaccharides from *C.indica* on the activities of NADH dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase in mitochondrial brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	CICP (200 mg/kg) + D-Gal	CICP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
NADH DH	168.55 ± 3.92^{e}	95.84 ± 2.66^{a}	120.97 ± 4.44^{b}	141.11 ± 4.67^{c}	154.83 ± 3.97^{d}
MDH	4.66 ± 0.12^{d}	3.34 ± 0.11^{a}	4.10 ± 0.13^{bc}	4.19 ± 0.09^{c}	4.01 ± 0.14^{b}
ICDH	3.31 ± 0.12^{d}	2.40 ± 0.10^{a}	2.88 ± 0.12^{b}	3.14 ± 0.13^{c}	3.46 ± 0.15^{d}

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT; Units: NADH DH - µM of NADH oxidized/min/mg protein; MDH- µM of succinate oxidized/min/mg protein; ICDH-µM/min/mg protein.

The results of the present study indicate that chronic administration of D-galactose caused an impairment in mitochondrial enzyme activities as indicated by decrease in the NADH dehydrogenase, MDH and ICDH activities. The observed alteration in mitochondrial enzymes activities could be involved in aging and its complications, which could be due to free radicals as well as reduction in both mitochondrial transcription and translation levels. Moreover, it has been reported that disruption of mitochondrial activity associated with inhibition of enzymes in the electron transport chain which, lead to increase in electron leakage from the mitochondria [48], production of ROS like the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). Mitochondrial dysfunction might be the key factor for the production of ROS, which leads to oxidative damage in D-galactose induced aging.

Effect of CICP on the levels of TAOC in D-gal induced mice

TAOC represents the free radical scavenging capacity of antioxidants. Decline of TAC level is an important part of the pathophysiological changes that occur during ageing, which is one of the reasons for the increased accumulation of ROS. D-galactose significantly decreased the TAOC level compared to control mice ($p<0.05$). CICP treatment (200 and 400 mg/kg b.wt) significantly enhanced the TAOC levels in the brain in a dose dependent manner. Vitamin E also reversed these altered changes to near normal levels (Fig. 4).

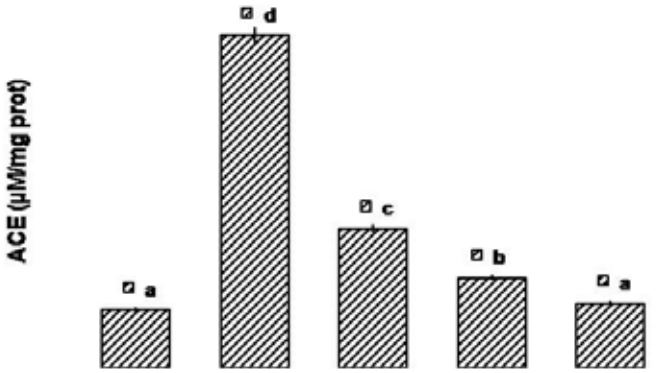

Figure 3. Effect of polysaccharides from *C.indica* on the activity of acetyl choline esterase in brain of D-gal induced mice. Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT

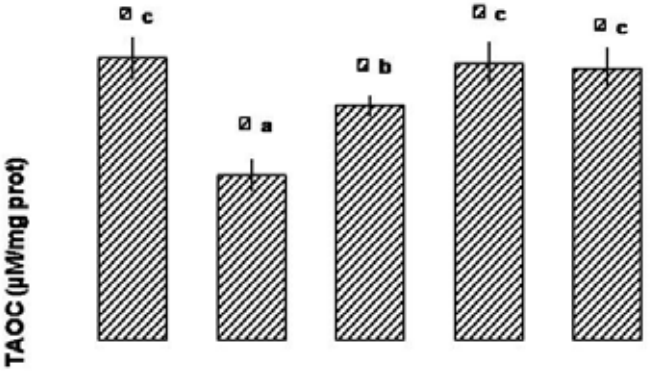

Figure 4. Effect of polysaccharides from *C.indica* on the levels of TAOC in brain of D-gal induced mice. Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT

TAOC, an indicator of enzymatic and non enzymatic antioxidants, reflects the total antioxidant capabilities. When antioxidant defenses are weakened, body cells and tissues become more prone to develop dysfunction and/or disease. The maintenance of adequate antioxidant levels is essential in preventing and managing a great number of conditions, so TAOC could be a reliable diagnostic biomarker. CICP which possessed strong antioxidant activity noticeably increased TAOC levels in galactose induced mice.Thus the observed beneficial effect of CICP might be either due to its antioxidant or free radical scavenging activity [49].

Effect of CICP on the activities of antioxidant enzymes in D-gal induced mice

As shown in Table 2 it is evident that SOD, CAT, GPx, GR, and GST activities in braindecreased significantly ($p<0.05$) in D-galactose induced mice compared with normal mice. Administration of CICP extract (200 and 400 mg/kg b.wt) increased the activity of antioxidant enzymes in a dose dependent manner. CICP at 400 mg/kg b.wt exhibited stronger antioxidant effect which was comparable to that of vitamin E. A vast number of evidence implicates that aging is associated with a decrease in antioxidant status and age dependent increases in lipid peroxidation [50], being in agreement with our present study. The major antioxidant enzymes, including SOD, GPxand CAT are regarded as the first line of the antioxidant defense system against reactive oxygen species generated *in vivo* during oxidative stress. SOD dismutates superoxide radicals to form hydrogen peroxide, which in turn is decomposed to water and

Table 2. Effect of polysaccharides from *C. indica* on the activities of enzymic antioxidants in brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	CICP (200 mg/kg) + D-Gal	CICP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
SOD	5.94 ± 0.20^{d}	3.63 ± 0.14^{a}	5.12 ± 0.13^{b}	5.77 ± 0.21^{d}	5.42 ± 0.18^{c}
CAT	12.04 ± 0.35^{c}	6.34 ± 0.23^{a}	11.12 ± 0.33^{b}	11.89 ± 0.52^{c}	12.02 ± 0.56^{c}
GPx	3.36 ± 0.10^{c}	1.64 ± 0.05^{a}	2.75 ± 0.07^{b}	3.24 ± 0.12^{c}	3.27 ± 0.14^{c}
GR	5.87 ± 0.21^{d}	3.60 ± 0.16^{a}	4.74 ± 0.21^{b}	5.66 ± 0.19^{cd}	5.43 ± 0.24^{c}
GST	421.17 ± 18.84^{d}	233.36 ± 9.48^{a}	389.45 ± 13.93^{b}	411.01 ± 11.03^{cd}	402.06 ± 17.98^{bc}

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT.

Units: SOD - inhibition of 50% nitrite formation/min/ mg protein; CAT- ìM of H2O2 consumed/min/ mg protein; GPx - ìM of glutathione oxidized/min/ mg protein; GR - µM of glutathione utilized/min/ mg protein; GST - µmole of CDNB-GSH conjugate formed/min/mg protein.

oxygen by GPx and CAT, thereby preventing the formation of hydroxyl radicals [51]. Therefore, these enzymes act cooperatively at different sites in the metabolic pathway of free radicals. Reduction in the activity of GPx in D-galactose induced mice indicates that the glutathione was consumed during the reaction with oxygen and peroxide radicals. Inhibition of this enzyme, which eliminates hydrogen peroxide and lipid peroxide leads to accumulation of these oxidants and thus the subsequent oxidation of lipids [52].

GR is the family of homologous proteins, whose members are dimeric, NADPH dependent and FAD containing enzymes. The increase in GR activity implies that CICP protects the brain from oxidative damage by GSH regenerated from its oxidized form (GSSG). The decrease in GST activity in D-galactose induced mice may favour the excretion of oxidised GSH, thereby maintaining the thiol redox status in tissues. The decreased activity of GST in the present study may be due to the decreased availability of GSH. The increased activity of GST in CICP treated aged mice hastens the detoxification of the lipid peroxides.

GST offers protection against LPO by promoting the conjugation of toxic electrophiles with GSH [53]. GST plays a physiological role in inactivating the detoxification of potential alkylating agents. GST activities were significantly reduced in D-galactose induced mice and were reverted to near normal after treatment with the CICP. The major antioxidant enzymes, including SOD, CAT and GPx are regarded as the ûrst line of the antioxidant defense systemagainst ROS *in vivo* during oxidative damage [54]. Non-enzymatic antioxidants, such as the total antioxidant capacity (T-AOC) and GSH level, also play a signiûcant role in intracellular antioxidant defense in the body [55]. The enhanced activities of SOD, and CAT as well as the increases in T-AOC and GSH levels in the aging mice could be very effective in clearing various types of oxygen free radicals and their products, thus reversing D-gal induced cognitive and motor performances deficits resulted from oxidative damage.

Effect of PFCI on the activities of ATPases in D-gal induced mice

Na^+K^+-ATPase, the main biomolecule for maintaining cation homeostasis, is responsible for generating and maintaining membrane potential necessary for neuronal excitability. Thus disturbance in its activity could have grave consequences for central nervous system (CNS) functioning [56]. Table 3 depicts the changes in the activities of Na^+K^+, Ca^{2+}, Mg^{2+}-ATPase in brain induced by D-gal. Treatment with D-gal showed a decline in Na^+K^+, Ca^{2+}, Mg^{2+} -ATPase activities ($p<0.05$), whereas the effect was significantly restored by CICPand vitamin E. CICP at a dose of 400 mg/kg b.wt exhibited more significant effect ($p<0.05$) than the dose of 200 mg/kg b.wt and vitamin E.

It has been reported that Na^+K^+-ATPase might play a relevant role in mechanism for learning [57], as well as in activity dependent synaptic plasticity, such as long term potentiation (LTP) [58]. As a result of oxidative damage, Na^+K^+-ATPase activity decreased age dependently in mice brain [59]. Our results showed that oxidative damage led to the decline of Na^+K^+-ATPase activity, whereas CICPcould protect Na^+K^+-ATPase.

Table 3. Effect of polysaccharides from *C.indica* on the activities of ATPase in brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	CICP (200 mg/kg) + D-Gal	CICP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
Na^+K^+ATPase	609.52 ± 19.62^c	374.55 ± 12.99^a	555.21 ± 16.88^b	593.02 ± 18.03^c	598.38 ± 27.93^c
Ca^{2+}ATPase	610.08 ± 16.37^c	323.49 ± 7.66^a	450.64 ± 20.15^b	588.10 ± 26.20^c	595.77 ± 15.98^c
Mg^{2+}ATPase	457.07 ± 16.35^d	291.86 ± 11.02^a	356.96 ± 15.96^b	414.84 ± 18.55^c	451.48 ± 13.00^d

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT.
Units: ATPases - nM Pi liberated/min/mg protein.

Effect of CICP on the levels of non-enzymic antioxidants in D-gal induced mice

The level of non enzymatic antioxidants (GSH and vitamin C) in D-gal induced mice decreased significantly ($p<0.05$) in brain as compared with the normal control group (Table 4).Treatment with CICP(200 and 400 mg/kg b.wt) significantly and dose dependently increased the non-enzymic antioxidant levels in brain. Glutathione is a major, non protein thiol in living organisms which performs a key role in coordinating the innate antioxidant defense mechanisms. It is involved in the maintenance of the normal structure and function of cells, probably by its redox and detoxification reactions [60].

Table 4. Effect of polysaccharides from *C.indica* on the levels of non enzymic antioxidants in brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	CICP (200 mg/kg) + D-Gal	CICP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
GSH	5.99 ± 0.16^{d}	2.23 ± 0.10^{a}	4.98 ± 0.20^{b}	5.88 ± 0.18^{cd}	5.68 ± 0.21^{c}
Vitamin C	5.83 ± 0.15^{d}	2.68 ± 0.10^{a}	4.17 ± 0.17^{b}	5.30 ± 0.27^{c}	5.73 ± 0.21^{d}

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT; Units: GSH, Vitamin C - µg/mg protein.

As an endogenous antioxidant, GSH plays a critical role in intracellular antioxidant defense in the brain. GSH scavenges ROS by directly reacting with it. On the other hand, GSH can prevent H_2O_2 induced hydroxyl radical formation. So, GSH level parallels the antioxidant defense capacity in the brain. Vitamin C is capable of maintaining sulfhydryl groups in reduced state in several redox reactions [61]. Remarkable decrease in the level of vitamin C in D-galactoseinduced mice is due to the increased utilization of vitamin C in antioxidant defense against increased reactive oxygen species or due to the decrease in GSH levels, since GSH is required for recycling of vitamin C. Vitamin C, a water soluble antioxidant and also pro oxidant is a potential and one of the most effective scavengers of oxygen free radicals and other derived species. It imparts its protection by undergoing oxidation to dehydroascorbate. For the back conversion to ascorbate, GSH is required. Consequently when GSH is reduced, there is fall in the level of vitamin C [62]. The increased level of vitamin C in aged mice treated with the CICP is due to its antioxidant property.

Effect of CICP on the levels of PCO and AOPPin D-gal induced mice

D-galactose treatment for 6 weeks significantly ($p<0.05$) raised the levels of protein carbonyl (PCO) and advanced protein glycation products (AOPP) in the brain of D-gal model group as compared to control mice (Table 5). CICP (200 and 400 mg/kg b.wt) treatment significantly attenuated the increase in PCO and AOPP levels as compared to control (D-galactose treated) group in a dose dependent manner.

Table 5. Effect of polysaccharides from *C.indica* on the levels of protein carbonyl and AOPP in mitochondrial brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	CICP (200 mg/kg) + D-Gal	CICP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
PCO	1.54 ± 0.03^{b}	2.28 ± 0.09^{e}	1.73 ± 0.04^{d}	1.64 ± 0.07^{c}	1.47 ± 0.05^{a}
AOPP	6.93 ± 0.15^{a}	10.69 ± 0.28^{d}	8.25 ± 0.21^{c}	7.07 ± 0.20^{a}	7.87 ± 0.23^{b}

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT.
Units: PCO - µM of carbonyl/min/mg protein; AOPP- µM/mg protein.

Effect of CICP on the levels of LPO and HPO in D-gal induced mice

MDA and TBARS levels are indexes of membrane damage and the elevated levels of MDA and TBARS result in reducing membrane fluidity inactivating membrane-bound proteins and changing the structure and function of the membranes [54].

The MDA production, a main index of lipid peroxidation and HPO was significantly increased ($p<0.05$) in brain of D-galactose induced mice when compared with the normal group (Table 6). Mice Treated with CICP(200 and 400 mg/kg b.wt) and vitamin E decreased ($p<0.05$) the MDA level in a dose dependent manner.The result suggested that administration of CICP extract (200 and 400 mg/kg b.wt) and vitamin E resulted in an effective inhibition of lipid peroxidation in D-galactose induced mice.

Table 6. Effect of polysaccharides from *C. indica* on the levels of lipid peroxidation and hydroperoxides in brain of D-gal induced mice

Parameters	Normal control	D-Gal (150 mg/kg)	CICP (200 mg/kg) + D-Gal	CICP (400 mg/kg) + D-Gal	Vit E (100 mg/kg) + D-Gal
LPO	1.50 ± 0.04^{a}	3.11 ± 0.12^{e}	1.98 ± 0.08^{d}	1.78 ± 0.04^{c}	1.62 ± 0.07^{b}
HPO	3.24 ± 0.08^{a}	6.49 ± 0.29^{d}	4.99 ± 0.69^{c}	4.15 ± 0.15^{b}	3.20 ± 0.14^{a}

Values are mean ± SD, n=6. Values within the same row not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT; Units: LPO - nM of MDA formed/min/mg protein; HPO - mM/g tissue.

Lipid peroxidation, a process induced by free radicals, leads to oxidative deterioration of poly unsaturated lipids. Under normal physiological conditions, only low levels of lipid peroxides occur in body tissues. The excessive generation of free radicals leads to peroxidative changes that ultimately result in enhanced lipid peroxidation [63]. MDA, a secondary product of lipid peroxidation, is used as an indicator of tissue damage [64]. Aging has been reported to be associated with increased disruption of membrane lipids leading to subsequent formation of peroxide radicals. In the present investigation, such a disruption of membrane lipids possibly accounted for the observed increase in MDA levels in the organs of D-galactose induced mice when compared to normal mice. In addition, insufficient levels of antioxidants to scavenge peroxy radicals during aging [65] could also have contributed to the elevated level of MDA in the D-galactose induced mice. Antioxidants are necessary for preventing the formation of free radicals and they inhibit some of the deleterious actions of reactive oxygen species on lipids, DNA and proteins.

CONCLUSION

The present results reveal that the administration of CICP in D-galactose induced rats helps to significantly elevate the activity of antioxidant enzymes, reduce the levels of lipid peroxidation products, and keep the balance of oxidative and antioxidative systems during severe oxidative stress in rats. Therefore, CICP should be further explored as a functional antioxidant food for the prevention of ageing-related diseases. The possible mechanism of the protective effect of CICP against oxidative stress in D-gal-induced ageing rats may be through its strong antioxidant activity. Nevertheless, our results need to be confirmed by more comprehensive and detailed human trials to determine whether these effects might be extrapolated to humans.In conclusion, present study highlights the potential role of CICP against D-galactose induced cognitive impairment, biochemical and mitochondrial dysfunction in mice.

REFERENCES

[1] Zhang M *et al.* (2007). Antitumor polysaccharides from mushrooms: A review on their isolation process, structural characteristics and antitumor activity. *Trends Food Sci. Tech.* 18: 4-19.

[2] Reiter RJ. (1995). Oxidative processes and antioxidative defense mechanisms in the aging brain. *FASEB J.* 9: 526-536.

[3] Bowling AC and Beal MF. (1995). Bioenergetic and oxidative stress in neurodegenerative diseases.*Life Sci*. 56: 1151-1171.

[4] Perry G *et al*. (2000). Oxidative damage in Alzheimer's disease: the metabolic dimension. *Int. J. Dev. Neurosci*. 18: 417-421.

[5] Haripriya D *et al*. (2005). Modulation of age-associated oxidative DNA damage in rat brain cerebral cortex, striatum and hippocampus by L-carnitine. *Exp Gerontol*. 40: 129-135.

[6] Sohal RS. (2002). Role of oxidative stress and protein oxidation in the aging process. *Free RadicBiol Med*. 33: 37-44.

[7] Turrens JF. (2003). Mitochondrial formation of reactive oxygen species. *J. Physiol*. 552: 335-344.

[8] Reddy PH. (2007). Mitochondrial dysfunction in aging and Alzheimer's disease: strategies to protect neurons. *Antioxid.Redox Signal*. 9: 1647-1658.

[9] Reddy PH and Beal MF. (2005). Are mitochondria critical in the pathogenesis of Alzheimer's disease? *Brain Res.Rev.* 49: 618-632.

[10] Zeevalk GD. (2005). Mitochondrial inhibition and oxidative stress: reciprocating players in neurodegeneration. *Antioxid.Redox Signal*. 7: 1117-1139.

[11] Kaplan LA and PesceAJ. (1996). Clinical Chemistry.Third edition, Mosby, Saint Louis, pp 954-955.

[12] Hsieh H *et al*. (2009). Soy isoflavones attenuate oxidative stress and improve parameters related to aging and alzheimer's disease in mice treated with D-galactose. *Food Chem. Toxicol*. 47: 625-632.

[13] Song X *et al*. (1999). Advanced glycation in D-galactose induced mouse ageing model. *Mech. Ageing Dev.* 108: 239-251.

[14] Baynes WJ. (2001). The role of AGEs in aging: causation or correlation. *Exp. Geront.* 36: 1527-1537.

[15] Xu XH and Zhao TQ. (2002). Effects of puerarin on D-galactose-induced memory deûcit in mice. *Acta Pharmacol. Sin*. 23: 587-590.

[16] Zhang C *et al*. (2004). Protective effect of tetramethylpyrazine on learning and memory function in D-galactoselesioned mice. *J Chin Med Sci* 19: 180-184.

[17] Lu J *et al*. (2007). Ursolic acid ameliorates cognition deûcits and attenuates oxidative damage in the brain of senescent mice induced by D-galactose. *Biochem Pharmacol* 74: 1078-1090.

[18] Zhang Q *et al*. (2005). D-Galactose injured neurogenesis in the hippocampus of adult mice. *Neurol Res*. 27: 552-556.

[19] Li HY *et al*. (2009). Antioxidant activities of extracts and fractions from *Lysimachia foenumgraecum Hance*. *Bioresource Technol*. 100: 970-974.

[20] Qiao DL *et al*.(2009). Antioxidant activities of polysaccharides from *Hyriopsis cumingii*. *Carbohydr. Polym*. 78:199-204.

[21] Sun F *et al*. (2009). Inhibitory effect of osthole on alcohol induced fatty liver in mice. *Dig. Liver Dis*.41: 127-133.

[22] Yuan JF *et al*. (2008). Antioxidant effects and cytotoxicity of three purified polysaccharides from *Ligusticum chuanxiong* Hort. *Carbohydr.Polym*. 74: 822-827.

[23] Cheng JJ *et al*. (2008). Properties and biological functions of polysaccharides and ethanolic extracts isolated from medicinal fungus, *Formitopsis pinicola*. *Process Biochem*. 43: 829-834.

[24] Lu J *et al*. (2007). Ursolic acid ameliorates cognition deficits and attenuates oxidative damage in the brain of senescent mice induced by D-galactose. *Biochem. Pharmacol*. 74: 1078-1090.

[25] Shinomol G and Muralalidara K. (2007). Differential induction of oxidative impairments in brain regions of male mice following subchronic consumption of khesari dhal (*Lathyrus sativus*) and detoxified *Khesari dhal*. *Neurotoxicology*. 28: 798-806.

[26] Ellman GL. (1961). A new and rapid calorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*. 7: 88-95.

[27] King TE and Howard RL. (1967). Preparations and properties of soluble NADH dehydrogenases from cardiac muscle. *Methods Enzymol*. 10: 275-284.

[28] Mehler A *et al*. (1948). The enzymatic mechanism of oxidation reductions between malate or isocitrate and pyruvate. *J. Biol. Chem*. 174: 961-977.

[29] Varley H. (1999). Practical Clinical Biochemistry. Arnold Heinemann Publishers Private Limited, 4, 52.

[30] BenzieI FF and Strain JJ. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant power" The FRAP assay. *Anal.Biochem*. 239: 70-76.

[31] Das S. (2000). Correlation between total antioxidant status and lipid peroxidation in hypercholesterolemia. *Curr. Sci*. 78: 486-487.

[32] Sinha AK. (1972). Colorimetric assay of catalase. *Anal.Biochem.* 47: 389-394.

[33] Ellman GC. (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82: 70-77.

[34] Beutler E. (1984). Glutathione in red blood cell metabolism. A manual of biochemical methods.Grune and Stratton, New York, pp. 112-114.

[35] Habig WH. (1973). Glutathione-S-transferase: The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249: 7130-7139.

[36] Bonting SL. (1970). Presence of enzyme system in mammalian tissues.Membranes and Ion Transport (Wiley-Interscience, London), pp. 25-28.

[37] Hsertan S and Pan H. (1983). Purification and characterization of two forms of a low affinity calcium ion ATPase from erythrocyte membrane. *Biochim. Biophys. Acta.* 755: 457-466.

[38] Ohnish T *et al.* (1982). Comparative study plasma membrane magnesium ion ATPase activity normal regenerating and malignant cells. *Biochim.Biophys. Acta*. 684: 67-74.

[39] Moron MS *et al.* (1979). Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat lung and liver. *Biochim. Biophys. Acta.* 582: 67-68.

[40] Omaye ST *et al.* (1979). Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. *Methods Enzymol.* 62: 1-11.

[41] Levine RL *et al.* (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* 186: 464-468.

[42] Kayali R *et al.* (2007). Male rats exhibit higher oxidative protein damage than females of the same chronological age. *Mech. Ageing Dev*. 128: 365-369.

[43] NiehiusWG and Samuelsson D. (1968).Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation. *Eur. J. Biochem.* 6: 126-130.

[44] Jiang ZY *et al.* (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal. Biochem.* 202, 384-389.

[45] Lowry OH *et al.* (1957). Protein measurement with the Folin's phenol reagent. *J. Biol. Chem.* 193: 265-275.

[46] Brandeis R *et al.* (1989). The use of the Morris water maze in the study of memory and learning. *Intl. J. Neurosci.* 48: 29-69.

[47] Muthuraju S. (2009). Acetylcholine esterase inhibitors enhance cognitive functions in rats following hypobaric hypoxia. *Behav. Brain Res.* 202: 1-4.

[48] Chen CF. (2006). Effects of D-galactose on the expression of hippocampal peripheral type benzo receptor and spatial memory performances in rats. *Psychoneuroendo.* 31: 805-811.

[49] Choi Y *et al.* (2007). Antioxidant activity of methanolic extracts from some grains consumed in Korea. *Food Chem*. 103: 130-138.

[50] Alvarado CA *et al.* (2006). Oxidative stress in leukocytes from young prematurely aging mice is reversed by supplementation with biscuits rich in antioxidants. *Dev. Comp. Immunol.* 30: 1168-1180.

[51] Yao CA *et al.* (2005). Chemosensory coding by neurons in the *Coeloconic Sensilla* of the *Drosophila Antenna. J. Neurosci.* 25: 8359-8367.

[52] Yoshikawa T *et al.* (1997). Free radicals and disease. In; Hiramatsu, M., Yoshikawa T, Inoue M. (Eds.), Food and Free Radicals, pp. 11-19.

[53] Jakoby WB. (1988). Detoxification, conjugation and hydrolysis. In: Liver Biology and Pathology. Arias, I.M., Jakoby, W.B., (Eds.), Raven Press, New York, pp. 375-385.

[54] Li XM *et al.* (2007). Effect of the *Lycium barbarum* polysaccharides on age related oxidative stress in aged mice. *J. Ethnopharmacol.* 111: 504-511.

[55] Zhang QB *et al.* (2003). *In vivo* antioxidant activity of polysaccharide fraction from *Porphyra haitanesis* (Rhodephyta) in aging mice. *Pharmacol. Res.* 48: 151-155.

[56] Mobasheri A *et al.* (2000). Na^+K^+-ATPase isozyme diversity; comparative biochemistry and physiological implications of novel functional interactions. *Biosci. Rep.* 20: 51-91.

[57] Sato S *et al.* (2000). Modulation of akt kinase activity by binding to hsp90. Proceedings of the National Academy of Sciences of the United States of America, 97: 10832-10837.

[58] Glushchenko TS and Izvarina NL. (1997). Na $^{+}K^{+}$ ATPase activity in neurons and glial cells of the olfactory cortex of the rat brain during the development of long term potentiation. *Neurosci. Behav. Physiol*. 27: 49-52.

[59] Gorini A *et al.* (2002). ATPases enzyme activities during ageing in different types of somatic and synaptic plasma membranes from rat frontal cerebral cortex. *Prog.Neuropsychopharmacol. Biol. Psychiatry*. 26: 81-90.

[60] Gueeri H. (1995). Influence on prolonged ethanol intake on the level and turnover of alcohol and aldehyde dehydrogenase and glutathione. *Adv. Exp. Med. Biol*. 23: 133-134.

[61] Frei B *et al.* (1989). Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci*. 86: 6377-6384.

[62] Allen RG. (1996). Oxygen reactive species and antioxidant responses during developments. The metabolic paradox by cellular differentiation. *Proc. Soc. Exp. Biol. Med*. 196, 117-129.

[63] Rikans LE and Hornbrook KR. (1997). Lipid peroxidation, antioxidant protection and aging. *Biochim. Biophys. Acta*. 1362: 116-127.

[64] Okhawa H *et al.* (1979). Assay of lipid peroxides in animal tissue by thiobarbituric acid reaction. *Anal. Biochem.* 95: 351-358.

[65] Sohal RS. (2002). Role of oxidative stress and protein oxidation in the aging process. *Free Radical Biol. Med.* 33: 37-44.

EFFECT OF CULINARY MEDICINAL MUSHROOMS, *PLEUROTUS OSTREATUS* AND *P. CYSTIDIOSUS* ON FASTING AND POSTPRANDIAL GLYCAEMIA IN HEALTHY VOLUNTEERS

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ABSTRACT

Pleurotus ostreatus (Jacq.Fr.) P. Kumm. and *P. cystidiosus* OK Miller are culinary-medicinal mushrooms grown worldwide. Our previous studies have reported the promising oral hypoglycaemic potential of *P. ostreatus* and *P. cystidiosus* in both normal and alloxan-induced diabetic rats as well as the anti-inflammatory activity of *P. ostreatus*.This study evaluates the effect of suspensions of freeze dried and powdered *P. ostreatus* and *P. cystidiosus* on the fasting and postprandial serum glucose levels in healthy volunteers at a dose of 50 mg/kg body weight, followed by a glucose load. Safety of the both mushrooms after long term consumption by healthy volunteers was also investigated. There was a significant fasting and postprandial serum glucose reduction in *P. ostreatus* or *P. cystidiosus* groups when compared with respective control groups ($p<0.05$). The percentage reduction in the fasting serum glucose levels for *P. ostreatus* and *P. cystidiosus* groups were 6.1% and 6.4% respectively and the postprandial serum glucose reductions were 16.4 % and 12.1 %. There were no significant differences in serum levels of liver enzymes, creatinine as well as estimated creatinine clearance before and after one month from the treatment. This indicated that the consumption of *P. ostreatus* and *P. cystidiosus* over a period of time did not cause any significant hepato cellular damage and detrimental effects in the renal system of the healthy volunteers. In conclusion, long-term consumption of *P. ostreatus* and *P. cystidiosus* appears to be effective as functional foods for glycaemic control.

Keywords: *Pleurotus ostreatus*, *Pleurotus cystidiosus*, hypoglycaemic, functional foods, glycaemic

INTRODUCTION

Diabetes mellitus is a chronic endocrine disorder characterized by hyperglycaemia, resulting from deficiency in insulin secretion, inaction of insulin or both [1]. The global prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030 [2]. In 2006, one in five adults in Sri Lanka has either diabetes or pre-diabetes while one-third of those with diabetes are undiagnosed [3]. Many different cultures of mushrooms have been used traditionally in the prevention and treatment of various diseases [4]. Recent studies have investigated and proven the traditional use of mushrooms as remarkable therapeutic agents. The main areas of medicinal studies include hepatoprotective, anti-inflammatory, anti-diabetic, anticancer and antimicrobial activity [5].

Two oyster mushrooms, *Pleurotus ostreatus* (Jacq.:Fr.) P. Kumm. and *P. cystidiosus* O.K. Miller (Pleurotaceae, higher Basidiomycetes) are culinary-medicinal mushrooms. These mushrooms are commonly known as American oyster and abalone respectively, were shown to possess antioxidant, antitumour, antinociceptive, antifungal, hypocholesterolaemic and hepatoprotective activity [6]. In our previous studies,we have demonstrated the anti-inflammatory activity of *P. ostreatus* [7] and the oral hypoglycaemic potential of *P. ostreatus* and *P. cystidiosus* [8] using animal models.

There is a lack of clinical evidence on *Pleurotus* mushrooms as an anti-diabetic agent despite the promising hypoglycaemic potential exhibited in animal models [9]. Since, promising acute and chronic oral hypoglycaemic potential of *P. ostreatus* and *P. cystidiosus* has already been established in both normal and alloxan-induced diabetic Wistar rats in our previous studies [8], there is a need to establish the effect in humans.

Hence, this study was designed to evaluate the effect of *P. ostreatus* and *P. cystidiosus* on fasting and postprandial glycaemia in healthy human volunteers. Functional foods based on medicinal mushrooms which are also called 'mushroom nutraceuticals' are becoming popularized [10]. Therefore, this study will also evaluate the safety of long term consumption of the two mushrooms.

MATERIALS AND METHODS

Place of study

This study was conducted at the Family Practice Centre and Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka.

Ethical clearance

Ethical clearance (No. 599/11) was obtained from the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka. Written informed consent was obtained from healthy volunteers.

Collection and preparation of mushrooms

Fresh mushrooms, *P. ostreatus* and *P. cystidiosus* were collected from a local farm. These were washed with water to remove soil particles and freeze-dried (Eyela, FD-5N, Japan) and ground with a commercial blender (Sonica, SA-317, China). Powdered samples of mushrooms were stored air-tight at 4 °C. The identification and authentication was done by studying the spore print and the shape of the cap and the stipe. Effect of long term administration of suspensions of freeze dried and powdered *P. ostreatus* and *P. cystidiosus* on fasting and postprandial serum glucose levels in healthy volunteers was studied.

A group of healthy volunteers were selected by an open advertisement (n=44) and fasting serum glucose levels were estimated using the glucose oxidase reagent kits (Biolabo reagents, France), following an overnight fast. All subjects received distilled water as the control at zero time. Thirty minutes later, 75 g of glucose in 300 ml of water was administered for two weeks. Two hours after the glucose load, postprandial serum glucose levels were measured. The subjects were divided into two groups and allocated to the two types of mushroom preparations *P. ostreatus* and *P. cystidiosus*. The two groups received the suspensions of freeze dried and powdered mushrooms at a dose of 50 mg/kg body weight daily for two weeks. After two weeks, the fasting serum glucose levels were assessed. Followed by this two groups received the respective mushroom suspension and glucose. Postprandial serum glucose levels were measured after two hours from the glucose load.

Effect of long term administration of suspensions of freeze dried and powdered *P. ostreatus* and *P. cystidiosus* on serum levels of key hepatic enzymes (ALT, AST, ALP and γ-GT), creatinine and creatinine clearance of healthy volunteers was studied. Base line values of alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), gamma glutamyltransferase (γ GT) and creatinine levels of healthy volunteers were measured. Creatinine clearance was calculated using Cockroft- Gault equation [1].Serum levels of ALT, AST, γ-GT and creatinine were measured by using the reagent kits (Biolabo reagents, France) and serum ALP levels were measured by using the reagent kits from Stanbio Laboratory, Texas.

The subjects were divided into two groups as *P. ostreatus* and *P. cystidiosus* and received the respective mushroom at a dose of 50 mg/kg body weight daily for two weeks. The subjects were monitored for one month for any adverse effects and at the end of one month serum levels of ALT, AST, ALP, γ GT and creatinine were determined. Creatinine clearance was calculated.

Statistical analysis

Statistical analysis was done using SPSS 17. 'Student's *t* test was used to analyse the results for statistical significance. Final results were presented as mean±SEM. p values <0.05 were considered as significant.

RESULTS AND DISCUSSION

Effect of long term administration of suspensions of freeze dried and powdered *P. ostreatus* and *P. cystidiosus* on fasting and postprandial serum glucose levels in healthy volunteers

This study evaluated the oral hypoglycaemic activity of *P. ostreatus* and *P. cystidiosus* on healthy human volunteers. Fig. 1 and Fig. 2 shows the fasting and postprandial serum glucose levels of healthy volunteers at the two groups of *P. ostreatus* and *P. cystidiosus*. The fasting serum glucose levels of *P. ostreatus* group was reduced by 6.1% and that of *P. cystidiosus* group by 6.4%. The percentage reduction in the postprandial serum glucose levels of *P. ostreatus* group was 16.4% and the group *P. cystidiosus* shows a reduction by 12.1%.

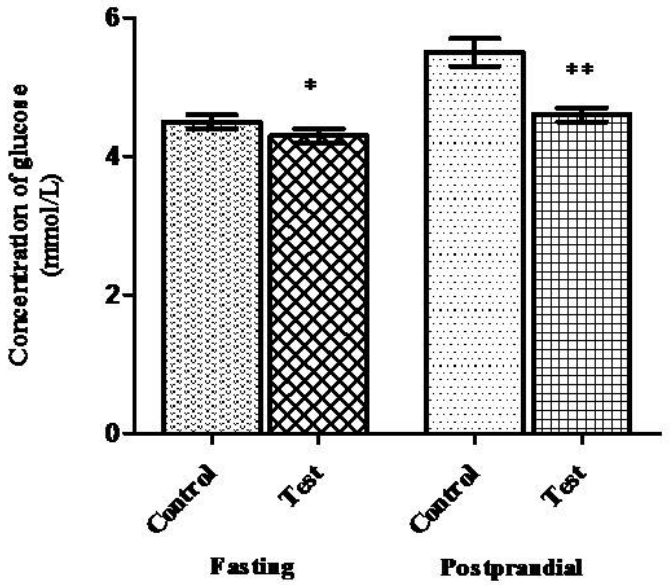

Figure 1. Effect of long term administration of suspensions of freeze dried and powdered *P. ostreatus* on fasting and postprandial serum glucose levels of healthy volunteers

Values are expressed as mean±SEM. $^{*}p < 0.05$ and $^{**}p < 0.001$ when compared with the control group.

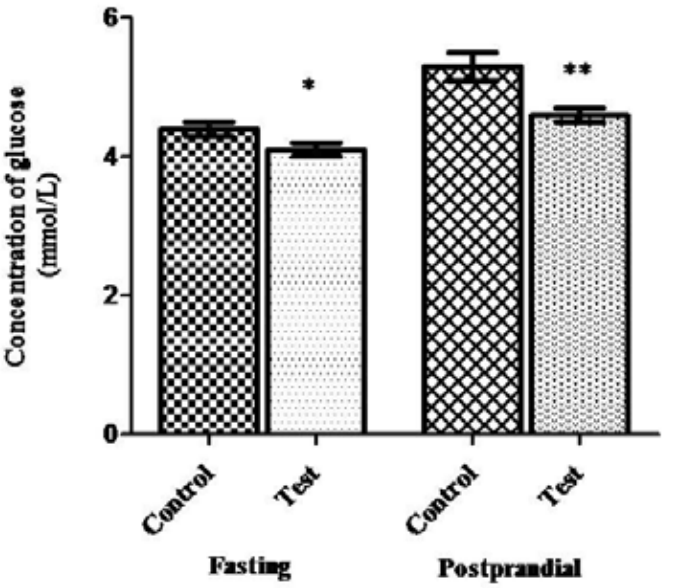

Figure 2. Effect of long term administration of suspensions of freeze dried and powdered *P. cystidiosus* on fasting and postprandial serum glucose levels of healthy volunteers

Values are expressed as mean±SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with the control group.

When consumed for 2 weeks, both fasting and postprandial serum glucose levels were decreased significantly in healthy volunteers. Hence, this study suggest that long-term consumption of *P. ostreatus* and *P. cystidiosus* may be beneficial to humans by improving the glycaemic status. It has been reported that *P. sajor-caju* significantly reduced blood glucose level in diabetic subjects [11]. Effect of long term administration of suspensions of freeze dried and powdered *P. ostreatus* and *P. cystidiosus* on serum levels of key hepatic enzymes (ALT, AST, ALP and γ-GT), creatinine and creatinine clearance of healthy volunteers.

The serum levels of key hepatic enzymes and creatinine as well as calculated creatinine clearance at base line and after one month from the treatment was represented at Table 1. There were no significant differences in serum levels of ALT, AST, ALP, γ-GT and creatinine as well as calculated creatinine clearance at baseline and after one month from the treatment (p>0.05). Hence, the consumption of *P. ostreatus* and *P. cystidiosus* over a period of time did not cause any hepato-renal damage in the healthy volunteers. According to our previous findings, the *P. ostreatus* and *P. cystidiosus* did not exert any toxic effects after long term administration to rats [12]. This study confirms the safety of long term consumption of *P. ostreatus* and *P. cystidiosus* by humans.

Table 1. Effect of long term administration of suspensions of freeze dried and powdered *P. ostreatus* and *P. cystidiosus* on serum levels of key hepatic enzymes (ALT, AST, ALP and γ-GT), creatinine and creatinine c learance of healthy volunteers

	Treatment (50 mg / kg/b.w.)	**ALT (IU/l)**	**AST (IU/l)**	**ALP (U/l)**	**γ-GT (IU/l)**	**Creatinine (mg/dl)**	**Creatinine clearance (mL/min)**
P. ostreatus	Base line	24.8±2.9	25.5±2.9	30.7±1.5	10.9±1.2	0.85±0.04	103.2±9.3
	After one month from the treatment	25.3±2.8	28.5±3.9	28.5±2.2	10.6±1.5	0.89±0.04	94.0±5.8
P. cystidiosus	Base line	23.8±2.8	29.8±2.6	32.1±2.4	15.8±2.5	0.84±0.06	117.6±14.1
	After one month from the treatment	24.2±2.6	29.5±2.2	33.2±2.9	15.6±2.1	0.89±0.05	104.2±9.6

Values are expressed as mean±SEM.

No statistically significant differences were observed between the base line and after one month values of tested parameters in each group.

CONCLUSION

The *P. ostreatus* and *P. cystidiosus* exerted significant hypoglycaemic effect in healthy volunteers challenged with glucose. The mushrooms are neither hepatotoxic nor nephrotoxic. Hence, this study confirms the suitability of *P. ostreatus* and *P. cystidiosus* as a hypoglycemic functional food for glycaemic control.

ACKNOWLEDGMENTS

The authors acknowledge the staff of the Family Practice Centre and Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka for providing the necessary facilities to carry out the study. Financial assistance by the University of Sri Jayewardenepura, Sri Lanka is gratefully acknowledged (Grant No: WCUP/PhD/04/2012).

REFERENCES

[1] Kumar P and Clark M. (2012). Clinical Medicine. A Textbook for Medical Students and Doctors, S. W. Saunders Company Ltd., London, United Kingdom, pp. 49-1045, ISSN.

[2] Wild S *et al.* (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*. 27(5): 1047-1053.

[3] Katulanda P *et al.* (2008). Prevalence and projections of diabetes and pre-diabetes in adults in Sri Lanka—Sri Lanka Diabetes, Cardiovascular Study (SLDCS). *Diabet Med*. 25(9): 1062-1069.

[4] Chang ST and Wasser SP. (2012).The Role of Culinary-Medicinal Mushrooms on Human Welfare with a Pyramid Model for Human Health. *Int. J. Med. Mushr.* 14(2): 95-134.

[5] Rai M *et al*. (2005). Therapeutic potential of mushrooms. *Nat. prod. Radiance*. 4(4): 246-257.

[6] Abeytunga DTU. (2011). Biological activities of *Pleurotus* mushrooms. In: Mushrooms: Types, properties and Nutrition, 329-350.

[7] Jayasuriya WJABN *et al.* (2012). Anti-inflammatory activity of *Pleurotus ostreatus* in Wistar rats. In: *Mushroom Science XVIII*.Eds. 118.

[8] Jayasuriya WJABN *et al*. (2012). Oral hypoglycaemic activity of culinary- medicinal mushrooms *Pleurotus ostreatus* and *P. cystidiosus* (Higher Basidiomycetes) in normal and alloxan-induced diabetic Wistar rats. *Int. J. Med. Mushr*. 14(4): 347-355.

[9] Deepalakshmi K and Mirunalini S. (2014). *Pleurotus ostreatus*: an oyster mushroom with nutritional and medicinal properties. *J. Biochem Tech.* 5(2): 718-726.

[10] Wasser SP and Didukh MY. (2004). Dietary Supplements from Culinary-Medicinal Mushrooms: A Variety of Regulations and Safety Concerns for the 21st Century. *Int. J. Med Mushr*. 6(3): 231-248.

[11] Agrawal RP *et al*. (2010). Effect of oyster mushroom on glycemia, lipid profile and quality of life in Type 2 diabetic patients. *Australian Journal of Medical Herbalism*. 22(2): 50-54.

[12] Jayasuriya WJABN *et al*. (2010). Safety and oral hypoglycaemic properties of *Pleurotus cystidiosus* in Wistar rats. In: Proceedings of the Annual Scientific Sessions, Faculty of Medical Sciences, University of Sri Jayewardenepura.

PROSPECTIVE ASPECTS OF MYCO-CHROME AS PROMISING FUTURE TEXTILES

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ABSTRACT

Natural dyes occupy significant position in various industries like food, pharmaceutical, and paper, etc. Natural dyes from plants and microbes are emerging as a new trend in current tech-savvy world due to the increasing awareness among the upcoming generation about synthetic colorants and its harmful effects. Apart from plants, natural dyes, pigments or colorants can also be extracted from insects, algae, bacteria, fungi and yeasts. Natural colorants are extracted using water or solvents, characterised and formulated as textile dyes, pigments or colorants. Application of microbial dyes, pigments or colorants for textile industry poses various challenges due to their low stability with varying pH and temperature; hence it demands for an intense investigation on microbial dyes so as to establish an eco-friendly textile in India and to extrude a novel dye for textile dyeing.

Keywords: fungi, mushrooms, dyes and pigments, textiles dyeing, industrial application

INTRODUCTI ON

Art and colour has been important since the very beginning of human existence. The natural pigment was the colour of the first art in cave paintings. The four basic pigments of our early heritage were yellow, red ochre, black and white [1]. Nature-identical colors are man-made pigments which are also found in nature. Examples are carotene, canthaxanthin and riboflavin. Synthetic colors are man-made colors which are not found in nature, these are often azo-dyes. Examples of inorganic colors are titanium dioxide, gold and silver. Although structurally very diversified and from a variety of sources, natural colorants can be grouped into a few classes, the three most important of which are: tetrapyrrols, tetraterpenoids and flavonoids. The terms "pigment" and "dye" are often used interchangeably. Strictly speaking, a pigment is insoluble in the given medium, whereas a dye is soluble. Thus, carotenoids are dyes in oil but pigments in water. This distinction may be difficult to maintain if nothing is assumed about the medium and in the following the term "pigment" will be used for colored substances in general [2].

Natural dyes, dye stuff and dyeing are as old as textiles themselves. Man has always been interested in colours; the art of dyeing has a long past and many of the dyes go back into prehistory. It was practised during the Bronze Age in Europe. The earliest written record of the use of natural dyes was found in China dated 2600 BC. Dyeing was known as early as in the Indus Valley period (2500 BC); this knowledge has been substantiated by findings of coloured garments of cloth and traces of madder dye in the ruins of the Indus Valley Civilization at Mohenjodaro and Harappa (3500 BC).

Synthetic dyes and environmental pollution

After the accidental synthesis of mauveine by Perkin in Germany in 1856 and its subsequent commercialization, coal-tar dyes began to compete with natural dyes. Synthetic colours were found technically more suitable than natural colours and became popular because for their fastness, available in a wide range of colours, low cost even at high concentration in low volumes [3]. The advent of synthetic dyes caused rapid decline in the use of natural dyes, which were completely replaced by the former within a century [4].

The Textile World News proclaims about the textile wastes, "Textile ranks sixth in toxic wastes" [5]. During the textile dyeing process up to 40% of dyes may remain unfixed to the fiber and contaminates the industrial wastewater. They are very stable and difficult to degrade. These dyes are resistant to microbial attack and are hardly removed from effluents by

conventional biological, physical or chemical treatments [6-7]. World effluent release ranges between 5,000 and 20,000 tons per year and causes a serious pollution as well as a non - negligible risk of toxicity against living organisms. Indeed, a survey of oral acute toxicity of 4,461 dyes, evaluated by the lethal dose for 50% of treated rats, has revealed that azo and cationic dyes are the most toxic and carcinogenic to human [8-10].

Classification of natural dyes

The natural dyes are classified based on their chemical structure, sources, method of application, colour, etc. They are classified into the following groups based on chemical structure like Indigoids, Anthraquinones, Alpha- naphthoquinones, Flavones, Dihydropyrans, Anthocyanidins and Carotenoids [11].

WHY WE NEED RESEARCH ON NATURAL DYES?

Pigments from natural sources have been obtained since long time ago, and their interest has increased due to the toxicity problems caused by those of synthetic origin. In this way the pigments from microbial sources are a good alternative.

NATURAL DYES FROM MICROBES

Many microorganisms, including algae, fungi and bacteria, also produce pigments. These pigments play a protective role, for example to absorb UV radiation or to quench oxygen free radicals. Other pigments have been suggested to have antibiotic properties. Several aerobic, gram-positive, pigmented, subsurface isolates exhibited greater resistance to UV light than the non pigmented strains [12]**.** Indigo is considered to be the oldest dye [13]. It was generally extracted from various species of plants initially. However, by the end of nineteenth century the commercial synthetic indigo almost completely replaced indigo production from the natural source. More recently, research work has been undertaken to find a way to replace the chemical synthesis of indigo by using bacterial systems [14].

Dyes and pigments from Bacteria

Bacteria are a good source of pigments which are mostly carotenoids, especially β-carotene in nature. *Streptomyces chrestomyceticus* subsp. *rubescens* has been employed to producce lycopene while zeaxanthin and lutein production from *Flavobacterium* sp. is gaining importance. Several studies have been reported from bacterial pigments.

Dyes and pigments from Fungi

Many species of fungi have attracted special attention because they have the capability of producing different coloured pigments showing high chemical stability [15]. Yellow pigments epurpurins (A – C) were isolated from *Emercicella purpurea* and azophilone derivatives, falconensins (A – H) and falcononsones (A1 and B2) were produced both by *E. falconensis* and *E. fruticulosa* [16].

Red and / or yellow pigments are efficiently produced by several strains of *Monascus* species which are commercially important [17-19]. Angkak or red fermented rice is produced from the fermentation of rice substrates by *Monascus* sp. is ground and used as a colorant. Pigments, produced from immobilized cultures of *Monascus*, are a mixture of six major related pigments (red, yellow and purple pigmented polyketides) and insoluble in acid. Anthraquinone pigments like catenarin, chrysophanol, cynodontin, helminthosporin, tritisporin and crythroglaucin are produced by microfungi *Eurotium* spp., *Fusarium* spp., *Curvularia lunata, Drechlera* spp., *Trichoderma* and *Aspergillus* strains [20].

Various workers have reported pigments from fungal isolates such as *Acrostalagmus* sp., *Alternaria alternata, Alternaria* sp., *Aspergillus niger*, *Bisporomyces* sp., *Cunninghamella*, *Penicillium chrysogenum, Penicillium italicum, Penicillium oxalicum*, *Penicillium regulosum*, *Phymatotrichum* sp [21].

Dyes and pigments from Mushrooms

A mushroom is the fleshy, spore-bearing fruiting body of a fungus and can be used for extraction of dyes and used for colouring purpose. The shingled hedgehog mushroom (*Sarcodon imbricatus*) and related species contain blue-green

Table 1: Mushrooms and colour shades obtained

Mushrooms	Color shades
Chanterelles	Mute Yellow
Oyster	Gray
Agaricus	Yellow-tan to gray green
Blewit	Grass Green
Maitake	Soft Yellow
Chicken of the Woods	Orange
Phaeolus	Orange, Yellow, Green, Red
Lepiota americana	pink to lavender

Dyeing and Paper-Making with Mushrooms Tradd Cotter Mushroom Mountain, LLC

pigments, which are used for dyeing wool in Norway [22]. The fruiting body of *Hydnellum peckii* can be used to produce a beige color when no mordant is used, and shades of blue or green depending on the mordant added [23]. *Phaeolus schweinitzii* (Dyer's Polypore) produces green, yellow, gold, or brown colors, depending on the material dyed and the mordant used (Book-Dyeing with Mushrooms) (Table 1). Extraction of anthroquinone dyes from a Polyporaceae member *Dermocybe sanguineus* showed potential dyeing properties on different fabric materials [24].

Laetiporus sulphureus (Bull.: Fr.) Murr. (Polyporales, Fungi) is a wood rotting basidiomycete growing on several tree species and producing shelf-shaped fruit bodies of pink-orange color, except for the fleshy margin, which is bright yellow. *Laetiporus* species contain a number of lanostane triterpenoids and other metabolites. Thus makes a great industrial application in food, textiles industry [25].

OUR EXPERIENCE WITH MYCOCHROME

Macro fungi (Mushroom) dyes (Myco-chrome) for future textiles

The work was initiated with isolation, followed by production of dyes from mycelial culture, simple and cost effective cultivation with successful fruiting body production, and extraction of dyes. It was followed by application of the mushroom dyes with cotton and silk yarns and fabrics. Pilot scale cultivation was successfully carried out and dyeing experiments were carried out at an industry for testing its suitability as natural dye.

An orange pigment from the fungi *Ganoderma applanatum*, *Coriolus versicolor* and *Amanita muscaria* was extracted from the basidiocarp and applied on the silk and cotton fabrics. The fungal pigment with different mordant like alum, copper, chromium, iron and tin developed color variation from orange to yellow and deep green shades in cotton and silk fabrics. Further, the dyed cotton and silk fabrics did not change the color in soap washing and sunlight drying [26]. The cost effective method of cultivation of *Ganoderma lucidum* and application of its dyes to cotton and silk yarns were done and the dyed yarns showed good fastness tests [27]. Cost effective method of cultivation of *Pycnoporus sanguineus* and application of its dyes to cotton and silk yarns and fabrics were done and the tested up to industrial level [28]. Based on the positive outputs from the above research, production was scaled up and industrial level of testing was carried out. Industrial dyeing was carried out at the site of the industry and natural dyeing trial was attempted in meters and the cotton fabric premordanted with various treatments and dyed with various mushroom dyes recorded fair to good wash fastness [29-30].

Microfungi dyes (Myco-chrome) for future textiles

Pigment from *Sclerotinia* sp. (pink) was exploited for textile dyes [31]. Pigments from selected microfungi (*Curvularia* sp. (green), *Alternaria* sp. (brown), *Phoma* sp. (pink), *Pestalopsis* sp. (purple) were exploited for textile dyes [32]. Production of dyes from *Pencillin, Phoma and Curvularia* was tried in optimized medium and continuous production was achieved. Further the production of pigments from selected microfungi is under progress. Simple extraction of dyes was carried out and the dyes were tested for their dyeability in cotton fabrics and developed variety of shades at an industry. The efficacy of the fabric (wash fastness) was tested to find out its suitability [33]. The promising outputs encouraged us to scale up towards precommercialization of microbial dyes to the textile industries.

ACKNOWLEDGEMENTS

The authors thank Department of Biotechnology and Department of Science and Technology for their financial support and Shri A.M.M. Murugappa Chettiar Research Centre, Taramani, Chennai for providing laboratory facilities.

REFERENCES

[1] Kremer G and Chemiker D. (2004). The History of Pigments Production- Use Possibilities.www.kremer-pigmente.com

[2] Ali Aberoumand. (2011). A review article on edible pigments properties and sources as natural biocolorants in foodstuff and food industry. *World J. of Dairy & Food Sci*. 6 (1): 71-78.

[3] Pattnaik P. *et al.* (1997). Biocolours: New generation additives for food. *Indian Food Industry*. 16: 21-32.

[4] Singh RV. (2001). Colouring plants – An innovative media to spread the message of conservation. *Down to Earth* 20 September, 25–27.

[5] Sastri AN and Miller DC. (1981). Biological monitoring of marine pollutants. *Academic press, London.* Vernberg FJ, Calabrese A Thurberg FP and Vernberg WB Eds.

[6] Trovaslet M *et al*. (2007). Potential of a *Pycnoporus sanguineus* laccase in bioremediation of waste water and kinetic activation in the presence of an anthraquinonic acid dye. *Enzyme Microb. Technol.* 41: 368 - 376.

[7] Vanhulle S *et al*. (2007). Overlap of laccases/cellobiose dehydrogenase activities during the decolorization of anthraquinonic dyes with close chemical structures by *Pycnoporus* strains. *Enzyme and Microbial Technol.* 40: 1723- 731.

[8] Clarke EA and Anliker R. (1980). Organic dyes and pigments. In: *The handbook of environmental chemistry.* Part A: Anthropogenic compounds. Hutzinger O. Ed. Springer Verlag, 3: 181 – 215.

[9] Coulson J. (1980). Naturally occurring colorants for food. In *'Developments in food colorants – I'* John Walford Ed. Applied science publication, England, pp. 121.

[10] Osman MY. (2005). Synthetic organic food colouring agents and their degraded products: Effects on human and rat cholinesterases. *Br. J. Biomed. Sci.* 61: 128-132.

[11] Vankar PS. (2000). Chemistry of natural dyes. *Resonance*. 5: 73-80.

[12] Arrage AA *et al.* (1993). Survival of Subsurface Microorganisms Exposed to UV Radiation and Hydrogen Peroxide. *Appl. Environ. Microbiol*. 59(11): 3545-3550.

[13] Ensley BD. (1983). Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. *Science*. 222: 167-169.

[14] Royoa JL *et al*. (2005). Stable long-term indigo production by overexpression of dioxygenase genes using a chromosomal integrated cascade expression circuit. *J. Biotechnol*. 116: 113-124.

[15] Hajjaj H *et al.* (2000). Kinetic analysis of red pigment and citrinin production by *Monascus ruber* as a function of organic acid accumulation, *Enzyme. Microb. Technol.* 27: 619-625.

[16] Mapari SAS *et al.* (2005). Exploring Fungal Biodiversity for the Production of Water-Soluble Pigments as Potential Natural Food Colourants. *Curr Opin in Biotechnol.* 16(2): 231-238.

[17] Fabre CE *et al*. (1993). Production and food application of the red pigment of *Monascus ruber. J. Food. Sci.* 58: 1099-1103.

[18] Hamdi M *et al.* (1995). A new process for red pigment production by *Monascus purpureus. Bioprocess Eng.* 17: 75-79.

[19] Blanc PJ. (1998). Les pigments rouges de *Monascus*. *Biofutur*. 183: 34-36.

[20] Durán *et al.* (2002). Ecological-friendly pigments from fungi. *Crit. Rev. Food Sci. Nutri.* 42: 53-66.

[21] Atalla *et al.* (2011). Production of textile reddish brown dyes by fungi. *Malaysian J. of Microbiol.* 7(1): 33-40

[22] Rice MC and Beebee D. (1980). Mushrooms for color. Eureka, CA: Mad River Press; pp. 153.

[23] Bessete A and Bessete AR. (2001). The rainbow beneath my feet: A mushroom dyer's field guide, Syracuse: Syracuse university press, pp. 118.

[24] Räisänen R. (2002). Anthraquinones from fungus *Dermocybe Sanguinea* as textile dyes. *Academic Dissertation*, University of Helsinki, Finland.

[25] Weber WS *et al*. (2004). Laetiporic acid, a new polyene pigment from the wood-rotting basidiomycete *Laetiporus sulphureus* (Polyporales, Fungi). *Tetrahedron Lett*. 45: 1075-1078.

[26] Perumal K. (2004). Isolation, extraction and dyeing performance of fungal pigment from *Ganoderma applanatum*, *Coriolus versicolor* and *Amanita muscaria*. *J. Mycol. Pl. Pathol.* 34: 690-691.

[27] Sumathi E. (2008). Cultivation, extraction of pigment from basidiomata of *Ganoderma lucidum* (Fr.) P. Karst and its dyeing performance with selected textile yarns. Ph. D. thesis, University of Madras, Chennai, India.

[28] Chandra Sekarenthiran S. (2010). Production, characterization of pigment from selected basidiomycetes - *Pycnoporus sanguineus* L. EX. Fries and *Coriolus versicolor* (L.) Quelet. and application of pigment in textile dyeing, Ph. D. thesis, University of Madras, Chennai, India.

[29] Perumal K. (2007). Scale up studies on production and utilization of fungal pigments (*Amanita muscaria*, *Coriolus versicolor* and *Ganoderma lucidum*) on textile dyeing. DBT-BT/PR4329/PID/06/186/2003 – 06. Project report. Department of Biotechnology. New Delhi.

[30] Perumal K. (2010). Yield enhancement, pilot scale production of *Coriolus versicolor* and *Ganoderma lucidum* and application of its pigments in selected dyeing industries. DBT-BT/PR/9471/PID/06/394/2007. Project report. Department of Biotechnology. New Delhi.

[31] Perumal K *et al*. (2009). Extraction and characterization of pigment from *Sclerotinia* sp. and its use in dyeing cotton. *Text. Res. J*. 79(13): 1178 - 1187.

[32] Perumal K. (2010). Hyper production of dyes/pigments from selected microfungi (*Curvularia lunata*, *Alternaria alternata*, *Sclerotinia* sp, *Pestalotia* sp, and *Phoma foveata*). An eco friendly product development for textile dyeing industry. DBT-BT/PR/7672/PID/06/323/2006. Project report. Department of Biotechnology. New Delhi.

[33] Perumal K. (2014). Development of cost effective process for enhanced dye production from selected micro fungi *Curvularia*, *Phoma*, *Pestalotiopsis* sp., large scale testing and formulation as industrial textile dyes". (DST/TSG/NTS/2010/75). Project report. Department of Science and Technology. New Delhi.

STATUS OF EDIBLE AND MEDICINAL MUSHROOM RESEARCH IN SRI LANKA

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ABSTRACT

Sri Lanka is an island situated 32 km away from southern most part of India. This paper discusses the present status of edible and medicinal mushroom research in Sri Lanka and suggests the future needs for a sustainable expansion of the mushroom sector. Commercially cultivable mushrooms were introduced to Sri Lanka in 1985, under the auspices of the UNDP and oyster mushrooms (*Pleurotus ostreatus*) are acclaimed as the first domesticated species. Although *Pleurotus* mushrooms are the preferred species in terms of production and consumption, straw, milky and button mushrooms are also popular though cultivated on lesser enormities. In view of the scarcity of mostly used softwood sawdust used for *Pleurotus* cultivation, several cost effective substrate mixtures have been formulated as potential alternatives. Novel technologies have been introduced for the cultivation of straw, button, milky and medicinal mushroom *Ganoderma lucidum*. Major pests of mushrooms have been identified and measures have been advocated to effectively control them through integrated management. Strategies are in place for efficient utilization of spent mushroom substrates. Several value added mushroom products have been produced to prolong the shelf life of mushrooms. As a future strategy, comprehensive ethno mycological studies are needed to explore the indigenous mushrooms in Sri Lanka and to investigate their nutritional and therapeutic properties. Accurate identification tools need to be formulated for the identification of mushrooms and to develop genetic relationship between different species. Introduction of exotic culinary and medicinal mushrooms, suitable for tropical climates is a perquisite for the expansion of the mushroom industry.

Keywords: culinary, medicinal, mushrooms, ethno mycological

INTRODUCTION

Sri Lanka is an island country situated 32 km away from southern most part of India. It lies between latitudes 5º 55'N and 9º 51'N and longitudes 79º 41'E and 81º 5'E, covering an area of 65,610 square kilometers. Despite its relatively small land area, Sri Lanka is blessed with exceptionally high bio diversity, similar to that of Western Ghats of India. Located in low latitudes and surrounded by the Indian Ocean, Sri Lanka shows very typical maritime-tropical temperature conditions. Edible and medicinal mushroom cultivation has come to light as one of the strategies in livelihood development programs to revitalize the country's economy, which was ravaged due to 30 years of civil strife. This paper discusses the present status of edible and medicinal mushroom research in Sri Lanka and suggests the future needs for the sustainable expansion of the mushroom sector.

Commercial mushroom cultivation was first introduced to Sri Lanka in 1985 under UNDP funded program. Sri Lanka Export Development Board took the initiative to set up state of the art spawn laboratories and mushroom houses for spawn production and mushroom cultivation. Subsequently, the Department of Agriculture as the state institution responsible for food crop production took over the responsibility of spearheading R&D activities connected to mushroom cultivation.

Cultivation Technology

Oyster mushrooms (*Pleurotus* species)

Pleurotus ostreatus is the most preferred cultivated species across the country, in terms of production and consumption. *P. cystidiosus* and *P. eous* can be ranked in the second and third places, respectively. Although technology was perfected by researchers [1] for the cultivation of *P. eryngii*, this has been confined to mid and up country. In Sri Lanka, for the cultivation of oyster mushrooms, soft wood saw dust has been popularly used as the basal medium. However, dwindling

supplies of sawdust during recent times makes it imperative to search for alternative substrates. Arulnandhy and Gayathri [2] reported significantly higher yields obtained from mixed substrates where sawdust and paddy straw were used at 1:1 ratio. The yields realized from single basal substrates were inferior. Lokuge and Rajapakse [3] included paddy husk in oyster mushroom substrate in an attempt to partially replace saw dust as reported by Hanai *et al*. [4]. Economical yields and biological efficiencies derived from this study reported significantly higher yields from mixed basal substrates (paddy husk and sawdust) compared to individual basal substrates. Bringing in slight modifications, the positive impact of utilizing banana pseudo stems for yield enhancement in oyster mushroom cultivation [5] was also reported by Rajapakse and Lokuge [6]. This technology was disseminated mainly in major banana growing areas of the dry zone where farm women were encouraged to use banana pseudo stem with sawdust in 1:1 ratio as oyster mushroom substrate for significantly higher yields. Preparation of different compost mixtures using 6 per cent well decomposed poultry manure or 2.5 per cent *Gliricidia* leaf powder to replaceone percent legume powder requirement, which is the current recommendation have been reported by Rajapakse *et al*. [7]. Amarasiri and Alahakoon [8] reported the advantage of adding *Lantana camara* leaf extract @ 20 per cent, to prepare paper beddings with 1 kg weight with a resultant 18 per cent yield increase. The above investigations enumerated the advantage of cost effective mixed basal substrates as growing media for oyster mushrooms ensuring higher yields.

Paddy straw mushroom

Outdoor cultivation is the common practice of paddy straw mushroom production in Sri Lanka. Rajapakse[2][9] developed an indoor cultivation method for paddy straw mushroom production using cotton waste and paddy straw and reported the significantly higher yields obtained at 5.38 kg/sqm and 4.71kg/sqm, respectively compared to 1.79 kg/sqm and 1.73 kg/sqm obtained from the same substrates using the outdoor cultivation method thus emphasizing the advantage of the indoor cultivation.

Button mushroom

The protocol developed for cultivation of button mushroom (*Agaricus bisporus*) by Wijesinghe *et al*. [10] was further improved with the expertise received from the Directorate of Mushroom Research (DMR) India.

Milky mushroom (*Callocybe indica*) and black ear mushrooms (*Auricularia* spp.)

Cultivation was first introduced by Udugama and Wickramarathna [11] using paddy straw as the main substrate.

Medicinal mushrooms

Cultivation protocol for indigenous *Ganoderma lucidum* was perfected by Rajapakse *et al*. [12]. Subsequently, Bandaranayaka *et al*. [13] reported the technology for the cultivation of domesticated *G. lucidum*.

Lentinus giganteus (*Pleurotus giganteus* (Berk.) Karunarathna & K.D.Hyde.)

This Sri Lankan endemic edible mushroom was identified as *Lentinus giganteus* (Berk.) by Udugama [14] and technology was developed for artificial cultivation [14] using sawdust. However, based on molecular evidences this was positioned under *Pleurotus* and renamed as *Pleurotus giganteus* by Karunaratna *et al*. [15].

Crop Protection

Pest infestations have been identified as one of the major constraint for the expansion of cottage mushroom enterprises in Sri Lanka. Thus the effectiveness of pest exclusion and sanitation methods has been identified as remedial measures. *Cyllodes bifacies* has been identified as the major pest of oyster mushrooms in the central region [16]. Although mycophagous mites have been recorded elsewhere in the world, mites that belong to only *Lasioseius* species is found in Sri Lanka [17]. According to Sirisena *et al.* [18] inclusion of citronella leaf powder in the saw dust based growth substrate @ 2.5 per cent, effectively controlled the beetle infestation. Rove beetle infestation (*Gyrophanena* spp.) was reported by Wegiriya [19] as the major pest problem in the southern region of Sri Lanka. Wickremasinghe *et al*. [20] identified four fungi namely

Aspergillus fumigates, Chetomium thermophium, Mucor pusillus and *Trichoderma harzianu*m from straw and oyster compost substrates which exhibited anti bacterial activity against three human pathogenic bacteria at relatively low (10-40 µg) concentrations. Significant anti bacterial activity exerted by *Volvariella volvacea* against *E.coli* was also reported [21]. Manikpurage *et al*. [22] observed anti oxidative activity as well as cytotoxicity of the *P. cystidiosus* extract against Hep-2 cancer cells. In addition, Manikpurage *et al*. [23] reported the existence of activity in *P. cystidiosus* against *C. gloesporioides* causing anthracnose. Pain alleviating properties of *P. cystidiosus* has also been validated [24].

Spent Mushroom Substrate (SMS)

Invitro investigations carried out by Rajapakse *et al*. [25] on oyster mushroom SMS reported the presence of antagonistic bacteria in spent mushroom substrate and their high efficacy against red leaf spot pathogen of *Alternantherasessilis*. Concurrent *invivo* study on SMS, *visa vis* cattle manure, poultry manure and recommended dosages of fertilizer when each applied @ 1 kg/sqm reported no significant difference between the 4 treatments implying the usefulness of SMS as a manure, particularly in urban agriculture. Furthermore mixing oyster mushroom SMS @ 50% w/w with sawdust in the growth substrate to attain significantly higher yields and its advantage to decrease the recommended legume powder amendment by 50% has also been reported by Karunatilaka and Rajapakse [26]

Mushroom Spawn

Rajapakse [27] introduced saw dust spawns for oyster mushroom cultivation and emphasized its cost effectiveness, particularly for the rural poor in Sri Lanka. However, the most sought after spawn type remained as grain spawns in Sri Lanka. Pathmashini *et al*. [28] tested four types of grains namely finger millet, paddy, sorghum, and maize for oyster mushroom cultivation and observed the highest biological efficiency and yield from the substrates inoculated with finger millet grain spawns thus reporting it as the best.

Value Addition

A mushroom sandwich spread with *Pleurotus ostreatus* mushroom powder and different levels of chicken flavor (w/w) was developed by Rajanayaka *et al*. [29] and the best ratio was reported [29] as 40:1, based on sensory properties and overall acceptability. A mushroom sausage with required sensory attributes was developed [30] using *P. ostreatus* mushroom with a shelf life of more than four months. Sarananda *et al*. [31] manufactured a rice flour biscuit incorporating different levels of *P. ostreatus* mushroom flour (w/w). Based on the nutritive value and overall acceptability, the five percent inclusion was reported as an acceptable level. Mushroom burgher was also developed by Farhana *et al*. [32] using *P. ostreatus* mushrooms.

Future strategy

In order to diversify the mushroom sector in Sri Lanka, it is necessary to introduce new mushroom varieties and also different species of the popular *Pleurotus* spp. Investigations are needed to formulate cost effective cultivation technologies for the new introductions and dissemination of proven technologies to the farmers. Systematic investigations on medicinal mushrooms are a necessity as the relevant information (anti oxidant, antimicrobial, anti tumor) is scarce in Sri Lanka. Ethno-mycological studies need to be carried out to explore the diversified indigenous macro fungi in Sri Lanka and to investigate their nutritional and therapeutic properties. Accurate identification tools need to be introduced for the identification of mushrooms and to develop genetic relationship among different species. Mushroom derived product development is to be further explored to popularize mushroom consumption.

CONCLUSION

Mushroom farming is a burgeoning agriculture based enterprise in Sri Lanka and has tremendous scope to improve the food and livelihood security of the people. Ministry of Agriculture has implemented several livelihood development programs during recent times, particularly to the reawakened northern region, which was devastated due to 30 years of civil strife.

Comprehensive scientific research is needed to enlighten the value of edible mushrooms and to dispel the mycophobic attitude from certain sections of the society.

ACKNOWLEDGEMENTS

The author wishes to express her sincere thanks to Dr. Abhaya Balasuriya, Senior Lecturer, Rajarata University of Sri Lanka for his valuable suggestions. Financial assistance provided by Department of Agriculture, Sri Lanka and KOPIA Sri Lanka Centre is also acknowledged.

REFERENCES

[1] Anon. (2009). Administrative report Department of Agriculture, Sri Lanka: 51.

[2] Arulnandhy V and Gayathri T. (2007). Identification of suitable and efficient substrate for the production of oyster (*Pleurotus ostreatus*) mushrooms Undergraduate research report, Dept. of Agric. Bio.Faculty of Agric. Eastern Univ, Sri Lanka.

[3] Lokuge KS and Rajapakse JC. (2010). Potential applicability of five agricultural wastes for oyster mushroom cultivation. *Proc. 1st Int. Sym. Faculty of Agric. Univ. of Ruhuna*. 39. 16 Nov, 2010.

[4] Hanai H *et al.* (2005). Stimulation of mycelia growth in several mushrooms species by rice husk. *Bio Sci Biotechnol. Biochem.* 69(1): 123-127.

[5] Jandaik CL. (1974). Artificial cultivation of the mushroom *Pleurotus sajor-caju* (Fr.) Singer. *Mush. J. Tropics.* 1(2): 34-35.

[6] Rajapakse JC and Lokuge KS. (2008). Utilization of banana pseudo stem for oyster mushroom (*Pleurotus ostreatus*) cultivation. *Proc. 2nd Int. Sym.of the Sabaragamuwa Univ. of Sri Lanka*. 8-12 July 2008.

[7] Rajapakse JC *et al.* (2007). The potential of using cost-effective compost mixtures for oyster mushroom (*Pleurotus* spp.) cultivation in Sri Lanka. *Trop.Agric. Res and Ext.* 10: 29-32.

[8] Alahakoon P and Amarasiri SL (1987). In Weed Research in Sri Lanka (Ed). HPM Gunasena (1992).

[9] Rajapakse P. (2011). New cultivation technology for paddy straw mushroom (*Volvariella volvacea*). Proc. 7th 1CMBMP: 446- 451.

[10] Wijesinghe MAK *et al*. (2000). (2000). Production of *Agaricus bisporus* white button mushroom under local condition. *Proc. Annual symposium of the Department of Agriculture, Sri Lanka*. 2: 345-347.

[11] Udugama S and Wickramarathna KW. (1991). Cultivation of two new mushrooms: Sukiri mushrooms (*Calocybe indica*) and black ear mushrooms (*Auricularia* spp.) Horticultural Crop R & Dev. Institute, Gannoruwa, Peradeniya, Sri Lanka.

[12] Rajapakse JC *et al*. (2010). Cultivation possibilities of some selected Sri Lankan indigenous mushrooms with special reference to *Ganoderna lucidum* (Fr.) Karst. *Proc. Annual Res. Sym. Univ. of Colombo, Sri Lanka*. May 12-13, 2010: 117.

[13] Bandaranayake EMLOK *et al.* (2012). Selection of suitable saw dust as growing media for Reishi mushroom (*Ganoderma lucidum*) cultivation. Annual academic session. Open University of Sri Lanka: 21-24 retrieved from digital.lib.ou.ac.lk 03.07.2014.

[14] Udugama S and Wickramarathna KW. (1991). Artificial Production of Naturally Occurring *Lentinus giganteus* (Uru Paha) a Sri Lankan edible mushroom. Retrieved from thakshana.nsf.ac.lk/SLAAS/1995 on 03.07.2014.

[15] Karunarathna SC *et al*. (2012). Current Status of Knowledge of Sri Lankan Mycota. *Current Research in Envt.& Appl. Myco.* 2(1): 18-29

[16] Gnaneswaran R and Wijayagunasekara HNP. (1999). Survey and identification of insect pests of oyster mushrooms (*Pleurotus ostreatus*) cultures in central province of Sri Lanka. *Tropical Agricultural Research and Extension*. 2(1): 21-25

[17] Wijesekara GAW. (2003). Development of pests management program for the cottage mushroom industry in Sri Lanka with emphasis on mites and nematode control. Terminal Report of the Sri Lanka Council for Agriculture Research Policy No. 12/446/333.

[18] Sirisena AA. (2008). Survey and management of pests of American oyster (*Pleurotus ostreatus*) mushroom cultivations in Matara district. Undergraduate research report, *Dept. of Agric. Bio.Faculty of Agriculture, Univ. of Ruhuna, Sri Lanka.*

[19] Wegiriya H. (2014). Per.comm. Dept. of Zoology, Faculty of Sc. Univ. of Ruhuna, Sri Lanka.

[20] Wickremasinghe R *et al*. (1999). Isolation and identification of fungi from mushroom compost and evaluation of their biological activity. *J. Natn. Sci. Foundation. Sri Lanka*. 27(1): 29-40.

[21] PereraWASW *et al.* (2001). Antibacterial activity of *Volvariella volvacea. J.Natn. Sci. Foundation. Sri Lanka*. 29(1&2): 61-68.

[22] Manikpurage IP *et al*. (2012). Antioxidant activity and cytotoxicity of the edible mushroom *P. cystidiosus* against Hep-2 carcinoma cells. *J. Natn. Sci. Foundation. Sri Lanka*. 40(2): 107 -114.

[23] Manikpurage IP *et al*. ((2009). An Oxidized Ergosterol from *Pleurotus cystidiosus* active against anthracnose causing *Colletotrichum gloeosporioides*. *Mycopathologia*. 167: 155-162.

[24] Kudahewa DD *et al*. (2008). Antinociceptive activity of *Pleurotus cystidiosus,* an edible mushroom in rats. *Pharmacognosy Mag*. Vol1(Iss.13).

[25] Rajapakse JC *et al*. (2012). Efficient utilization of spent mushroom substrate with special reference to urban agriculture. *Proc. Indo-Sri Lanka Biotech. Conf:* 19. 12-13 March 2012, Colombo, Sri Lanka.

[26] Karunatilaka HKA and Rajapakse JC. (2012). Potential applicability of post mushroom substrate as an alternative nutrient media for oyster mushroom (*Pleurotus* spp) cultivation. Poster presented at the Int. Symp. Faculty of Agriculture, Univ. of Ruhuna, Sri Lanka.

[27] Rajapakse P *et al*. (2010). Identification of low cost spawn production for oyster mushrooms. *Proc. Annual symposium of the Department of Agriculture, Sri Lanka*. 12.

[28] Pathmashini L *et al*. (2008). Efficacy of different spawn types on sawdust media. *Trop. Agric. Res. & Ext*. (11): 55-59.

[29] Rajanayaka TUSM *et al*. (2013). Development of mushroom (*Pleurotus ostretus*) sandwich spread and determination of its quality parameters. Research report submitted to Faculty of Agriculture, Univ. of Ruhuna, Sri Lanka.

[30] Sarananda KH and Jayatilaka WD. (2008). Development of mushroom based sausage. MSc Thesis submitted to Univ. of Sri Jayawardanapura, Sri Lanka.

[31] Sarananda KH and Premaratne MPT. (2008). Manufacturing of mushroom incorporated rice biscuits and its quality as affected by packging. MSc. Thesis submitted to Univ. of Peradeniya, Sri Lanka

[32] Farhana MZKF. (2006). Development of mushroom burgher. Proc. of the 16th Annual Student Research session. University of Peradeniya, Sri Lanka:13-14.

MUSHROOM SCIENCE IN CUBA: TOWARDS NEW OPPORTUNITIES FOR DEVELOPING FUNCTIONAL FOODS/NUTRACEUTICALS

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ABSTRACT

Mushroom science in Cuba allows the valorization of agricultural by-products into functional foods/nutraceuticals for human consumption to address objectives of sustainability and biotechnological development. Much research work done in Cuban eastern region has been performed on the *Pleurotus* genus, whose cultivation has increased greatly during the last few decades. *Pleurotus* species, like many edible and medicinal mushrooms, are a good source of immunomodulators and "host defense potentiators" (HDPs). In this context, dietetic supplements with a high therapeutic potential acting on the immune system and formulated from refined or partially refined mushroom extracts, or from dried mycelia/fruiting bodies biomass are referred as "mushroom immunoceuticals". The present study examined the synergy exerted by the structural diversity of biomolecules found in *Pleurotus* crude extracts and powders on immune responses of both immunocompetent and immunodeficient Balb/c mice. *Pleurotus* derived-products could potentiate the host defense mechanisms *in vivo* and should be promising for further pharmacological studies. The effects on cell immunity are especially valuable in the prophylaxis of tumors, immunodeficiencies and as co-adjuvant in chemotherapy. The results also demonstrate that not only mushrooms but also their mycelia may be a good candidate for nutraceuticals production. Through this immunological "window" we are assisting to a revolution in mushroom science characterized by the diversity of compounds found in mushrooms and on the other hand, by the possibilities given by the abundance of specific molecular targets. An extended knowledge of the immuno-enhancing activity of *Pleurotus* nutraceuticals would be useful in understanding their potential applications for immunonutrition and immunotherapy.

Keywords: *Pleurotus*, edible and medicinal mushrooms, functional foods, nutraceuticals, immunomodulating activity

INTRODUCTION

Today the well-being of humankind faces unprecedented challenges involving inadequate regional food supplies, deficiency in new insight into healthy eating, diminishing quality of health, and increasing environmental deterioration. Therefore, we live in an age of human health crises, especially when considering the leading killer diseases of our time such as cancer, HIV/AIDS and the upsurge of hypertension, diabetes, cardiovascular disorders and obesity worldwide. The magnitude of these problems is set to increase as the world's population continues to grow [1]. A cost analysis made in 2010 at Harvard´s University suggested that if present health tendencies are not reverted, the costs due to medical services associated to chronic non-transmissible diseases will rise to 47 USD trillions in the next 20 years [2]. Cuba is not an exception in this international scene and Table 1 shows the statistics for the main causes of mortality caused by these diseases in 2013 [3].

Taking into account that the main health determinants involve the environment, the life-styles, human biology and the medical assistance and that of these factors diet is the determinant to which all of us are daily exposed, the challenge now is to drastically change the food habits [4]. In this context, the use of mushrooms, past and present, and practices, represent an important cultural heritage as they have been used since immemorial times as food and medicine according to traditional ecological knowledge transmitted along generations; its current use is supported by scientific evidences [5, 6]. Mushrooms have long been valued as highly tasty/nutritional foods with an established history of use in traditional oriental therapies and modern clinical practice in several Asian countries [7, 8].

In the last five years, the consumption of mushrooms, either as whole mushroom or extracted supplements has increased [9]. Most mushroom-derived preparations (extracts, powders and tablets) are usually included in the following categories of products: dietary supplements, functional foods, nutraceuticals, nutriceuticals, phytochemicals and design foods [10, 11]

Table 1. Selection of the main causes of mortality for all groups of age in Cuba (2013)*

Diseases	Number of deaths	Mortality(number of deaths/ 100 000 inhabitants)
Cancer	22 868	204.8
Cardiovascular diseases	22 651	202.9
Cerebrovascular diseases	9 011	80.7
Influenza and pneumonia	6 091	54.6
Diabetes	2 246	20.1
VIH/SIDA	353	3.2

*Adapted from the Cuban Health Statistical Annals (2013).

and immunoceuticals [12]. Therefore, the significant impact of mushroom cultivation and mushroom products on long-term food nutrition, health enhancement and human welfare in the 21st century could be considered globally as a non-green revolution [13].

The verification of beneficial effects pointing mainly to the reduction of risk factors for chronic non-transmissible diseases is the major issue in the science of functional foods. Medicinal effects have been demonstrated for many traditionally used mushrooms, including extracts of species from genera *Auricularia, Flammulina, Ganoderma, Grifola, Hericium, Lentinus* (*Lentinula*), *Pleurotus, Trametes* (*Coriolus*), *Schizophyllum,* and *Tremella* [9, 14].

The number of recognized mushroom species has been reported to be 14,000 which account for 10% of the estimated 140 000 mushroom species. Of the recognized mushroom species, about 7,000 (50%) are considered to possess varying degrees of edibility and approximately 700 are considered to be safe species with medicinal properties [9, 15]. Therefore, mushrooms represent a major and yet largely untapped source of powerful new functional and pharmacological products [16].

Strictly speaking, mushroom biodiversity in Cuba is not estimated. The Cuban National Botanical Garden presented a list of 97species belonging to 59 genera, 31families and 11orders, standing out for their representativeness the families Polyporaceae and Coprinaceae. In the study, 17 species were identified as edible and six possessed medicinal properties [17]. Thus the evaluation of the genetic and phenotypic biodiversity and the effects of variations in environmental factors on the quality of products (edible mushroom and active ingredients) are needed.

Unlike Latin American countries, edible mushrooms are not part of the culinary tradition of the Cuban people. Until a few decades, *Pleurotus* was the only genus of mushroom cultivated in Cuba for human consumption. Its cultivation began in 1988 at the Cuban Research Institute for Sugar Cane Derivatives (ICIDCA, Havana) using sugar cane bagasse as substrate [18]. Taking into account that Cuban eastern mountainous region produces about 80% of the high quality coffee in the island, and as a result large amounts of organic by-products like coffee pulp are generated, the Center for Studies on Industrial Biotechnology (CEBI, Santiago de Cuba) began to use these lignocellulosic materials as substrates for *Pleurotus* cultivation [19].This is an efficient biotechnological process for their bioconversion and recycling as a sustainable model for rural production in agreement with the "cluster thought" of agriculture in the 21st century [1].Other activities in Cuban eastern region generate several by-products (cocoa shells, coconut husks) also used as substrates for mushrooms production both in CEBI experimental unit and in a rural mushroom farm [19].

Recently, the Ministry for Foreign Investment and Economic Cooperation of Cuba and the Food and Agriculture Organization of the United Nations (FAO) signed a Technical Cooperation Project devoted to increase the cultivation of edible fungi, mainly *Volvariella volvacea* using rice straw as a substrate.The main executor of the project is the Institute of Fundamental Research in Tropical Agriculture (INIFAT, Havana), and now is part of the urban agriculture program of that institution.

Why mushroom research in Cuba? The Cuban Social Policy guidelines for Health declare: *"the need to pay the highest attention to the development of natural and traditional medicine"* as well as *"to strengthening promotion and prevention actions that delay or prevent the onset of chronic non-transmissible diseases and their sequelae"*. In this context, the CEBI´s Biotechnology of Edible and Medicinal Mushrooms group is aimed *"To carry out innovative research/ development activities in Biotechnology of Edible and Medicinal Mushrooms aimed to obtain new natural products with potential applications as dietetic supplements and/or biopharmaceuticals, thus achieving a favorable impact on public health"*.

Why *Pleurotus* genus? This genus comprises some of the most popular *Basidiomycetes* edible mushrooms whose cultivation has increased greatly throughout the world during the last few decades [20]. Its popularity has been expanded due to its vigorous growth on a variety of agroforestry substrates and for the production of a high nutritional value-food [21] containing compounds with therapeutic effects [22]. On the other hand, recent studies on *Pleurotus* spp. have shown a number of pharmacological activities, such as antitumour, immunomodulatory, antioxidant, anti-inflammatory, hypocholesterolaemic, antihypertensive, antihyperglycaemic, antimicrobial and antiviral activities [23].

With the view of developing new therapeutic agents to potentiate host resistance to cancer and infectious disease, such as AIDS, there has been an upsurge of interest in immunomodulating substances from medicinal mushrooms [16, 24]. *Pleurotus* species, like many edible and medicinal mushrooms, are a good source of immunomodulators and substances considered as "host defense potentiators" (HDPs) as judged by their immunostimulating properties. Several molecules able to augment or complement a desired immune response have been isolated from *Pleurotus* spp., particularly polysaccharides. These compounds stimulate different cell populations of the immune system, for instance, macrophages, Natural Killer (NK) cells, T cells, and also modulate cytokine system [24, 25]. The study of the synergy exerted by the vast structural diversity of biomolecules found in *Pleurotus* crude extracts, powders and other preparations on immune responses deserves special attention [26].

In Cuba, the implementation of technologies for the cultivation of *Pleurotus* spp. on agricultural substrates, in addition to food generation for human consumption opened new research activities towards mushroom immunoceuticals. In addition to the use as functional foods, *Pleurotus* fruiting bodies and mycelia obtained under Good Manufacture Practices can be applied in the formulation of nutraceuticals and biologically active products.

Through different examples of recent research made in our laboratory, this paper illustrates the effects of *Pleurotus* sp. crude extracts and powders on immune responses of both immuno competent and immuno deficient Balb/c mice. An extended knowledge of the immuno-enhancing activity of *Pleurotus* functional foods/ nutraceuticals would be useful in understanding their potential applications for immunonutrition and immunotherapy.

MATERIALS AND METHODS

Mushroom material

Pleurotus sp. strain (CCEBI-3024) is deposited at the Culture Collection of the Center for Studies on Industrial Biotechnology (CEBI, Cuba). The strain was maintained on slants with solid medium of potato dextrose agar (PDA) incubated at 5 °C.

Preparation of *Pleurotus*-derived products

Pleurotus sp. cultivation was performed by solid-state fermentation of mushroom spawn on pasteurized coffee pulp used as substrate in plastic bags of 2 kg (30-40 cm) [19]. The fruiting bodies were harvested, sliced into small pieces and dried at 45 °C for 24 h. The dried material (*Pleurotus*-DP) was milled, and the resulting powder was preserved away from light and humidity in plastic bags for further use.

For obtaining the *Pleurotus* fruiting bodies cold water-extract (CW-E), the collected carpophores were exhaustively washed with distilled water and sliced into 1 cm^2 pieces. They were weighed and 5 ml of distilled water was added per gram of biological material. The extraction was made at 20 °Cwith continuous stirring at 100 rpm for three hours and the final extracts were collected by centrifugation and filtration. The extracts were stored at -20 °C and freeze dried. They are mainly composed of 43% of carbohydrate and 35% of protein.

The preparation of *Pleurotus* mycelium hot-water extract (HW-E) started with the inoculation of mycelium in Erlenmeyer flasks, which contained YPG medium (yeast-peptone-glucose). The flasks were incubated at 27 °C with continuous stirring at 100 rpm for 15 days. After the submerged fermentation was carried out, mycelia were collected by centrifugation at 4000 rpm and washed twice with distilled water. Isolated mycelia, suspended in 200 g (wet weight)/L of distilled water, were extracted with boiling water for 10 h and the final extracts were collected by centrifugation and filtration. The extracts were stored at -20 °C and freeze dried. The major components of HW-E were carbohydrate (76.8%) and protein (12%).

Mycochemical profile of *Pleurotus*-derived products

The fpowder of ruiting bodies was extracted with hot-water to obtain an aqueous extract for assessing its mycochemical profile. The metabolites contained in *Pleurotus*-derived products were estimated qualitatively [27].

Laboratory animals

Balb/c mice purchased from the National Center for Production of Laboratory Animals (CENPALAB, Havana) were used. Experiments were done under conventional sanitary conditions and animals were maintained at controlled temperature and humidity throughout the investigation ensuring the optimal interval for the specie. The administration of products was made daily in the morning between 9-10 am. The research was approved by the institutional Ethical Committee (University of Oriente) and has been performed in accordance with Cuban legislation and the National Research Council Guidelines for the Care and Use of Laboratory Animals.

Effect of oral administration of *Pleurotus* fruiting bodies powder to immune competent Balb/c mice

Both female and male Balb/c mice of eight weeks weighing between 18-25 g were used. Mice were fed a standard diet and acidified water *ad libitum*. Twenty mice were randomly divided into four groups (n=5), two groups belonging to each sex. The groups identified as *Pleurotus*-DP females and *Pleurotus*-DP were administered orally for 14 days with 0.2 ml of a freshly prepared suspension of fruiting bodies powder in saline solution, equivalent to a dose of 1000 mg/kg of body weight, as a supplement of the standard pelleted diet. Control-females and -males groups were fed with the conventional diet throughout the investigation. On day 15, blood samples were collected from the orbital vein of mice for total and differential leukocyte counts. For differential counts, a blood sample was placed onto glass slides, fixed with methanol, and then stained with Giemsa solution.

In a parallel-conducted experiment, the cell-mediated immune response was assessed by the delayed-type hypersensitivity reaction (DTH). Animals were immunized by an intradermal (i.d.) injection of 50 µl of 5 mg/ml bovine serum albumin (BSA) emulsified in Complete Freund Adjuvant (CFA) (Sigma, St. Louis, MO) at two sites on the abdomen. Eight days after immunization, the mice were rechallenged by injection of 20 µl of 5 mg/ml BSA into one rear foot pad, while the other rear foot pad received a comparable volume of phosphate buffered saline (PBS). Measurements of foot pad swelling were taken at 24, 48 and 72 h after challenge by use of micrometer (Mitutoyo, Tokyo, Japan). The magnitude of the DTH response was determined as the differences in foot pad thickness between the antigen and PBS injected foot pads [28].

Effect of oral administration of *Pleurotus* fruiting bodies cold water-extract (CW-E) to malnourished Balb/c mice

Female Balb/c mice, weighing 20 g, were housed individually at 23 °C with a 12-hour/12-hour light/dark cycle. Thirty mice that were starved for 3 days and had free access to salted water were studied. After this time, blood was collected from the orbital vein of 10 mice and the animals were killed (M group). The others were re-fed *ad libitum* for 8 days with commercial pelleted diet (M-DCgroup) or with the commercial diet and the *Pleurotus* fruiting bodies cold water-extract (CW-E) administered orally at a dose of 100 mg/kg of body weight per day (M/CW-E group). A control group of 10 mice was fed with commercial diet throughout the study.

After the small intestine was collected, the segment correspondent to jejunum was rinsed thoroughly with ice-cold saline solution, opened, and blotted dry. The mucosa was scraped with a glass slide and weighed separately. Jejunal mucosa was homogenized with ice-cold phosphate-buffered saline with a pH of 6.0 (1:3 w/v). Total protein and DNA were quantiûed by the methods of Lowry *et al.* [29] and Burton [30], respectively. Humoral immune response was evaluated through an

immunization protocol with sheep red blood cells (SRBC) as antigen. Three groups, comprised of five mice, were designed: M-DC, M/CW-E and control as described above. After the starvation (day 0) mice were injected intraperitoneally (i.p.) with 0.2 ml of a 25% SRBC saline solution. After 7 days from the first injection, blood samples of 50 µl were drawn from the orbital plexus to measure antibody titresby a haemagglutination (HA) reaction. The reciprocal serum dilution, which just gave agglutination, was considered to be the titre. At this time, mice received the second immunization and on day 14, antibody titres were determined.

Effect of intraperitoneal (i.p.) administration of *Pleurotus* mycelium hot-water extract (HW-E) to cyclophosphamide-treated or whole-body irradiated mice

Cyclophosphamide (CY)-treated mice.Fifteen male mice (20-25 g) were divided into two groups. *Pleurotus* mycelium hot-water extract (HW-E) was administered intraperitoneally (i.p.) at 100 mg/kg for 7 days to ten Balb/c mice and cyclophosphamide (CY) USP 23 for injection, obtained from JSLYP (China), at 100 mg/kg was given i.p. on the fifth day. The control group, comprised of five mice, was injected i.p. with physiological saline. On the eighth day, blood was collected from the orbital vein and animals were then bled to death.

Whole-body irradiated mice

Male mice were randomly allocated into two groups (n= 10) for eventual whole-body irradiation with a ^{60}Co source Theratron teletherapy unit (Siemens, Erlanger, Germany) in the Oncological Hospital "Conrado Benítez" (Santiago de Cuba, Cuba) at a dose rate of 0.43 Gy/min for 20 min (date of exposure to be designated Day 0). For the analyses of effects of the mushroom-derived materials, one group of mice was administered the extract intraperitoneally (i.p.) at a dose of100 mg/kg in a volume of 0.2 ml on days -10 to -6 and -2 to +1 with respect to the irradiation. Mice in the control group (n=10) were injected with saline solution in place of the extract; non-irradiated mice were used as negative controls. All mice were euthanized by cervical dislocation 24 h after the final administration of extract or saline and tissues/bloods isolated for analyses.

In both experiments, the blood specimens were analyzed for white blood cell count. Moreover, femoral bone marrow cells were withdrawn with Hanks' solution and counted with a Neubauer chamber (Germany).The effects of the extract on *in vivo* phagocytic activity was estimated by measuring carbon clearance in peripheral blood (as an index of the phagocytic activity of liver and spleen) (see reference [24] for details).

Statistical analysis

The results were expressed as mean ± standard deviation (SD). One-way analysis of variance and *post hoc* Tukey's tests or Kruskal-Wallis rank test followed by the Student-Newman-Keuls test was applied to determine the significance of differences between treatments. The Student's *t*-test was used to compare the two means in the experiments related to the effects of HW-E administration in cyclophosphamide-treated or whole-body irradiated mice.Differences at $p< 0.05$ were accepted as significant. The software Statgraphics Plus v. 5.1 (Statistical Graphics Corporation, 1994-2001) was used in the analysis.

RESULTS AND DISCUSSION

In the last decade numerous reports have been published on preclinical studies and clinical trials related to the functionality and bioactive properties of edible mushrooms and their nutraceutical derivatives, including the immune modulatory effects. The 77% of the products were obtained from the fruiting bodies, commercially grown or harvested from the wild, 21% come from the mycelium and about 2% of filtered culture media [9].

Dried *Pleurotus* mushroom would become an attractive alternative for the development of functional foods and nutraceutical preparations. The powder evaluated in this work contained in terms of dried weight: carbohydrate (55%), protein (25%), fat (4%), total fibre (7.5%), ash (7.57%) and total phenols (138 mg/100 g) with an overall energy value of 336 kcal/100 g. Differences in biosynthesis patterns of cell molecular components in distinct stages of the vital cycle [31] would explain the dissimilarities in biochemical composition of fruiting bodies and mycelium extracts.

Table 2. Mycochemical profile of *Pleurotus* sp. fruiting bodies and mycelium derived aqueous extracts*.

Metabolites	Assays	Fruiting bodies hot-water extract	Fruiting bodies cold-water extract	Mycelium hot-water extract
Alkaloids	Dragendorff	++	+++	++
	Wagner	+	++	+
Terpenoids	Solkowski	-	-	-
	Lieberman-Burchard	+	-	-
Carbohydrates/Glycosides	Molisch	++	+	++
Reducing Sugar	Fehling	++	+	+
	Benedict	+	+	+
Phenols and tannins	$FeCl_3$	+	+	+
Amino acids	Ninhydrin	++	+	++
Flavonoids	Concentrated H_2SO_4	+	+	+
	Rosemheim	+	+	+

*The mycochemicals contained in the aqueous extracts were estimated qualitatively according to Harbourne (1984). Three replicates were used for each assay. (-) none, (+) present, (++) mild, (+++) marked.

Although in our study polysaccharides appear to be the most important bioactive component in *Pleurotus*-derived preparations with respect to immunomodulation, the presence in varying amounts of different secondary metabolites could lead to a synergy in the immune enhancing activity (Table 2). The result of the mycochemical test shows that both *Pleurotus* fruiting bodies and mycelium extracts contain alkaloids, phenolic compounds like flavonoids and tannins, reducing sugars and amino acids.

Fats and oil were generally absent in the extracts due to the polarity of the solvent used in their preparation. Among the mentioned bioactive components identified in aqueous extracts, phenolic compounds have been studied for their antioxidant properties, and they also play an important role in cancer prevention [32]. Reducing sugars are structural constituents of beta-D-glucans, components of mycetes' cell walls with a well-documented immunity-stimulating effect [33]. On the other hand, amino acids are the structural units of proteins that may be associated to polysaccharides to form immunomodulating complexes, like PSP isolated from the mycelium of *Trametes versicolor* [34]. Moreover, fungal immunomodulatory proteins, purified from medicinal mushrooms comprise a group of novel proteins which possess immunomodulatory properties and have a strong potential of being applied to food or pharmaceutical products for commercial development [35].

Immune system is a very complex homeostatic system consisting of a network of interacting cells, tissues and organs. It allows the organism to exist within itself and maintains a surveillance to recognize components considered non self. Among higher fungi investigated for immunomodulating effects, several mushroom species demonstrate great potential and some of them are already commercially developed [36].

The oral administration of *Pleurotus* fruiting bodies powder to immune competent Balb/c mice independently of animal sex led to a significant increase in total leukocyte counts with respect to controls ($p<0.05$) between the interval considered as normal for this rodent strain ($6\text{-}17x10^9$/L)(Fig. 1). These findings would be related with the stimulation of the production of white blood cells precursors in bone marrow by hematopoietic cytokines as part of the action mechanism of metabolites contained in mushrooms preparations.

Although leukocyte populations (lymphocytes and neutrophils) increased in Balb/c mice treated with *Pleurotus* powder as a diet complement, a differential pattern was showed depending on animal sex. In this context, lymphocyte counts were

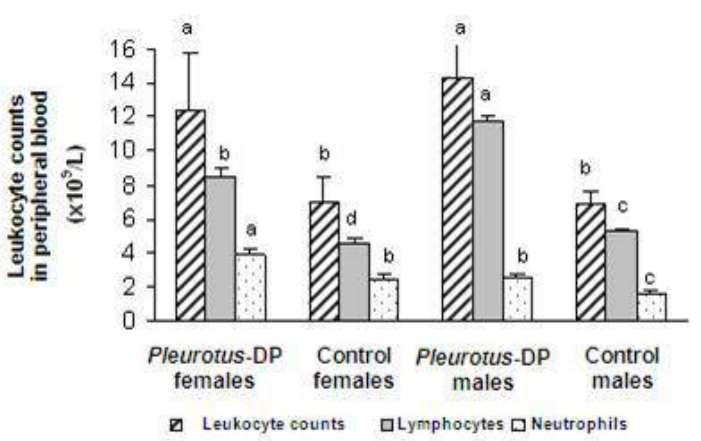

Figure 1. Effect of oral administration of *Pleurotus* fruiting bodies powder on leukocyte counts of immune competent Balb/c mice
All values are given as the arithmetic mean ± standard deviation of 10 mice. Different letters indicate significant differences among the groups (Kruskal-Wallis, Student-Newman-Keuls).

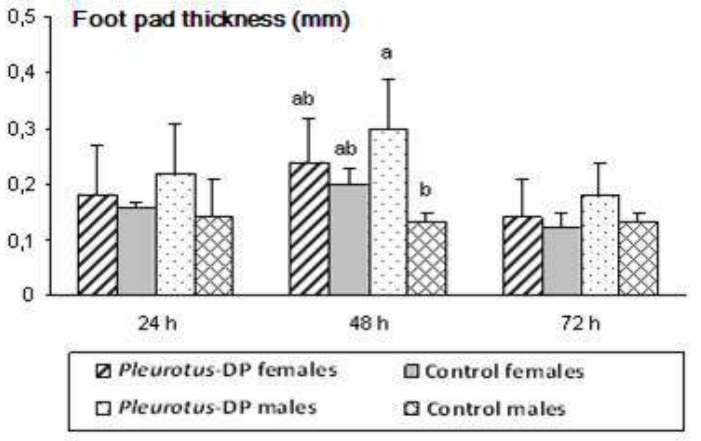

Figure 2. Effect of oral administration of *Pleurotus* fruiting bodies powder on the delayed-type hypersensitivity response (foot pad thickness) of immunocompetentBalb/c mice
All values are given as the arithmetic mean ± standard deviation of 5 mice. Different letters indicate significant differences among the groups (Kruskal-Wallis, Student-Newman-Keuls, $p<0.05$).

higher in males receiving the mushroom preparation while neutrophils exhibited superior values in females ($p<0.05$). The magnitude of delayed-type hypersensitivity reaction (DTH) in male mice, particularly at 48 h after antigen rechallenge ($p<0.05$) (Fig. 2), may reflect the induction of $CD4^+$ Th1 cells and the activation of macrophages by cytokines: tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) [30].

Because most chronic disease states are associated with fasting, which contributes to the establishment of malnutrition,we investigated the effect of *Pleurotus* fruiting bodies cold water-extract (CW-E)on the immunonutritional recovery of malnourished mice.The intestinal tract is an important interface between the organism and the environment. In protein-energy malnutrition, the adaptive responses and defense mechanisms of the gut mucosa are altered [37]. After 72 h of food

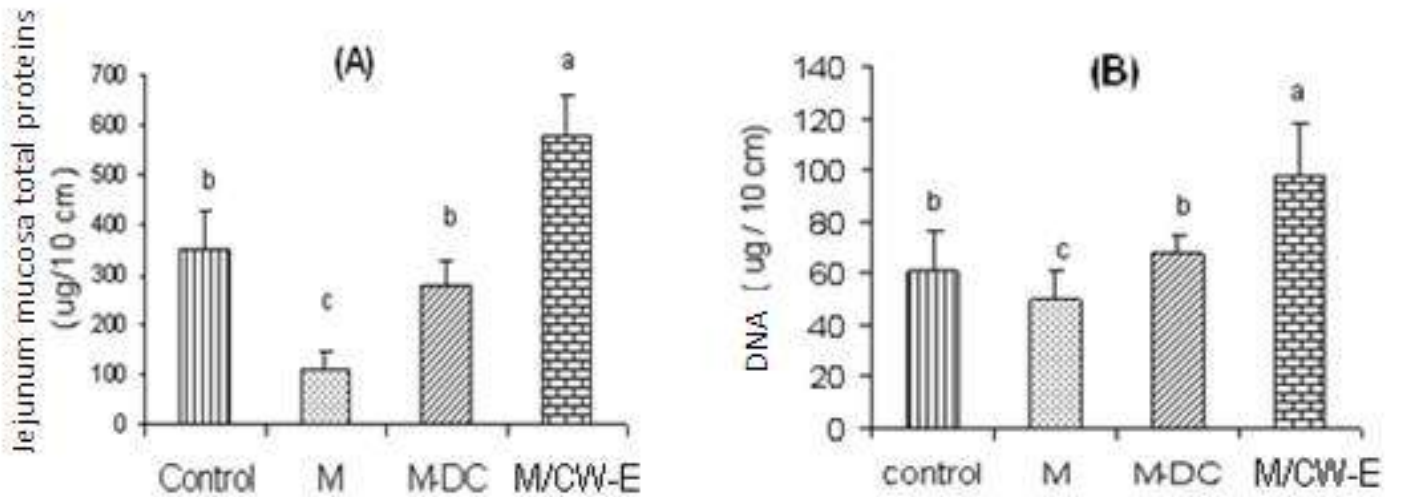

Figure 3. Effect of starvation and refeeding with commercial diet supplemented (M/CW-E) or not (M-DC) with *Pleurotus* fruiting bodies cold water-extract on protein and DNA contents in the jejunum of Balb/c mice.
All values are given as the arithmetic mean ± standard deviation of 10 mice. Different letters indicate signiûcant differences among the groups according to the Tukey test ($p < 0.01$).

deprivation, fasted mice (group M) showed decreases in mucosal DNA and protein contents (Fig. 3). Recovered M-DC mice exhibited a trend toward increased DNA and protein contents, but CW-E refed animals showed values in both gut mucosal protein and DNA higher than control mice ($p<0.05$). The increased DNA content in mice supplemented with CW-E might be associated with the stimulation of protein synthesis, cell division and turnover of enterocytes.

Another effect of CW-E on the immune system was a potentiation of the humoral response, which was determined by measuring antibody titres to SRBC, a T-dependent antigen (Fig. 4). The secondary response (day 14) in CW-E group evoked antibody titres higher than M-DC and controls ($p<0.05$). These findings suggested the stimulation of the functional abilities of Th (T-helper cells) and/or expanded pools of memory B cells by CW-E.

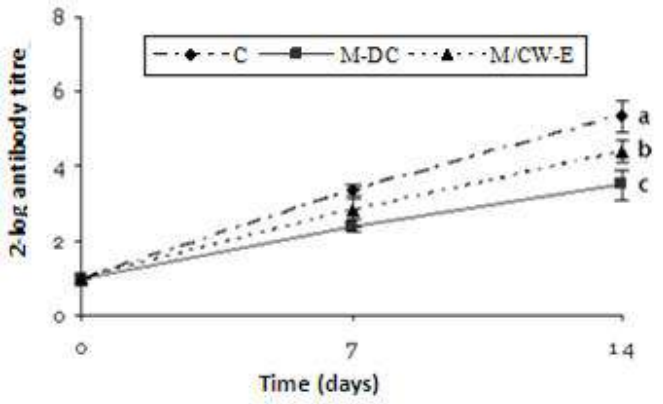

Figure 4. Effect of starvation and refeeding with commercial diet supplemented (M/CW-E) or not supplemented (M-DC) with *Pleurotus* fruiting bodies cold water-extract on humoral immune response against a T-dependent antigen (sheep red blood cells, SRBC).
All values are given as the arithmetic mean ± standard deviation of 5 mice. Different letters indicate signiûcant differences among the groups in Tukey test, $p < 0.05$.

These findings suggest that oral administration of edible mushrooms derived products would stimulate the immune system after their absorption in the gastrointestinal tract and the activation of gut-associated lymphoid tissues, thus integrating different elements of the immune function.An enhancement in Th1 response through intestinal epithelial cells and the suppression of ovalbumin (OVA)-sensitized allergy in mice was reported by Bouike *et al.*[38] as result of the oral treatment with extract of *Agaricus blazei* Murill.

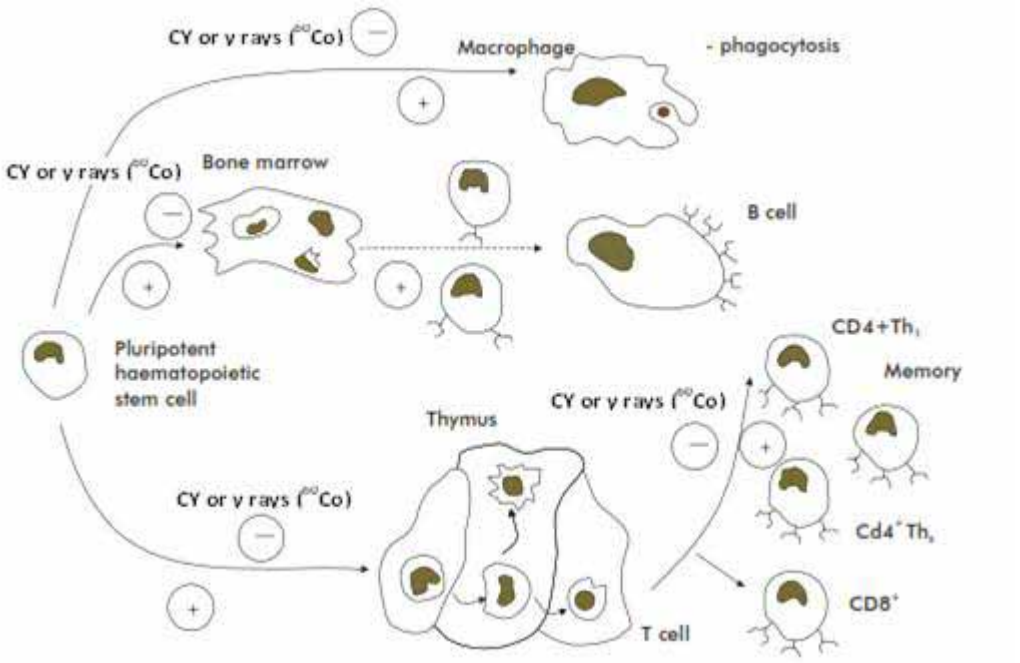

Figure 5. Alterations in the immunological response during cyclophosphamide (CY) or whole-body irradiation (^{60}Co) treatments and the immunoenhancing effects of a mycelium hot-water extract from *Pleurotus* sp. (HW-E) in Balb/c mice. The sign (–) indicates an inhibitory effect of the suppressive treatments and the sign (+) the immunomodulating action of HW-E in immunodeficient mice

The chemotherapy and radiotherapy in cancer treatment contribute to further depression of the immune system. Use of immunomodulating therapeutic agents can solve these problems and efforts to find new immunomodulators are on-going [39]. For that reason, we studied the effects of intraperitoneal administration of *Pleurotus* mycelium hot-water extract (HW-E) to cyclophosphamide-treated or whole-body irradiated mice (Fig. 5).

Cyclophosphamide is probably the most common antineoplastic used in cancer chemotherapy; however, cyclophosphamide shows potent immunosuppressing properties [24]. As expected, cyclophosphamide severely impaired the mice hematopoietic tissue, but *Pleurotus* HW-E was found to have an active protective effect. HW-E increased bone marrow cellularity ($4.1x10^6$ *vs.* $1.5x10^6$ per femur in saline control group, $p< 0.05$), the white blood cell counts ($7.6x10^9$ *vs.* $4.8x10^9$cells/L, $p< 0.05$) and enhanced the monocyte-macrophage system as judged by the shorter rate of carbon clearance (4.23 *vs.* 6.18 min, $p< 0.05$). The stimulant effect on hemopoiesis and cell immune response exerted by a *Pleurotus* fruiting bodies powder administered in a prophylactic schedule to cyclophosphamide treated mice was reported by Morris *et al.*[40].

On the other hand, the radioprotective effect exerted by mycelium HW-E was evident by increases in bone marrow cellularity ($5.1x10^6$ *vs.* $1.1x10^6$/femur in saline-control mice, $p<0.05$), leukocyte counts ($10.5x10^9$ *vs.* $4.5x10^9$/L, $p<0.05$) and the stimulation of macrophage phagocytic activity demonstrated by a faster rate of carbon clearance (1.62 *vs.* 2.01, $p<0.05$). Hence, this extract may be a candidate therapeutic agent with radioprotective activity for hematopoiesis damage, particularly to cells involved in immune function.

Although current knowledge of the role of *Pleurotus*-derived products in the prophylaxis and treatment of diseases are still largely at the empirical level, results obtained in this study demonstrate that not only *Pleurotus* mushrooms but also their mycelia obtained by submerged fermentation may be an interesting renewable resource for developing functional foods, nutraceuticals and new therapeutic agents with immunomodulating activity.

CONCLUSION

Through this immunological window in mushroom biotechnology, we are assisting to a revolution in nutrition and pharmacology. Therefore, attempts to "domesticate" the immune system for the benefit of man, in addition to specific vaccines and antibodies, would find in mushrooms new and unlimited possibilities of exogenous molecules. Our findings provide the basis for submitting the first Cuban dietetic supplement designed from mushroom material "NUTRISETAS®" to the national regulatory agency. Two important challenges for Cuban researchers involved in mushroom science are: (i) the evaluation of both nutritional and pharmacological potentialities of wild mushroom strains and (ii) the developing of new therapies and/or clinical assays based on mushrooms derived preparations alone or combined with traditional therapies. To accomplish these goals in present and future investigations we have to keep in mind the optimization of technologies/ production techniques as well as total quality assurance.

ACKNOWLEDGEMENTS

This work was supported by the University of Oriente under the Institutional project 9615; the FSP Coopération Scientifique Franco-Cubaine, project "Biotechnologie de *Pleurotus* sp. à Cuba et diversification de sa Culture pour des Applications Environementales et Nutraceutique"; and the IRD under the Programme Jeunes Equipes AIRD (JEAI) "Diversification de la Technologie de Culture de *Pleurotus* sp à Cuba et sa Diffusion dans la zone Caraïbe Cari SETA" This work has been also supported by the Belgian Development Cooperation through VLIR-UOS (Flemish Interuniversity Council).

REFERENCES

[1] Chang S and Wasser SP. (2012). The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health. *Int. J. Med. Mush.* 14: 95-134.

[2] Bloom DE *et al.* (2011). The global economic burden of non-communicable diseases. In: *World Economic Forum*. Available from: http://www.weforum.org/reports/global-economic-burden-non-communicable-diseases.

[3] Cuban Ministry of Public Health. (2014). *Annual Statistical Health 2013.*Direction of Medical Registers and Health Statistics, p. 38, ISSN 1561-4425.

[4] Oz M. (2011). The Oz diet: no more myths, no more fads, what you should eat and why. *Time* Sept 12: 30-35.

[5] Pereira E *et al.* (2012). Towards chemical and nutritional inventory of Portuguese wild edible mushrooms in different habitats. *Food Chem.* 130: 394-403.

[6] Roupas P *et al.* (2012).The role of edible mushrooms in health: Evaluation of the evidence. *J. Funct. Foods* 4: 687-709.

[7] Zaidman B *et al.* (2005). Medicinal mushrooms modulators of molecular targets as cancer therapeutics. *Appl. Microbiol. Biotechnol.* 67: 453-468.

[8] Ulziijargal E and Mau JL. (2011). Nutrient compositions of culinary-medicinal mushroom fruiting bodies and mycelia. *Int. J. Med. Mush.* 13: 343-349.

[9] Wasser SP. (2010) Medicinal mushroom science: history, current status, future trends, and unsolved problems. *Int. J. Med. Mush.* 12:1-16.

[10] Chang S and Buswell J. (1996). Mushroom Nutriceuticals. *World J. Microb.Biotech.* 12: 473-476.

[11] Wasser SP. (2004). Dietary supplements from culinary-medicinal mushrooms: A variety of regulations and safety concerns for the 21th Century. *Int. J. Med. Mush.* 6: 231-248.

[12] Petrova R *et al.* (2005). Potential role of medicinal mushrooms in breast cancer treatment: current knowledge and future perspectives. *Int. J. Med. Mush.* 7:141-155.

[13] Chang ST. (1999). Global impact of edible and medicinal mush-rooms on human welfare in the 21st century: nongreen revolution. *Int. J. Med. Mush.* 1:1-7.

[14] Wasser SP and Weis AL. (1999). Medicinal properties of substances occurring in higher basidiomycetes mushrooms: current perspectives (review). *Int. J. Med. Mush.*1: 31-62.

[15] Hawksworth DL. (2001). Mushrooms: the extent of the unex-plored potential. *Int. J. Med. Mush.* 3:333-337.

[16] Wasser SP. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl. Microbiol. Biotechnol.* 60: 258-274.

[17] Cabarroi M *et al.* (2008). Hongos del Jardín Botánico Nacional de Cuba. I. *Basidiomycota.Revista del Jardín Botánico Nacional* 29: 161-169.

[18] Klibansky MM *et al.* (1993). Production of *Pleurotus ostreatus* mushrooms on sugar cane agrowastes. *Acta Biotechnol.*13: 71-78.

[19] Bermúdez RC *et al.* (2001). Cultivation of *Pleurotus* on agricultural subtrates in Cuba. *Micol. Apl. Int.* 13:25-29.

[20] Martínez-Carrera D and López-Martínez de Alva L. (2010) Historia del cultivo comercial de hongos comestibles en México II: éxitos y fracasos durante el período 1991-2009. In: *Hacia un Desarrollo Sostenible del Sistema de Producción-Consumo de los Hongos Comestibles y Medicinales en Latinoamérica: Avances y Perspectivas en el Siglo XXI.* Martínez-Carrera D. *et al.* Eds. pp. 513-551.

[21] Bermúdez RC *et al.* (2003). Influencia de la luz en la calidad proteica de *Pleurotus ostreatus*var. *florida*. *Rev. Cubana Invest. Bioméd.* 22: 226-231.

[22] Gunde-Cimerman N. (1999). Medicinal value of the genus *Pleurotus* (Fr.) P. Karst.(Agaricaless.I., Basidiomycetes). *Intl. J. Med. Mush.* 1: 69-80.

[23] Gregori A *et al.* (2007). Cultivation techniques and medicinal properties of *Pleurotus* spp. *Food Technol. Biotechnol.* 45: 238-249.

[24] Morris HJ *et al.* (2003). Immunomodulating effects of the hot water extract from *Pleurotus ostreatus* mycelium on cyclophosphamide treated mice. *Micol.Apl. Int.* 15: 7-13.

[25] Patel Y *et al.* (2012). Medicinal properties of *Pleurotus* species (Oyster Mushroom): a review. *World J. Fungal Plant Biol.* 3: 1-12.

[26] Llauradó G *et al.* (2013). Phytochemical screening and effects on cell-mediated immune response of *Pleurotus* fruiting bodies powder. *Food Agric. Immunol.* 24: 295-304.

[27] Harbourne HB. (1984). Phytochemical methods: a guide to modern technique of plant analysis. 2nd ed. London: Chapman and Hall.

[28] Kim YS *et al.* (1998). Targeting the IL-15 receptor with an antagonist IL-15 mutant/$F_{cã}$ 2a protein blocks delayed-type hypersensitivity. *J. Immunol.* 160: 5742-5748.

[29] Lowry H *et al.* (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275.

[30] Burton K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA. *Biochem. J.* 62: 315-323.

[31] He X and Seleen J. (2004). Chemical analysis as a quality control method for medicinal mushroom and fungi extracts. *Int. J. Med. Mush.* 6: 253-261.

[32] Dubost NJ *et al.* (2007). Quantification of polyphenols and ergothioneine in cultivated mushroom and correlation to total antioxidant capacity. *Food Chem.* 105: 727-735.

[33] Rop O *et al.* (2009). Beta-glucans in higher fungi and their health effects. *Nutr.Rev.* 67: 624-631.

[34] Hobbs C. (2004). Medicinal value of Turkey tail fungus *Trametesversicolor* (L.: Fr) pilát (Aphyllophoromycetideae). a literature review. *Int. J. Med.Mush.* 6: 195-218.

[35] Ou CC *et al.* (2009). Stability of fungalimmunomodulatory protein, FIP-gts and FIP-fve, in IFN-ã production. *Food Agric. Immunol.* 20: 319-332.

[36] Poucheret P *et al.* (2006) Biological and pharmacological activity of higher fungi: 20-year retrospective analysis. *Cryptogamie, Mycologie* 27(4):311-333.

[37] Cummings JH *et al.* (2004). PASSCLAIM-gut health and immunity. *Eur. J. Nutr.* 43 S2: II 118- 173.

[38] Bouike G *et al.* (2011).Oral treatment with extract of *Agaricus blazei* Murill enhanced Th1 response through intestinal epithelial cells and suppressed OVA-sensitized allergy in mice. *Evid-Based Compl. Altern.Med.* Article ID 532180, 11 p.

[39] Zhuang SR. (2009). Effect of citronellol and the Chinese medical herb complex on cellular immunity of cancer patients receiving chemotherapy/radiotherapy. *Phytother. Res*. 23: 785-790.

[40] Morris HJ *et al.* (2011) Immunomodulating properties of *Pleurotus* sp. fruiting bodies powder on cyclophosphamide treated mice. In: Mush. Biol. Mush. Prod. Savoie J.M. *et al.*Eds. 7, 329-338.

IN VITRO AND *IN VIVO* ANTIDIABETIC ACTIVITY OF *CALOCYBE INDICA*

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ABSTRACT

In vitro and *in vivo* antidiabetic activity of milky mushroom (*Calocybe indica*) exhibited significant results for its α-amylase (89.49 ± 3.54 % at 1.0 mg/ml) and α-glucosidase inhibitory activity (67.30 ± 2.93 % at 1.0 mg/ml) in a dose-dependent manner. The methanolic extract showed significant activity ($p < 0.05$) at the tested dose level (200 mg/kg b.w) which was comparable to glibenclamide, a standard antidiabetic drug. Presence of phytochemicals namely phenols, flavonoids, saponins and tannins which may be responsible for such antidiabetic activity.

Keywords: *Calocybe indica*, antidiabetic activity, phytochemicals, phenols, flavonoids.

INTRODUCTION

Diabetes mellitus is a life threatening chronic metabolic disorder caused by lack of insulin and insulin dysfunction, characterized by high levels of glucose in the blood. It is characterized by hyperglycemia and alteration in carbohydrate, protein and lipid metabolism caused by defects in insulin production or action [1,2]. Contributory factor in the pathogenesis of diabetes also comprises of oxidative stress [3,4].

Plants including fungi are the main source of natural compounds used as medicine and they have attracted considerable interest because of their wide variety of bioactive metabolites. Mushrooms are being developed as nutriceuticals which garner their essence and to make their consumption easy. Further, scientific validation of traditional knowledge bears evidence of the many positive effects of consuming mushrooms on human health [5-7]. The investigation of the local species may yield mycochemicals with novel medicinal properties that can be used for the development of therapeutic agents in diabetes and for other ailments [8, 9].

MATERIALS AND METHODS

The fruiting bodies of *Calocybe indica* were obtained from Mushroom Unit, Department of Biology, Gandhigram Rural Institute - Deemed University, Gandhigram, Dindigul, TamilNadu, India. Sample preparation [10], qualitative phytochemical analysis [11], *invitro* antidiabetic activity namely α-amylase [12] and á-glucosidase[12] inhibitory activity and *in vivo* antidiabetic activity namely evaluation of alloxan induced diabetic rats were carried [13].

Animal studies: Animal experiments were carried out according to the guidelines of the Committee for the purpose of control of experiments on animals and approved by the Institutional Animal Ethics Committee (Reg. No.: CPCSEA/265).

Statistical analysis: The results were expressed as mean values and standard deviation (SD). Linear regression analysis was used to calculate IC_{50} value. Data were analyzed using One-way Analysis of Variance (ANOVA) followed by Turkey's multiple comparison post hoc tests using SPSS software 16.0 versions. Values of $p<0.05$ were considered as statistically significant.

RESULTS

Qualitative phytochemical screening: Methanolic extract of *Calocybe indica* were qualitatively analyzed and presented in Table 1.Among the various phytochemicals assessed, maximum values of phenols and saponins; minimum values of flavonoids and lowest values of tannins were recorded.

Table 1. Qualitative phytochemical screening of *Calocybe indica*

Test for extract	Inference
Phenols	+++
Flavonoids	++
Saponins	+++
Tannins	+

Key: "+++" Occurrence very High concentration; "++" Occurrence High concentration; "+" Occurrence Low concentration.

***In vitro* antidiabetic activities:** The methanolic extract of *Calocybe indica* showed significant inhibition of á-amylase (89.49±3.54 at 1000 µg/ml) and á-glucosidase inhibitory activity (67.30±2.93 at 1000 µg/ml) in a dose-dependent manner and the concentrations required for 50% of the inhibition (IC_{50}) were 38.06 ± 0.82 and 281.27 ± 6.69 µg/ml, respectively (Table 2).

Table 2. *In vitro* antidiabetic activities of *Calocybe indica*- Inhibition of α-amylaseand α-glucosidase inhibitory activity

Sample concentration (µg/ml)	á-amylase inhibition activity (%)	IC_{50} value (µg/ml)	α-glucosidase inhibition activity (%)	IC_{50} value (µg/ml)
200	31.34 ± 4.56		6.94 ± 0.33	
400	48.55 ± 1.66		20.77 ± 0.33	
600	56.88 ± 1.37	38.06 ± 0.82	41.49 ± 0.64	281.27 ± 6.69
800	81.70 ± 1.13		52.04 ± 0.33	
1000	89.49 ± 3.54		67.30 ± 2.93	

Data represent the mean ± S.E.M (n = 3) (p< 0.05)

***In vivo* antidiabetic study:** *In vivo* antidiabetic study revealed the significant reduction of blood glucose, serum cholesterol, serum triglyceride, LDL levels and significant increase of HDL level in *Calocybe indica* treated diabetic rats in14 days trials (Table 3).

Table 3. *In vivo* antidiabetic study - blood glucose, serum cholesterol, serum triglyceride, LDL and HDL levels in *Calocybe indica* treated diabetic rats in14 days trials

Experimental groups	Blood glucose (mg/dl)	Serum cholesterol (mg/dl)	Serum triglyceride (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
Group - É	81.40 ± 3.22[b]	34.00 ± 1.74[b]	33.40 ± 3.45[b]	22.00 ± 2.10[b]	24.60 ± 1.47[b]
Group - ÉÉ(120 mg/kg b.w)	512.0 ± 15.29	84.0 ± 4.94	123.00 ± 6.63	58.80 ± 3.22	10.20 ± 1.12
Group-ÉÉÉ(200 mg/kg b.w)	320.0 ± 14.40[b]	99.20 ± 5.23	93.20 ± 4.61[a]	42.20 ± 1.24	14.00 ± 0.65
Group - IV(400 mg/kg b.w)	124.40 ± 7.84[b]	32.20 ± 2.49[b]	38.20 ± 1.90[b]	23.60 ± 1.90[b]	25.80 ± 0.98[b]

Each value represents the Mean ± SD (n = 5) [a]p< *0.05*, [b]p< *0.01* Vs Diabetic control (ANOVA followed by Turkey's multiple comparison test). Group I – Control; Group II – Diabetic; Group III – *Calocybeindica*; Group IV – Glibenclamide; Group V – HDL - High density lipoprotein; LDL - Low density lipoprotein; b.w - Body weight.

DISCUSSION

Calocybe indica is a good source of extractable phytochemicals with inhibitory potentials against key enzymes namely, á-amylase and á-glucosidase linked to Type 2 diabetes. *In vitro* tests can play a very important role in the evaluation of antidiabetic activity of drugs as initial screening tools, where the screening of large number of potential therapeutic candidates may be necessary. They might provide useful information on the mechanism of action of therapeutic agents [14-17].

Therapeutic approach for treating Type 2 diabetes mellitus is to decrease the post-prandial glucose levels. This could be done by retarding the absorption of glucose through the inhibition of the carbohydrates hydrolyzing enzymes, á-amylase and á-glucosidase, present in the small intestinal brush border that are responsible for the breakdown of oligosaccharides; disaccharides into monosaccharides suitable for absorption [14, 18-20]. Inhibitors of these enzymes, like acarbose, delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise [14, 15].

Jumepaeng *et al.* [21] reported á-amylase inhibitory activity was significantly higher as compared to acarbose drug currently administrated for controlling glucose levels in diabetic patients. Natural products from plants have shown lower inhibitory activity against á-amylase activity and stronger inhibitory activity against non insulin dependent diabetes mellitus (NIDDM) with minimal side effects. This is a positive result, since as explained earlier, the excessive inhibition of á-amylase results in abnormal bacterial fermentation of undigested carbohydrates in the colon, which inturn results in abdominal digestion, flatulence, meteorism and possibly diarrhea. On the other hand, it was found that dates extract strongly inhibits the activity of á-glucosidase [22, 23].

The presence of inhibition to á-glucosidase activity in the extracts of *Calocybe indica* mushroom could be caused by the presence of carbohydrate and protein (flavanols) which are suspected to be the competitive inhibitors for á-glucosidase enzyme. This is appropriate with the substrate of á-glucosidase, which is a food starch and carbohydrate (glycogen) [24].

In the present study, *in vitro* antidiabetic studies revealed the inhibition of á-amylase and á-glucosidase activity. The percentage inhibition at 200, 400, 600, 800 and 1000 µg/ml concentrations of *Calocybe indica* on á-amylase and á-glucosidase showed a concentration dependent reduction in percentage inhibition. Therefore, the antidiabetic effect of *C. indica* might attribute to its inhibitory effect against á-amylase and á-glucosidase that retarding the digestion of carbohydrate to delay the postprandial rise in blood glucose.

In the *in vivo* studies, blood glucose levels were assessed from 0 and 14th days in normal rats; diabetic induced rats; mushroom extracts treated rats and also glibenclamide treated rats. There is a significant reduction in all antidiabetic parameters on 14th day in the rats treated with *Calocybe indica* mushroom extracts. In the *in vivo* studies, alloxan induced diabetic rats showed significant increase in the levels of blood glucose than the diabetic rats ($p<0.05$). Blood glucose level was measured in normal and diabetic rats on day 0 and 14th day of drug treatment. After treatment with both species at 200 mg/kg b.w, the blood glucose levels on day 14th were significantly reduced compared to those on day 0 ($p<0.01$). The glibenclamide treated rats also showed significant reduction in serum glucose level ($p<0.05$). *C. indica* and glibenclamide administration attenuated hyperglycemia, while no significant changes were observed in normal and diabetic groups ($p>0.05$).

Mushrooms have been shown to be useful in supporting healthy cholesterol levels and have been shown to improve circulation; also they have been shown to help in maintaining blood sugar balance *via* blood sugar lowering effects, elevation of plasma insulin levels and enhanced liver metabolism of glucose and increase cellular insulin sensitivity [25]. Hyperglycemia caused by diabetes is known to be a cause of oxidative stress that leads mainly to enhanced production of mitochondrial ROS. Oxidative stress induced by hyperglycemia leads to the activation of stress sensitive signaling pathways, which worsen both insulin secretion and action, and promote the development of Type 2 diabetic mellitus [26-29]. Fasting hyperglycemia is a hallmark of diabetic mellitus. It has been postulated but is still debated that the fasting hyperglycemia in noninsulin dependent diabetic mellitus arises from the hepatic over production of glucose [30].

Wi *et al*. [31] suggested that the post absorptive hyperglycemia in Streptozotocin diabetic rats is largely due to decreased peripheral glucose clearance, while increased hepatic glucose output might also be a contributing factor at a very high Streptozotocin dose. Krishna *et al*. [32] stated that polysaccharide extracted from *Pleurotus citrinopileatus* showed blood glucose lowering effect in rats. These findings suggest that mushrooms are promising antidiabetic nutriceuticals, but there is lack of enough clinical evidences. Khan *et al*. [33] have reported that oral administration of *Pleurotus ostreatus* given to rats leads to blood glucose lowering effect in both insulin–dependent and insulin–independent diabetic conditions. Antidiabetic effects of ethanolic extract of *Pleurotus ostreatus* on alloxan induced diabetic rats was extensively studied and reported as an effective antidiabetic regimen [34].

CONCLUSION

The methanolic extract of *Calocybe indica* with its significant antidiabetic activity in rats, suggests its therapeutic potential for the prevention and control of diabetics; moreover, the mushroom species can be used as an easily accessible source of natural antidiabetic and as a possible food supplement or in pharmaceutical industry. However more intensive and extensive investigations are needed to exploit their valuable therapeutic potentials and the chemical characteristics of the antidiabetic components in the extracts should be further investigated.

ACKNOWLEDGEMENTS

The authors express their sincere thanks to The Principal, and Head, Department of Pharmacology, The Periyar College of Pharmaceutical Sciences, Tiruchirapalli, for providing laboratory facilities.

REFERENCES

[1] King *et al*. (1998). Global burden of diabetes 1995–2025: prevalence, numerical estimates and projections. *Diabetes Care*. 21 (9): 1414–1431.

[2] Rushita *et al*. (2013).Effect of *Pleurotus citrinopileatus* on blood glucose, insulin and catalase of streptozotocin induced Type 2 diabetes mellitus rats. *J. Anim. Plant Sci*. 23 (6): 1566–1571.

[3] Murugan P and Pari L. (2006). Antioxidant effect of tetrahydrocurcumin in streptozotozin nicotinamide induced diabetic rats. *Life Sci*. 79 (18): 1720–1728.

[4] Sonawane *et al*. (2013). Hypoglycemic effect of Phansomba (*Phellinus badius* Berk Ex Cooke) G. Cunn, on alloxan induced diabetic rats. *J. Nat. Remed*. 13 (1): 29-34.

[5] Cragg *et al*. (1997). Natural products in drug discovery and development. *J. Nat. Prod*. 60: 52-60.

[6] Wong *et al*. (2012). Neurogenerative potential of Lions mane mushroom, *Hericiumerinaceus* (Bull.: Fr.) Pers., (Higher Basidiomycetes) in the treatment of peripheral nerve injury. *Int J. Med. Mushrooms*.14: 427-446.

[7] Hur *et al*. (2012). *In vitro* anti-inflammatory activity of *Russula virescens* in the macrophage like Cell line RAW 264.7 activated by lipopolysaccharide. *J. Nutr. Food Sci*. 2 (4): 1–4.

[8] Chang ST and Mshigeni KE. (2001). Mushroom and their human health: their growing sig*nificance as potent dietary supplements. The University of Namibia,* Windhoek. 1 (79): 1188-1194.

[9] Wang *et al*. (2012). *In vitro* and *in vivo* comparisons of the effects of the fruiting body and mycelium of *Antrodia camphorata* against amyloid β-protein induced neurotoxicity and memory impairment. *Appl. Microbiol. Biotecnol*. 94: 1505–1519.

[10] Suffness M and Douros J. (1979). Drugs of plant origin. In: *Methods in Cancer Research*, Academic Press, New York. DeVitaV.T. and Busch H. Eds. 26, 73–126.

[11] Harborne JB. (1973). *Phytochemical methods: A guide to modern techniques of plant analysis. Published by Chapman and Hall, an imprint of Thomson Sciense*, London, UK.

[12] Miller GL. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Ann. Chem*. 31: 426–428.

[13] Phuong *et al*. (2004). Regression trees for regulatory element identification. *Bioinformatics*. 20 (5): 750–757.

[14] Laar *et al*. (2005). α-glucosidase inhibitors for patients with Type 2 diabetes: Results from a Cochrane systematic review and meta analysis. *Diabetes Care*. 28: 166–175.

[15] Cheng A and Fantus I. (2005). Oral antihyperglycemic therapy for Type 2 diabetes mellitus. *Can. Med. Assoc. J.* 172: 213–226.

[16] Piparo *et al*. (2008). Flavonoids for controlling starch digestion: Structural requirements for inhibiting human α-amylase. *J Med Chem*. 51: 3555-3561.

[17] Sabitha *et al*. (2012). *In vitro* α-glucosidase and α-amylase enzyme inhibitory effects in aqueous extracts of *Abelmoscus esculentus* (L.) Moench. *Asian Pac. J. Trop Biomed*. S162–S164.

[18] Lobovitz H. (1997). α-glucosidase inhibitors. *Endocrinology and Metabolism of North America*. 26: 539–551.

[19] Inzucchi SE. (2002). Oral antihyperglycemic therapy for Type 2 diabetes. *Scientific Review*. 287 (3): 360–372.

[20] Thorat *et al*. (2012). *In vitro* models for antidiabetic activity assessment. *Int. J. Res. Pharm Biomed Sci*. 3 (2): 730–732.

[21] Jumepaeng *et al*. (2012). Determination of antioxidant capacity and α-amylase inhibitory activity of the essential oils from Citronella grass and lemongrass. *Int. Food Res. J*.20 (1): 481–485.

[22] Bischoff H. (1994). Pharmacology of á-glucosidase inhibition. *Eur. J. Clin Invest*. 24: 3–10.

[23] Sulaiman *et al*. (2010). *Organized retailing of fresh fruits and vegetables: Is it really helping producers. CRISP working paper 2010-001. Centre for Research on Innovation and Science Policy,* Hyderabad.

[24] Davis SN and Granner DK. (2001). Insulin, oral hypoglycemic activity agents and the pharmacology of endocrine pancreas. In: Goodman and Gilman's: *The pharmacological basis of therapeutics*, 11thEdn. *McGraw-Hill Medical Publication Division:* New York. Brunton *et al*. Eds. 1706–1707.

[25] Rajeswari P and Krishnakumari S. (2013). Potent antihyperglycaemic activity of *Calocybe indica* in streptozotocin induced diabetic rat's antihyperglycemic activity of *Calocybe indica. Int. J. Pharm Pharm Sci*. 5 (2): 512–515.

[26] Brownlee M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature*. 414: 813–820.

[27] Evans *et al*. (2002). Oxidative stress and stress activated signaling pathways: a unifying hypothesis of Type 2 diabetes. *Endocr Rev*. 23: 599–622.

[28] Jay *et al*. (2006). Oxidative stress and diabetic cardiovascular complications. *Free Radical Bio. Med*. 40: 183–192.

[29] Kowluru *et al*. (2007). Small molecular weight G-protein, H-Ras, and retinal endothelial cell apoptosis in diabetes. *Mol. Cell Biochem*. 296: 69–76.

[30] Defronzo RA and Ferranini E. (1991). Insulin resistance: a multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidaemia and artherosclerotic cardiovascular disease. *Diabetes Care*. 14: 173–194.

[31] Wi *et al*. (1998). Reduced glucose clearance as the major determinant of post absorptive hyperglycemia in rats. *Am. J. Physiol Endocrinol Metab*. 274(2): 257–264.

[32] Krishna *et al*. (2009). Characterization of Dichotomoceras in the Oxfordian of Kachchh. *J. Geol. Soc. India*.74: 469–479.

[33] Khan *et al*. (2011). *Pleurotus sajor-caju* and *Pleurotus florida* mushrooms improve some extent of the antioxidant systems in the liver of hypercholesterolemic rats. *Open Nutraceuticals J*. 4: 20-24.

[34] Johnny I and Okon J. (2013). Antidiabetic effect of *Pleurotus ostreatus* (Jacq ex Fr) Kumm, mushroom on alloxan induced diabetic rats. *Indian J. Pharm. Biol. Res*.1 (1): 31–36.

MYCORRHIZAL, ENTOMOPATHIC AND NOVEL MUSHROOMS

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ABSTRACT

Mushrooms have always been a source of good health and are being used since time immemorial as medicines. They are pro-biotic i.e. they help our body strengthen itself and fight off illness by maintaining physiological homeostasis. Mycorrhizal, entomopathic and novel mushrooms are gaining popularity since last few decades worldwide. Mycorrhizal mushrooms comprise a specific group of edible fungal species that form symbiotic associations with their host plants and includes *Tricholoma matsutake, Tuber melanosporum, Tuber magnatum, Lactarius deliciosus, Lyophyllum shimeji, Tuber borchii, Tuber formosanum, Rhizopogon rubescens,Terfezia laveryi, Boletus edulis, Cantharellus cibarius, Lactarius hatsutake, Lactarius akahatsu* and *Morchella* spp. However, currently only *Tuber melanosporum* and *Tuber uncinatum* have been cultivated commercially, although some success has been achieved with *Lactarius deliciosus, Lyophyllum shimeji, Tuber borchii, Tuber formosanum, Rhizopogon rubescens, Morchella esculenta* and *Terfezia laveryi*. Entomopathic fungi usually attach to the external body surface of insects in the form of microscopic spores. The most common and important entomopathic fungus is *Cordyceps (Ophiocordyceps)*. *Cordyceps sinensis* has long been used in folk medicine and is known to have remarkable medicinal properties. It has been determined that there is perhaps a greater biodiversity of compounds within different strains of this single species. Due to the great difference in the concentration of native compounds, a wide range of quality is found in *Cordyceps* cultivated from different strains and utilizing different culture methodology. Due to its peculiar characteristics, habitat, morphology and being a store house of medicinal properties, it is a highly prized mushroom. *Ganoderma lucidum* has gained wide popularity in recent years as a dietary supplement, not only in China and Japan, but also in North America and other parts of the world. The reason it attracts international attention as a valuable Chinese herb is due to the wide variety of its biological activities such as antitumor, immunomodulatory, cardiovascular, respiratory, anti hepatotoxic and antinociceptive effects. The diversity in the biological actions may be attributed to the fact that it is composed of different chemical entities including triterpenoids, polysaccharides, alkaloids, amino acids, peptides, inorganic elements, steroids, fatty and organic acids. *G. lucidum* products with different triterpenes and polysaccharides or combinations of these two groups are most likely to result in different pharmacological activities. However, there is lot more to be explored of this wonderful gift of nature and requires the attention of scientific community to exploit this mushroom to the benefit of mankind.

Keywords: edible ecto-mycorrhiza, entomopathic mushroom, *Cordyceps sinensis*, novel mushrooms, *Ganoderma lucidum*, medicinal uses, basic and applied research

INTRODUCTION

The world of mushrooms has always been fascinating and mystic to man owing to their sudden appearance in numbers, groups, rings, bunches and also in isolation as a single attractive and imposing structure. Man has been fascinated with this biological entity since time immemorial and references about the mushrooms are available in most ancient literatures like Vedas and Bible. Mushrooms have always been a source of good health and have been used since time immemorial as medicines. During the past 50 years, several major advancements in medicine came from lower organisms such as molds, yeasts and mushrooms (fungi). In fact, the first antibiotic was extracted from the fungi. Penicillin, tetracycline and auromycine were derived from molds and were regarded as wonder drugs for infectious and communicable diseases. Their medicinal significance has been widely appreciated throughout the world and are thus extensively used in the form of nutriceuticals, nutraceuticals and pharmaceuticals. The studies suggest that mushrooms are pro-biotic i.e. they help our body strengthen itself and fight off illness by maintaining physiological homeostasis by restoring the balance of the body. The compounds present in mushrooms are regarded as Host Defense Potentiators (HDPs) which have immune system enhancement properties and are currently extensively used for treatment of cancers and other malignancies and to increase general body health.

MYCORRHIZAL MUSHROOMS

Edible ecto-mycorrhizal mushrooms (EEM) comprise a specific group of edible fungal species that form symbiotic associations with their host plants (Smith and Read, 1997). This includes about 200 common edible ecto-mycorrhizal mushroom species that are widely eaten in the Northern Hemisphere, although many more, particularly in Africa and South America, have to be recorded. A few species have well established worldwide markets in excess of US$ 2 billion, while many others are locally important (Hall *et al*. 2003). EEMs are not only gourmet foods, they are also a livelihood for many people who collect the mushrooms from their natural habitats or cultivate them. *Tricholoma matsutake* (matsutake) in Japan, and truffles such as *Tuber melanosporum* (Périgord black truffle) and *Tuber magnatum* (Italian white truffle) in France and Italy, are also an important part of the culture, while others have medicinal properties (Yun and Hall, 2004). Unlike saprobic edible mushrooms, with few exceptions, the market of EEMs is supplied from what can be harvested from natural forests. Unfortunately, harvests of many edible mushrooms have declined over past century, because of changes in their natural environment caused by various natural and social factors (Hall and Wang, 2002). Despite numerous scientific publications and the establishment of thousands of hectares of plantations, the downward trend in EEM production continues. Collection of ecto-mycorrhizal and other edible mushrooms in native forests for food and recreation is a popular activity in many cultures and countries. When harvest of edible mushroom is recreational, rather a source of income, generally there is little impact on the environment. However, large-scale commercial harvesting, which generates significant income, can cause serious environmental problems in forests, especially when some commercial harvesters disrespect both the mushrooms and their forest environment. As a consequence, the management of commercially harvested areas has become a major concern. For example, *Tuber melanosporum* harvests have fallen from around 2000 tons in the 1900s to sometimes less than 100 tons annually. Similarly, current matsutake production in Japan is just 5% of 1940s harvests. The decline in the availability of EEMs and increased demand has encouraged research into developing technologies for the cultivation of EEMs as well as methods for the sustainable productivity in natural forests. Currently only *Tuber melanosporum* and *Tuber uncinatum* have been cultivated commercially, although some success has been achieved with *Lactarius deliciosus*, *Lyophyllum shimeji*, *Tuber borchii*, *Tuber formosanum*, *Rhizopogon rubescens*, *Morchella esculenta* and *Terfezialaveryi*. Host plants have also been infected under sterile conditions in the laboratory or greenhouse with *Boletus edulis*, *Cantharellus cibarius*, *Lactarius hatsutake* and *Lactarius akahatsu*, although fruiting bodies have not yet been produced in the field. However, despite many years of study, attempts to cultivate species such as *Tricholoma matsutake* and *Tuber magnatum* have met with failure (Wang and Hall, 2004). The management of plantations for better EEM production is an important issue and over the years a huge knowledge has been accumulated. However, we still do not know why some truffières consistently produce large quantities of truffles, while others produce nothing. Obviously a greater understanding of this and other species of EEMs is needed. Some technologies have been developed for the cultivation of a few EEM and management of existing EEM-producing forests to maximize their production. Unfortunately, these efforts do not seem to have had an effect on diminishing production. The exploitation of new sources of EEMs, especially in developing countries, will provide income for these countries and would find ready markets in developed countries. However, over picking has to be avoided, and conservation and management are needed if EEM are not to be damaged. Mycologists facing these issues will experience considerable challenges in years to come.

ENTOMOPATHIC FUNGI

Entomopathic fungi usually attach to the external body surface of insects in the form of microscopic spores (usually asexual). Under the right conditions of temperature and humidity, these spores germinate, grow as hyphae and colonize the insect's cuticle; eventually they bore through it and reach the insects' body cavity (hemocoel). Then, the fungal cells proliferate in the host body cavity, usually as walled hyphae or in the form of wall-less protoplasts (depending on the fungus involved). After some time the insect is usually killed and new propagules are formed in or on the insect if environmental conditions are again right. The entomopathic fungi include taxa from several of the main fungal groups and do not form a monophyletic group. Many common and important entomopathic fungi are in the order Hypocreales of the Ascomycota: the asexual (anamorph) phases *Beauveria*, *Metarhizium*, *Nomuraea*, *Paecilomyces=Isaria*, *Hirsutella* and the sexual (teleomorph) state *Cordyceps (Ophiocordyceps)*.

Cordyceps sinensis: *Cordyceps* is a fungus of subphylum Ascomycotina, class Pyrenomycetes, order Clavicipitales and family Clavicipitaceae and includes more than 300 species found worldwide. A new classification of *Cordyceps* species has been suggested on the basis of chemo-taxonomy of partial nucleotide sequence of 18S rDNA obtained from four different species. *Cordyceps* species are parasitic, mainly on insects and other arthropods. Some of these are also parasitic on other fungi like the subterranean, truffle-like *Elaphomyces* and also on spiders. The mycelium invades and eventually replaces the host tissue, while the elongated fruiting body (stroma) may be cylindrical, branched, or of complex shape. The genus has a worldwide distribution and most species have been described from Asia (notably China, Japan, Korea and Thailand). *Cordyceps* species are particularly abundant and diverse in humid temperate and tropical forests. Some *Cordyceps* species are sources of important biochemical substances like cordycepin which has very high medicinal properties. The species that parasitizes the vegetable caterpillar, *Cordyceps sinensis*, is the most famous amongst all the species of *Cordyceps* and has been considered a precious ingredient of high medicinal importance.

1. Ethno-Mycological and Traditional Uses

Cordyceps sinensis, a parasitic fungus in the alpine regions has been highly valued in Traditional Medicinal System of China, Nepal, Tibet and India. In interior mountain areas, it is also locally known as 'Yarsha Gamboo', Keera ghas' and 'Keera jhar'. The Tibetan name 'Yarsha Gamboo' means 'summer-grass winter-worm'. Tibetans believe that during winter time it lives as a 'worm' and later, as metamorphosis occurs at the start of the spring season, this worm transforms into a kind of 'grass'. Two distinct phases have been recognized during the entire transformation process. Firstly, the 'grass' starts growing form the head of the larvae. The worm at this particular stage, appears to be white, is alive and can be seen moving over the ground. The blade-like part can be seen protruding out from the head of the insect like a tiny horn. This horn like structure continues to grow further. Ultimately, the worm or the insect dies and transforms into a brownish-yellow coloured 'root' like structure of the grass.

2. The mushroom and its host

C. sinensis is an entomophagous fungus of the family Clavicipitaceae. It parasitizes a range of grass root boring caterpillars, most commonly the Thitarodes (*Hepialus armoricanus*, family Hepialidae). In all, around 40 species of *Hepialus* moth have been recognized in the Tibetan Plateau region and around 30 of these species can be infected by *C. sinensis*. The mycelium of the fungus grows in soil and colonizes the buried larvae (caterpillar) of this moth. The caterpillar becomes mummified by the growth of the mycelium and hence is given the name, "caterpillar fungus". It has been reported that *C. sinensis* has evolved and developed a special adaptation to improve chances of reproductive success. Reproduction is highly host-specific. Every single spore fragments into around 32 million propagules. These tiny propagules get attached to the larval stage of the insect. The larvae is then forced to move closer to the surface of ground (non-infected larvae will not hibernate close to the ground surface). The mycelium, which is composed of white thread like structures called hyphae, grows inside the body of the insect in the form of a cottony mesh. The hyphae fill the interior of the entire caterpillar and mummify it, leaving behind the larval exoskeleton filled with only the white mycelium of fungus. When alpine grasses start sprouting, a fruiting body develops which, surprisingly, always emerges out from the head of the caterpillar (larvae). This fruiting body is usually 5-10 cm long, brown colored and club-shaped. The propagules present on the fruiting body are dispersed by the wind and can attach to new host insects. The fruiting body resembles grass sprouting but the difference is the colour which is dark blue to black.

3. Natural Habitats

Native occurrence of this entomophagus fungus is mostly confined to the high Himalayan Mountains in Tibet, Nepal and India, at an altitude ranging from 3000 to 5000 metres. The most common occurrence of this fungus is between 3500 and 4500 metres elevation in cold and arid environment. *C. sinensis* is endemic to the Tibetan Plateau including the adjoining high altitude areas of the Central and Eastern Himalayan range (covering areas of Nepal, Bhutan and Uttarakhand, Sikkim, Himachal Pradesh and Arunachal Pradesh in India). It is found in the high altitudes of Pithoragarh, Uttarakhand (Bhatt, 2012) and other provinces at locations above 7000 feet. It is also common in the grasslands and shrub lands of the Tibetan Plateau including west Sichuan, North Yunnan and major areas of Qinghai and West Gansu. The distribution of *C. sinensis* is limited to those areas where the average annual precipitation is above the range 350-400mm. This caterpillar fungus thrives very well in sub-alpine and alpine

grasslands or meadows and in open dwarf shrub lands. Extensive research has been conducted on the ecology, collection, utilization, trade route, management and significance and species diversity of *C. sinensis* in various parts of India, Tibet, Michigan, Bhutan, China and Korea.

4. Collection and trade of *C. sinensis*

Years back this fungus was collected and traded from Tibet to China in exchange for tea and other commercial goods like silk, grains, etc. The most appropriate time for harvesting of this fungus starts with the arrival of the spring season, in about starting of May. Local villagers and nomads search for the fungus in the grasslands and shrub lands. But the harvesting is slightly a difficult process as only the stroma or the grass-like part of the fungus is visible over the surface which too is quite short, not longer than 2-5cm and has to be lifted out with the help of a sharp knife. Extreme care has to be taken while pulling out the fungus from the ground surface because if the stroma breaks off from the head of the larvae, it directly affects the commercial value of the mushroom resulting in a decline in its market rate.

During the harvesting season, all other activities come to a standstill as everyone is focused on gathering more and more of the fruiting bodies. This has often led to many blooded wars in these areas. The Ministry of Population and Environment, Govt. of Nepal has banned its collection, trade and transportation. However, in Tibet it is an open trade. In some areas of Tibet, even the schools announce vacations for 15-20 day in late May so that the students can also help in the collection and harvesting of this precious mushroom. The daily collection may vary from 250g to as much as 5kg.

During recent years, caterpillar mushroom has emerged out be an important cash crop traded on a large scale and a new source of income in the rural areas in the higher altitude regions usually above 3000-5000 metres. In the river valley of Gori Ganga, India alone, the number of fungus gatherers at alpine habitats has increased about four-fold since the year 2000. During 2002, nearly 900 persons went to seven different alpine habitats in search of the fungus (average 128 persons per habitat) and collected about 200 g of the fungus per day. High price of the fungus very often make the transaction secretive in the local market while due to cross-boundary trade between countries, the rest of the trade is under the surface (Sharma, 2004). Cost of the fungus at the final destination (brokers in national and international markets) was much higher than the price paid to the field gatherers. It is believed that in the International market the fungus may fetch a price between one and two million ₹ per kg (US$ 20,000-40,000). The amount paid varies among the trade channels which start from the wild material gatherers in the field, then to the brokers and agents who collect the dried material from the various locations and sell it at a higher price. However, rapid and immediate marketing of this fungus is not required as the fungus is usually sold and consumed in a dried form. Its small size and easy storage conditions make the transportation much easier.

The fruiting bodies of the *Cordyceps sinensis* were collected from a high altitude (3500-4500m) at different locations viz. Nahardevi, Martoli, Ralam, Laspa, Milam, Chetribugyal, Chiplakot, Malpa Top, Tola Top, Burphu and Panchachuli in district Pithoragarh, Uttarakhand, India (Singh *et al*., 2009). The ascocarps were 4.0-7.0 cm in length, mostly erect, slightly swollen at tip; emerged single, double or triple from the head of larvae. Caterpillar cadaver varied 3.0-4.0 cm in size, having eight pairs of legs with fine transverse wrinkles.

5. Composition of *Cordyceps sinensis*

The pharmacological and medicinal significance of *Cordyceps sinensis* is mainly due to its bioactive ingredients. The composition properties and structure-activity relationship of these components have been under extensive scrutiny by many researchers and have eventually been rediscovered and modified with respect to the present scenario of disease and abnormalities. It contains a wide variety of potentially important constituents, including polysaccharides, ophiocordin (an antibiotic compound), cordycepin, cordypyridones, nucleosides, bioxanthracenes, sterols, alkenoic acids and exopolymers, etc. The constituents of *C. sinensis* were thoroughly studied and a crystalline substance Cordycepic acid was isolated and identified, which was later identified as D-mannitol. Cordycepin and cordycepic acid are regarded as the most important constituents of this fungus and owe high medicinal significance.

6. Medicinal uses of *Cordyceps sinensis*

In the recent past, a variety of medicinal preparations in the form of tablets, capsules and extracts from mushrooms for the treatment of various kinds of ailments, diseases and disorders have been produced and marketed. In 1991, the value of world mushroom crop was estimated to be around 8.5 billion dollars and in the same year 1.2 billion dollars were estimated to have been generated from medicinal products from various medicinal mushrooms. Herbal medicinal preparations from different mushrooms have become a growing business in various parts of the world as for instance, Bhutan is an emerging market for *Cordyceps* and its usage has shown a tremendous increase since last few years.There are data of clinical trials that support the efficacy of *C. sinensis* as a medicinal herb, especially for disorders related to the liver, kidney and immune system. A number of studies indicate that *C. sinensis* (and also its mycelial extract) possess certain anti-cancer, anti-metastatic and immuno-stimulating properties. It is also reported to have anti-oxidant activity (Singh *et al.*, 2007).

Since ages, *C. sinensis* has been regarded as panacea of life, imparting youth, vigor and longevity. Other important functions include activation of the immune responses, controlling the blood sugar levels, treatment of Hepatitis B, improvement of the respiratory functions, improvement in the functioning of the heart, maintaining the levels of cholesterol, reduction of the tumor size in cancer patients**,** protection against free radical damage**,** reduction of fatigue, combats sexual dysfunction, helping in organ transplantation, improvement in the functioning of kidney and adrenal gland etc. (Singh *et al.*, 2008a).

7. Morphological and Physiological characteristics

Isolation of the fungus was done using fresh ascocarp along with larva cadaver on Sabouraud's dextrose agar with yeast extract (SDAY) medium. The hyphe of the fungus was aerial cottony white to creamish or yellowish, septate, branched, dense and 1-3 µm wide. Colony was found initially white and later on pink red or orange and reverse cream to purplish red in colour on potato dextrose agar (PDA) medium; initially cream with lined depressions, later dark orange and from reverse dark tan in colour on sabouraud's dextrose agar with yeast extract (SDAY) medium; initially light pink which changed to purplish red and from reverse blood red colour on malt extract agar (MEA); initially creamish yellow, later light purple and dark tan colour from reverse on oat meal agar (OMA) and initially light yellow with purplish red margin, finally dark purplish red and dark tan colour in reverse on czapek dextrose agar (CDA) medium. *C. sinensis* showed the maximum and minimum growth on 12th day (44.93 and 32.60mm) on SDAY and CDA media, respectively (Arora, 2008). The fungus also showed maximum (44.87mm) and minimum growth (34.27mm) at 15 ^{0}C and 25 ^{0}C, respectively and it declined below and above 15 ^{0}C. The maximum growth (44.93mm) of the fungus was reported to be at pH 6.

Addition of carbon sources significantly affects the mycelial growth of *C. sinensis* (Arora, 2008). Disaccharides (sucrose, maltose and lactose) produced a higher mycelial growth as compared to the monosaccharides (glucose and fructose). Amongst the carbon sources tested, sucrose gave a maximum dry weight of the mycelia (5.15 g/l) followed by maltose and lactose while check produced a minimum dry weight (2.83 g/l) (Table 1).

Vitamins played the significant role in the mycelial growth of *C. sinensis*. Folic acid (12.42 g/l) out yielded all the vitamins tested, while the pyridoxine produces the minimum (5.39 g/l) which was even lower than that of check. The D-biotin, thiamine and nicotinic acid were at par in terms of yield and next to folic acid. However, riboflavin was found to be at par with the check. Micro and macro elements (minerals) produced significantly higher mycelial yield as compared to that of check. Addition of micro elements was more effective in terms of mycelial dry weight per litre as compared to macro elements.Amongst the micro and macro elements the highest yield was observed in case of $ZnCl_2$ (7.87 g/l) and $CaCl_2$. $2H_2O$ (5.55 g/l), respectively (Table 2).

Table 1. Effect of the carbon and nitrogen sources on the mycelial growth of *C. sinensis*

C-sources	Dry wt. (g/l)	N-sources	Dry wt. (g/l)
Monosaccharides		**Inorganic**	
Glucose	3.74	Potassium nitrate	3.33
Fructose	3.18	Sodium nitrate	2.75
Disaccharides		Ammonium nitrate	2.52
Sucrose	5.15	Ammonium chloride	2.30
Maltose	4.72	Ammonium sulphate	2.16
Lactose	4.25	Urea	1.67
Check (no carbon)	2.83	**Organic**	
—	—	Beef extract	5.24
—	—	Check (no nitrogen)	1.85
CD at 5%	0.26	CD at 5%	0.31

Table 2. Effect of the vitamins and minerals on the mycelial growth of *C. sinensis*

Vitamins	Dry wt. g/l	Minerals	Dry wt. (g/l)
Folic acid	12.42	**Micro**	
D-biotin	9.40	$CuSO_4.5H_2O$	6.60
Thiamine	8.88	$FeSO_4.7H_2O$	7.13
Nicotinic acid	8.94	$MnCl_2.4H_2O$	6.20
Riboflavin	7.66	$ZnCl_2$	7.87
Pyridoxine	5.39	**Macro**	
Check (SDY)	7.42	$CaCl_2.2H_2O$	5.55
—	—	KH_2PO_4	4.50
—	—	K_2HPO_4	4.67
—	—	$MgSO_4.7H_2O$	4.20
—	—	NaCl	4.87
—	—	Check (no mineral)	3.87
CD at 5%	0.72	CD at 5%	0.13

8. Nutritional and Medicinal characteristics

The fungus was multiplied using different cereal grains viz. wheat, rice and maize grains. It was observed that the wheat grains were colonized by the fungus in minimum period of 25 days while on maize grains took 39 days maximum and on rice 31 days for colonization. Maximum (6.48 g) and minimum (2.36 g) weight loss was observed in wheat and maize grains, respectively. Whereas, the weight loss in case of rice was 4.65 g. It showed that the fungus grew well and nutritional utilization of the fungus from wheat and rice grains was higher.

Arora and Singh (2009) reported much higher amount of sugar in the mycelia in SDY broth (63.1%) as compared to that in the fruiting bodies (24.2%) while protein content was higher in fruiting bodies (28.6%) as compared to the mycelia (8.2%). The lipid content was 3.15% in fruiting bodies and 2.95% in the mycelium of *C. sinensis* (Table 3).

Table 3. Total soluble sugars, proteins and lipids in fruiting bodies and mycelium

Source	Content %		
	Total soluble sugar	**Protein**	**Lipid**
Fruiting body	24.2	28.60	3.15
Mycelium	63.10	8.2	2.95

Detection of adenosine, cordycepin and ergosterol from fruiting bodies and mycelia of *C. sinensis* by HPLC analysis are depicted in Figs. 2,3,4,5 and 6. Spectrum was taken at ë max-254 nm and the retention times were noted for the standard- adenosine and cordycepin from 50 ppm to 100 ppm. Retention time of adenosine and cordycepin were found to be 6.895 and 7.611 minute, respectively (Fig. 1). While the hot water extract of fruiting bodies were found to contain both adenosine and cordycepin (Fig. 2) but in case of *C.sinensis* mycelia, adenosine was detected while cordycepin was undetected (Fig. 3). The retention time of standard ergosterol was around 19.194 minute (Arora *et al.*, 2008) and its presence in fruiting bodies and in the mycelia of *C. sinensis* was confirmed (Figs. 4, 5 & 6) .

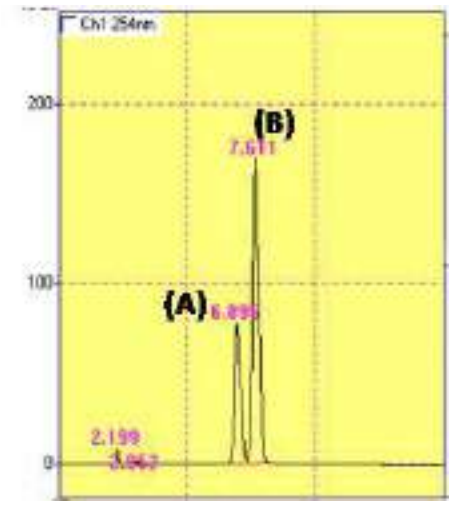

Figure 1. Chromatogram of standard adenosine (A) and cordycepin (B) at 100ppm

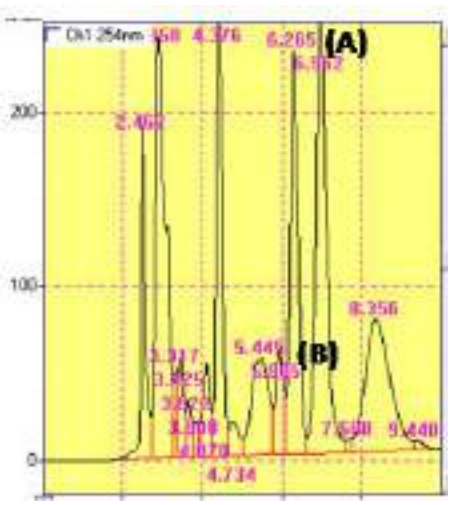

Figure 2. Chromatogram of purified extract of fruiting bodies of *C. sinensis* showing retention time were 6.895 and 7.611 min

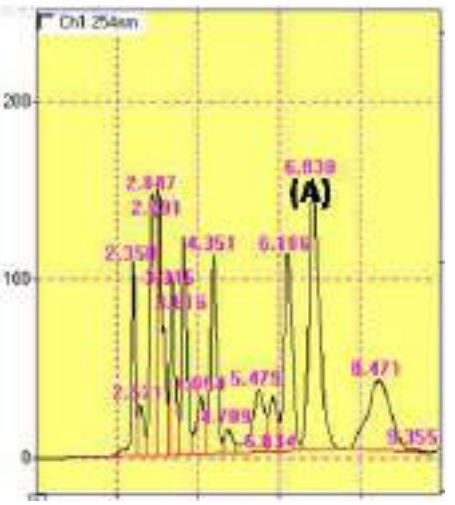

Figure 3. Chromatogram of mycelium of *C. sinensis* showing adenosine (A) adenosine (A) and cordycepin (B)

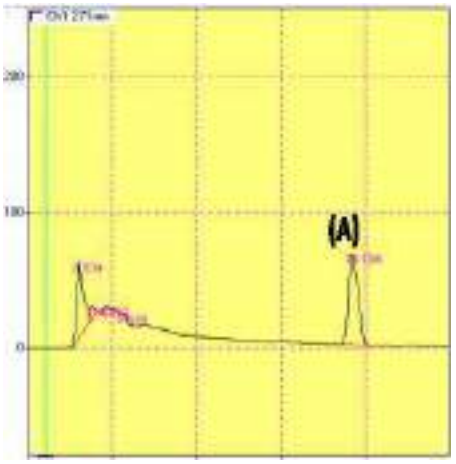

Figure 4. Chromatogram of standard-ergosterol (A) at 100ppm. Retention time was 19.194

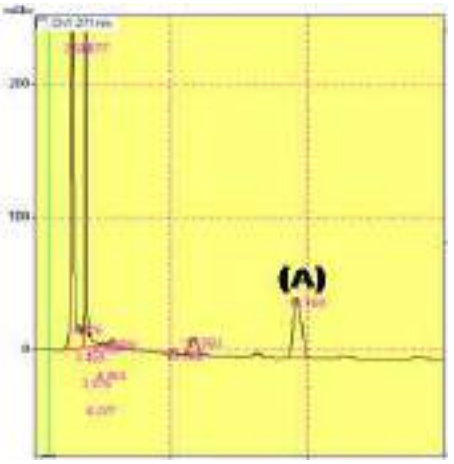

Figure 5. Chromatogram of purified extract of fruiting bodies of *C. sinensis* showing ergosterol (A)

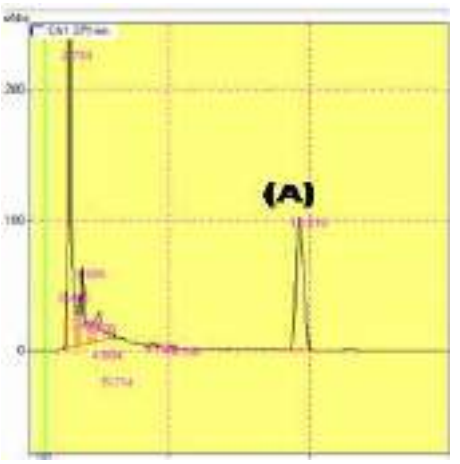

Figure 6. Chromatogram of mycelium of *C. sinensis* showing ergosterol (A)

Singh *et al*. (2007) determined anti-oxidative properties like reducing power and chelating activity on Fe^{2+} from the ethanolic extracts of *G. lucidum* and *C. sinensis* and reported that reducing power of *G. lucidum* and *C. sinensis* extracts increased with an increase in concentration and was 2.50 and 1.97 at 1.0 mg/ml, respectively (Fig .7). At the same concentration *G. lucidum* howed higher reducing power than *C. sinensis.* In addition, the reducing power of both *G. lucidum* and *C. sinensis* extracts were higher than that of L-ascorbic acid (0.71).

The *G. lucidum* and *C. sinensis* extracts showed chelating activity on Fe^{2+} in a concentration dependent manner (Fig. 8). The *G. lucidum* extract exhibited higher chelating activity on Fe^{+2} than *C. sinensis* extract. However, chelating activity of *G. lucidum* and *C. sinensis* extracts with concentration of 1.0 mg/ml (47.2 and 41.6%) was higher than that of EDTA (34.9%) and acetic acid (30.9%). The authors report that *G. lucidum* showed higher anti-oxidative properties than *C. sinensis*, probably due to presence of different compounds in the fruiting bodies.

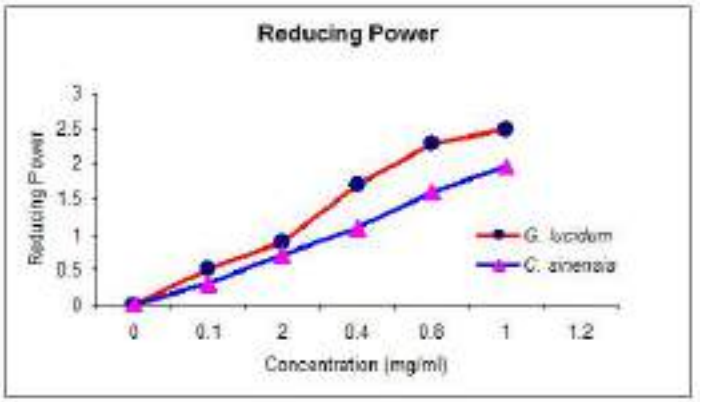

Figure 7. Reducing power of *G. lucidum* and *C. sinensis*

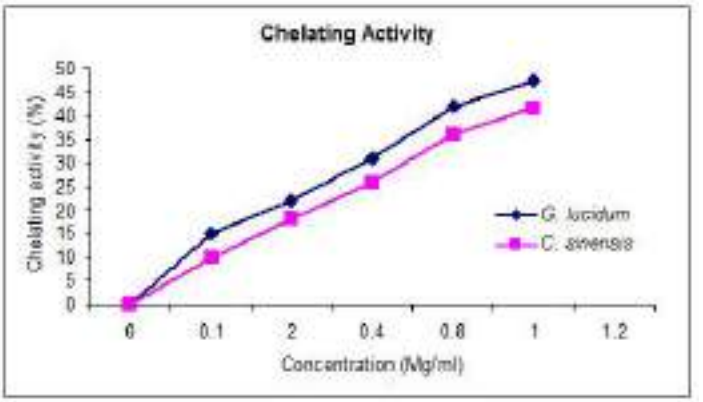

Figure 8. Chelating activity of *G. lucidum* and *C. sinensis* on Fe^{2+}

NOVEL MUSHROOMS

Edible and medicinal mushrooms can produce a variety of biologically active compounds and can be therefore described as a novel class of nutraceuticals which are widely used as dietary supplements (Jiang and Sliva, 2010). Recent epidemiological studies from Asia demonstrated that mushroom intake protects against cancer, specifically gastrointestinal (GI) cancer and breast cancer. The anticancer activities of mushrooms were mainly linked to the modulation of the immune system by branched polysaccharides (glucans), glycoproteins or peptide/protein-bound polysaccharides. Moreover, mushrooms contain minerals, vitamins (e.g., thiamin, riboflavin, ascorbic acid, and vitamin D), amino acids, and other organic compounds. Some of these natural mushroom compounds demonstrated specific activity against aberrantly activated signaling pathways in cancer cells and were able to modulate specific molecular targets in the cell function including cell proliferation, cell survival and angiogenesis. Mycophyto Complex (MC) is a dietary supplement consisting of a mixture of six varieties of mushroom mycelia, including *Agaricus blazei*, *Cordyceps sinensis*, *Coriolus versicolor*, *Ganoderma lucidum*, *Grifola frondosa* and *Polyporusum bellatus* and additional β-1,3-glucan isolated from the yeast, *Saccharomyces cerevisiae*. Interestingly, these specific mushrooms have been linked to different health promoting or disease preventing functions.

1. ***Agaricus blazei:*** *A. blazei* is traditionally believed to treat many common diseases like atherosclerosis, hepatitis, hyperlipidemia, diabetes, dermatitis and cancer (Firenzuoli *et al*., 2008). The polysaccharide phytocomplex was suggested to be responsible for the immuno-stimulant and anti-tumor properties of *A. blazei*, probably through an opsonizing biochemical pathway. In addition, recent studies demonstrated anticancer activities of *A. blazei* through the induction of apoptosis by the activation of caspase-3 in prostate cancer cells and inhibition of constitutively active NF-κB in leukemic cells. Moreover, *A. blazei* demonstrated anti-metastatic effect through the inhibition of MMP-9 in melanoma cells.

2. ***Coriolus versicolor:*** *C. versicolor* is an obligate aerobe that is commonly found year-round on logs, stumps, tree trunks, and branches. The fungus occurs throughout the wooded temperate zones of Asia, Europe and North America and may be the most common shelf fungus in the Northern Hemisphere. *C. versicolor* contains biologically active, structurally different protein-bound polysaccharide-K (PSK) and polysaccharopeptide (PSP), which were approved in Asia for immunotherapy or as biological response modifiers (BRMs) (Cui and Chisti, 2003). In addition to the immuno-modulatory activity, extracts of *C. versicolor* demonstrated direct effects on a variety of cancer cells. Therefore, *C. versicolor* induced apoptosis of breast cancer cells through p53 and Bcl-2 dependent and independent mechanisms suppressed cell proliferation and induced apoptosis of leukemia cells by mechanisms including inhibition of transcription factor NF-κB and down-regulation of expression of COX-2. Moreover, direct cytotoxic effect of PSK, through the cell cycle arrest at G0/G1 phase and induction of apoptosis associated with the caspase-3 expression, was reported in various tumor cell lines derived from leukemia, melanomas, fibrosarcomas and cervix, lung, pancreas and gastric cancers.

3. ***Ganoderma lucidum:*** *Ganoderma* is one of the members of Ganodermataceae family systematized under the order Aphyllophorales of phylum Basidiomycota. The fungus has very large climatic diversity and occurs in natural habitat of almost all around world from temperate to tropical regions. Of the 250 known species of *Ganoderma*, several species are used for medicinal purposes. Fruiting bodies of the *G. lucidum* contain polysaccharides, triterpenoids, adenosine, germanium, protein (LZ-8), amino acids etc., and found to have anti tumor and immuno-modulating effect (Singh *et al.,* 2007 and 2008b). Because of its perceived health benefits this species has gained wide popularity, not only in China and Japan but also in Korea, North America, Malaysia, India and other parts of the world. A number of products prepared from *G. lucidum* are sold throughout the world as dietary supplements. The estimated global turnover of *G. lucidum* products was approximately $2.16 billion (Lai *et al.,* 2004).

   Uttarakhand a hill state of India is gifted with a rich medicinal mushroom flora that includes *Ganoderma* (Singh *et al.*, 2007). The fungus occurs in natural habitat of almost all parts of the zone A and B of the state spread over 100-1500m in height. *Ganoderma* is composed of a vast number of bioactive compounds of pharmacological activities appear to be triterpenes, polysaccharides and adenosines. *G. lucidum* isolated from different altitudes in Uttarakhand, India were designated as GL-1, GL-2, GL-3, GL-4, GL-5, GL-6, GL-7, GL-8, GL-9 and GL-10.

Table 4. Yield performance of isolates over different substrate

Sl. No.	Isolate	Av. yield 'g'/500g dry substrate								
		WS	WS+ RB 5%	BE%	SD	SD+ RB 5%	BE%	CP	CP+ RB 5%	BE%
1	GL-1	103.00	111.00	22.20	64.0	81.10	16.2	27.50	38.50	7.70
2	GL-2	93.00	93.00	18.60	56.5	71.00	14.2	24.50	31.25	6.25
3	GL-3	75.25	85.60	17.12	43.0	52.75	10.5	24.00	32.33	6.46
4	GL-4	71.50	79.25	15.85	33.2	54.50	10.9	20.50	35.00	7.00
5	GL-5	67.50	75.60	15.12	37.1	57.66	11.5	18.25	21.50	4.30
6	GL-6	55.10	74.10	14.82	33.3	51.20	10.2	15.75	21.50	4.30
7	GL-7	60.31	71.25	14.25	31.0	47.00	9.40	14.20	25.25	5.05
8	GL-8	68.80	71.00	14.20	40.0	46.20	9.20	14.50	21.00	4.20
9	GL-9	61.20	69.50	13.90	32.2	45.10	9.02	15.00	19.10	3.82
10	GL-10	39.50	42.25	8.45	27.0	31.50	6.30	19.00	29.33	5.86

RB = Rice bran; WS = Wheat straw; SD= sawdust; CP= Coir pith

CD at 5% ; Isolate a= 2.40; Substrate b= 2.82 and Interaction a x b=5.12

The results revealed that all the substrate, either alone or with 5% rice bran resulted in the development of fruit bodies of all the isolates of *G. lucidum* and all the substrate varied significantly from each other in terms of yield (Table 4). Wheat straw supplemented with 5% rice bran out yielded all other substrate. However, the lowest biological efficiency was obtained on coir pith alone or with rice bran. Among the isolates, the isolates GL-1 GL-2 and GL-3 gave significantly higher yields 111.00g, 93.00g, 85.60g/500g dry WS substrate, respectively though, the yield varied significantly from each other (Singh *et al.*, 2009). Mishra and Singh (2008) reported wheat straw as a better substrate as compared to sawdust which is widely used for cultivation of *G. lucidum.*

Mishra and Singh (2010) reported that total soluble and non reducing sugar content varied from 14.58–14.88 and 14.55-14.80%, respectively among the different wild isolates of the fungus. However, protein content varied from 21.0-27.0% in wild and their cultivated isolates. The wild and their cultivated isolates showed presence of ganoderic acids-A, C & H. These ganoderic acids were higher in wild isolates as compared to their cultivated isolates (Singh *et al.*, 2012). Wild isolates of *G. lucidum* showed higher content of glucosaminoglycan (Singh *et al.*, 2008b), however, the genetic makeup of the wild and cultivated isolates was the same.

4. ***Grifola frondosa:*** *G. frondosa* is a popular culinary mushroom originally recognized in Japan and Korea. The anticancer activities of *G. frondosa* were originally described more than 30 years ago and are associated with the presence of 1,3-ß glucan named grifolan LE (Kodama *et al.*, 2002). 1,3-branched-1,6-ß-glucan isolated from *G. frondosa*, was associated with the stimulation of natural killer (NK) cells and activation of macrophages and differentiation of T-cells. The direct anticancer effect was demonstrated on the gastric cancer cells by the induction of apoptosis via caspase-3-dependent and independent pathways.

CONCLUSION

Nature is the source of all the raw materials that we need. About 2–3 decades ago, most of the drugs were of herbal origin. A variety of reasons under pin why people like to use natural medicines as it is evident that patients are getting even more distressed after using chemically synthesized drugs, rather than natural means like medicinal mushrooms that can conquer life claiming diseases, leaving no side effects on human health. To maintain proper growth, the pharmaceutical industries need innovation and access to high output rate on low-cost materials with reasonable safety. The combination of modern chemistry with bio-based starting materials, like, bio-metabolites, offers the scope for revolutionizing mushroom based pharmaceutical industries. In the near future, bio-metabolites (cordycepin, polysaccharides, ganoderic acids etc.) extracted from medicinal mushrooms will have a role that compares with that of oil and gas crackers today. Therefore, bio-metabolites of medicinal mushrooms will be the key future driving force in the realm of green pharmacology and pharmacognosy.

REFERENCES

[1] Arora RK. (2008). Collection, isolation and characterization of caterpillar mushroom-*Cordyceps sinensis* (Berk. Sacc.) of Uttarakhand. Ph.D. thesis, G.B. Pant University of Agriculture and Technology, Pantnagar.

[2] Arora RK *et al.* (2008). Determination of bio-active compounds in medicinal mushroom, *Cordyceps sinensis. Mushr. Res.* 17: 51-98.

[3] Arora RK and Singh RP. (2009). Biochemical characterization of caterpillar mushroom (*Cordyceps sinensis*). *Mushr. Res*. 18: 39-41.

[4] Bhatt N. (2012). Economic implications of a fungus *Cordyceps sinensis* on scheduled tribes of high altitutes of Pithoragarh, Uttarakhand, India. *Mushr. Res*. 21: 43-47.

[5] Cui J and Chisti Y. (2003). Polysaccharopeptides of *Coriolus versicolor* physiological activity, uses, and production. *Biotechnol. Adv.* 21: 109-122.

[6] Firenzuoli F *et al.* (2008). The medicinal mushroom *Agaricus blazei* Murrill: review of literature and pharmacotoxicological problems. *Evid. Based Complement Alternat. Med.* 5:3-15.

[7] Hall IR *et al.* (2003). Edible and poisonous mushrooms of the world. Timber Press, Portland, Oreg.

[8] Hall IR and Wang Y. (2002). Truffles and other edible mycorrhizal mushrooms—some new crops for the Southern Hemisphere. Edible mycorrhizal mushrooms and their cultivation. *In:* Proceedings of the 2nd International Conference on Edible Mycorrhizal

Mushrooms, Christchurch, New Zealand, 3–6 July 2001. I.R. Hall, Y. Wang, E. Danell, and A. Zambonelli (Eds). New Zealand Institute for Crop & Food Research Limited, Christchurch.

[9] Jiang J and Sliva D. (2010). Novel medicinal mushroom blend suppresses growth and invasiveness of human breast cancer cells. *Int. J. Onco*. 37:1529-1536.

[10] Kodama N *et al*. (2002). Effects of D Fraction, a polysaccharide from *Grifola frondosa* on tumor growth involve activation of NK cells. *Biol. Pharm. Bull*.25:1647-1650.

[11] Lai T *et al*. (2004). Global marketing of medicinal Ling Zhi mushroom, *Ganoderma lucidum* (W. Curt.:Fr.) Lloyd (Aphyllophoromycetedeae) products and safety concerns. *Int. J. Med. Mushr.* 6:189-194.

[12] Mishra KK and Singh RP. (2010). Cultural and biochemical variability amongst indigenous *Ganoderma lucidum* (Reishi mushroom) isolates from Uttarakhand. *Mushr. Res*. 19 (2): 74-81.

[13] Mishra KK and Singh RP. (2008). Evaluation of substrate for production of *Ganoderma lucidum* (W. Curt.: Fr.) P. Karst (Aphyllophoromycetideae). *Int. J. Med. Mushr.* 10(4): 379-383.

[14] Sharma S. (2004). Trade of *Cordyceps sinensis* from high altitudes of the Indian Himalaya: conservation and biotechnological priorities. *Curr. Sci*. 86: 1614–1619.

[15] Singh RP *et al*. (2007). Anti-oxidative properties of *Ganoderma lucidum* and *Cordyceps sinensis*. *Mushr. Res*. 16:19-22.

[16] Singh RP *et al*. (2009). *Ganoderma lucidum*: Their collection, characterization, cultivation and medicinal componenets. *In:* Proceedings of the 5th International medicinal mushroom conference. Sept. 5-8, Nantong, China. pp. 571-578.

[17] Singh RP *et al*. (2009). *Cordyceps sinensis*: Their collection, characterization and medicinal componenets. *In:* Proceedings of the 5th International medicinal mushroom conference. Sept. 5-8, Nantong, China. pp. 661-670.

[18] Singh RP *et al*. (2012). Medicinally important terpenoids of *Ganoderma lucidum* isolated from Uttarakhand, India. *Mushr. Res*. 21: 79-81.

[19] Singh RP *et al*. (2008a). Caterpillar fungus (*Cordyceps sinensis*): A review. *J. Ecofriendly Agri*. 3(1): 1-15.

[20] Singh RP *et al*. (2008b). Extraction and quantification of glucosaminoglycan from *Ganoderma lucidum*. *J. Ecofriendly Agri*. 3(2): 180-182.

[21] Smith S and Read D. (1997). Mycorrhizal symbiosis. 2nd ed. Academic Press, London.

[22] Wang Y and Hall IR. (2004). Matsutake—a natural biofertilizer? *In:* Handbook of microbial fertilizers. M. Raid (Ed.). Haworth Press, Binghamton, N.Y.

[23] Yun W and Hall IR. (2004). Edible ectomycorrhizal mushrooms: challenges and achievements. *Can. J. Bot*. 82: 1063-1073.

ROLE OF COPPER ON GROWTH, DEVELOPMENT AND PHYSIOLOGICAL EFFECT OF *PLEUROTUS NEBRODENSIS*

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ABSTRACT

Pleurotus nebrodensis is an important rare edible fungus with independent intellectual property rights in China, has commercialized and large scale culture firstly realized in 1990s in Beijing, is a widely popular product in the market, and has current yearly yield over 200,000 tonnes. This paper focuses on influence of copper on growth and development of *P. nebrodensis* and on activities of extracellular *laccase* and manganese peroxidase; and meantime, and change of flow rate of H^+ and Ca^{2+} on mycelia surface under the action of copper is determined, to make physiological effect of copper clear. It is shown by research result that, copper supplemented substrate can obviously promote growth rate (4.35 mm/d) of *P. nebrodensis* mycelia at a content of 50 mg/kg, significantly different with that of the control group. At copper content of 50 mg/kg, growth cycle and biological efficiency of *P. nebrodensis* are 166.22 days and 50.66%, prolonged by 16 d and improved by 4.61%, respectively. In growth period of *P. nebrodensis* mycelia, laccase has high activity, and even higher (significantly different with that of the control group) when the copper content in the substrate is 25-50 mg/kg, but has activity decreased when the copper content is over 50 mg/kg. In growth period of *P. nebrodensis* fruiting body, manganese peroxidase has activity higher than that in growth period of mycelia, and has highest activity at copper content of 50 mg/kg, indicating certain correlation with yield. The content of copper in the substrate can change flow direction of extracellular H^+ and Ca^{2+} on mycelia surface. All these results will provide scientific basis for culture techniques and theory of *Pleurotus nebrodensis*.

Keywords: *Pleurotus nebrodensis*, mycelial growth, yield, enzyme activity, ion flow

INTRODUCTION

Pleurotus nebrodensis belong to *Pleurotus*, Pleurotaceae, Agaricales, Basidiomyceta. It is a rare edible fungus, is called Bailinggu or Aweigu in China [1]. *P. Nebrodensis*was firstly described and recorded by Inzengae in 1863, and names as *Agaricus nebrodensis*, and was renamed as *P. nebrodensis* (Inzengae Quelet) in 1886. Recent researches indicate that, *P. nebrodensis* in China is an independently evolving branch of *Pleurotus eryngii* and should be named as *P. eryngii* var. *tuoliensis* [2-3].

P. nebrodensis was successfully cultivated in China in 1983 for the first time, and is popular in the market as the fruiting body is large, white, tender and delicious and rich in nutrition. Meanwhile, research on medicinal value of *P. nebrodensis* has attracted increasing attention [4]. At present, artificial culture techniques of *P. nebrodensis*are generalized in most area of northern China, and a good deal of culture experience has been obtained. Despite the great market value of *P. nebrodensis* products, low unit production and long culture period increases production risk and restricts the production and development of *P. nebrodensis.* In recent years, researches on *P. nebrodensis* mostly focus on strain identification [5-8], culture substrate selection and the like, and research reports on micronutrient element utilization and physiology are few. Microelement plays a very important role in substance and energy metabolism in growth and development process of edible fungi, not only is the constituent of cell structure and multiple enzymes and enzyme activator, but also participates in biochemical reaction in cells. Microelement can promote growth of fungi in *Pleurotus*. For example, manganese element supplemented in culture substrate can promote mycelial growth and increase yield and can also promote lignin degradation [9]. Beelman [10] found that accumulation of Cu can improve yield and quality of *A. bisporus*, and increase of Cu content in basidiomycete may increase activity of tyrosinase and cause browning of fruiting body. There is a significant difference in inherent levels of Cu in different strains of basidiomycete, but *P. nebrodensis* does not accumulate Cu and disease resistanceis not correlated with Cu content inbasidiomycete [11].

In the present research, divalent Cu (in the form of copper salt) at different concentrations was added into conventional culture medium for *P. nebrodensis*, activities of manganese peroxidase and laccase in the whole growth cycle were determined, differences between enzyme activities and yields in different culture media were compared, and change of flow rate of H^+ and Ca^{2+} on mycelia surface was determined by NMT, to make effect of copper on growth and development of *P. nebrodensis*clear, to find optimal Cu content for growth and development, to learn physiological effect of Cu, and to provide scientific basis for improvement in culture medium, promotion in growth, increment in yield and establishment of culture techniques and theory of *P. nebrodensis*.

MATERIALS AND METHODS

Experiment strain

Pleurotus nebrodensis 00485 provided by China Center for Mushroom Spawn Standards and Control ((CCMSSC).

Main reagents and apparatuses

$CuSO_4 \cdot 5H_2O$ and H_2O_2 (analytically pure), HAC-NaAC buffer solutions (0.1 mol/l acetic acid-sodium acetate, self made) with pH 4.6, pH 5.0 and pH 5.8, ABTS (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma).

UV-2800H ultraviolet spectrophotometer (Unico (Shanghai,China) Instrument Co., Ltd) and LK-S3D thermostat water bath (Beijing Likang Company, China).

Experimental Methods

Spwan: Culture of stock strain was cultured on potato dextrose agar (PDA) in an incubator at 25±1°C for 12 days. Culture process of mother spawn included preparing culture medium from wheat 1000 g, gypsum 10 g and lime 3 g, sterilizing by conventional method, bagging, inoculating by aseptic operation, and culturing in an incubator at 25±1°C for 15 days. Liquid medium was made using the following ingredients: peeled potato (200g), dextrose(20g), peptone (3g) and warm tap water (1200ml), natural pH. The liquid spawn substrate was autoclaved for 45min at 121°C, cooled and inoculated with mycelial plugs from PDA (5 mm diameter; 5plugs/flask). Shake culture was performed at rotation speed 130/min, 22°C in dark for 13 days. The substrate standard formulation for production substrate (63% moisture) included cotton seed hulls (80% d.w.), bran(15%), corn flour (3%), calcium carbonate (1%) and calcium sulfate (1%).

Experiments of liquid culture at different copper contents: The liquid medium was added with copper ion (copper sulfate) at different concentrations, loaded in 1000 ml triangular flasks at an amount of 500 ml, sterilized and cooled, and inoculated with *P. nebrodensis* 00485. Addition of Cu^{2+} content is shown in Table 1. Mycelia were respectively collected by filtering the fermentation liquid, oven dried at 45°C and weighed. Dry weights of mycelia from different flasks were compared. The experiment was completely randomized with 5 replicates per treatment.

Table 1. Design of additive content of Cu in experiment of liquid culture

Treatments	CK	A1	A2	A3	A4	A5	A6	A7	A8
Cu^{2+}(mg/l)	0	50	100	150	200	250	300	350	400
CuSO4·5H2O (mg/l)	195.32	390.64	585.96	783.28	978.6	1173.92	1369.24	1566.56	195.32

Experiments of production at different copper contents: The materials of the culture substrate were mixed and added with Cu^{2+} solution with different Cu^{2+} contents according to Table 2, and bagged in 17 cm×33 cm×0.05 cm high pressure polypropylene plastic bags at a amount of 350 g, with 45 bags for each Cu^{2+} content. The bagged substrate was sterilized at high pressure at 121°C by conventional method, cooled, and inoculated with mother spawn by aseptic operation. The culture and fruiting management was performed according to the literature [12].

Table 2. Design of additive content of Cu in experiment of production

Treatments	CK	C1	C2	C3	C4
Cu^{2+}(mg/kg)	0	25	50	100	150
CuSO4·5H2O (mg/l)	0	97.66	195.32	390.64	585.96

Mycelia growth rate, bagful time, low temperature stimulation time and harvesting date were recorded during the experiment. Average growth rate was calculated by randomly selecting 5 substrate bags from each formulation, marking mycelia growing height by drawing a line on the bag 10 days after inoculation, and marking once every 5 days for 3 times in total. Bud appearing time was recorded when the first fruiting bud appeared. Harvesting time was recorded as the number of days from the beginning of harvesting and terminal of harvesting. The weight of each fruiting body was recorded during the harvesting. The biological efficiency was calculated based on fresh weight of fruiting body from the first harvesting.

Biological efficiency = (fresh weight of fruiting body/dry weight of culture substrate)×100%.

Enzyme activity determination: Sample was collected 20 days after inoculation, and once every 15 days. Sample was collected 2 cm below inoculation surface in elevation direction. Extraction of extracellular enzyme was performed by weighing frozen sample 20.0 g with an analytical balance, placing in a 200 ml beaker, adding distilled water 100 ml, extracting in thermostat water bath at 30 °C for 1 h, filtering with two layers of filter paper, collecting filtrate as crude enzyme solution, and setting the volume at 100 ml in a volumetric flask.

Determination of laccase (Lac) activity was performed according to Robert Bourbonnais method, which was modified according to the speed of the laccase oxidizing ABTS. The reaction was performed at 25 °C in a 5 ml system containing 2 mL of 0.l mol/l sodium acetate buffer solution (pH 5.0) containing 0.5 mmol/l ABTS, 2.9 ml of distilled water, and 0.1 ml of crude enzyme solution for 4 min. Absorbance at 420 nm was detected. The enzyme unit is defined as the amount of the enzyme that increases absorbance per minute by 0.1. Determination of manganese peroxidase (MnP) activity was performed in a 5 mL system containing 0.11 mol/l pH4.5 sodium lactate buffer solution 4 ml, 10 mmol/l manganese sulfate 0.25 ml, 10 mmol/l guaiacol 0.25 ml and crude enzyme solution 0.25 ml at 30 °C for 30 min with reaction started by dropwise addition of H_2O_2 (6 mmol/l) 0.25 ml. Absorbance at 240 nm was detected. The enzyme unit is defined as the amount of the enzyme that increases absorbance per minute by 0.1.

Determination of ion flow on mycelia surface by NMT: The determination was performed 15 d after inoculation for the first time, and performed in the whole growth period of *Pleurotus nebrodensis* at the same frequency as that of enzyme activity determination. The flow of two ions (Ca^{2+} and H^+) was determined each time, with the same sampling location as that of enzyme activity determination. Five parallel tests were performed at each sampling location.

Statistical analysis

Experimental data was analyzed by statistical software Microsoft Excel and SPSS. Multiple comparison between average of each group was analyzed by least significant difference method (LSD), and variance analysis was performed for analysis of growth potential of *P. nebrodensis* mycelia and yield of fruiting body.

RESULTS AND ANALYSIS

Determination on biomass of liquid culture under different copper ion contents

Copper ion content in culture medium has significant influence on biomass of *P. nebrodensis* under liquid culture condition (result shown in Fig. 1). When copper ion content in the culture medium is less than 100 mg/l, biomass of *P. nebrodensis* increases with the increase of copper ion content

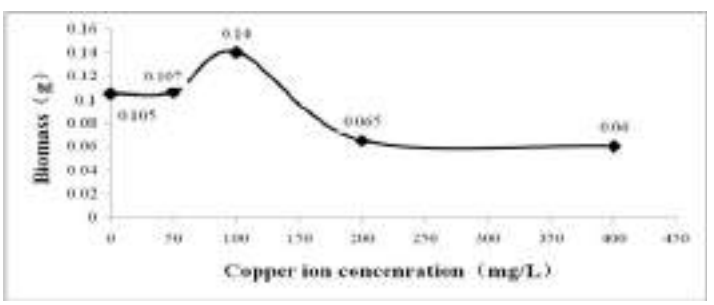

Figure 1. Comparison of biomass of *P. nebrodensis* at different Cu contents in liquid media

and reaches maximal value of 0.14 g/l at copper ion content of 100 mg/l; When copper ion content in the culture medium is greater than 100 mg/l, biomass of *P. nebrodensis* gradually declines, and the biomass has no significant change when the copper ion content is 250-400 mg/l.

Mycelial growth rate and bagful time

Experimental results (shown in Table 3) indicate that, copper ion has little effect on mycelial vigor but can promote mycelial growth. The copper ion can promote mycelial growth at the content of 25 mg/kg, showing no significant difference with that of control group. At copper ion content of 50 mg/kg, mycelial growth rate (4.35 mm/d) is obviously increased, significantly different with that of control group at $p<0.05$ and having no significant difference with that of control group at $P<0.01$, and the bagful time is shortened by 7 days. The copper ion at content of 100mg/ kg has certain promoting effect on growth of *P. nebrodensis* mycelia, showing significant difference with that of control group at $p<0.05$, but the growth of mycelia becomes slow. Hence, copper ion can promote mycelial growth at low content, but suppress mycelial growth at high content.

Table 3. Comparison of mycelial growth rate, bagful time and mycelialvigor in different copper concentration

Treatments	Bagful time/d	Mycelial growth rate Average(mm/d)	Mycelial vigor Significance of difference		
			0.05	**0.01**	
CK	71.29	3.49±0.38	a	A	+++
C1	68.61	3.89±0.24	ab	A	+++
C2	64.74	4.35±0.38	b	A	++++
C3	66.34	4.22±1.13	b	A	+++
C4	65.48	4.02±2.00	ab	A	++++

Note: The data is test average ± SD (n=30). The alphabet shows significance of difference at $p<0.05$ or $p<0.01$. + indicates low mycelial vigor, ++ indicates general mycelial vigor, +++ indicates good mycelial vigor, and ++++ indicates vigorous mycelial growth.

Yield, culture cycle and biological efficiency

It is shown by result analysis that, addition of copper ion at certain content can shorten culture cycle of *P. nebrodensis* and improve biological efficiency. At copper content of 50 mg/kg, growth cycle and biological efficiency of *P. nebrodensis* are 166.22 days and 50.66%, prolonged by 16 d and improved by 4.61%, respectively, when compared with those of CK. Analytic result indicates no statistically significant difference between the test formulations and the CK in average unit yield at ($p<0.01$). (Table 4).

Table 4. Comparison of yield, culture cycle and biological efficiency among different formulas

Treatments	Culture cycle	Average yield/g	Yield Significance of difference		Biological efficiency / %	Ratiowith biological efficiency of CK
			0.05	**0.01**		
CK	175.86	161.18	a	A	46.05%	/
C1	174.22	161.87	a	A	46.25%	0.2%
C2	166.22	177.30	a	A	50.66%	4.61%
C3	169.47	175.88	a	A	50.25%	4.2%
C4	170.17	169.88	a	A	48.54%	2.49%

Note: The data is test average ±SD (n=30). The alphabet shows significance of difference at $p<0.05$ or $p<0.01$.

Manganese dependent peroxidase and laccase

Research result shows that (Fig. 2) in the whole growth and development process of *P. nebrodensis*, *laccase* has an activity trend of high in mycelia growing period, sequential slow decline in post-ripening period, temperature difference stimulation period, bud appearing period and fruiting body growing period, and sharp drop after harvest. Manganese dependent peroxidase has small activity fluctuation in mycelia growing period and post-ripening period, evident activity increase in temperature difference stimulation period, high activity level at early stage of bud appearing period, gradual activity decrease in late stage of fruiting body growing period, and the same activity as that in mycelia growing period (Fig. 2). Change trend of laccase and manganese peroxidase in activity is in coincidence with that in research of role of manganese on *P. nebrodensis*.

It is found by analysis combining Table 4 and Fig 2 that, experimental group with high activity of manganese dependent peroxidase has high yield. The difference of peroxidase activity between different experimental groups can be considered as the difference in yield. The bigger the difference in enzyme activity is, the bigger the difference in yield, and vice versa.

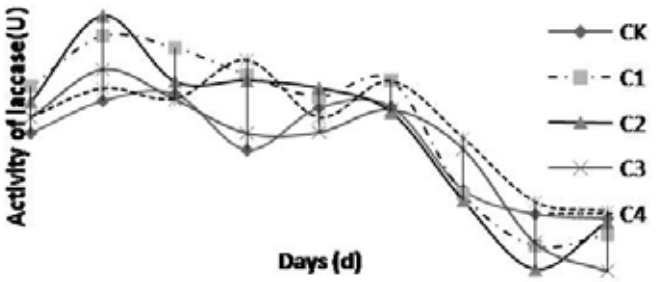

Figure 2. Comparison of the Lac in different periods among different experimental groups

Figure 3. Comparison of the MnP in different periods among different experimental groups

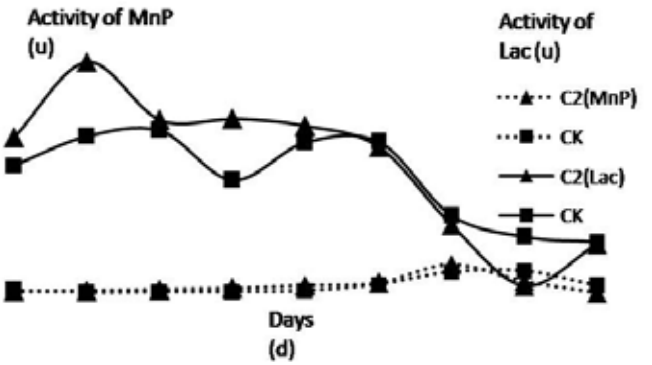

Figure 4. Comparison of activity change of the two enzymes in different period both C2 and CK

Change of H^+ and Ca^{2+} flux

Copper ion has maximal influence on growth rate, culture cycle and biological efficiency at concentration of 50 mg/kg, thus treatment C2 is selected for determining flux of H^+ and Ca^{2+} on mycelia surface (Fig. 5 and 6). Flux change of H^+ and Ca^{2+} on mycelia surface has no correlation with content of added copper ion. It is estimated that outward flow rate of H^+ may has no direct influence on change of Ca^{2+} flow rate, but the addition of copper ion can directly changed ion flow rate and direction in *P. nebrodensis* mycelia. The mechanism and path of these influences needs further research.

CONCLUSION AND DISCUSSION

It is shown by research result that, addition of copper at certain content can promote growth of *P. nebrodensis*. Copper can obviously promote growth rate (4.35 mm/d) of *P. nebrodensis*, shorten culture cycle (166.22 days) and yield (177.30 g). Whereas, result of research on *P. eryngii* by Rodriguez Estrada and Royse DJ [11] showed that copper at content of

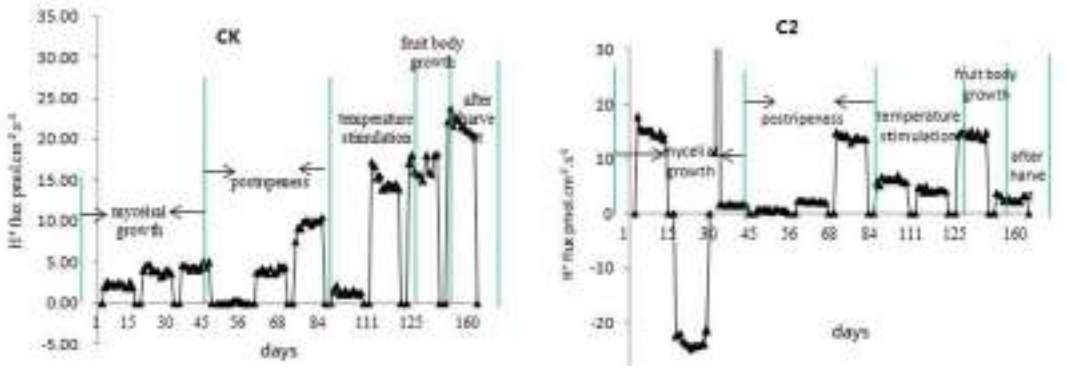

Figure 5. Change curve of H^+ flux on surface of mycelia of C2 group and CK group in different periods

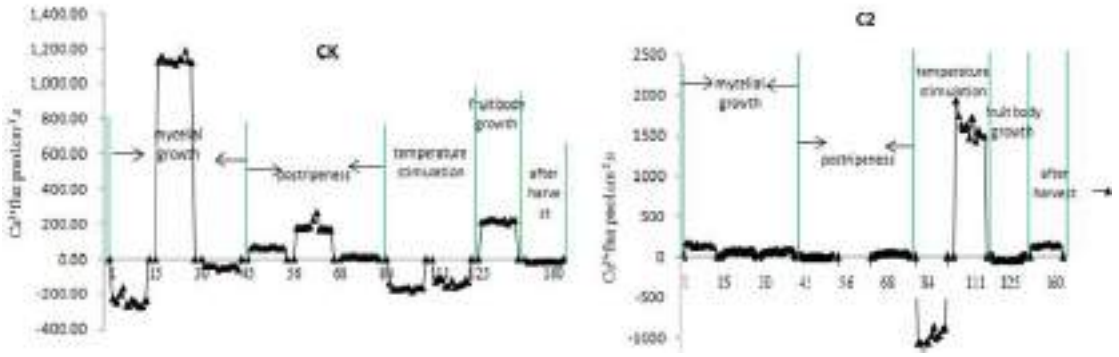

Figure 6. Change curve of Ca^{2+} flux on surface of mycelia of C2 group and CK group in different periods

150-250 μg/g (high content) can significantly reduce yield of *P. eryngii*, causes severe pathogenicity rate, but has low influence on biological efficiency, and average size and amount of *P. eryngii* fruiting body. It is also found that different strains have different copper tolerances, which is correlated with copper content in culture substrate [11]. Ling Yafei [13] found in the research on effect of mineral element, vitamins and hormone on growth of *Agrocybe aegirita* mycelia that, addition of copper can promote growth of *A. aegirita* mycelia but growth of *A. aegirita* mycelia has little demand on copper. Preliminary study on nutritive and physiological property of *Sparassis crispa* shows that addition of copper can obviously promote growth of *S. crispa* mycelia [14]. It is supposed that different edible fungi or different strains may have different tolerance on copper, which might be associated with gene of strains and copper content in culture substrate.

In enzyme activity experiment, laccase has highest activity in mycelia growing period, and sequential slow decline in post-ripening period, temperature difference stimulation period, bud appearing period, fruiting body growing period, and harvest period, which is concordant with the findings of Feng Zuoshan [15]. But in the research on change law of extracellular enzyme activity in growth and development process of *P. nebrodensis* by Zhou Changqing, *laccase* has low activity in mycelia growing period and post-ripening period, and significant activity increase in temperature difference stimulation period, which is completely different with the results of the research, and needs further confirmation. Manganese dependent peroxidase has low activity in mycelia growing period and post-ripening period, gradually increasing activity in temperature difference stimulation period, highest activity in bud appearing period, and gradually decreasing activity with the growth of *P. nebrodensis* fruiting body, which is in agreement with the results of research on *L. edodes* by Pan Yingjie [16]. Manganese dependent peroxidase has identical activity change trend in different strains, and has activity increase in early stage of fruiting body formation and activity decrease after harvesting of fruiting body. Relation between the manganese dependent peroxidase and formation of fruiting body deserves further study and discussion.

ACKNOWLEDGMENTS

This work was financially Supported by Special Fund for China Agriculture Research System for Modern Edible Fungi Industry (CARS-24), and Popularization and Application Program of Modern Agricultural Bio-industrial Developmentin Shenzhen (NYSW20130326B010022).

REFERENCES

[1] Dai YC *et al*. (2010). A revised checklist of edible fungi in China. *Mycosystema* 29: 1-21 (in Chinese).

[2] Kawai G *et al*. (2008). Taxonomic position of a Chinese *Pleurotus* "Bai-Ling-Gu": it belongs to *Pleurotus eryngii* (DC.: Fr.) Quél. and evolved independently in China. *Myco Science* 49:75-87.

[3] Mou CJ *et al*. (2004). A New Variety of *Pleurotus eryngii* and Its Cultural Characters. *Mycosystema* 6: 153-156.

[4] HuiLv *et al*. (2009). Nebrodeolysin, a novel hemolytic protein from mushroom *Pleurotus nebrodensis* with apoptosis-inducing and anti-HIV-1effects. *Phytomedicine* 16: 198–205.

[5] Choi DB *et al*. (2006). Studies on culture and biological activities of *Pleurotus nebrodensis* in zenga. *Korean J. Chem. Eng*. 23: 241-246.

[6] Zhang JX *et al*. (2004). RAPD and IGS Analysis of *Pleurotus nebrodensis* Cultivars in China. *Mycosystema* 23: 514-519. (in Chinese)

[7] Yao FJ*et al*. (2004). Study on Characteristics of Mating-Type System in *Pleurotus nebrodensis*. *Mycosystema* 24: 539-542. (inChinese)

[8] Feng Z *et al*.. Optimization of *Pleurotus nebrodensis* Culture Substrate Using Mixture Design. *Acta Edulis Fungi* 18: 26-29 (in Chinese)

[9] Niess A and Grabbe K. (1990). Response of the oyster mushroom (*Pleurotus ostreatus*) to manganese supply. In: Proceedings of the Fourth International Mycological Congress. Regensburg, Germany. Abstract IIE-246/4.

[10] Beelman RB *et al*. (1995). Relationship between copper accumulation and yield and quality of fresh mushrooms. *Mush Sci*. 14: 765-770.

[11] Rodriguez E and Royse DJ. **(**2007). Yield, size and bacterial blotch resistance of *Pleurotus eryngii* grown on cottonseed hulls/oak sawdust supplemented with manganese, copper and whole ground soybean. *Bioresource Technology*. 98: 1898-1906

[12] Hu Qingxiu. (2005). The manual on high quality mushroom products planting technology. Beijing: Chinese Agriculture Science Technology Press. 153-159.

[13] Ling Yafei. (2000). Effects of the Mineral Elements, Vitamin and Hormone on Mycelial Growth of *Agrocybe cycoindracea*. *Journal of Zhejiang Wanli University* 13: 26-30. (in Chinese)

[14] Fu SJ and ZHang LQ. (1998). A Preliminary study on natrition physiological characterisitics of thelephora ganbajun zang mycelia. *Journal of Microbiology* 18: 32-32. (in Chinese)

[15] Feng ZSH *et al*. (2014). Activities of ectoenzymes and respiratory enzymes and ionic flus of *Pleurotus nebrodensis* throughout culture process. *Mycosystema* 34: 341-354.(in Chinese)

[16] Pan YJ. (1995). The biodegradation law of lignocellulose during the growth of *Lentinus edodes*. *Acta Edulis Fungi* 2: 18-22 (in Chinese).

ORANGE DYE FROM *PYCNOPORUS* SP. FOR TEXTILE INDUSTRIES

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ABSTRACT

Colour plays a vital role in life of each and every one through industries like textiles, paint, food, clothing, art and cosmetics. The traditional natural dyes from plants were quickly replaced by synthetic dyes ever since the discovery of synthetic organic dye, mauveine by William Henry Perkin in 1856. Thousands of synthetic dyes have been prepared with several advantages like low cost, vast range of new colours and ability to impart better properties upon the dyed materials. Pigments from microbes especially fungi are considered as a good alternative to hazardous synthetic dyes. The work deals with orange dye from *Pycnoporus* sp. and its application as mushroom dye for textile industries. The work was initiated with isolation, followed by production of dyes from mycelial culture, simple and cost effective cultivation with successful fruiting body production, and extraction of dyes. It was followed by application of the mushroom dyes with cotton and silk yarns and fabrics. Pilot scale cultivation was successfully carried out and dyeing experiments were carried out at an industry for testing its suitability as natural dye.

Key words: *Pycnoporus* sp. dyes, textiles

INTRODUCTION

Color is the most pleasing attribute of any article; red color exudes warmth, increases pulse rate and respiration, whereas, blue or green color suggests cool and peaceful environment and encourages relaxation [1]. Although structurally very diversified and derived from a variety of sources, natural colorants can be grouped into a few classes, the most important of which are: anthocyanins, anthraquinones, tetrapyrrols, tetraterpenoids and flavonoids. The terms “pigment” and “dye” are often used interchangeably. After the accidental synthesis of mauveine by Perkin in Germany in 1856 and its subsequent commercialization, coal-tar dyes began to compete with natural dyes.

Synthetic colors became popular because of their fastness, wide range of colors, low cost even at high concentration in low volumes [2]. During the textile dyeing process up to 40% of dyes may remain unfixed to the fibre and act as contaminant in the industrial wastewater. It is known that 90% of reactive dyes entering activated sludge sewage treatment plants will pass through unchanged and be discharged into rivers [3]. They are very stable and difficult to degrade. These dyes are resistant to microbial attack and are hardly removed from effluents by conventional biological, physical or chemical treatments [4, 5].

Textile industry is the major consumer of dyes and the international market potential for textile dyes is US$ 10 billion. The potential for colorants and intermediates is US$ 24 billion of which dyes and pigments constitute $17 billion (71%) and dye intermediates $7 billion (29%). China is the major player with 25% share in production of dyes and India with 7-8% in global trade, which is expected to become 10% in a few years. India and China are the net exporters of dyes as they consume less than what they produce. China is robust in producing dyes since domestic consumption is 60-70% while exports are 30 - 40% and it is converse with India consuming 30-40% in domestic market and exporting 60-70% [6].

The biosphere is under constant threat by the textile dyeing industries due to release of environmental pollutants. Considerable research work is being undertaken around the world on the application of natural dyes. The industries are continuously looking for cheaper, more environment friendly routes to existing dyes in order to minimize the damage to the environment [7-9]. It has been well known that a variety of plants, animals and microorganisms produce pigments [10-13].

Natural dyes are derived from naturally occurring sources such as plants (eg., indigo and saffron), insects (eg., cochineal beetles and lac scale insects), animals (eg., some species of mollusks or shellfish), minerals (eg., ferrous sulfate, ochre and clay) and from variety of microorganisms [14-27].

The fruit bodies of some macromycetes/mushrooms (e.g., agaric) have been used less but they can produce a range of pink, blue, yellow, red, and brown (boletol blue) *Boletus luridus, Cortinarius* spp, *Hydnellum* spp. and *Hygrocybe* spp.) using mordant such as alum or iron [28].

In 1974, Miriam C. Rice published the first book concerning dyeing with fungi and since then, the custom has spread all over the world. *Laetiporus sulphureus* (Bull.: Fr.) Murr. (Polyporales, Fungi) is a wood rotting basidiomycete growing on several tree species and producing shelf-shaped fruit bodies of pink-orange colour, except for the fleshy margin which is bright yellow. *Laetiporus* species contain a number of lanostane triterpenoids and other metabolites and has a great industrial application in food, textiles industry [29-30]. Extraction of anthroquinone dyes from a Polyporaceae member *Dermocybes anguineus* showed potential dyeing properties on different fabric materials [31].

An orange pigment from the fungi *Ganoderma applanatum, Coriolus versicolor* and *Amanita muscaria* was extracted from the basidiocarp and applied on the silk and cotton fabrics [20].The cost effective method of cultivation of *Ganoderma lucidum* and application of its dyes to cotton and silk yarns were done and the dyed yarns showed good fastness tests [22]. Cost effective method of cultivation of *Pycnoporus sanguineus* and application of its dyes to cotton and silk yarns and fabrics was done and tested up to industrial level [27].

Pycnoporus is a slow-growing saprophytic fungus belonging to the class Basidiomycetes of the family Polyporaceae. It is a known white-rot fungi of wide occurrence in tropical and subtropical countries. It grows on decaying logs and tree stumps, which causes decay of certain types of wood in the forests of tropical and subtropical areas [32-34].

Pycnoporus is being intensively studied because of the metabolites produced by it and reported to synthesize some substances with medical applications [35-36], and to produce various enzymes of industrial applications such as invertase [37], tyrosinase [38], α-amylase [39], xylanase and β-glucosidase [33] and laccase [34]. Among the enzymes produced by *Pycnoporus*, laccase is of particular interest because of its effectiveness in numerous biotechnological applications [40]. The laccase secreted by *Pycnoporus* has demonstrated its ability on the decolourization of azo and triphenyl methane dyes [41].

The work was initiated with isolation, followed by production of dyes from mycelial culture, simple and cost effective cultivation with successful fruiting body production, and extraction of dyes. It was followed by application of the mushroom dyes with cotton and silk yarns and fabrics. Pilot scale cultivation was successfully carried out and dyeing experiments were carried out at an industry for testing its suitability as natural dye.

MATERIALS AND METHODS

Pycnoporus were inoculated on PDA medium for a period of 7 days for the development of pure culture. The mycelial disc (2 mm in diameter) of *Pycnoporus* was inoculated at the centre of the petriplate containing 20 ml of medium and incubated at 30±2 ^{o}C. Optimization studies like pH and temperature were undertaken for the successful production of dyes [27].

Pycnoporus was subjected to spawn development using different substrates such as sorghum grains, maize grains, paddy husk, combination of maize grains with paddy husk (1:1) and sorghum grains with paddy husk (1:1).The locally available lignocellulosic wastes substrates such as sugar cane bagasse, saw dust, paddy straw and wood shavings were collected from the surrounding places and brought to the laboratory. The collected substrates were transported to the laboratory and shade dried. The infected or contaminated parts of the substrates were discarded and healthy lignocellulosic substrates were further processed for mushroom beds preparation and maintained [27]. Standardization of various parameters was done for the successful cultivation and the biological efficiency was calculated.

Various extraction procedures using different polar and nonpolar solvents were followed and the pigment was extracted. Cotton and silk fabric was treated with alum, tannic acid and dyed with the dyes of *Pycnoporus*. The wash fastness of the fabric was recorded.

RESULTS AND DISCUSSION

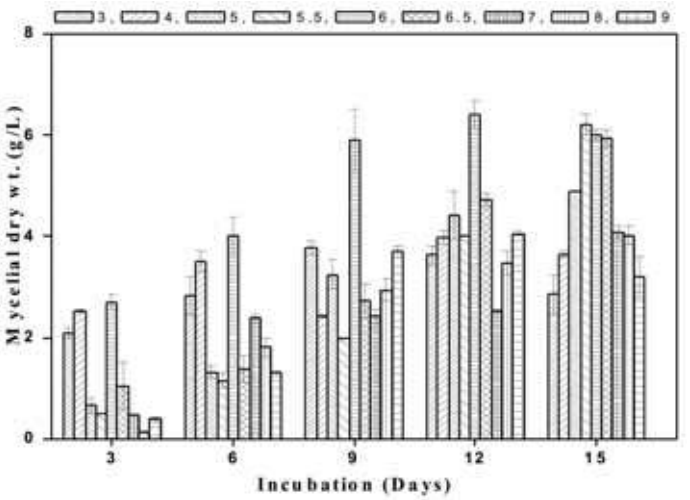

Figure 1. Optimization of growth (dry wt) of *Pycnoporus* in PDB at various pH

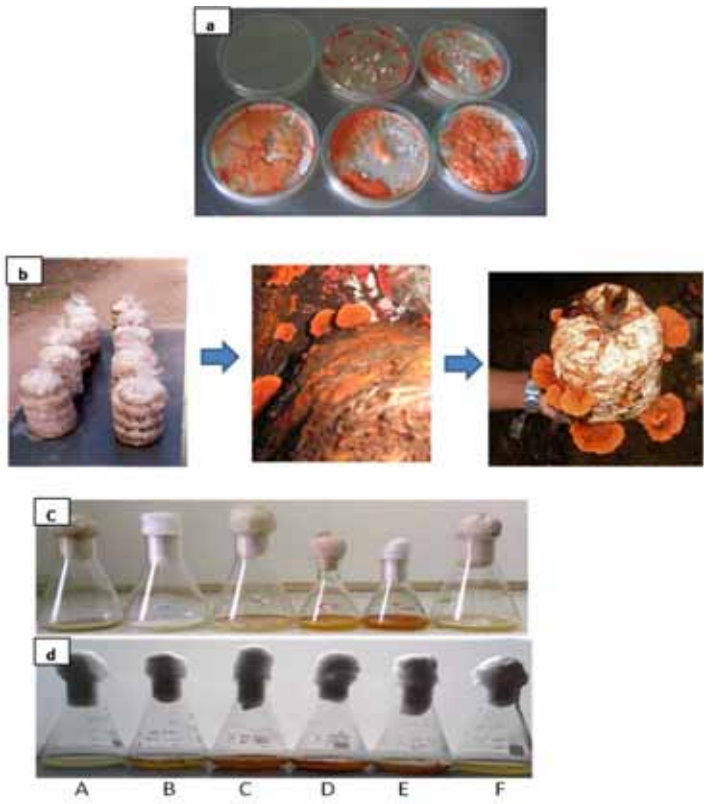

Plate 1. Production of *Pycnoporus* on solid and liquid media **a)** Growth of *Pycnoporus* in solid media; **b)** Cultivation of *Pycnoporus* in locally available lignocellulosic substrates; **c and d –** Optimization of *Pycnoporus* at Various pH and temperature; **c** - A – PDB Control, B - pH 3, C - 4, D - 5, E - 6, F -7; **d** -A - PDB Control, B - 20, C - 25 , D - 30, E - 35, F – 40 !

Pycnoporus mycelium was grown on PDA medium for a period of 7 days (Plate 1a). The mycelial growth of *Pycnoporus* was studied in PDB medium maintained at pH 3.0, 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0 and 9.0.The pH had influence on growth of *Pycnoporus***.** The maximum mycelial growth (6.42 ± 0.2 g dry weight/l) was recorded on PDB at pH 6.0 on 12thday (Fig.1). The mycelial growth of *Pycnoporus* significantly reduced at pH 8.0 to 9.0. The pH 6.0 and temperature 30 °C was found to be optimum for the growth of *Pycnoporus* (Plate1 c, d).

Mycelial spawn development of *Pycnoporus*on maize grains initiated on 3rd day after inoculation.The mycelium densely colonized maize grains within 20 days as compared to other substrates which required 30 days. The optimum temperature for spawn growth was 25±2 °C.

Locally available lignocellulosic agriculture residues such as paddy straw, sugar cane bagasse, saw dust and wood shavings were individually utilized as substrate for cultivation of fruiting bodies of *Pycnoporus*. The mycelial growth started on 2nd day and complete mycelial colonization was recorded on 12th day of incubation. The appearance of pin heads and the matured fruiting body of *Pycnoporus* is shown in Plate 1b. Successful pilot scale cultivation was carried out using simple and cost effective cultivation methods. The research finding revealed that *P. cinnabarinus* can be cultivated successfully on different substrates like wheat straw, saw dust, bird seeds and wheat flour [42]. Wild fruit body of *P. cinnabarinus* was collected from the Coorg region in western ghat. The spawn was prepared on sorghum grains and substrate (saw dust in combination with wood chips) supplemented and successful cultivation was reported [43].

Successive pigment was extracted using simple, cost effective methods and the fruiting bodies of *Pycnoporus* and the recovered dye was applied to treated cotton and silk fabrics. Control fabric was maintained without any treatment and also fabrics dyed without any mordant treatments recorded fair to good wash fastness properties (Plate 2 a & b).

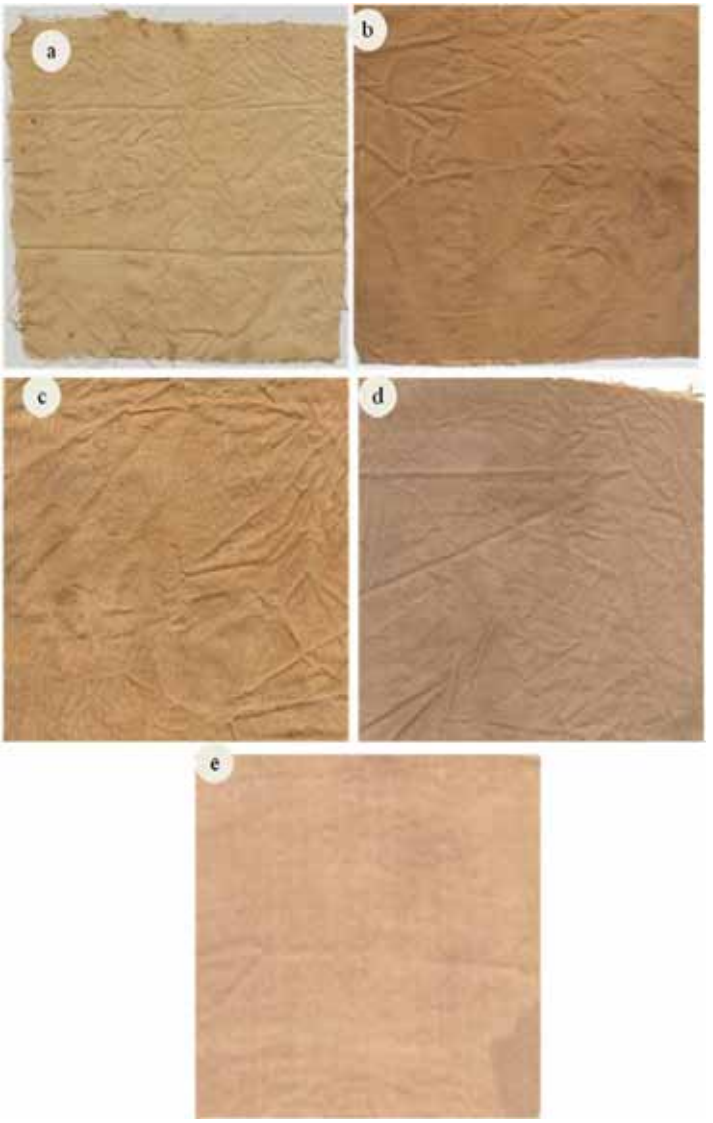

Plate 2a. Dyeing of cotton fabrics with *Pycnoporus* pigment premordanted with alum and tannic acid. **a)** Cotton + Alum +Pigment, **b)** Cotton + Tannic acid +Pigment, **c)** Cotton + Alum + Tannic acid +Pigment, **d)** Cotton + Alum + Tannic acid +Pigment + water washed, **e)** Cotton – Alum + Tannic acid +Pigment + soap washed

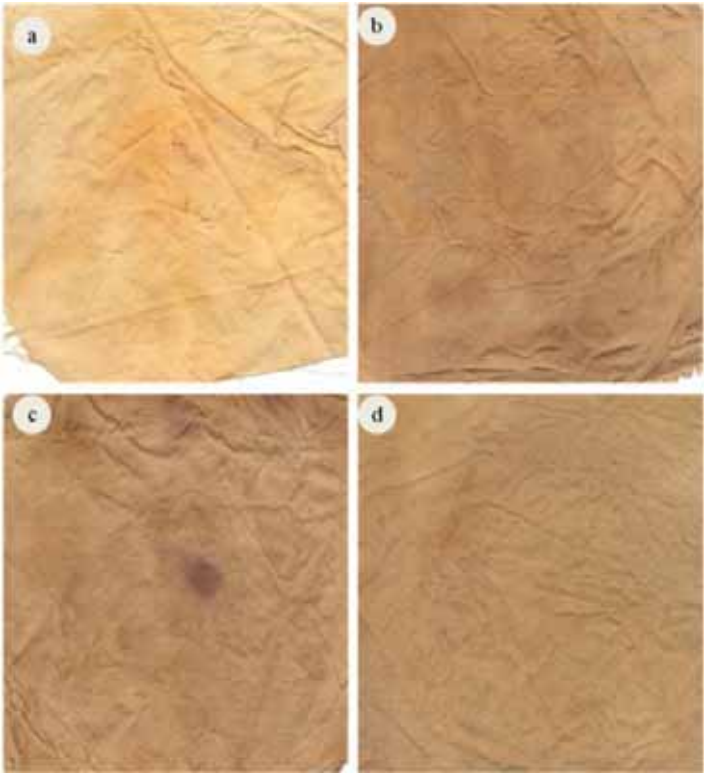

Plate 2b. Dyeing of silk with pigment of *Pycnoporus* premordanted with alum and tannic acid. **a-** Alum +Pigment, **b-** Tannic acid +pigment, **c-** Alum+ tannic acid+ pigment, **d-** Alum+ tannic acid+ washed

CONCLUSION

The work highlights the experience with the orange dye from *Pycnoporus* sp. and its application as mushroom dye for textile industries. Many interesting results were obtained and it is hoped that at least some of them will find applications in the future.

ACKNOWLEDGEMENTS

The authors thank Department of Biotechnology for their financial support and Shri A.M.M. Murugappa Chettiar Research Centre, Taramani, Chennai for providing laboratory facilities.

REFERENCES

[1] Joshi VK *et al*. (2003). Microbial Pigments. *Indian J. of Biotechnol.* 2: 362-369.

[2] Pattnaik P *et al*. (1997). Biocolours: New generation additives for food. *Indian Food Industry.* 16: 21-32.

[3] Pierce J. (1994). Colour in textile Effluents-The Origins of the Problem. *J. Soc. Dyers Colours.* 110: 131-134.

[4] Trovaslet M *et al*. (2007). Potential of a *Pycnoporuss anguineus* laccase in bioremediation of waste water and kinetic activation in the presence of an anthraquinonic acid dye. *Enzyme Microb. Technol*. 41: 368-376.

[5] Vanhulle S *et al*. (2007). Overlap of laccases/cellobiose dehydrogenase activities during the decolorization of anthraquinonic dyes with close chemical structures by *Pycnoporus* strains. *Enzyme and Microbial Technol.* 40: 1723-731.

[6] Murthy KS. (2010). Dyes and Dye intermediates global scenario. Present and future. *Colourage*. 57(8): 85-89.

[7] Gupta D. (2001). Dyeing studies with hydroxy lanthraquinones extracted from Indian madder. Part 2: dyeing of nylon and polyester withn or damn canthal. *Coloration Technol*. 117: 33-336.

[8] Durán N *et al*. (2002). Ecological-friendly pigments from fungi. *Crit. Rev. Food Sci. Nutri.* 42: 53-66.

[9] Kamel MM *et al*. (2005). Ultrasonic assisted dyeing III. Dyeing of wool with lac as a natural dye. *Dyes and Pigments.* 65: 103-110.

[10] Lauro GJ. (1991). A primer on natural colours. *Cereal Foods World.* 36: 949-953.

[11] Masahiro KO *et al*. (1994). Production and release of anthraquinone pigments by hairy roots of madder (*Rubiatinctorum* L.) under improved culture conditions. *J. Ferment. Bioeng.* 77: 103-106.

[12] Kim JK *et al*. (1995). Novel antimutagenic pigment produced by *Bacillus licheniformis* SSA3. *J. Microbiol. Biotechnol*. 5: 48-50.

[13] Kim CH *et al*. (1998). Production of red pigment by *Serratia* sp. KH-95 and its cultural properties. *Kor. J. Biotechnol. Bioeng.*13: 431-437.

[14] Cross BE and Edinberry MN. (1972). Pigments of *Gnomonia erythrostoma*. Part I. The structures of erythrostominone, deoxyerythrostominone, and deoxyerythrostominol. *J. Chem. Soc. Perkin*. 3: 380 - 390.

[15] Mizukami H *et al*. (1978). Variation in pigment production in *Lithospermum erythrorhizon* callus cultures. *Phytochem*. 17: 95-97.

[16] Papageorgiou VP *et al*. (1979). Studies on the relationship of structure to antimicrobial properties of naphthoquinones and other constituents of *Alkannatinctoria*. *Planta Med*. 35: 56-60.

[17] Ryu BH *et al*. (1989). Production of purplish-red pigment in mixed culture of *Streptomyces propurpuratus* ATCC 21630 and *Bacillus* sp. R-89. *Kor. J. Appl. Microbiol. Bioeng*. 17: 327-333.

[18] Parisot D *et al*.(1990). Naphthoquinone pigments related to fusarubin from the fungus *Fusarium solani* (Mart.) *Sacc. Microbios*. 64: 31- 47.

[19] Yongsmith B *et al*.(1994). Production of yellow pigments in submerged culture of a mutant of *Monascus* sp. *J. Ferment. Bioeng*. 78: 223 -228.

[20] Perumal K *et al*. (2004). Isolation, extraction and dyeing performance of fungal pigment from *Ganoderma applanatum*, *Coriolus versicolor* and *Amanita muscaria*. *J. Mycol. Pl. Pathol*. 34: 690 -691.

[21] Perumal K *et al*. (2009). Extraction and characterization of pigment from *Sclerotinia* sp. and its use in dyeing cotton, *Text. Res. J*. 79(13): 1178 - 1187.

[22] Sumathi E. (2008). Cultivation, extraction of pigment from basidiomata of *Ganoderma lucidum* (Fr.) P. Karst and its dyeing performance with selected textile yarns. *Ph. D. thesis*. University of Madras, Chennai, India.

[23] Velmurugan P *et al*. (2010b). Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather. *Carbohydrate Polymers.* 79: 262 - 268.

THE PROSPECT OF ROT FUNGI IN CONTROLLING OF *TRICHODERMA* SPP. IN MUSHROOM CULTIVATION

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ABSTRACT

Trichoderma spp. are often encountered in mushroom cultivation. They could contaminate substrates at the time of spawning or after. The heavy infections could reduce the yield considerably. The JPA isolate is a rot fungus that was found in oil palm plantations. Based on the initial detection by paper disc method, the methanolic extract of JPA mycelia cultivated in 1.5% of malt extract could inhibit the growth of *Bacillus subtilis*. Based on the bioautography test, the crude extract gave the inhibition zone as well with the R_f in a range of 0.7-0.8. The antagonistic test of JPA isolate with *Trichoderma* sp. S2-2 was conducted in the baglogs containing 500 g of oil palm empty fruit bunches (OPEFB) supplemented with 15% bran, 1.5% lime and 1.5% gypsum. Both isolates were inoculated at both sides of the baglogs simultaneously. Based on this test, JPA isolate could inhibit the growth of *Trichoderma* sp. S2-2 and covered the colony of *Trichoderma* sp. S2-2 and the OPEFB substrates after 8 weeks of incubation. Furthermore, the methanolic extract of the OPEFB substrates inoculated only with JPA isolate were tested against *Trichoderma* sp. S2-2 by paper disc method. The results showed that the extracts could inhibit the growth of *Trichoderma* sp. S2-2 with formation of very thin *Trichoderma* colony zones or clear zones around the paper discs containing the extracts. Based on ITS1 and ITS4 squences data, JPA isolate is *Lentinus* sp. but the data do not match closely with any species registered in GenBank. Further research is the development of the extract formula and test it in mushroom cultivation. The mycelial extracts isolated from other tropical *Lentinus*, especially *Lentinus cladopus* will also be tested.

Keywords: control, JPA isolate, rot fungi, *Trichoderma*

INTRODUCTION

Indonesian society has known wild mushrooms as food since ancient times. Straw mushroom is the first wild mushrooms cultivated in Indonesia. At present many mushrooms have been cultivated in Indonesia, among others, button mushroom, ear mushroom, shiitake mushroom, etc. But white oyster mushrooms are widely cultivated and accepted by society in Indonesia since 20 years.

Generally mushroom cultivation in Indonesia is still done traditionally or moderately. Only a few large companies that cultivate mushrooms, especially button mushroom, using the latest equipment and methods. Consequently, mushroom cultivations in Indonesia have often pest and disease problems. In the pocket book of Indonesian Ministry of Agriculture [1] for the farmers, they suggested applying of prevention, physical and chemical controls using such as lime, dichloros, dicofol, malathion, diflubenzuron for control of undesirable insects while 0.5% formalin solution, benomyl, carbendazim for control of fungal diseases although over time, the mushroom insects and pathogens have developed resistance to the pesticides.

Trichoderma spp. is the cause of green mould disease in mushroom cultivation in many countries and led to losses of up to tens of millions of dollars. Likewise, *Trichoderma* is often complained by farmers in Indonesia. Generally these fungi are not controlled by the farmers and losses are never counted. Farmers leave the contaminated substrates at growing house and near the farm, consequently the fungal spores spread around the house and reinfection occurred easily on new bags and cultivation equipment.

Trichoderma can be prevented by applying strict sanitation, but if heavy infection has already occurred then the proper fungicide should be applied to the spawn, casing or compost. A number of fungicides are recommended for control of *Trichoderma* such as Environ [2], Prochloraz, mixture of Prochloraz and Carbendazim [3], Thiabendazol [4] and Imidazole

[5]. Many chemical compounds are no longer effective and there is emergence of resistant strains. Therefore, growers have to improve disease prevention and research is to be carried out for alternative control methods.

Many researchers have conducted studies on *Trichoderma* in order to control these fungi in mushroom cultivation. Antagonistic bacteria against *Trichoderma* spp. have been found [6, 7]. The brown strains of *Agaricus bisporus* were highly resistant to green mold caused by TA4 [8]. Effort was made to obtain metabolites that can inhibit the growth or induce resistance to *Trichoderm*a but the Lysing Enzymes of *Trichoderma harzianum* could not increase the resistance of *A. bisporus* against *T. aggressivum* [9].

The purpose of this research is to determine the ability of the colony and secondary metabolites of JPA isolate in inhibiting the growth of *Trichoderma*. JPA isolate is a wood rot fungus that was found in oil palm plantation. In addition, the position of JPA isolate was investigated based on phylogenetic analyses of ITS sequences.

MATERIALS AND METHODS

Isolates

JPA isolate and *Trichoderma* sp. S2-2 were isolated from fruiting body grown on root and soil of oil palm plantation, respectively. Both isolates were stored on agar media of PSA (200 g potatoes, 20 g sucrose, 15 g agar and 1 l distilled water) at room temperature.

Direct confrontation between colonies

Confrontations between JPA isolate and *Trichoderma* sp. S2-2 were investigated in 35 x 20 cm sized polyethylene bags (baglogs) containing 500 g of oil palm empty fruit bunches (OPEFB) supplemented with 15% bran, 1.5% lime and 1.5% gypsum. Initially, OPEFB was chopped, water soaked for 24 hours and drained in a closed plastic bags which were hung for 6 hours to gain 75% moisture content. Afterwards all bags were sterilized, spawned with grain spawns with four different treatments of inoculations, i.e. the substrates were inoculated with JPA isolate (JPA); with *Trichoderma* sp. S2-2 (T); with both isolates (JPA+T) and with *Trichoderma* sp. S2-2 but the sterile substrates were previously fully colonized by JPA isolate [(JPA)+T]. All substrates were incubated at room temperature (in a range of 28-30 ^{o}C) for 8 weeks. Ten replications were used for each growing trial.

Extraction of mycelia and substrates of JPA isolate

Mycelia were obtained from ten liquid cultures of JPA isolate. Each culture consisted of 1.5% of malt extract. One of 7 mm inoculum was inoculated on the surface of each culture containing 100 ml of media in 250 ml of Erlenmeyer flask. Cultures were incubated at room temperature at static condition for 4 weeks. Substrate samples were obtained from ten substrates, each containing of 500 g OPEFB supplemented with 15% bran, 1.5% lime, 1.5% gypsum and inoculated with JPA isolate, then incubated at room temperature for 8 weeks. Substrate samples were obtained as well from mixture substrate containing *Paraserianthes falcataria* sawdust and OPEFB with proportion 1:1, respectively. Mycelia extraction is done as follows: mycelia were separated from the culture filtrates with filter paper. Mycelia of each culture was ground with mortar and then extracted twice with methanol at a concentration of 5%, respectively and agitated overnight on a rotary shaker for each extraction. The mycelial methanolic extract was separated from residues with fritted glass filter no. 3 and then dried under vacuum, with a 30 ^{o}C water bath and a rotary evaporator, then redissolved in methanol. The mycelial ethanolic extract was obtained as well with the same procedure. Substrate extraction is done as follows: substrates were ground by the hammer mill and extracted twice with methanol at a concentration of 10%, respectively and left overnight for each extraction. The further procedure was similar to those described in the section on mycelial extraction.

Activity test

The extract activities were tested by paper disc method against *Bacillus subtilis* and *Trichoderma* sp. S2-2. The test media were tryptone glucose yeast (TGY) (tryptone 5 g, yeast extract 5 g, glucose 1 g, K_2HPO_4 1 g, agar 7.5 g, distilled water 1 l) for *B. subtilis* and (PSA for) *Trichoderma* sp. S2-2. The cell suspensions of *B. subtilis* were obtained by growing 100 µl of stock suspension on 100 ml of TGY medium which was then 25 ml poured on Petri dish and incubated at room temperature for 15 days. Each Petri dish was then added with 3 ml of sterile distilled water, the suspension was

then scrapped with glass rod, filtered with sterile cotton and pasteurized three times at 60°C for 30 min.The cell suspensions of *Trichoderma* sp. S2-2 were obtained by growing one of 7 mm inoculum on PSA and incubated at room temperature for 5 days. Each Petri dish was then added with 10 ml of sterile distilled water, the suspension was then scrapped with glass rod, filtered with sterile cotton. One percent of each extract were poured on 12 mm paper discs. The controls were the discs with similar solvent without the extract. The solutions were dried by air in order to remove solvent. The discs were sterilized with UV ray (254 nm) for 30 min and then placed on the surface of test media containing 1-3 x 10^5 cells of target microbes per ml. These test media were then incubated at 10°C for 3-4 hours in order to diffuse of extracts into agar media. After incubation for 24 hours at 35°C for *B. subtilis* and for 2 days at room temperature for *Trichoderma* sp. S2-2, activities of extract were estimated by measuring the diameters of inhibition zones which had been reduced by the diameters of disc.

Detection of active compounds

Detection was done by bioautography method using analytic thin layer chromatography (TLC) and *B. subtilis* [10]. The medium for *B. subtilis* was TGY. One hundred microliter of this cell suspension were added to 100 ml of TGY medium and then medium was poured on TLC plate. TLC was carried out with silica gel plates (Merck 60 F254, 0.1 mm thick, 20x5 cm). The mycelial methanolic extracts were deposited as spots and developed in a butanol-acetic acid-water mixture (3:1:1, vol:vol:vol). Dry chromatograms were covered with 15 ml TGY medium containing *B. subtilis* and then incubated at 10 °C for 3-4 hours in order to diffuse of extracts into agar media. The locations of inhibition zones were estimated by measuring the R_f values of inhibition spots.

Molecular character of JPA isolate

Two-week-old JPA isolate colony cultured in 100 ml of 1.5% malt extract and 0.5% peptone in 250 ml Erlenmeyer flask at room temperature and static conditions was used to extract genomic DNA. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) and performed according to the instructions of the manufacturer with slight modifications. DNA concentrations were estimated visually in 1.2% agarose gel. The PCR reactions were performed in a 25 ì l volume (8.8 µl ddH_2O; 5 µl 5x Q5 buffer; 5 µl 5x Q5 Enhancer; 1µl dNTPs; 1 µl 10 pmol ITS 1–F; 1 µlpmol ITS4-R 10; 3 µl DNA extract; 0.2 µl Tag HF). The thermal cycles consisted of 95 °C for 2 min, followed by 35 cycles at 95 °C for 45 s, 58 °C for 1 min, 72 °C for 1 min, with a final extension step of 72 °C for 7 min and storage 15 °C for 20 min. The PCR products were verified by staining with ethidium bromide on 1.5% agarose electrophoresis gels stained with ethidium bromide in 1x Tris-boric acid EDTA buffer. ITS 1 and ITS 4 were used to sequence both strands of DNA molecules at PT. Genetika Science Indonesia. Phylogenetic analysis was performed using Mega 5.5.

RESULTS AND DISCUSSION

Direct confrontation between colonies

The results showed that the antagonistic interaction had occurred between *Trichoderma* sp. S2-2 and JPA isolate or vice versa when both isolates were simultaneously inoculated on each side of polybags (JPA+T treatment) (Fig. 1). It depended on the period of colonization, at 30 days of interaction, *Trichoderma* sp. S2-2 colonized 75% of baglog volumes while JPA isolate colonized only 25% of bag log volumes. It seems *Trichoderma* sp. S2-2 inhibited the growth of JPA isolate, because if only JPA isolate was grown on the substrates (JPA treatment), it took 35 days for JPA isolate to fully colonize the substrates. At 45 days of interaction, JPA isolate started to give antagonistic interaction against *Trichoderma* sp. S2-2 because JPA isolate colonized 50% of baglog volumes and covered of *Trichoderma* sp. S2-2 colonies which were previously occupied 75% of baglog volumes. Furthermore, at 60 days of interaction, JPA isolate colonized 75% of baglog volumes, whereas *Trichoderma* sp. S2-2 colonized only 25% of baglog volumes. So, JPA isolate could inhibit the growth of *Trichoderma* sp. S2-2 and covered the colonies of *Trichoderma* sp. S2-2 and the OPEFB substrates. The sterilization of the colonized substrates [(JPA)+T treatment] could damage the active metabolites which were produced probably by JPA isolate. Therefore *Trichoderma* sp. S2-2 could grow on such sterile substrates which were previously full colonized by JPA isolate and it took 22 days for 100% substrate colonization. It also took 26 days for 100% substrate colonization for only *Trichoderma* sp. S2-2 grown on the substrates (T treatment).

Extract Activities of mycelia and OPEFB substrates of JPA isolate against *Bacillus subtilis* and *Trichoderma* sp. S2-2

The previous results showed that JPA isolate could inhibit the growth of *Trichoderma* sp. S2-2 on OPEFB substrates. So there is a possibility that JPA isolate had the ability to produce active compounds that inhibit the growth of *Trichoderma* sp. S2-2. Tests showed that the extract of OPEFB and mixture substrates could inhibit the growth of *B. subtilis* which was used as a sensitive test microbe (Fig. 2, 3). The same results were shown by methanolic and ethanolic extract of mycelia, even the activities were higher. Further tests were conducted against *Trichoderma* sp. S2-2. The OPEFB substrates inoculated only with JPA isolate were tested against *Trichoderma* sp. S2-2 and the results showed that the OPEFB substrate extracts could inhibit the growth of *Trichoderma* sp. S2-2 with formation of very thin *Trichoderma* sp. S2-2 colony zones (Fig. 3B) or clear zones around the paper discs containing the extracts (Fig. 3C). The clear zones were not rounded with diameter of zones in a range of 20-38 mm. In the next test the methanolic extract of mycelia will be tried against *Trichoderma* sp. S2-2. In addition the methanolic extract of JPA mycelia could inhibit the growth of *Ganoderma boninense* as well (unpublished).

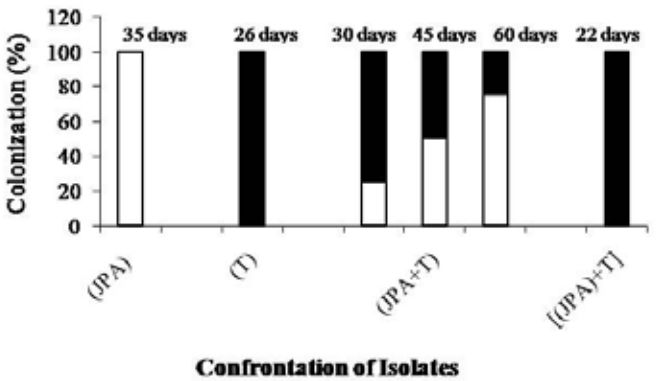


Figure 1. The oil palm empty fruit bunches substrates were colonized by JPA isolate (JPA) (blank bars); *Trichoderma* sp. S2-2 (T) (black bars); both isolates (JPA+T) and *Trichoderma* sp. S2-2 but the sterile substrates were previously full colonized by JPA isolate [(JPA)+T].

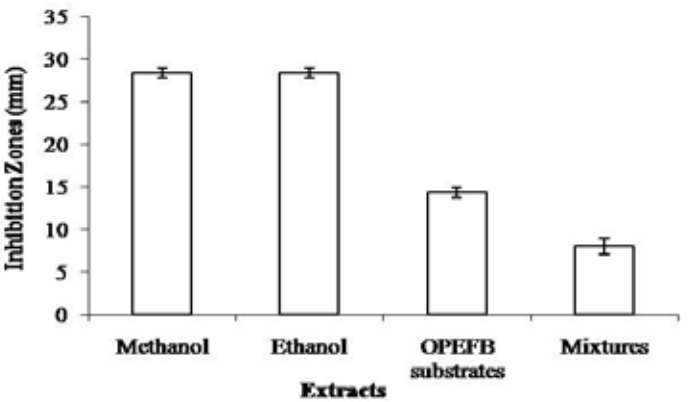
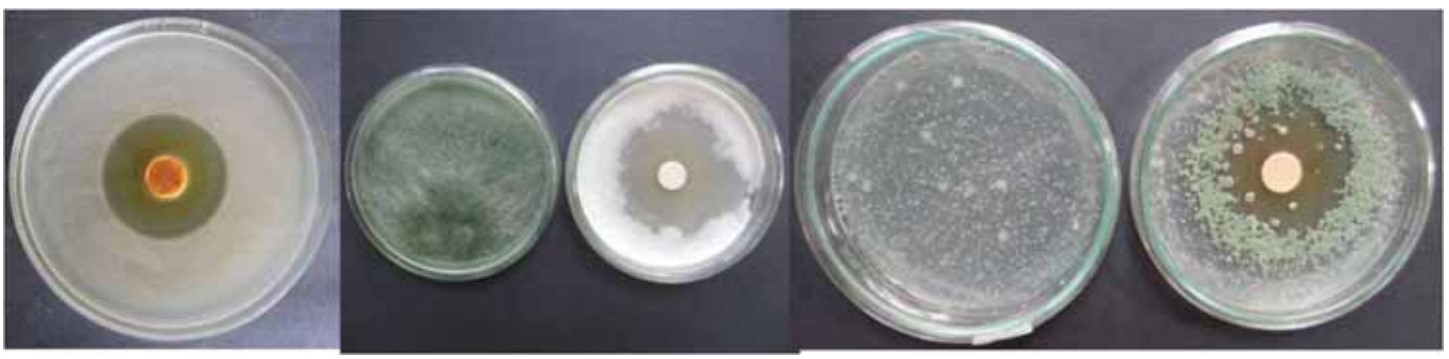


Figure 2. Mycelial extract, OPEFB substrate extractand mixture extract activities of JPA isolate against *Bacillus subtilis*



A **B** **C**

Figure 3. Mycelialextract activity of JPA isolate against *Bacillus subtilis* (A), OPEFB substrate extract activities of JPA isolate against *Trichoderma* sp. S2-2(B and C)

Detection of active compounds

Based on the bioautography test, the mycelial crude extract of JPA isolate gave the inhibition zone against *B. subtilis* as well with the R_f in a range of 0.7-0.8 (Fig. 4). This active compound should be separated and tested against *Trichoderma* sp. S2-2. The characterization of this compound is in progress.

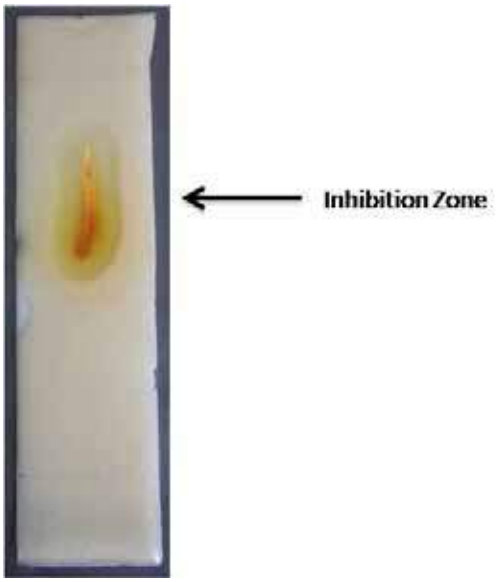


Figure 4. Detection of active compound from mycelial crude extract of JPA isolate on thin layer chromatogram. The activity was tested against *B. subtilis*

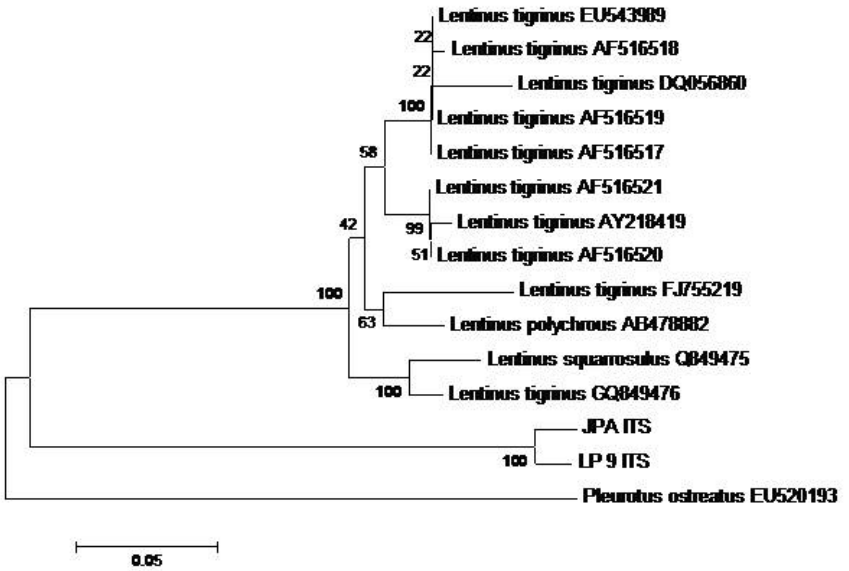


Figure 5. Phylogenetic relationships among JPA isolate and some *Lentinus* and *Pleurotus ostreatus* EU520193

Molecular character of JPA isolate

JPA isolate was grown on root of oil palm trees. Based on morphological characters of fruiting body, JPA isolate was similar to *Lentinus* or *Pleurotus* characters with white pileus, short and off-centre stipe (unpublished). Therefore we investigated the position of JPA isolate based on phylogenetic analyses of the nrITSsquences. Based on ITS sequences data of JPA isolate, they did not match closely with any species presently lodged in GenBank but there were phylogenetic relationships among JPA isolate with some selected species of *Lentinus*. JPA isolate was positioned in the same clade as some selected species of *Lentinus* and the tree is rooted with *Pleurotus ostreatus* EU520193 (Fig. 5). JPA isolate is in need of further research including morphological and cultural identification. For this purpose, JPA isolate will be attempted to cultivate to obtain its fruiting body.

Future research is to test JPA isolates as inducers of resistance in white oyster mushrooms by determining of laccase enzyme activity after confrontation between the two fungi. Increased laccase activity is a way to overcome another antagonist by *Pleurotus*. The laccases activity increase in *Pleurotus ostreatus,* induced by antagonistic bacteria *Bacillus* spp such as *Paenibacillus polymyxa* [11].

CONCLUSION

JPA isolate was potential as source of antimicrobial compounds and their active compounds could probably be used as biopesticides in control of mushroom diseases.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Department of National Education of Indonesia, in the projects of Fundamental Research, as well as to The Fourth State-Owned Estate (PTPN IV) for financial support of this research. Further, we are grateful to our technicians: Iwa, Engkus and R. Heri Jumhaer who were involved and helped us during research.

REFERENCES

[1] Indartiyah N *et al*. (2012). Jamur. In: Tanaman Obatdan Jamur, pp 121-149, ISBN 978-602-8591-21-8.

[2] Abosriwil SO and Clancy KJ. (2002). A protocol for evaluation of the role of disinfectants in limiting pathogens and weed moulds in commercial mushroom production. *Pest Manag. Sci*. 58: 645-652.

[3] Abosriwil SO and Clancy KJ. (2003). A mini-bag technique for evaluation of fungicide effects on *Trichoderma* spp. in mushroom compost. *Pest Manag. Sci*. 60: 350-358.

[4] Rinker D and Alm G. (2008). Management of casing *Trichoderma* using fungicides. *Mush. Sci.* XVII: 589-600.

[5] Romaine CP *et al*. (2008). Emergence of benzimidazole-resistant green mould, *Trichoderma aggressivum*, on cultivated *Agaricus bisporus* in North America. *Mush. Sci*. 17: 510-523.

[6] Bhatt N and Sing RP. (2002). Casing soil bacteria as biocontrol agents against the mycoparasitic fungi of *Agaricus bisporus*. In: *Proc. 4th Int. Conf. Mush. Biol. Mush. Prod*.: 1-9.

[7] Gyorfi J and Geosel A. (2008). Biological control against *Trichoderma* species in *Agaricus* cultivation. In: *Proc. 6th Int. Conf. Mush. Biol. Mush. Prod.*: 158-164.

[8] Anderson MG *et al*. (2001). Yield comparison of hybrid *Agaricus* mushroom strains as a measure of resistance to *Trichoderma* green mold. *Plant Dis*. 85: 731-734.

[9] Savoie JM and Mata G. (2003).*Trichoderma harzianum* metabolites pre-adapt mushrooms to *Trichoderma aggressivum* antagonism. *Mycol*. 95: 191-199.

[10] Sudirman LI *et al*. (1994). Purification of antibiotics produced by *Lentinus squarrosulus* and preliminary characterization of a compound active against *Rigidoporus lignosus*. *Curr. Microbiol*. 29: 1-6.

[11] Velazquez-Cedeno M *et al*. (2008). Role of *Bacillus* spp. in antagonism between *Pleurotus ostreatus* and *Trichoderma harzianum* in heat-treated wheat-straw substrates. *Bioresource Tech*. 99: 6966-6973.

TOXICOLOGICAL EVALUATION OF *INOCYBE VIROSA*

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ABSTRACT

Western Ghats of the Indian sub-continent harbours great fungal diversity with a wealth of mushroom flora, a few genera of which have been found to be poisonous. A new endemic species, *Inocybe virosa*, reported in the Kerala state, is an addition to the group of poisonous mushrooms and is the focus of the present study. HPLC technique was employed for the qualitative analysis and the quantitative estimation of muscarine in *Inocybe virosa*. The HPLC data revealed a concentration of 0.3 mg/g of the hydro-ethanol extract. Further, the toxicological evaluation was carried out *in vitro* by subjecting the mushroom extract to different digestive enzymes and pH variations, simulating the *in vivo* digestion conditions. The toxin was present in the digested fraction and was identified using the chromatographic technique. The digestate obtained on *in vitro* digestion was studied for its cytotoxicity on intestinal Caco-2 cell line and its *in vivo* toxic potential was verified in mice. On oral administration, characteristic symptoms of toxicity viz., perspiration, lacrimation and salivation were observed. As commonly found in the species of Inocybaceae, the basidiomata of this new endemic *Inocybe virosa* too contains muscarine in a concentration which causes undesirable effects and hence, is not recommended for consumption.

Keywords: *Inocybe virosa*, muscarine, Caco-2 cell line, cytotoxicty, Inocybaceae

INTRODUCTION

Mushrooms have been a coveted food item. But mycophagy or mushroom eating is inextricably linked to mushroom poisoning [1]. Mushroom foraging involves a high risk of confusion, wherein the poisonous mushrooms are mistaken for their edible look-alikes. Macroscopic features cannot be the basis to differentiate poisonous mushrooms as it is the chemical nature of the mushroom which determines its edibility or toxicity.

The Indian sub-continent harbours great fungal diversity and in particular, the Western Ghats, recognised as a biodiversity hotspot, has a wealth of mushroom flora. Studies undertaken to explore the diversity of the macro-fungi in the Western Ghats have indicated the occurrence of a few genera of poisonous mushrooms [2-4]. A survey on the agaric flora of the Kerala state has reported a new endemic species, *Inocybe virosa*, previously undescribed in the literature [5].

Among the seven types of mushroom poisoning syndromes [6], muscarine syndrome is mainly due to consumption of *Inocyb*e and *Clitocybe* species containing the toxin muscarine which cannot be made non-toxic by cooking, freezing or any other means of processing [7]. The manifestations of the syndrome occur within 15-30 minutes after consumption of the mushrooms. The peripheral parasympathetic nervous system gets activated by muscarine as it mimics the action of acetylcholine at the muscarinic acetylcholine receptors. The toxin stimulates the exocrine glands and causes the characteristic symptoms viz., perspiration, salivation and lacrimation. The poisoning also results in constriction of pupils, blurred vision, muscle spasms, nausea, vomiting, diarrhoea, abdominal pain, slow heart-beat and a drop in blood pressure [8]. Muscarine syndrome can also be fatal in the case of ingesting large quantities of the causative species as profound activation of the peripheral parasympathetic nervous system may end in convulsions and finally death [7].

The presence of the toxin muscarine has been identified in different matrices such as mushroom carpophores and food samples as well as in biological samples like urine using various chromatographic techniques. Brown *et al*. [9] detected muscarine in a few *Inocybe* species by paper chromatography using Thies and Reuther's reagents. Gas chromatography was used to detect muscarine and its isomers in a few selected species of *Inocybe* [10]. The identification of muscarine in *Inocybe napipes* by TLC/MS and LC/SIMS was described by Unger *et al*. [11]. Hydrophilic interaction liquid chromatography (HILIC) in combination with electrospray ionization (ESI) tandem mass spectrometry (MS/MS) on a TSK-Gel Amide 80 column was developed by Chung *et al*. [7] to analyse muscarine along with the cyclopeptide mushroom

toxins in food samples. A Liquid Chromatography/Mass Spectrometry method was developed for rapid and specific quantification of muscarine in human urine by Barbora *et al*. [8].The HILIC-ESI-MS-MS method developed for simultaneous identification of several mushroom toxins, was modified by Naoki *et al*. [12] to LC-TOF-MS using a PFP (Penta fluoro phenylpropyl) column.

So far, muscarine has been identified using the liquid chromatography technique in combination with mass spectrometric analysis. Thus, an attempt has been made in the present work to detect muscarine using HPLC independent of mass spectrometry. The method was used for the identification of muscarine in an *Inocybe* species. On establishing the presence of the toxin muscarine in the new species, *Inocybe virosa*, a preliminary toxicological evaluation, *in vitro* and *in vivo* of the mushroom sample was carried out.

MATERIALS AND METHODS

Identification of muscarine in *Inocybe virosa*

Sample preparation: The 50% hydro-ethanol extract of the dried sample of *Inocybe virosa* (Tropical Botanical Garden Trivandrum Herbarium, Accession no. 14191) was prepared. It was purified using the Solid Phase Extraction (SPE) cartridge (Oasis HLB, 1cc) preconditioned with methanol [12]. The extract was dissolved in Milli-Q water and eluted through the cartridge. About 1ml of methanol was then eluted on the same cartridge and the elute was used for the HPLC analysis.

HPLC analysis: Muscarine was determined by HPLC using the JASCO HPLC system fitted with a UV/VIS multi-wavelength detector. Separation was carried out on a HiQ SiL C18 column (250 mm × 4.6 mm, particle size 5µm). The absorbance was monitored at 235 nm. The mobile phase comprised of three solvents: A- buffer containing ammonium formate (2 mM) and formic acid (5 mM) at pH 3.5, B-Acetonitrile and C-Methanol at a flow rate of 0.2 ml/min The gradient program used is as follows: 0 to 8 min- 4% A and 96% B from; 9-30 min- 10% A, 78% B and 12% C; this ratio of solvents was maintained for the next 20 min; 10% A, 70% B and 20% C for the last 10 min [7]. The standard, (+)-Muscarine (iodide salt) was purchased from Cayman Chemical (Purity ≥95%).

In vitro digestion model

The *in vitro* digestion model applied to mushroom samples by Cristina *et al*. [13] is the method adopted for the hydro-alcohol extract of *Inocybe virosa* with a few changes. 100 g of the sample was mixed with sufficient volume of water and heated to boiling. The sample was then cooled and mixed with 10 ml of volunteer's saliva and 10 ml of phosphate buffer (0.08 M pH-6.7) and ground for 5 min on a mortar with a pestle. The pH was then set to 2 with 6 M HCl and pepsin (5 mg per gram homogenate) was added. The mixture obtained was incubated for 2 h at 37 ºC with slow stirring at 60 rpm in a rotary shaker. The sample was subjected to further digestion by adding 20 ml of pancreatic solution (0.08 g pancreatin and 0.5 g bile salts in 20 ml of 0.1 m $NaHCO_3$) and the pH was adjusted to 7.5 with 0.5 M NaOH. To the digested samples, 1 g of $NaHCO_3$ was added for maintaining the pH. The mixture was incubated again for 2 h at 37 ºC with slow rotary stirring at 60 rpm. On complete digestion of the sample, a mixture of a liquid supernatant (LS) and a solid precipitate was obtained and separated.

Cytotoxicity of the digestate

Cell lines: Caco2, a human colorectal adenocarcinoma cell line obtained from NCCS, Pune, was used to assess the cytotoxicity of the digestate obtained on *in vitro* digestion. The cells were grown and maintained using Minimum Essential Medium (MEM) (Sigma-Aldrich) supplemented with 10% foetal bovine serum (HiMedia), 1% penicillin-streptomycin (Sigma-Aldrich), 1.5 g/l Sodium bicarbonate and 110 mg/l Sodium pyruvate (Sigma-Aldrich) at 37 ºC in a humidified atmosphere containing 5% CO_2.

Colorimetric MTT (tetrazolium) assay: The cytotoxic effect was evaluated using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay according to the method published by Mosmann *et al*. [14]. The cells were grown at approximately 5 x 10^5cells per plate in 12 well plate. The digestate was added at 0.5 and 1% in triplicates and incubated over night at 37 ºC under 5% CO_2.The medium present in the wells were replaced by 100 µl MMT. The absorbance was read at 570 nm after 2 hours. Per cent cell death was calculated using the formula: Per cent cell death = (sample OD value – digestate standard OD value)/ (cells only value – digestate standard OD value) ×100

In vivo toxicity study of *Inocybe virosa*

The *Inocybe virosa* extract was tested for its *in vivo* toxic effects on male albino wistar mice (weighing 25-30 gm).The extract was orally administered at 4 different dosages (25, 50,100 and 200 mg/kg body weight (bwt). Each dosage was given to three mice along with three control mice which were administered only water. The behavioural pattern was observed for 4 hours.

RESULTS AND DISCUSSION

Estimation of muscarine in *Inocybe virosa* by HPLC

Muscarine is a characteristic component of the genus *Inocybe* [15] and in fact, the presence of muscarine in Inocybaceae is an ancestral trait [16]. The new *Inocybe* species, *Inocybe virosa*, which by its nomenclature indicates its toxic nature (*virosa*-full of poison), required an analysis for the occurrence of muscarine in its fruiting bodies. Thus an easy and rapid HPLC procedure was employed to test and confirm the presence of muscarine in *Inocybe virosa*, as shown in Fig. 1. The toxin content was quantitatively estimated in the hydro-ethanol extract of dried mushroom sample and it was found to be 0.3 mg/g of the extract.

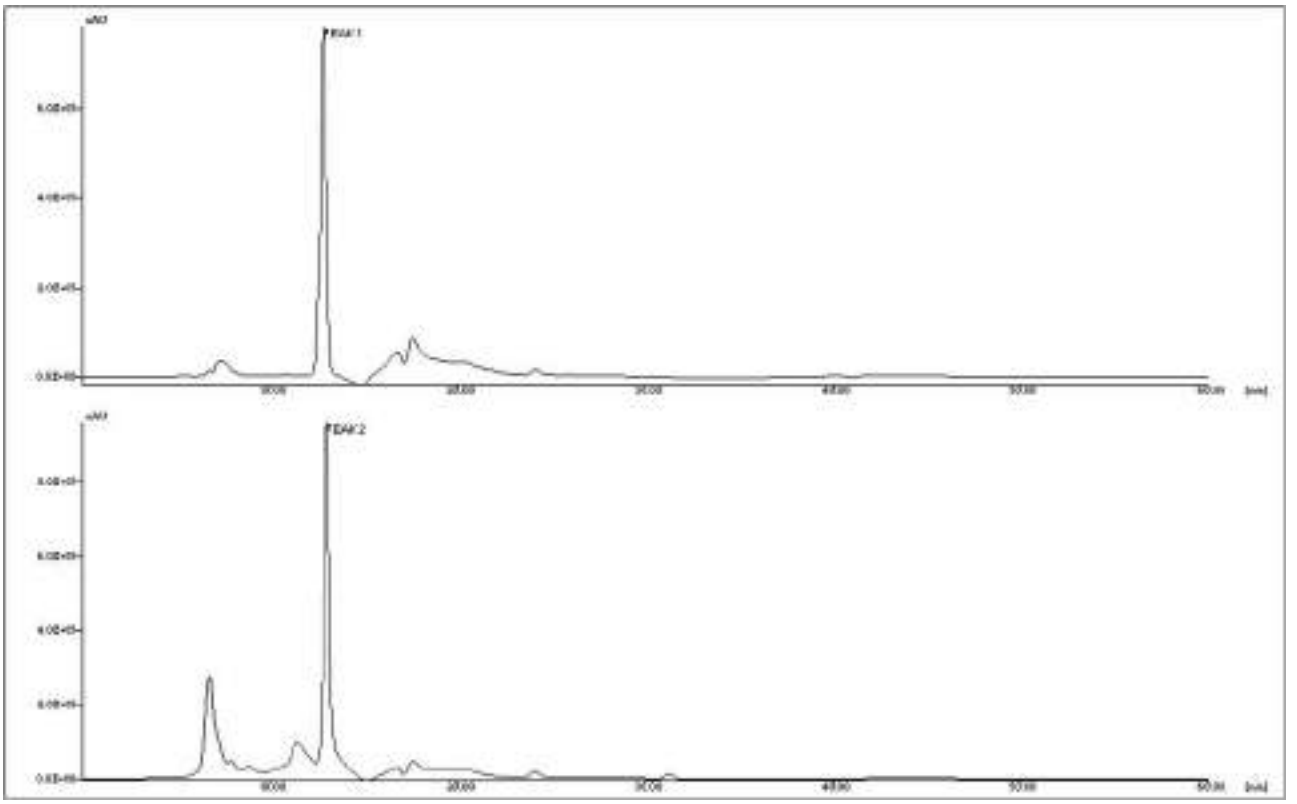

Figure 1. HPLC identification of the presence of Muscarine. PEAK 1: Muscarine Standard; PEAK 2: Muscarine in hydro-ethanol extract of *Inocybe virosa*

Cytotoxic effects of muscarine in the digestate

Since mushrooms are usually consumed along with food, an attempt was made to analyse the effect of digestive enzymes and the extreme pH variations of the digestion process on the compound of interest, muscarine. An *in vitro* digestion model simulating digestive conditions in the mouth, stomach and small intestine was followed. The large intestinal tract was not considered, since during *in vivo* food digestion it is mainly involved in the absorption process [17]. The *in vitro* digestion model for studying the digestibility of the toxin, in combination with Caco-2 cells (representative of the intestinal barrier.), is a useful approach to assess the risk of ingesting the mushroom *Inocybe virosa* containing muscarine.

Muscarine is a fast-acting poison [7] and has a short latency period (symptoms start within 5 hours of consumption) [18]. The initial phase in muscarine poisoning is the gastrointestinal toxicity, reflected in its symptoms like vomiting, diarrhoea and abdominal pain. The liquid supernatant obtained is the bio-accessible fraction which is most likely to be absorbed by the intestinal enterocytes.

Thus, the damaging effects of the bio-accessible fraction was estimated in terms of the cell death caused which is measured by the MTT assay (Fig 2).The extent of damage in case of exposure to 1% of the LS was greater in comparison to 0.5%, evident from the morphological changes observed (Fig 3). A mixture of the digestive enzymes used for the *in vitro* digestion was also applied to the cells to assess their effect on the cells independent of the toxin.

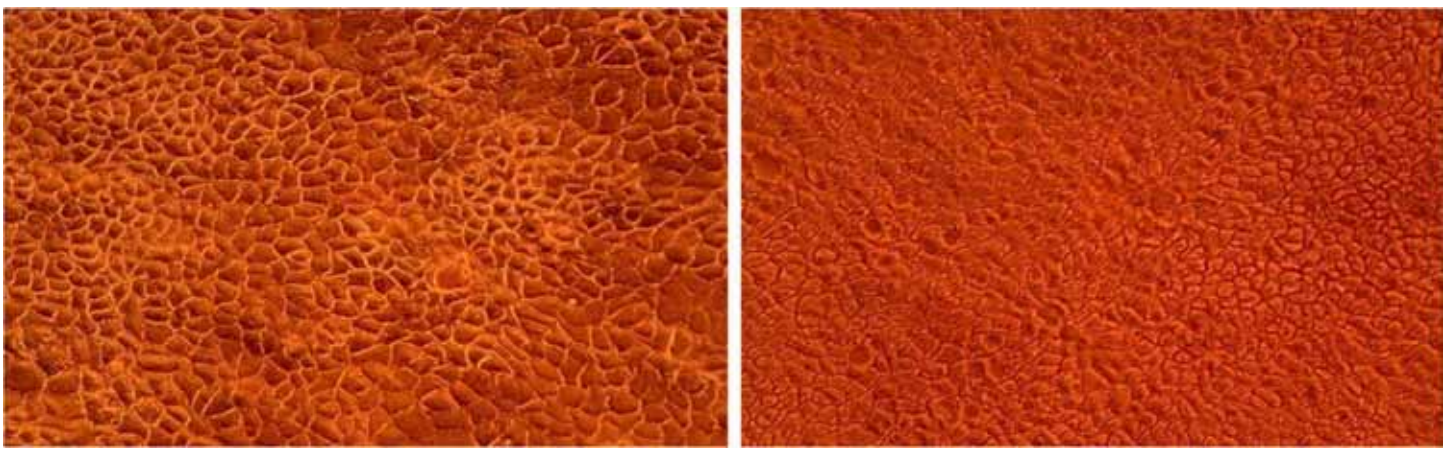

Figure 2. MTT assay to determine the viability of the Caco2 cells upon the exposure to the muscarine digestate. 1-Control, 2-Enzyme treated, 3-0.5% exposure (TEST 1) and 4-1% exposure (TEST 2) of the digestate

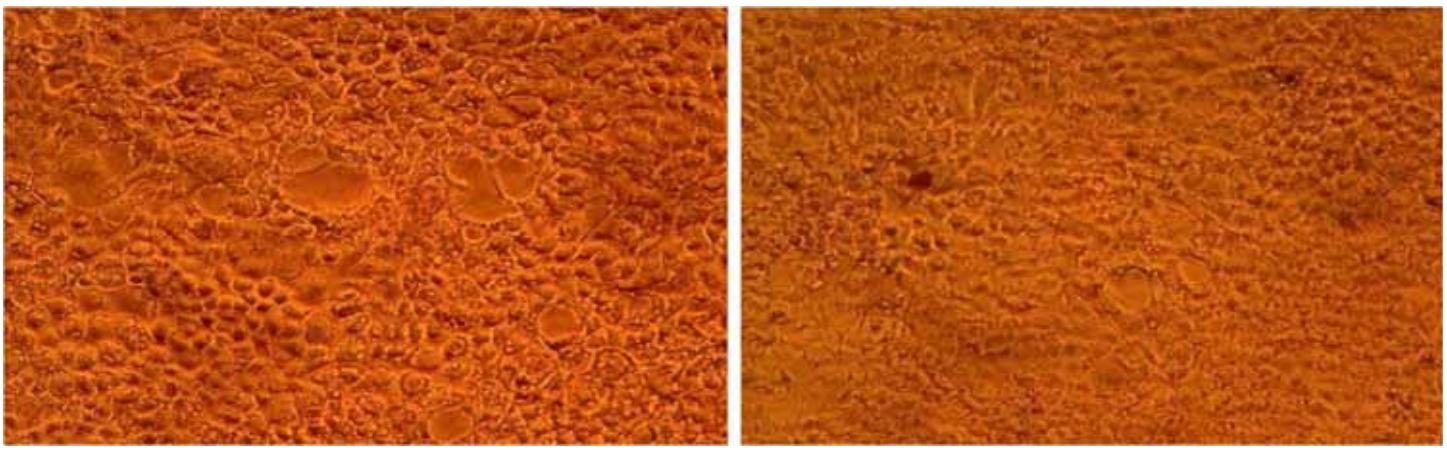

Figure 3. Effect of the digestate on the morphological structure of the cells

In vivo toxicity study

The toxic nature of the mushrooms containing muscarine is well reported [19, 20] and validated by cases of poisoning [21, 22]. A dose dependent study was conducted on mice to assess the effect of consumption of *Inocybe virosa*. The effect of different dosages of the extract is summarized in the table below:

Table 1. Dose-dependent study of *Inocybe virosa* extract

Dosage	Onset of symptoms	Reaction
25mg/Kg bwt	Within 10 minutes	Slight salivation and lacrimation not very evident
50 mg/Kg bwt	Within 10 minutes	Moderate salivation; evident lacrimation
100 mg/Kg bwt	Within 5 minutes	Profuse salivation and pronounced lacrimation
200 mg/Kg bwt	Within 5 minutes	Profuse salivation and pronounced lacrimation
Control	Nil	Nil

All the characteristic symptoms of muscarine poisoning were evident in the study. With an increase in the concentration of the extract administered, the severity of the symptoms was also found to be increasing correspondingly.

CONCLUSION

As commonly found in the species of Inocybaceae, the fruiting body of *Inocybe virosa*, a new and endemic to the Western Ghats of India, also contains muscarine toxin. The muscarine content in the mushroom has been estimated and its toxic effects have been evaluated by this preliminary study. To date, there are no reports available on the toxicity of this mushroom in humans or animals since the mushroom is new. However, to avoid food poisoning due to this mushroom, further studies are warranted in these lines.

ACKNOWLEDGEMENTS

We would like to thank Dr. C. K. Pradeep, Scientist, Plant Systematics & Evolutionary Science Division, Jawaharlal Nehru Tropical Botanic Garden & Research Institute (JNTBGRI) Palode, Thiruvananthapuram, Kerala for the taxonomic identification of the *Inocybe virosa* sample. The constant encouragement provided by Dr. H V Batra, Director, Defence Food Research Laboratory, Mysore is gratefully acknowledged.

REFERENCES

[1] Kumar M and Kaviyarasan V. (1976). Few common poisonous mushrooms of Kolli Hills, South India. *Mycotaxon*. 3(3), 363.

[2] Farook AV *et al.* (2013). A checklist of agarics (gilled mushrooms) of Kerala State, India. *Mycosphere.* 4(1): 97-131.

[3] Thiribhuvanamala G *et al*. (2011). Biodiversity, Conservation and Utilisation of Mushroom Flora from the Western Ghats Region of India. In Mushroom Biology and Mushroom Products. Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products, Arcachon, France, 4-7 October, 2011. Volume 1. Oral presentations. (pp. 155-164). Institut National de la Recherche Agronomique (INRA).

[4] Swapna S *et al.* (2008). Diversity of macrofungi in semi-evergreen and moist deciduous forest of Shimoga District, Karnataka, India. *J Mycol Pl Pathol*. 38(1): 21-26.

[5] Vrinda KB and Pradeep CK. (1996). A new *Inocybe* (Cortinariceae) from Kerala State, India. Mycotaxon (USA).

[6] http://www.botany.hawaii.edu/faculty/wong/BOT135/Lect18.htm

[7] Chung WC *et al.* (2007). Separation of Polar Mushroom Toxins by Mixed-Mode Hydrophilic and Ionic Interaction Liquid Chromatography-Electrospray Ionization-Mass Spectrometry. *J. Chromatog. Sci*. 45(2): 104-111.

[8] Merová B *et al*. (2011). Determination of muscarine in human urine by electrospray liquid chromatographic-mass spectrometric. *Journal of Chromatography.* 879(25): 2549-2553.

[9] Brown JK *et al*. (1962). Paper chromatographic determination of muscarine in *Inocybe* species. *J. Pharmaceu. Sci*. 51(9): 853-856.

[10] Catalfomo P and Eugster CH. (1970). Muscarine and muscarine isomers in selected Inocybe species. *Helvetica Chimica Acta*. 53(4): 848-851.

[11] Unger SE *et al*. (1981). Identification of quaternary alkaloids in mushroom by chromatography/secondary ion mass spectrometry. *Analytical Chem*. 53(7): 976-981.

[12] Yoshioka N *et al*. (2014). A simple method for the simultaneous determination of mushroom toxins by liquid chromatography-time-of-flight mass spectrometry. *Forensic Toxicology*. 32(1): 89-96.

[13] Soler-Rivas C *et al*. (2009). Effect of cooking, in vitro digestion and Caco-2 cells absorption on the radical scavenging activities of edible mushrooms. *Intl. J. Food Sc. Tech*. 44(11): 2189-2197.

[14] Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*. 65(1): 55-63.

[15] Catalfomo P and Eugster CH. (1970). Muscarine and muscarine isomers in selected Inocybe species. *Helvetica Chimica Acta*. 53(4): 848-851.

[16] Kosentka P *et al*. (2013). Evolution of the toxins muscarine and psilocybin in a family of mushroom-forming fungi. *PloS one*. 8(5): e64646.

[17] Versantvoort CH *et al*. (2005). Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. *Food Chemi. Toxicol*. 43(1): 31-40.

[18] De Oliveira and Paulo. (2009). Mushroom poisoning. *Medicina Interna*. 16(4): 232-238.

[19] Malone MH *et al*. (1961). A bioassay for muscarine activity and its detection in certain Inocybe. *Lloydia*. 24(4): 204.

[20] Clark ED and Smith CS. (1913). Toxicological studies on the Mushrooms *Clitocy beilludens* and *Inocybe infida*. *Mycologia*. 5(4): 224-232.

[21] George P and Hegde N. (2013). Muscarinic toxicity among family members after consumption of mushrooms. *Toxicol. Intl*. 20(1): 113.

[22] Lurie Y *et al*. (2009). Mushroom poisoning from species of genus *Inocybe* (fiber head mushroom): a case series with exact species identification. *Clinical Toxicol*. 47(6): 562-565.

IDENTIFICATION OF *TOLYPOCLADIUM PARADOXUM* AND *OPHIOCORDYCEPS YAKUSIMENSIS* FROM CHINA

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ABSTRACT

Two *Cordyceps* species pathogenic to cicada were collected from Zhejiang province, eastern China and identified using morphological and molecular approaches. Morphological characters showed that they were similar to *Tolypocladium paradoxum* and *Ophiocordyceps yakusimensis* respectively. Phylogenetic analyses of *18S-28S rDNA* internal transcribed spacer ITS4/ITS5 sequence or nuclear ribosomal large subunit (26S rDNA) D1/D2 domain sequences analyses further confirmed the morphological identification results respectively

Keywords: *Tolypocladium paradoxum*, *Ophiocordyceps yakusimensis*, *Cordyceps*, phylogeny

INTRODUCTION

The genus *Cordyceps* was earlier classified in the Clavicipitaceae, Clavicipitales, and then reclassified into three families, viz., *Clavicipitaceae*, *Cordycipitaceae* and *Ophiocordycipitaceae* of Hypocreales [1]. Its members are mostly invertebrate pathogens, some of them infect other fungi, mainly *Elaphomyces*, and a few species infect plants [2]. Since its discovery as vegetable fly (well explained by W. Watson in 1763) which was later on classified by Fries as *Cordyceps sobolifera* (the most popular cicada fungus), many interesting specimens have been found [3] and some species are also recently found in China [4-7].

Isaria cicadae (Vegetable cicada), mainly produced in southern forest areas of China, is considered as a famous Chinese herbal medicine. A survey was conducted on the distribution of *Isaria cicadae* resources in Zhejiang bamboo groves and *Isaria cicadae* was found growing on *Platylomia pieli*. Two other types of *Cordyceps* sp. were also found.

To clarify the taxonomy of these two species, detailed studies on the morphology of their teleomorphs and anamorphs were carried out, and comparison was made on their phylogenetic relationships using sequences of two regions of rDNA [internal transcribed spacer ITS4–5.8S-ITS5 or large subunit (LSU) D1/D2].

MATERIALS AND METHODS

Materials

The specimens13AJ6 and 13AJ28 were collected in Anji County, Zhejiang Province, China in 2013, and the isolates obtained were named as BA0332 and BA0351 respectively. The specimens and isolates are deposited in the Mycological Collection of BioAsia Institute of Life Sciences.

Morphological examinations

Morphological observations were conducted by light microscopy. To measure teleomorphic structures such as perithecia, asci, and part spores, fresh materials were mounted in one drop of water on glass slides and observed with an Olympus BX51 microscope using CellSens Dimension software. Microscopic examinations of anamorphic characters were made from cultures maintained on Czapek agar and potato sucrose agar (PSA) at 25 ^{o}C in the dark for more than 14 d.

DNA Extraction

For DNA isolation, the stromata were washed with distilled water and cut to collect contamination-free tissue. Cultured isolates were incubated in potato sucrose broth (PSB) in shake flask at 25 °C for 1 week to collect mycelium. Total DNA was extracted, using N96 Plant Genomic DNA Kit (Tiangen, Beijing, China), according to the manufacturers' instructions.

PCR

The internal transcribed spacer (ITS) of rDNA was amplified by polymerase chain reaction (PCR) using Taq DNA Polymerase (Tiangen, Beijing, China) as a single fragment with the standard primer pairs ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [8] for specimen 13AJ6 and isolate BA0332. Amplification was performed as follows: pre-denaturation at 95°C for 5 min, denaturation 94 °C for 30s, annealing 56 °C for 30s, extension at 72 °C for 50s, 30 PCR cycles, and then extension at 72 °C for 7 min. The divergent D1/D2 domain of the LSU rDNA gene was amplified with primers NL1 (52 -GCATATCAATA AGCGGAGGAAAAG-32) and NL4 (52 -GGTCCGTG TTTCAAGACGG-32) (Cletus and Christie, 1998)[9] for specimen 13AJ28 and isolate BA0351. Amplification was performed as follows: 36 PCR cycles with annealing at 52 °C, extension at 72 °C for 2 min, and denaturation at 94 °C for 1 min.

The PCR products obtained were purified with a Gel/PCR DNA fragments extraction kit (Tiangen, Beijing, China).

Molecular Phylogenetic Analyses

The sequences were obtained from both the type specimen and strains, and submitted to GenBank blasting. Sequences were edited in ClustalX ver. 1.83 software [10]. ClustalX was used to generate the evolutionary distances and the similarity values and to perform the neighbor-joining (NJ) analysis from *K*nuc values. A Neighbor Joining tree (NJ tree) was constructed using Mega 4. The percentages expressed above the branches were frequencies with which a given branch appeared in 1000 bootstrap replications [11]. Species included in this study and other GenBank accession numbers of internal transcribed spacer ITS4–5.8S-ITS5 or large subunit (LSU) D1/D2 were shown in Table 1.

RESULTS AND DISCUSSION

Morphological characters of *Tolypocladium paradoxum* (Kobayasi) Quandt,Kepler & Spatafora

Stroma raising from host of head, fleshy, dark brown or purple brown, erect, solitary, 1-2 root, mostly 1 root, unbranched, clavate or oblate, surface war, fertile portion clavate, long 26-48 mm, thick 4-11 mm; stem cylindrical shape, light brown, long 9-45 mm, thick 5-7 mm, without infertility top (Fig. 1 A).

Perithecia ovate to elliptic, brown, 520-690 × 250-360 µm, vertical deep buried. The wall thickness 23.0-33.5 µm, orifice nearly circular, diameter 34-80 µm (Fig. 1 B). Ascus serpentine, a pointed end, (200) 300-340 × 6-11 µm; ascus cap hat shaped, deeply lobed, orange, 4.2-8 µm high, 5.5-9 µm thick (Fig. 1 C). Ascospore ribbon shaped, numbering 8, which fractured after maturing. Secondary ascospores short rectangular, colorless, 4-7 × 2-3 µm.

In the medium of PSA, colonies are round velvet, pale yellow or abaxially white, the diameter reached 29.1mm after 14 d at 25□ (Fig. 2 A). On Czapek medium white, it reached 19.4mm after 14 d at 25□ (Fig. 2 B). Conidiophores erect, separated 1-2 branches, solitary or wheel was born in the fertile hypha, 13.2-52.5×1.2-2.6 µm, sporogenous cells cone, located in the top coremium, 5.0-10.8 ×1.2-2.3 µm. Conidia polyhedron like, 2.5-4.7 × 2.4-3.7 µm (Fig. 2 C).

There are a few *Elaphocordyceps* species known to be pathogenic on cicada, namely *E. inegoensis*, *E. paradoxa*, and *E. toriharamontana*. However, *E. inegoensis* has perithecia superficial or apparently half-immersed, stalks cylindrical, almost with same diameter with fertile part. *E. toriharamontana* has perithecia rectangularly immersed, oblong, with somewhat long neck (Kobayasi and Shimizu, 1963)[12]. The genus *Elaphocordyceps* and *Chaunopycnis* are emended

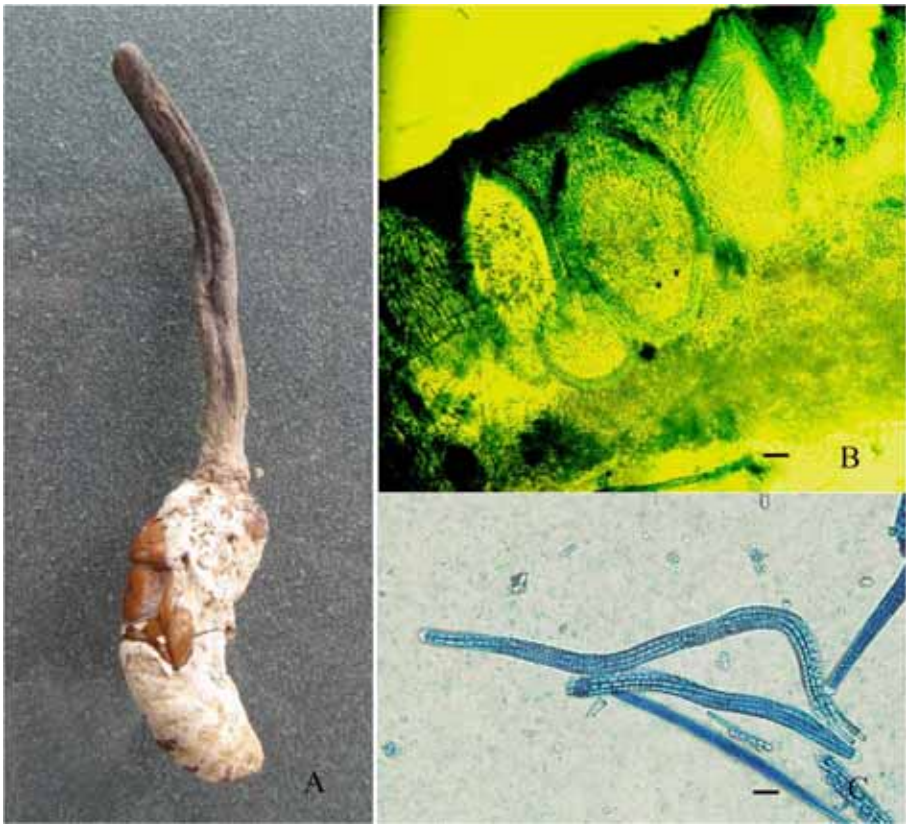

Figure 1. A: Specimen of AJ6; B: Perithecia Bar=100 µm; C: Ascus Bars=10 µm

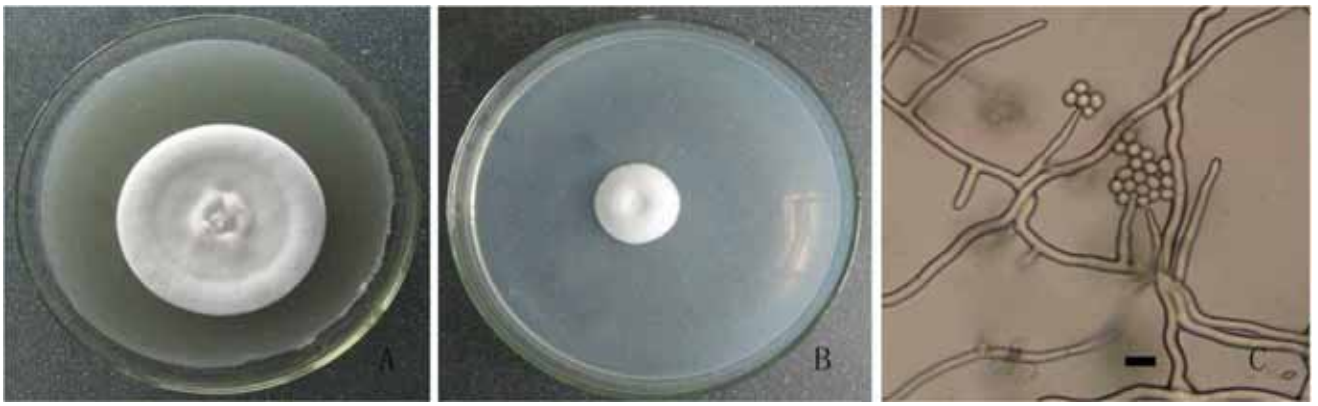

Figure 2. Colonies of the isolate BA0351 on PSA and Czapek media. A: Colony on PSA medium; B: Colony on Czapek medium; C:Conidiogenous structure of BA0351, Bar=10 µm

as new combinations within the genus *Tolypocladium* [13] recently, here we named the specimens 13AJ6 and isolate BA0332 as *Tolypocladium paradoxum.*

Morphological characters of *Ophiocordyceps yakusimensis* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora

Stroma 8-11 cm long, 1.5-2 mm thick, stalk simple or dichotomous with short branch on the upper part, slender cylindric, irregularly contorted (Fig. 3 A). Perithecia vertical buried, spindle shaped or long elliptic, long 620-790 µm, wide 120-200 µm, wall thickness of 22-40 µm; orifice circular, diameter 48-97 µm, center slightly convex (Fig. 3 B). Ascus cylindrical, the lower end acuminate, 180-300 × 3-6.5 µm; ascus cap is flat globose, 3-5 ×5-7 µm (Fig. 3 C). Secondary ascospores short column, 2-3.5× 1-1.8 µm.

Colonies grow slowly on PSA, smooth, gray white, the old culture produced a large number of conidia, appearance silt like yellow, diameter 9.7 mm after 14 days at 25 °C (Fig. 4 A). On Czapek medium, colonies smooth, gray white, 4.9 mm after 14 days at 25 °C (Fig. 4 B).

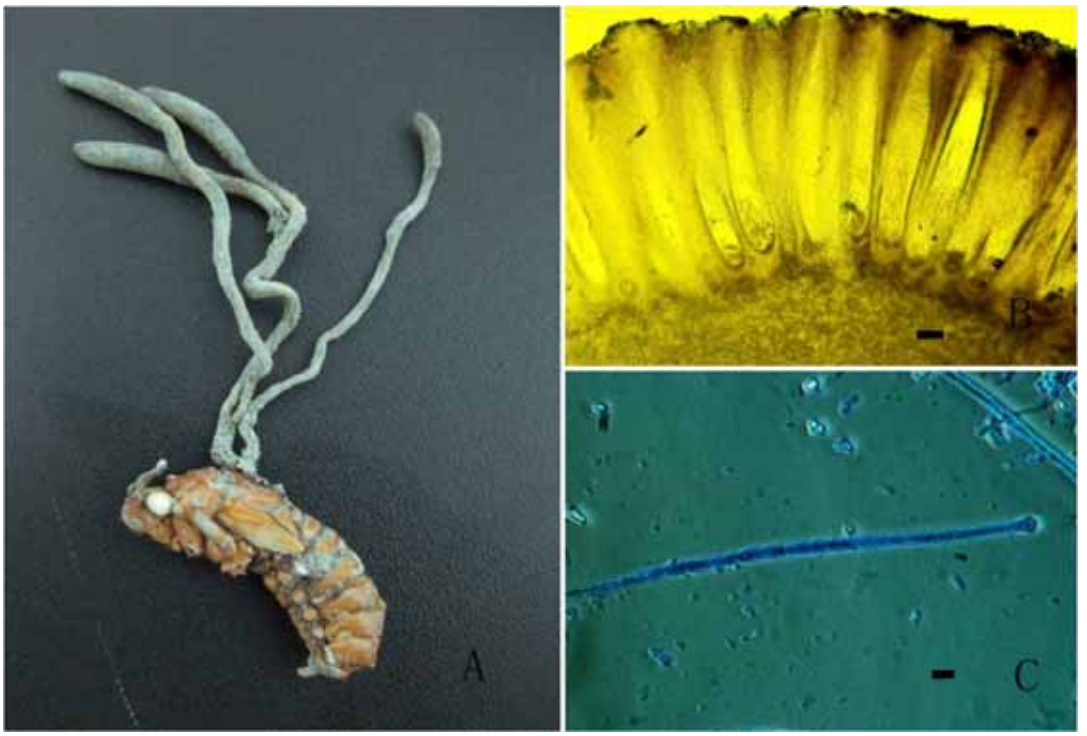

Figure 3. A: specimen of AJ28; B: perithecia Bar=100 µm; C: ascusBar=10 µm

Conidiophores sympodial branched, surface rough, 75-261 × 3.8-6.8 µm. Sporogenous cells ellipsoid, with dilated base, 5.0-10.9 × 2.5-5.3 µm, and a slender neck, 1.50-2.50 µm (Fig. 4C). Conidia are solitary, long shuttle shape, both ends slightly, long oval, colorless, 5.4-10.0×2.5-4 µm (Fig. 4 D).

There are a few *Ophiocordyceps* species known to be cicada pathogens, namely *O. sobolifera*, *O. longissima*, *O. heteropoda*, and *O. yakusimensis.* However, *O. sobolifera* stroma is generally single on head of host, fertile part not distinct from stalk, stromata clavate, usual with conidial form as side branches. *O. longissima* fertile part is distinct from stalk, perithecia ovoid. *O. heteropoda* stroma is thick cylindrical, fertile part ovoid, ascospore 6-7.7 × 0.9-1 µm.

Phylogenetic relationships of specimen/ isolateBA0332 *Tolypocladium paradoxum*

DNA fragments of approximately 600 to 700 bp were amplified by PCR using primers ITS4 and ITS5. The fragment contained ITS4-5.8S-ITS5 regions of rDNA. A fragment of ITS4-5.8S-ITS5 rDNA nucleotides was submitted to GenBank. A BLAST search in NCBI (www.ncbi.nih.gov/blast) showed these sequences to be most similar to *Tolypocladium* sp. KF696558.1 (99%) and *Elaphocordyceps paradoxa* KF356192.1 (99%). A phylogenetic tree was generated from 7 aligned sequences with an equal character. This tree showed that both the specimen and the fungal isolate locate in the same clade with other reference fungal sequences from *Tolypocladium* sp. KF696558.1 and *Elaphocordyceps paradoxa* KF356192.1 (Fig. 5).

Phylogenetic relationships of isolate BA0351*Ophiocordyceps yakusimensis*

DNA fragments of approximately 600 to 700 bp were amplified by PCR using primers NL1and NL4. The fragment contained the LSU D1/D2 regions of rDNA. A fragment of LSU rDNA nucleotides was submitted to GenBank. A BLAST search in NCBI showed this sequence to be most similar to *Ophiocordyceps yakusimensis* KF836760.1 (99%). A phylogenetic tree was generated from 8 aligned sequences with an equal character. This tree showed that both the specimens and the fungal isolate locate in the same clade with other reference fungal sequences *Ophiocordyceps yakusimensis* KF836760.1 (Fig.6). The tree shows *Ophiocordyceps yakusimensis, Metacordyceps yakusimensis, Ophiocordyceps longissima* and *Ophiocordyceps sobolifera* have very closely relatedness.

Figure 4. Colonies of the isolate BA0351 on PSA and Czapek media. A: Colony on PSA medium; B: Colony on Czapek medium; C: Conidiogenous structure of BA0351,D: Conidia of BA0351; Bar=10 µm

CONCLUSION

Morphological characters and phylogenetic analyses confirmed that the two species of *Cordyceps* were *Tolypocladium paradoxum* and *Ophiocordyceps yakusimensis*.

ACKNOWLEDGEMENTS

We would like to acknowledge Zhou L and Zhou JG for collecting the specimens.

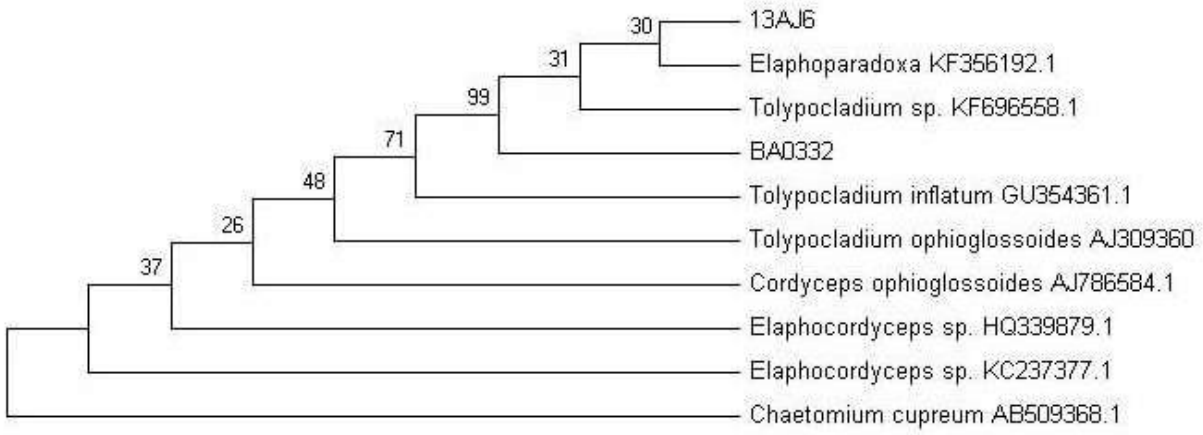

Figure 5. Phylogenetic tree of *Tolypocladium paradoxum* speciemen 13AJ6 and isolate BA0332 and its allies using Clustal X by NJ analysis based on ITS4-5.8S-ITS5 rDNA sequences.

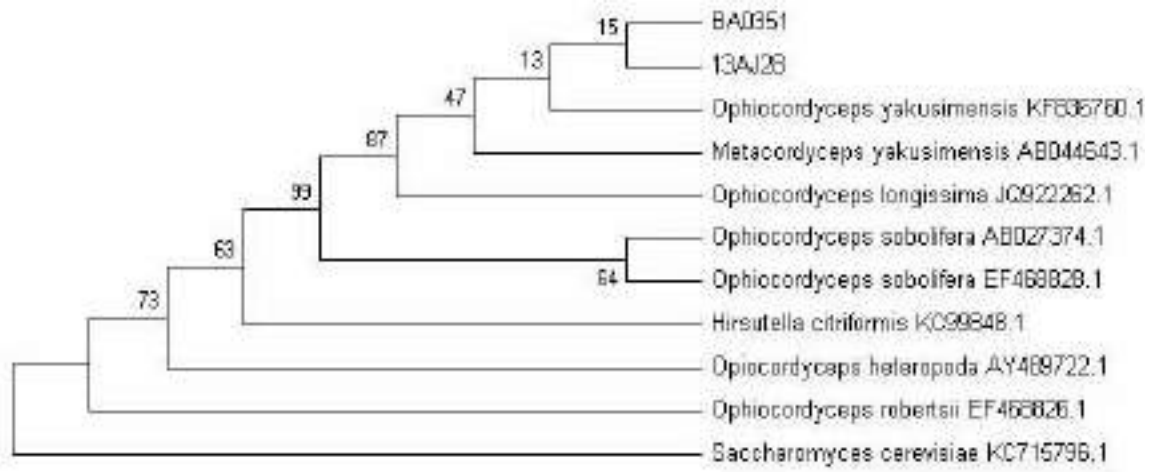

Figure 6. Phylogenetic tree of *Ophiocordyceps yakusimensis* specimen 13AJ28 and isolate BA0351 and its allies using NJ analysis based on LSU D1/D2 rDNA sequences

Table 1 *Cordyceps* and related species and their NCBI accession numbers used in this study.

Species	**GenBank accession no.**	
	Internal transcribed spacer ITS4/ITS5 sequence	**Large subunit (LSU) D1/D2 rDNA sequences**
Tolypocladium sp. YPC-2013	KF696558.1	
Cordyceps ophioglossoides	AJ309360	
Elaphocordyceps sp. 20124794d	HQ339879.1	
Elaphocordyceps paradoxa	KF356192.1	
Tolypocladium inflatum strain KVL 07-62	GU354361.1	
Cordyceps ophioglossoides	AJ786588.1	
Elaphocordyceps sp.20124521a	KC237377.1	
13AJ6	This study	
BA0332	This study	
Chaetomium cupreum	AB509368.1	
Ophiocordyceps yakusimensis strain BA0024		KF836760.1
Ophiocordyceps sobolifera strain KEW 78842		EF468828.1
Ophiocordyceps longissima strain EFCC 6814		JQ922262.1
Ophiocordyceps sobolifera		AB027374.1
Metacordyceps yakusimensis		AB044643.1
Ophiocordycepsbertsii strain KEW 27083		EF468826.1
Ophiocordyceps heteropoda		AY489722.1
13AJ28		This study
BA0351		This study
Hirsutella citriformis strain INIFAP-Hir-2		KC911848
Saccharomyces cerevisiae strain JN1N-17		KC715796.1

REFERENCES

[1] Sung GH *et al.* (2007). Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Studies in Mycology* 57: 5-59.

[2] Kobayasi Y (1982). Keys to the taxa of the genera *Cordyceps* and *Torrubiella. Trans. mycol. Soc. Japan*. 23: 329-364.

[3] Sato H *et al.* (2012). Reassessment of type specimens of *Cordyceps* and its allies,described by Dr. Yosio Kobayasi and preserved in the mycologicalherbarium of the National Museum of Nature and Science (TNS).Part 3: *Cordyceps*s. l. on Cicadidae. *Mycoscience.* 53: 402–408.

[4] Li CR *et al.* (2001). The genus *Cordyceps* and its allies from Anhui I. *Mycosystema*. 20: 29-30.

[5] Liang ZQ *et al.* (2002). Some entomogenous fungi from wuyishan and zhangjiajie nature reserves I. *Cordyceps*. *Mycosystema*. 2: 162-166.

[6] Li CR *et al.* (2006). *Hisutella heteropoda* sp. nov. and its telemorph, a new variety of *Cordyceps heteropoda* . *Mycosystema*. 25: 163-168.

[7] Li CR *et al.* (2010). *Metacordyceps guniujiangensis* and its *Metarhizium* anamorph: a new pathogen on cicada nymphs. *Mycotaxon*. 111: 221-231.

[8] White TJ *et al.* (1990). Ampliûcation and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322, in MA Innis *et al*. (eds.). PCR protocols: a guide to methods and applications. *San Diego, Academic Press*

[9] Cletus PK and Christie JR. (1998).Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek.* 73: 331-371.

[10] Su JX *et al.* (2012). Phylogenetic placement of two enigmatic genera, *Borthwickia* and *Stixis*, based on molecular and pollen data, and the description of a new family of *Brassicales*, *Borthwickiaceae*. *Taxon*. 61: 601–611.

[11] Tamura K *et al.* (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24: 1596-1599.

[12] Kobayasi Y and Shimizu D. (1963). Monographic Studies of *Cordyceps* 2. Group parasitic on Cicadidae *BulL.Nat. Sci. Mus*. 6: 286-314.

[13] Quandt CA *et al.* (2014). Phylogenetic-based nomenclatural proposals for *Ophiocordycipitaceae* (*Hypocreales*) with new combinations in *Tolypocladium*. *IMA Fungus.* 5: 121–134.

TAXONOMY, SOCIOBIOLOGY, NUTRITIONAL AND NUTRACEUTICAL POTENTIAL OF TERMITOPHILOUS AND LEPIOTOID MUSHROOMS FROM NORTH WEST INDIA

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ABSTRACT

The present paper deals with biology and the evaluation of nutritional and nutraceutical potential of wild edible lepiotoid and termitophilous mushrooms from North West India. The studies reveal that presence of appreciable amount of most of the essential nutrients. Out of the total wild mushrooms analysed, *Termitomyces medius* possessed maximum (46.2%) amount of protein followed by *T. badius* (44%) whereas *T. striatus* possessed the least amount(12.95%) on dry weight basis. Maximum percentage of fat was in *Macrolepiota procera* (3.4%) followed by *T. mammiformis* (3.3%). All other mushrooms had considerably low percentage of crude fat per 100 g of dry sample. The crude fibre content was highest in *T. mammiformis* (8% of dry weight) while *T. badius* and *M. rhacodes* possessed minimum percentage of crude fibre (2.5%). The carbohydrate content was highest in *T. striatus* was evaluated to contain (60.27%) followed by *T. mammiformis* (47.65%) while lowest carbohydrate content was in *T. microcarpus* (33.5%) and *T. medius* (33.3%). The overall energy value was maximum in *M. rhacodes* (364.7 kJ) as compared to the species of *Termitomyces* and *Macrolepiota*, evaluated. Amongst the minerals, Fe content was maximum in *T. mammiformis* (673 mg/100 g dry weight) followed by *T. radicatus* (482 mg/100 g) and minimum in *T. striatus* (82 mg/100 g). In addition *Termitomyces* species are quite rich in Mg. *T. medius* (330 mg/100 g) had maximum amount of Mg followed by *T. heimii* (287 mg) and minimum in *T. microcarpus* (6 mg/100 g). Mn (13mg/100 g) and Ca (204mg/100 g) content was highest in *T. medius* and minimum amount of Manganese (1 mg/100 g) was recorded in *M. dolichaula* and *T. microcarpus* (6 mg), respectively. Cu was maximum in *T. striatus* (11 mg/100 g) followed by *T. radicatus* (9 mg/100 g) and *T. badius* (7 mg /100g). As compared minimum quantity of this element was in *T. mammiformis* (4 mg). Zn was maximum (94.3 mg/100g) in *M. rhacodes* followed by *T. microcarpus* (79.5 mg/100 g), whereas minimum quantity of Zn was recorded in *T. radicatus* (35.9 mg/100 g). The nutraceutically important nutrient Se was maximum in *T. microcarpus* (123.2 mg/100g) followed by *T. heimii* (113.10mg/100 g) whereas minimum amount of this element was recorded in *T. striatus* (46.5 mg/100g). The dry mushroom samples were also evaluated for the presence of heavy metals. Traces of some heavy metals, viz. Hg, As and Cd were detected in *T. radicatus, T. medius, T. badius, T. microcarpus* and *M. rhacodes* while Cr, Ag, and Pb were found to be absent in these mushrooms. Phenolic content in *T. microcarpus* (25.85 mg) was maximum, followed by *T. mammiformis* (22.5 mg) and minimum in *M. dolichaula* (5.9 mg). Flavonoids were found in small amount ranging between 1.36 mg/g in *T. microcarpus* and 2.02 mg/g in *M. rhacodes*. The β-carotene content was found in very low amount in different species ranging from 1.1 μg/g in *T. heimii* to 1.5 μg/g in *T. badius.* Lycopene was found to be least in these species ranging from 1.03 μg/g in *T. heimii* to 1.27 μg/g in *T. striatus.* Alkaloids were found in very small concentration ranging between 0.046 mg/g in *T. radicatus* and *T. heimii* and 0.103 mg/g in *M. dolichaula.* Substantially low amount of phenolic content, carotenoids, alkaloids and flavonoids detected in these mushrooms account for their antioxidant property. Maximum content of vitamin A (retinol) in the analysed mushrooms was observed in *Lepiota humei* (0.17 mg/ 100 g) followed by *T. heimii* (0.12 mg/ 100 g) and minimum in *M. dolichaula* (0.075 mg/100 g). As compared thiamine (Vitamin B1) content ranged from 0.75 mg/100 g in *M. rhacodes*to 0.21 mg/100 g in *T. heimii* and the riboflavin (Vitamin B2) content was maximum in *T. heimii* (0.25 mg/100 g) and minimum in *M. rhacodes* (0.13 mg/100 g). Vitamin C (Ascorbic acid) was maximum in *T. reticulatus* (1.45 mg/100 g) and minimum in *L. humei* (0.18 mg/100 g). The nutritional and nutraceutical constituents in these mushrooms are comparable to commonly cultivated mushrooms. Their culinary credentials also compares well with the commonly consumed vegetables. These mushrooms need to be explored further with a view to conserve them and domesticate them so that these can be fruitfully used for human welfare.

Keywords: taxonomy, sociobiology, nutraceutical potential, termitophilous mushrooms, lepiotoid mushrooms

INTRODUCTION

The lepiotoid and termitophilous mushrooms are practically important for their edibility and nutritional profile, medicinal utility and unique flavour man has been hunting for wild mushrooms since times immemorial [1, 2]. Their fresh fruit bodies are sold in the markets of Thailand, along road sides in Old World Tropics [3, 4] and many parts of India [5-8].

Furthermore these mushrooms are also reported to have high medicinal attributes. These properties make them important from the human health point of view. In the trade and manufacture of valuable compounds from these mushrooms, scientists from African countries and South-East Asian countries like South Africa, Thialand, Old Tropics, Tanzania, China and North America are doing lot of work [9-15].

In view of above investigations for evaluation of nutritional and nutraceutical prospects of these species of lepiotoid and termitophilous mushrooms collected from different localities of North part of India was initiated. The results of which are presented in this manuscript.

MATERIALS AND METHODS

Fully mature samples of all wild edible lepiotoid and termitophilous mushrooms were collected from North West India during monsoon season, and ethnomycological information with respect to each one of them was collected using questionnaires, personal observations, and inter-views with old and experienced persons and local informants. Among the interviewees, about 30%– 40% were aware of the ethnomycological uses, out of which a majority (80%) were the elders (above 60 years of age). The specimens were dried at 45 °C for preserva-tion. The dried samples were used for nutritional and nutraceutical studies. The analysis of each collected sample for nutritional attributes was carried out following standard protocols.

Standard techniques of analysis as published by AOAC from time to time for determining the proximate composition of mushrooms (carbohydrates, crude fat, proteins, fibers, ash, moisture content, minerals, heavy metals and vitamins) were employed.

The per cent carbohydrate content present in the mushroom samples was estimated by substracting the total components excepting carbohydrates from 100 g of mushroom samples as per the following relation after Crisan and Sands [16].

Carbohydrate % = 100 - (Protein + Fat + Fiber + Ash Content + Moisture Content)

So as to determine the fat content in the wild samples of edible mushrooms, solvent extraction method as given by AOAC [17] was followed. The per cent crude fat in the mushroom was determined by employing the following relation.

$$\text{Crude Fat}\,\% = \frac{\text{Weight of Ether Soluble Material}}{\text{Weight of the Sample}} \times 100$$

For the calculation of crude protein content in the form of per cent nitrogen, following relation as given by AOAC [18] was followed.

$$\text{Nitrogen}\,\% = \frac{\text{Titrate Volume} \times 0.00014 \times \text{Volume made}}{\text{Aliquot taken} \times \text{Weight of the Sample}} \times 100$$

The fiber estimation was done according to the method given by Maynard [19] and calculated as

$$\text{Crude Fiber}\,\% = \frac{\text{Loss of Weight on Ignition}}{\text{Weight of the Sample}} \times 100$$

The ash content was determined as residue after incineration by following the protocol given by AOAC [20] and calculated by using the following the formulation

$$\text{Crude Ash \%} = \frac{\text{Weight on Ash (g)}}{\text{Weight of the Sample}} \times 100$$

The moisture content estimation was done according to the method given in AOAC [20] and calculated as

$$\text{Moisture Content \%} = \frac{\text{Loss of Weight (g)}}{\text{Weight of the Sample}} \times 100$$

Quantitative estimation of minerals and heavy metals was done by the method of Jackson [21]. Vitamins were estimated by the methods given in Indian pharmacopeia [22]. In the extraction procedure for neutraceutical evaluation the fruiting bodies were air-dried in a Lyophilizer (Ly-Christ Alpha1-2) and powdered before analysis. The dried samples (5 g) were extracted by stirring with 100 ml of methanol at 25 ± ºC at 150 rpm for 24 hr. and filtered through Whatman No. 4 paper. The residue was then extracted with two additional 100 ml portions of methanol, as described earlier. The combined methanolic extracts was evaporated at 40 ± ºC to dryness and re-dissolved in methanol at a concentration of 50 mg/ml, and stored at 4 ±ºC for further use for extraction of different nutraceutical components.

Phenolics (Singleton and Rossi [23]): Phenolics were determined by a Folin–Ciocalteu assay. The extract solution (1 ml) was mixed with Folin–Ciocalteu reagent (5 ml, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/l, 4 ml). The tubes were vortex mixed for 15 seconds and allowed to stand for 30 minutes at 40ºC for colour development. Absorbance was then measured at 765 nm. Gallic acid was used to obtain the standard curve (0.0094 – 0.15 mg/ml), and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

Flavonoids (Yoo *et al*. [24]): For flavonoids quantification, the extract sample concentrated at 2.5 mg/ml (0.5 ml) was mixed with distilled water (2 ml) and $NaNO_2$ solution (5%, 0.15 ml). After 6 min, $AlCl_3$ solution (10%, 0.15 ml) was added and allowed to stand further for 6 minutes. NaOH solution (4%, 2 ml) was added to the mixture, followed by distilled water until a final volume of 5 ml was obtained. The mixture was properly mixed and allowed to stand for 15 minutes. The intensity of pink colour was measured at 510 nm. (+). Catechin was used to make the standard curve (0.015 – 1.0 m M) and the results were expressed as mg of (+)-chatequin equivalents (CE) per g of extract.

Carotenoids (Barros *et al.* [25]): For β-carotene and lycopene determination, the dried methanolic extract (100 mg) was vigorously shaken with 10 ml of acetone–hexane mixture (4:6) for 1 minute and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505 and 663 nm. The amount of β-carotene and Lycopene was calculated according to the following equations: For β-carotene (mg/ 100 ml) = $0.216 \times A_{663} - 0.304 \times A_{505} + 0.452 \times A_{453}$ and lycopene (mg/100 ml) = $-0.0458\ A_{663} + 0.372\ A_{505} - 0.0806\ A_{453}$. The results were expressed as ìg of carotenoid/g of extract.

Alkaloids (Maxwell *et al*. [26]): The alkaloids were extracted from 5 g of each of the dried powdered mushroom samples using 100 ml of 10 % acetic acid, which was left to stand for 4 hrs. The extract was filtered to remove cellular debris and then concentrated to a quarter of the original volume. To this concentrate, 1 % Ammonium solution was added drop-wise until the formation of precipitate. The alkaloids thus obtained were dried to a constant weight at 65 ºC in an oven. The percentage of alkaloids was calculated by using formula.

$$\text{Percentage alkaloids \%:} = \frac{\text{Weight of residue}}{\text{Weight of thesample}} \times 100$$

Three samples of each species wereanalyzed for statistical analysis and all the assays were carried out in triplicate. The results are expressed as mean val-ues and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA).

RESULTS

A. Nutritional Compositions

The results of the studies carried out on the 7 species of *Termitomyces* viz., *T. badius, T. heimii, T. mammiformis, T. medius, T. microcarpus, T. radicatus, T. striatus, T. reticulatus* and 3 species of *Macrolepiota* viz., *M. dolichaula, M. procera, M. rhacodes* on a dry weight basis are presented in Table 1.

Proteins: The results show that maximum protein content was found in *T. medius* (46.2%), followed by *T. badius* (44%), while *T. striatus* (12.95%) contained the lowest amount of protein.

Table 1. Proximatecomposition of wild ediblemushroomsfrom North India

S.No.	Name of Species	Carbohydrate (%)	Crude Fat (%)	Proteins (%)	Fibers (%)	Ash (%)	Moisture (%)
1	*Macrolepiota dolichaula*	56.2 ± 0.10	3.2 ± 0.20	19.95 ± 1.35	4.85 ± 0.18	7.3 ± 0.15	8.5 ± 1.47
2	*M. procera*	60.82 ± 0.11	3.4 ± 0.08	19.95 ± 1.06	5.1 ± 0.22	1.93 ± 0.06	8.8 ± 0.62
3	*M. rhacodes*	68.19 ± 0.17	2.9 ± 0.11	16.45 ± 0.54	2.5 ± 0.01	2.16 ± 0.14	7.8 ± 0.62
4	*Termitomyce sbadius*	39.0 ± 0.17	2.2 ± 0.10	44.00 ± 0.10	2.5 ± 0.01	6.6 ± 0.03	5.7 ± 0.22
5	*T. heimii*	36.2 ± 0.72	1.65 ± 0.19	40.95 ± 0.84	5.0 ± 0.11	8.6 ± 0.05	7.6 ± 0.23
6	*T. mammiformis*	47.65 ± 0.02	3.3 ± 0.17	23.45 ± 0.04	8.0 ± 0.26	9.9 ± 0.09	7.7 ± 0.06
7	*T. medius*	33.3 ± 0.37	2.0 ± 0.05	46.2 ± 0.02	7.5 ± 0.04	5.0 ± 0.20	6.0 ± 0.17
8	*T. microcarpous*	33.55 ± 0.11	2.5 ± 0.01	37.45 ± 0.45	5.0 ± 0.11	15.6 ± 0.06	5.9 ± 0.24
9	*T. radicatus*	41.07 ± 0.03	1.8 ± 0.12	40.00 ± 0.20	4.8 ± 0.04	6.3 ± 0.08	6.03± 0.02
10	*T. striatus*	60.27 ± 0.20	3.25 ± 0.06	12.95 ± 0.05	4.1 ± 0.15	12.13 ± 0.33	7.3 ± 0.18

Carbohydrate: Carbohydrate percentage was found to be maximum in *M. rhacodes* (68.19%), *M. procera* (60.82%), while *T. medius* (33.3%) contained the lowest amount of carbohydrate.

Crude Fat: Maximum amount of crude fats was evaluated in *M. procera* (3.4%). The percentage was comparable in *M. dolichaula* (3.2%), followed by *T. mammiformis* (3.3%) and *T. striatus* (3.25%) and least in *T. heimii* (1.65%)

Fibers: The fiber percentage on dry weight basis in termitophilous mushrooms range from 2.5% - 8% which is substantially high in comparison to species of *Macrolepiota*, where *M. procera* has been evaluated to contain 5.1% fibers in comparison to 4.85% in *M. dolichaula*and 2.5% in *M. rhacodes.*

Ash: Termitophilous mushrooms contained substantially high percentage of ash content which ranged from 5.0% in *T. medius*to 15.6% in *T. microcarpus* as compared to lepiotoid mushrooms where *M. dolichaula* contained maximum (7.3%) and minimum in *M. procera* (1.93%).

Moisture: During the present investigation the moisture percentage in air-dried samples ranged between 5.7% (*T. badius*) to 7.7% (*T. mammiformis*) in termitophilous mushrooms in comparison to 8.8% in *M. procera*.

Minerals: Out of 10 wild samples examined for estimation (Table 2), maximum amount of Fe (673 mg) was recorded in *T. mammiformis* followed by *T. radicatus* (482 mg) while *T. striatus* (82 mg) contained lowest amount of Fe. Other seven species also possessed significant levels of the mineral element. Mg was maximum in *T. medius* (330 mg) followed

by *T. heimii* (287 mg) where as minimum quantity of Mg (6 mg) was recorded in *T. microcarpus.* Maximum amount of Ca (204 mg) was recorded in *T. medius* followed by *T. radicatus* (109 mg) whereas minimum quantity was documented in *M. dolichaula* (5 mg). Cu was maximum in *T. striatus*(11 mg) followed by *T. radicatus* (9 mg/100 gm dry wt.) and *T. badius* (7 mg) where as minimum quantity of this element was detected in *T. mammiformis* (4 mg). Mn was maximum in *T. medius* (13 mg) followed by *T. radicatus* (10 mg). Minimum amount of Mn (1 mg) was recorded in *M. dolichaula.* Zn was maximum (0.09 mg) in *M. rhacodes* followed by *T. microcarpus* (0.08 mg), whereas minimum quantity (0.04 mg) of Zn was recorded in *T. radicatus*. Maximum amount of Se (0.12 mg) was recorded in *T. microcarpus* followed by 0.11 mg in *T. heimii* whereas minimum amount (0.05 mg) was recorded in *T. striatus.*

Table 2. Macro and Micro mineral elements in twenty wild species (mg/100 g of the sample)

S. No.	Name of Species	Ca	Cu	Fe	Mg	Mn	Se	Zn
1	*Macrolepiotadolichaula*	5 ± 0.72	5 ± 0.92	241 ± 1.73	143 ± 1.00	1± 0.23	0.10 ± 0.05	0.08 ± 0.01
2	*M. procera*	14 ± 0.6	9 ± 0.32	276 ± 0.87	254 ± 2.00	5 ± 0.30	0.08 ± 0.03	0.06 ± 0.03
3	*M. rhacodes*	28 ± 1.40	217 ± 0.50	248 ± 1.74	217 ± 0.50	3 ± 0.53	0.06 ± 0.03	0.09 ± 0.02
4	*Termitomycesbadius*	24 ± 1.42	7 ± 1.48	144 ± 0.90	205 ± 1.05	3 ± 0.32	0.08 ± 0.02	0.06 ± 0.02
5	*T. heimii*	28 ± 1.35	6 ± 0.09	388 ± 0.74	287 ± 0.50	5 ± 0.27	0.1 ± 0.02	0.07 ± 0.01
6	*T. mammiformis*	30 ± 1.00	4 ± 0.46	673 ± 1.00	277 ± 0.50	2 ± 0.53	0.07 ± 0.01	0.06 ± 0.01
7	*T. medius*	204 ± 0.50	7 ± 1.84	454 ± 1.00	330 ± 1.00	13 ± 0.79	0.07 ± 0.01	0.06 ± 0.01
8	*T. microcarpous*	24 ± 1.42	6 ± 0.30	86 ± 1.00	6 ± 0.20	3 ± 0.53	0.12 ± 0.02	0.08 ± 0.02
9	*T. radicatus*	109 ± 1.00	9 ± 0.32	482 ± 1.50	272 ± 1.98	10 ± 0.15	0.09 ± 0.01	0.04 ± 0.01
10	*T. striatus*	15 ± 0.22	11 ± 1.08	82 ± 0.82	191 ± 1.00	2 ± 0.28	0.05 ± 0.02	0.07 ± 0.0

Determination of heavy metals: Traces of some heavy metals viz., As, Pb,Ag, Hg, Cd and Cr were found to be present in some of the presently investigated samples (Table 3). Arsenic (As) content was found to be maximum in *T. striatus* (0.0185 mg) followed by *M. rhacodes* (0.0074 mg), *T. mammiformis* (0.0037 mg) and *T.medius* (0.00010 mg) and *T. badius* (0.00002 mg). This heavy metal was not detected in the remaining species of *Macrolepiota* and *Termitomyces*. Other heavy metals including Pb, Ag, Sb and Cr were not detected in any of the evaluated species of *Termitomyces* and *Macrolepiota*. During evaluation the presence of Hg was detected and its concentration ranged from 0.062 - 0.087 mg in the dry samples of *M. dolichaula, M. procera, M. rhacodes* and 0.016-1 mg in all the seven species

Table 3. Qualitative analysis for heavy metals in *Termitomyces* and *Macrolepiota* species

S.No.	Name of Species	As	Pb	Ag	Hg	Sb	Cr	Cd
1	*Macrolepiota dolichaula*	ND*	ND	ND	0.062	ND	ND	0.0019
2	*M. procera*	ND	ND	ND	0.087	ND	ND	0.0019
3	*M. rhacodes*	0.0074	ND	ND	0.069	ND	ND	0.0014
4	*Termitomyces badius*	0.00002	ND	ND	0.096	ND	ND	0.0045
5	*T. heimii*	ND	ND	ND	0.018	ND	ND	0.0040
6	*T. mammiformis*	0.0037	ND	ND	0.043	ND	ND	0.0027
7	*T. medius*	0.00010	ND	ND	0.10	ND	ND	0.0039
8	*T. microcarpous*	ND	ND	ND	0.094	ND	ND	0.00488
9	*T. radicatus*	ND	ND	ND	0.016	ND	ND	0.0022
10	*T. striatus*	0.0185	ND	ND	0.099	ND	ND	0.0017

of *Termitomyces* evaluated. Cadmium (Cd) was another heavy metal which ranged from 0.0014 – 0.0019 mg in all the 3 species of *Macrolepiota* and 0.0017 – 0.00488 mg in the 7 species of *Termitomyces* evaluated.

Vitamins: Amongst the evaluated taxa (Table 4) *T. reticulatus* (1.45 mg) shows maximum amount of Ascorbic Acid followed by *T. radicatus* (0.96 mg) and minimum in *L. humei* (0.18 mg/100 g). As for other vitamins are concerned, *M. rhacodes* (0.80 mg) possessed highest amount of Thiamine, *T. heimii* (0.12 mg, 0.25 mg) contained maximum content of retinol and riboflavin, respectively.

Table 4. Vitamins in lepiotoid and termitophilous mushrooms (mg/100 g of the sample)

S.No.	Name of Species	Vitamin A (Retinol)	Vitamin B1 (Thiamine)	Vitamin B2 (Riboflavin)	Vitamin C (AscorbicAcid)
1	*M. dolichaula*	0.07 ± 0.01	0.75 ± 0.05	0.13 ± 0.02	0.48 ± 0.02
2	*M. rhacodes*	0.09 ± 0.00	0.80 ± 0.04	0.13 ± 0.03	0.36 ± 0.01
3	*T. heimii*	0.12 ± 0.01	0.21 ± 0.01	0.25 ± 0.01	0.24 ± 0.01
4	*T. mammiformis*	0.11 ± 0.01	0.28 ± 0.01	0.21 ± 0.01	0.30 ± 0.0
5	*T. radicatus*	0.10 ± 0.02	0.42 ± 0.04	0.20 ± 0.01	0.96 ± 0.03
6	*T. reticulatus*	0.01 ± 0.00	0.26 ± 0.02	0.23 ± 0.01	1.45 ± 0.10
7	*Lepiota humei*	0.16±0.01	0.49±0.01	0.20±0.03	0.18±0.01

Nutraceutical Studies

Evaluation for the presence of phenolics, flavonoids, carotenoids and alkaloids in the wild edible lepiotoid and termitophilous mushrooms was done by employing standard biochemical techniques (Table 5).

Table 5. Phenolic compounds in wild *Termitomyces* and *Macrolepiota* species (On dry weight basis)

Sr. No.	Species	Phenolic compounds (mg/g)	Flavonoids (mg/g)	β-carotene (µg/g)	Lycopene (µg/g)	Alkaloids (mg/g)
1	*T. microcarpus*	25.85±0.10	2.02±0.17	0.28±0.01	0.14±0.02	0.056±0.03
2	*T. badius*	15.00±0.50	1.49±0.08	0.50±0.03	0.19±0.01	0.052±0.03
3	*T. medius*	16.41±0.01	1.51±0.01	0.17±0.00	0.04±0.00	0.053±0.09
4	*T. striatus*	15.04±0.02	1.38±0.02	0.39±0.01	0.27±0.02	0.050±0.16
5	*T. heimii*	21.32±0.33	1.72±0.02	0.11±0.02	0.03±0.02	0.046±0.04
6	*T. mammiformis*	22.49±0.50	1.86±0.15	0.27±0.02	0.06±0.01	0.077±0.05
7	*T. radicatus*	20.14±0.05	1.67±0.18	0.29±0.00	0.09±0.01	0.046±0.08
8	*M. dolichaula*	5.90±0.58	1.76±0.20	0.12±0.02	0.05±0.01	0.103±0.01
9	*M. procera*	11.0±0.87	1.46±0.04	0.29±0.07	0.07±0.00	0.048±0.03
10	*M. rhacodes*	16.81±0.05	1.36±0.03	0.26±0.01	0.12±0.02	0.053±0.02

Evaluation for Phenolic compounds: Total phenolic content of the edible termitophilous and lepiotoid mushrooms was determined colorimetrically using Folin - phenol method. Maximum amount of phenolic content was documented in *T. microcarpus* (25.85 mg/g) followed by *T. mammiformis* (22.49 mg/g) and minimum amount of phenolic content was evaluated in *M. dolichaula* (5.9 mg/g). Out of the examined species, all species of *Termitomyces* contained substantial amount of phenolics.

Flavonoids: Flavonoids are also polyphenolic compounds that are ubiquitous in nature. The quantity of flavonoids ranged from 1.36 mg/g in *M. rhacodes* to 2.02 mg/g in *T. microcarpus*.

Carotenoids: The β-carotene content was found in very low amount in different species ranging from 0.11 μg/g in *T. heimii*to 0.50 μg/g in *T. badius*. Lycopene content ranged from 0.03 μg/g in *T. heimii* to 0.27 μg/g in *T. striatus*.

Alkaloids: *M. dolichaula* contained 0.103 mg/g of alkaloids which was maximum in comparison to all other evaluated species of *Macrolepiota* and *Termitomyces*.

DISCUSSION

The nutritional analysis carried out on these mushroom samples showed that they are all rich in proteins, fibers, moisture and carbohydrate contents, in contrast to low fat levels, which make them suitable to incorporate into low caloric diets. These results are in agreement with different studies conducted by various workers [25, 27, 28]. In case of *Termitomyces* species evaluated by Mukiibi [29] and Zakia *et al.* [30] the carbohydrate content has been reported to range from 54.2% - 62%. Various medicinal uses were report-ed by Nakalembe *et al.* [31] in many species of termi-tophilous mushrooms (*T. microcarpus*, *T. auranti-cus*, *T. eurrhizus*, *T. clypeatus*, and *T. tyleranus*). Oboh and Shodehinde [32] found a higher protein content in *T. mammiformis* in comparison to other edible mushrooms of Nigeria, and more protein content was found in the pileus than the stipe (28.6% and 24.8%, respectively). Masamba and Kazombo-Mwale [33] evaluated 3.9% protein while working with *T. letestui*. Nabubuya *et al.* [34] studied the nutritional properties of *T. microcarpus* and evaluated 25.48% protein content. Mukiibi [29] and Zakia *et al.* [30] documented high percentage (6.8% - 15.6%) of dry ash in different species of *Termitomyces* including *T. microcarpous* (14.1%). Crisan and Sands [16] documented an average mushroom to contain approximately 90% moisture when fresh and 10% - 12% when air-dried.

Alkaloids were also found in very small concentrations ranging from 0.046-0.077 mg/g which is higher than reported earlier in *Schizophyllum* commune (0.015%) and *Polyporus* spp. (0.013%) [37].

Total phenolics evaluated in species were significantly higher than several mushroom species such as *Lycoperdon perlatum*, *Clavaria vermicularis, Marasmius oreades*, *Russula delica*, *Morchella conica*, *Pleurotus pulmonarius, Ganoderma lucidum* and *Coriolus versicolour* has been documented by a number of workers [35-39]. Present findings are in line with those of Barros *et al*. [25], Ramesh *et al.* [36] on their research on bioactive compounds.

Olfeti *et al*. [41] documented the presence of P (8.8 mg), K (23.9 mg), Na (1.1 mg), Ca (1.4 mg) and Mg (1.8 mg) in *M. procera* which is low from present study.

There are several reports about the accumulation of high concentrations of heavy metals in mushrooms [42-45]. Elekes *et al.* [44] recorded the bio-accumulation of some heavy metals in the fruit-ing body of wild growing mushrooms. Slávik *et al*. [46] observed the highest amount of total mercury content is 176.40982 mg/kg dry matter in two fruit bodies of *M. procera* which is much more than our present investigation. Falandysz [47] determined Selenium in fruit body of *Macrolepiota* spp., with an average range of 5 to < 10 μg/g dw and *M. rhacodes* with < 10 μg/g dw which is less then present findings. All species of *Macrolepiota* and *Termitomyces* were evaluated to possess some traces of heavy metals, though within the permissible limits of human consumption [48]. The results obtained in case of vitamins are in agreement with the similar nature of work from elsewhere [25, 27, 28, 49, 50].

In view of their composition, these mushrooms needs to be used directly in diet so as to promote health, taking advantage of the additive and synergistic effects of all the bioactive compounds present in them. Therefore, wild edible mushrooms such as species of *Termitomyces* and *Macrolepiota* a need to be domesticated for their large scale production and subsequent use as natural nutrition sources.

Figure 1. *Termitomyces microcarpus*

Figure 2. *Termitomyces mammiformis*

Figure 3. *Termitomyces radicatus*

Figure 4. *Termitomyces medius*

Figure 5. *Termitomyces heimii*

Figure 6. *Termitomyces striatus*

Figure 7. *Termitomyces badius*

Figure 8. *Termitomyces reticulatus*

Figure 9. *Macrolepiota rhacodes*

Figure 10. *Macrolepiota procera*

Figure 11. *Macrolepiota dolichaula*

Figure 12. *Lepiota humei*

REFERENCES

[1] Chang ST and Miles G. (2004). Mushroom: cultivation, nutritional value, medicinal effects and environmental impact (2nd Ed.) CRC Press, Boca Raton, Fla. pp. 455.

[2] Adejumo TO and Awosanya OB. (2005). Proximate and mineral composition of four edible mushroom species from South Western Nigeria. *Afr. J. Biotechnol*. 4(10): 1084-1088.

[3] Jones EBG *et al*. (1994). A fungus foray to Chiang Mai market in Northen Thailand. *The Mycologist.* 8: 87-90.

[4] Turnbull E and Watling R. (1999). Some records of *Termitomyces* from old world rain forests: 731-738.

[5] Harsh NSK *et al*. (1993a). Forest fungi and tribal economy- a case study in Baiga tribe of Madhya Pradesh. *Indian J. Trop. Biodiv*. 9: 270-279; Harsh NSK *et al*. (1993 b). Market Potential of wild edible fungi in Madhya Pradesh. *Indian J. Trop. Biodiv*. 1: 93-98.

[6] Bhaben T *et al.* (2011). Wild edible fungal resources used by ethnical tribes of Nagaland India. *Indian J. Traditional Knowledge*. 10 (3): 512-515.

[7] Atri NS *et al*. (2005). Systematics and Sociobiology of Termitophilous mushrooms from Punjab. In: Dargan, JS, Atri, NS and Dhingra, GS, eds.TheFungi-Diversity and Conservation in India. Bishen Singh Mahendra Pal Singh: 159-182.

[8] Atri NS *et al*. (2012). Nurtitional and sociobiological aspects of termitophilous mushrooms from North India. *Int. J. Med. Mush.* 14(5): 471–479.

[9] Kurtzman RH. (1975). Mushrooms as a source of food protein. In: Mriedman, M. ed. Protein nutritional quality of Foods and Feeds", Part 2, Marcel Dekker, Inc., New York.

[10] Chang ST and Buswell JA. (1996). Mushroom nutraceuticals. *World J. Microb. Biotechnol*. 12: 1-79.

[11] Cheung PCK. (1997). Dietry fibre content and composition of some edible fungi determined by two methods of analysis. *J. Sci. Food Agaric*. 73: 255-260.

[12] Wasser SP. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl. Microbiol. Biotechnol.* 60 (3): 258-74.

[13] Wasser SP and Weis AL. (1999). Medicinal properties of substances occurring in higher basidiomycetes mushrooms: current perspectives (review). *Int. J. Med. Mush.* 1: 31–62.

[14] Helpern GM and Miller AH. (2002). Medicinal Mushrooms, ancient remedies for modern ailments. M. Evans and Company, New York.: 172.

[15] Ferreira ICFR *et al*. (2010). Compounds from wild mushrooms with antitumor potential. *Anti-Cancer Agents Med. Chem.*10: 424-436.

[16] Crisan EV and Sands A. (1978). Nutritional value. In (eds. Chang, S.T. and Hayes, W.A.) The biology of cultivation of Edible Mushrooms. New York: Academic Press. pp. 251-293.

[17] AOAC International. (1995). Official methods of analysis of AOAC International. 2 vols. 16th edition. Arlington, VA, USA, Association of Analytical Communities.

[18] AOAC (Association of Official Analytical Chemistry). (1990). Official methods of analysis of the Association of Official Analytical Chemists. 15th ed. Arlington: 1105-1106.

[19] Maynard AJ. (1970). Extraction methods and separationprocesses. In: Joslyan, AM *methods of Food Analysis*. 2ndedn. Academic Press. New York: 141-155.

[20] AOAC International. (2003). *Official methods of analysis of AOAC International*. 17th edition. 2nd revision. Gaithersburg, MD, USA, Association of Analytical Communities.

[21] Jackson ML. (1967). *Soil Chemical Analysis.* New Delhi: Prentice Hall of India Ltd.

[22] IP. (1996). *Indian Pharmacopeia*. Addendun 2000. The controller of publications, Govt. of India, Ministry of Health and Family Welfare, Delhi, India.

[23] Singleton VL and Rossi JA. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic*. 16: 144-158.

[24] YooYM *et al*. (2008). Pectolinarin and pectolinarigenin of Cirsiumsetidens prevent the hepatic injury in rats caused by d-galactosamine via an antioxidant mechanism. *Biological and Pharmaceutical Bulletin.* 31: 760–764

[25] Barros L *et al*. (2007). Total phenols, ascorbic acid, beta carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. *Food Chem.*103(2): 413-419.

[26] Maxwell A *et al*. (1995). 3B amino S spirosalane steroidal alkaloids from *Solanumtriste*. *J. Nat. Prod*. 58: 625-628.

[27] Barros L *et al*. (2008). Wild and commercial mushrooms as source of nutrients and nutraceuticals. *Food and Chem. Toxicol*. 46: 2742–2747.

[28] Ouzouni PK *et al.* (2009). Nutritional value and metal content of wild edible mushrooms collected from West Macedonia and Epirus. Greece. *Food Chem*. 115: 1575–1580.

[29] Mukiibi J. (1973). The nutritional value of some Uganda mushrooms. *Acta Hortic*. 33: 171-175.

[30] Zakia B *et al*. (1964). Amino acids of edible mushrooms, *Lepiota* and *Termitomyces* species. *Indian J. Chem*. 2: 380 -391.

[31] Nakalembe I *et al.* (2009). Indigenous knowledge and usage of wild mushrooms in Mid–Western, Uganda. *Afric. J. Ani. Biomed. Sci*. 4 (2): 63-73.

[32] Oboh G and Shodehinde SA. (2009). Distribution of nutrients, polyphenols and antioxidant activities in the pileus and stipes of some commonly consumed edible mushrooms in Nigeria. *Bull. Chem. Soc. Ethiop*. 23(3): 391-398.

[33] Masamba KG and Kazombo-Mwale R. (2010). Determination and comparison of nutrient and mineral contents between cultivated and indigenous edible mushrooms in Central Malawi. *Afri. J. Food. Sci.* 4(4): 176-179.

[34] Nabubuya A *et al.* (2010). Nutritional and hypocholesterolemic properties of *Termitomyces microcarpus* mushrooms. *Afri. J. Food Agri. Nutri. Dev*. 10 (3): 2235-2257.

[35] Ferreira ICFR *et al.* (2009). Antioxidants in wild mushrooms. *Current Med. Chem.* 16: 1543-1560.

[36] Ramesh CH and Pattar MG. (2010). Antimicrobial properties, antioxidant activity and bioactive compounds from six wild edible mushrooms of western ghats of Karnataka, India. *Pharmacognosy Research.* 2: 107-111.

[37] Qui U and Lui Y (2000). Fruitbody production in Basidiomycetes. App Microbio Biotech. 54: 141 -152.

[38] Mau J *et al.* (2002). Antioxidant Properties of Several Medicinal Mushrooms. *J. Agri. Food Chem.* 50: 6072-6077.

[39] Türkoðlu A *et al.* (2009). Antioxidant and Antimicrobial Activity of *Russuladelica* Fr: An Edible Wild Mushroom. *Eurasian J. Analytical Chem.* 2(1): 54-63.

[40] Kumari D *et al.* (2011). Antioxidant activity of three species of wild mushroom Cantharellus collected from north-western Himalaya, India. *Int. J Agri. Bio*. 13(3): 415-418.

[41] Olfati J *et al.* (2009). Identification and chemical properties of popular wild edible mushrooms from northern Iran. *J. Hort. Forest.* 1(3): 48-51.

[42] Kalaè P *et al.* (1991). Concentration of lead, cadmium, mercury and copper in mushroom in the vicinity of a lead smelter. *Sci. Tot. Env.* 105: 109-119.

[43] Isiloglu M *et al.* (2001). Heavy metal contents in some macrofungi collected in the North western part of Turkey. *Enr. Cont. Toxi*. 41: 1-7.

[44] Elekes CC *et al.* (2010). The bioaccumulation of some heavy metals in the fruiting body of wild growing mushrooms. *Not. Bot. Hort.Agrobot. Cluj*. 38(2): 147-151.

[45] Higgs DJ *et al.* (1972). Effect of cooking on selenium content of food. *J. Agric. Food Chem*. 20: 678–680.

[46] Slávik M *et al.* (2013). The content of mercury in edible mushrooms from middle spiš area. *J Microbiol, Biotech Food Sci*. 2 Suppl 1: 2115-2124.

[47] Falandysz J and Gucia M. (2008). Bioconcentration factors of mercury by Parasol Mushroom (Macrolepiotaprocera). *Environ Geochem Health*. 2008; 30 Suppl 2: 121-125.

[48] FAO/WHO Standards, (1976). List of maximum levels recommended for contaminants by the Joint FAO/WHO Codex Alimentarius.

[49] Heleno SA *et al*.(2009). Study and characterization of selected nutrients in wild mushrooms from Portugal by gas chromatography and high performance liquid chromatography. *Microchemical Journal*. 93: 195–199.

[50] Grangeia C *et al*. (2011). Effects of trophism on nutritional and nutraceutical potential of wild edible mushrooms. *Food Res. Inter*. doi:10.1016/j.foodres.2011.03.006.

BIODIVERSITY EXPLORATION OF MILKY MUSHROOM (*CALOCYBE INDICA* P&C) - CONCEPT TO COMMERCIALIZATION

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ABSTRACT

Indian mushroom industry is witnessing a fabulous change in recent years with respect to the types and strains cultivated. *Calocybe indica* P&C popularly known as milky mushroom is relatively new to world mushroom lovers. This species is highly suitable for commercial exploration in the warm humid tropical zones around the world. Purkayastha and Chandra in1974 [1] identified the fungus for the first time from India. Limited attempts were made in cultivation of this species until 1990s. During 1992 an isolate of *Calocybe indica* was collected near a coconut tree (*Cocos nucifera*) at Coimbatore, Tamil Nadu, India. This isolate was found to out yield hither to known cultivated mushrooms around the globe (with an average bio-efficiency of 142 per cent in paddy straw). Further, this tropical edible mushroom variety possessed incomparable shelf life. During 1994-1998 the technology was severally field tested under University Adoptive Research Trials (ART) and Multi Location Trials (MLT) involving mushroom farmers in the entire state. Finally, the technology for commercial cultivation and the new variety APK-2 has been introduced for the first time in the world from Tamil Nadu Agricultural University, Coimbatore, India during 1998**.** From the concept to commercialization, the journey was very tough and challenging. Systematic studies were undertaken during 1992-94 to standardize the physical, physiological, biochemical and cultural requirements for the commercial cultivation of milky mushroom. Continuing with the efforts through a research project funded by ICAR to develop milky mushroom hybrids (2003-2006); and also through ICAR - All India Coordinated Research Project on Mushroom (2000-2014), we have made sustained efforts to collect 25 wild isolates from different habitats and locations in Tamil Nadu. Many of these isolates were found to have mycorrhizal association with the finer roots of *Cocos nucifera, Borosus flabellifer, Peltaphorum ferrugenum, Delonix regia* and *Tamarindus indicus*. Sometimes they have also been found exclusively humicolous. Cultures of at least five isolates have been deposited in the National Repository (NCBI Gen accession No.AY636067) at the Directorate of Mushroom Research (DMR), ICAR, Solan, Himachal Pradesh, India. At present, the annual milky mushroom production in Tamil Nadu state alone is approximately 530 tonnes worth of Rs. 8.0 crore. More than 120 milky mushroom growers are distributed throughout the state. Horizontal spread of the technology is done through ICAR-AICRP, KVKs and State extension functionaries.

Keywords: Milky mushroom, *Calocybe indica* P&C, biodiversity, variety APK-2, commercial prospects

INTRODUCTION

Mushroom production is the best biotechnology process for integrated agro-waste management in rural areas. As an integral part of secondary agriculture, mushroom growing helps to create sustainable rural employment, in addition to addressing protein malnutrition. Indian mushroom industry is witnessing a tremendous change in recent years with respect to the types and strains cultivated. *Calocybe indica* P&C popularly known as milky mushroom is relatively new to world mushroom lovers. This species is highly suitable for commercial exploration in the warm humid tropical zones around the world. Purkayastha and Chandra in1974 [1] identified the fungus for the first time from India. Limited attempts were made in cultivation of this species until 1990s. Milky mushroom (*Calocybe indica*) is a tropical edible mushroom with incomparable shelf life and yield, relatively new to the world mushroom lovers. The technology for commercial cultivation and the new variety APK-2 has been introduced for the first time in the worldfrom Tamil Nadu Agricultural University, Coimbatore by Krishnamoorthy *et al*. during 1998[2]. As on date, button mushroom (*Agaricus bisporus*) available in the market has certain common features with that of milky mushroom. However, cultivation of button mushroom requires huge investment on infrastructure, which is not normally affordable to small farmers. Although some seasonal growers in north India cultivate button mushroom, availability of quality compost and spawn is a major constraint. Further, this mushroom prefers to grow

only under low temperature conditions (15-20 °C). Hence, year around cultivation in a small scale is much difficult. Moreover, button mushroom needs to be carefully transported in cold chain to reach distant markets. Many a times, before it reaches the consumers, lose its original shape, becomes brown, flattened and the market quality is totally lost. Milky mushroom var. APK-2 has several advantages as compared to button mushroom. The technology is much flexible for a small farmer to a big industry. Obviously, there is no doubt that this Indian origin mushroom will have greater stake holders in the world market very soon.

MATERIALS AND METHODS

Field surveys have been conducted since 1992 during South West monsoon (June to September) and North East monsoon (October to December) months in the tropical plains of Tamil Nadu, India. Field data related to the habit and habitat of occurrence of milky mushroom was recorded. Collected specimens have been identified both by morphological and molecular techniques. Cultures have been maintained in PDA slants and some of them have been deposited in the National Repository maintained at DMR, Solan, Himachal Pradesh, India. Sorghum grain spawn was prepared and commercial cultivation techniques have been perfected over years (1992-1998) following standard methods described by Krishnamoorthy [9].

RESULTS AND DISCUSSION

Calocybe indica is popularly known as Dhuthichatta in India.Approximately 20 species of *Calocybe* have been described worldwide by Pegler [3] and Singer [4]. This genus has wide distribution from temperate to tropical climate, although it loves to grow under warm humid weather. *Calocybe* is mainly a grassland species, saprophytic in nature and sometimes ectomycorrhizal. Milky mushroom normally grows on humus soil on road side and forest areas. Almost about four decades ago, *Calocybe indica* was a wild edible mushroom reported from India by Purkayastha and Chandra [1]. Although success was achieved in the induction of fruit bodies of *C. indica* in culture by Purkayastha [5], only limited attempts were made on its commercial cultivation until 1998 by Purkayastha and Nayak [6]; Doshi *et al*. [7] and Pandey and Tewari [8]. Meanwhile, Krishnamoorthy [9] collected an isolate of *Calocybe indica* near a coconut (*Cocos nucifera*) tree adjacent to a sugarcane field near Coimbatore, Tamil Nadu, India. Pure cultures of the specimen were made and preliminary attempts have been made to perfect the commercial production techniques.

From the concept to commercialization, the journey was very tough and challenging. In fact, the initial laboratory work was started in the Mushroom Research Laboratory, Department of Plant Pathology, TNAU, Coimbatore during 1992 as a part of Ph.D thesis work by the author. Systematic studies were undertaken during 1992-94 to standardize the physical, physiological, biochemical and cultural requirements for the commercial cultivation of milky mushroom. With the limited literature scan (only seven at that time, all Indian work and most of the work was reported by Purkayastha and Chandra [1,5,6] from West Bengal, India); and also with minimum experience in working with edible mushrooms, several attempts were made to induce the fruiting bodies of milky mushroom with paddy straw as the basal substrate. More specific attempts were made to design the cropping room for cultivation. However, no significant achievement was possible. It seemed to be very complex. At this crucial stage, the first author was transferred and posted to one of the Regional Research Stations of TNAU at Aruppukottai, during the hot summer month of May-1994. The ambient temperature at that time was around 42 °C. Minimum facilities were available to continue the research work. Luckily, a 15'x12' size room with glass windows all around in the first floor of the residential quarters was available to continue the research. The side walls of the room were partly soaked due to the seepage of water from the overhead tank. Enough sunlight passed through the glass window in to the room from morning to evening. With all hesitation and gloomy mind set, some of the mushroom beds (at case running stage) transported from the TNAU main campus at Coimbatore were dumped inside the new and the only facility available. For the next ten days, the room was completely closed. Surprisingly, and as a blessing in disguise, robust mushrooms each weighing 0.5 to 1.6 kg with milky white colour; and definite shape were found growing on the casing surface. This was the turning point in commercializing milky mushroom technology. Till this stage, the actual cultural requirements viz., high temperature (30-35 °C); high humidity (>85%) and increased levels light intensity were not known to the research team. Later, the blue light requirement of the milky mushroom fungus was established which paved the way to design simple

Table 2. Wild isolates of milky mushroom collected during the survey (1992-2014)

Code No.	Habit and Habitat	Soil type	Weight (g)	Texture	Colour	Pileus		Stipe	
						Diameter (cm)	Thickness (cm)	Length (cm)	Bredth (cm)
WC1	Humicolous	Black loam	250	rough	brownish white	9.3	1.7	3.1	3.0
WC2	Mycorrhizal - *Cocos nucifera*	Black loam	75	smooth	milky white	4.6	1.8	2.5	2.5
WC3	Mycorrhizal - *Cocos nucifera*	Red loam	900	rough	pale to white	13.5	3.0	10.0	2.7
WC4	Humicolous	Clay	78	smooth	white	5.2	2.4	2.8	2.6
WC5	Humicolous	Clay	800	scaly	white	15.8	2.7	8.7	4.0
WC6	Mycorrhizal - *Cocos nucifera*	Black loam	85	smooth	dull white	5.4	2.5	3.0	2.8
WC7	Mycorrhizal - *Cocos nucifera*	Black loam	1100	scaly	white	19.7	2.0	6.5	6.7
WC8	Mycorrhizal - *Cocos nucifera*	Red loam	2010	scaly	white	37.0	3.4	15.0	6.5
WC9	Mycorrhizal - *Cocos nucifera*	Red sandy	1080	smooth	white	17.5	2.5	8.0	6.0
WC10	Mycorrhizal - *Borossus flabellifer*	Black loam	700	rough	white	11.4	2.5	9.7	2.4
WC11	Mycorrhizal - *Delonix regia*	Black loam	120	smooth	milky white	8.0	2.2	4.2	2.4
WC12	Mycorrhizal - *Cocos nucifera*	Red sandy	150	rough	white	5.4	3.3	3.8	3.0
WC13	Humicolous	Black loam	55	smooth	dull white	6.2	2.4	2.8	2.4
WC14	Mycorrhizal - *Tamarindus* indicus	Black loam	200	rough	white	5.5	3.0	3.0	2.5
WC15	Humicolous	Black loam	660	smooth	white	5.0	2.6	10.4	3.0
WC16	Humicolous	Red loam	510	scaly	white	5.2	3.0	3.6	2.5
WC17	Mycorrhizal - *Cocos nucifera*	Red loam	140	smooth	white	4.8	2.8	3.0	2.2
WC18	Mycorrhizal - *Cocos nucifera*	Red sandy	620	rough	dull white	10.0	3.2	12.1	5.2
WC19	Mycorrhizal - *Cocos nucifera*	Red loam	65	smooth	milky white	6.5	3.0	2.8	4.6
WC20	Humicolous	Black loam	720	smooth	white	7.0	3.2	8.4	3.5
WC21	Humicolous	Black loam	540	smooth	white	8.0	3.6	7.4	3.2
WC22	Mycorrhizal - *Peltaforum* sp	Black loam	85	smooth	white	3.5	2.0	3.0	2.2
WC23	Mycorrhizal - *Cocos nucifera*	Red loam	400	scaly	pale to white	7.5	3.2	6.5	2.9
WC24	Humicolous	Black loam	7500	smooth	dull white	47.0	5.4	42.0	4.4
WC25	Mycorrhizal - *Cocos nucifera*	Red loam	820	smooth	pale to white	12.5	3.0	7.0	3.2

commercial mushroom growing sheds with natural ventilation. The period during 1995 -1998 was very much crucial during which the technology was severally field tested under University Adoptive Research Trials (ART) and Multi Location Trials (MLT) involving several mushroom farmers in the state over years (1994-1998). Finally, the first ever variety of milky mushroom *C. indica* var. APK-2 was released for commercial cultivation during 1998 by Tamil Nadu Agricultural University, Coimbatore, India by Krishnamoorthy *et al.* [2, 10, 11].

Milky mushroom var.APK-2 was found to out yield hither to known cultivated mushrooms around the globe (with an average bio-efficiency of 142 per cent (Table 1) in paddy straw). Its milky white color and robust nature are appealing to all. Hence, it is known as "Milky mushroom". Continuing the efforts through a research project funded by ICAR to develop milky mushroom hybrids (2003-2006); and also through ICAR - All India Coordinated Research Project on Mushroom (since 1982), we have made sustained efforts to collect more than 25 wild isolates from different locations in Tamil Nadu (Table 2). Many of these isolates were found to have association with the finer roots of *Cocos nucifera, Borosus flabellifer* and *Tamarindus indicus*. Pure cultures of at least five isolates have been deposited in the National repository (NCBI Gen accession No.AY636067) at National Research Centre for Mushroom (NRCM), ICAR, Solan, Himachal Pradesh, India (presently ICAR-Directorate of Mushroom Research). Some of these isolates were found to out yield APK-2 milky mushroom.

Table 1. Comparative yield performance of *Calocybe indica* var. APK2 in selected mushroom farms (MLT)

Name of the Mushroom Farm	Days for spawn run		Days for pinhead formation	Number of pinheads formed	Number of buttons harvested	Yield (g/ 500g of substrate)	Average weight (g/button)	Bio-efficiency (%)
	50%	100%						
A Prince 5 Mushroom Farm, Sooriampalayam,	7.51^{a}	14.54^{a}	23.67^{ab}	92.46^{b}	13.96^{a}	701.91^{c}	50.28^{b}	140.38
Sakthi Mushroom farm, Nasianur	7.86^{ab}	15.07^{ab}	24.12^{b}	82.59^{c}	12.78^{bc}	663.27^{d}	51.90^{b}	132.65
Maga Mushroom Farm, Coimbatore	9.52^{d}	14.54^{a}	23.17^{ab}	90.00^{b}	12.67^{c}	601.60^{e}	47.48^{c}	120.32
Annai Mushroom Farm Gopichettipalayam	8.43^{c}	15.39^{b}	22.94^{a}	93.47^{b}	12.85^{bc}	774.51^{b}	60.27^{a}	154.90
Sujji Mushroom Farm Kumarapalayam	7.94^{b}	15.22^{ab}	22.84^{a}	101.49^{a}	13.37^{ab}	817.50^{a}	63.28^{a}	163.50

Means followed by a common letter are not significantly different at five per cent level according to DMRT.
Mean of four replications

Panoramic view of a Milky mushroom farm variety with an average bio-efficiency ranging 167-187 % (Table 3). Milky mushroom is a close relative of the highly praised and excellent edible fungus known as St.George's Mushroom (*C. gambosa* (Fr.) Sing.) in Europe. However, the sporophores of *C. i ndica* are attractive milky white in colour with incomparable shelf life (even up to 10 days without refrigerated storage). Commercial production of milky mushroom is much flexible from home growing to big industry.

Milky mushroom var. APK-2 released from TNAU, Coimbatore (1998)

A wild isolate (WC11) found to have mycorrhizal relationship with *Delonix regia*

Wild isolate (WC9) collected near *Cocus nucifera*

A wild isolate (WC24) weighing 7.5 kg

Small mushroom ! Big hope !!

Panoramic view of a Milky mushroom farm

Table3. Comparative yield performance of selected wild isolates of *C. indica* (2013-14)

Culture code	Days for spawn run		Days for pinhead formation	Number of pin-heads formed	Number of buttons harvested	Yield (g/ 500g of substrate)	Average weight (g/button)	Bio-efficiency (%)
	50 %	100 %						
WC 2	8.5[c]	15.0[b]	23.3[c]	105.7[c]	15.4[a]	835.9[b]	54.4[b]	167.0
WC 6	8.0[b]	15.0[b]	22.6[b]	106.5[c]	15.7[a]	909.8[c]	58.1[c]	182.0
WC 19	8.0[b]	14.0[a]	22.1[b]	115.4[b]	16.9[b]	934.4[d]	62.7[d]	186.9
APK 2	7.5[a]	14.5[a]	21.5[a]	145.1[a]	15.3[a]	738.5[a]	48.3[a]	147.7

Means followed by a common letter are not significantly different at five per cent level according to DMRT.
Mean values recorded in five different mushroom farms.

CONCLUSION

At present, the annual milky mushroom production in Tamil Nadu is approximately 530 t worth of INR 8.0 crore, More than 120 milky mushroom growers are distributed throughout the state of Tamil Nadu, India. Due to the horizontal spread of the technology through ICAR – All India Coordinated Research Project on Mushroom (functioning in 14 different centres in India including TNAU, Coimbatore with the headquarters at ICAR-DMR, Solan, HP) since 1998, the technology is spreading fast throughout the country for the past few years, especially in Kerala,Karnataka, Andhra Pradesh, Chhattisgarh, Orissa, Bihar and West Bengal. The average production capacities of individual units vary from 15 kg to 200 kg per day. In addition to regular growers, hundreds of home growers are also engaged in the production of milky mushroom throughout India. Regular training, hands on support and Agribusiness Incubation opportunities are provided by TNAU to the milky mushroom growers.

ACKNOWLEDGEMENTS

The author profusely thanks Tamil Nadu Agricultural University, Coimbatore, India for the physical and laboratory support and Indian Council of Agricultural Research, New Delhi for the financial support rendered through ICAR-Adhoc scheme (2002-05); and continuous further support through ICAR-AICRP on Mushroom.

REFERENCES

[1] Purkayastha RP and Chandra A. (1974). New species of edible mushroom from India. *Trans. Br. Mycol. Soc*. 62**:** 415-418.

[2] Krishnamoorthy AS *et al*. (1998). APK 2 Milky mushroom – *Extension Bulletin*, RRS, TNAU, Aruppukottai. India. p 12.

[3] Pegler. (1983). Agaric flora of the lesser Antilles. *Kew Bull. London. Addl. ser. IX*.

[4] Singer R *et al*. (1983). The ectotrophically mycorrhizal fungi of the neotropical low lands especially central Amazonia. *Nova Hedwigia Beih*. 77: 1-352.

[5] Purkayastha RP. (1976). A new method of cultivation of cultivation of *Calocybe indica*–an edible mushroom. *Taiwan Mushroom* 3: 14-18.

[6] Purkayastha RP and Nayak D (1981). Development of cultivation method and analysis of protein of promising edible mushroom, *Calocybe indica* P&C. *Mushroom Sci*. 11**:** 697-713.

[7] Doshi A *et al*. (1989). Cultivation of summer mushroom. *Calocybe indica* (P&C) in Rajasthan. *Mushroom Sci*. 12: 395-400.

[8] Pandey M and Tewari RP. (1993). 1993. Milky mushroom grown on paddy straw. *The Hindu.* Dec. 93: 24.

[9] Krishnamoorthy AS. (1995). Studies on the cultivation of milky mushroom, *Calocybe indica* P&C, *Ph.D. Thesis*, TNAU, India. 124 p.

[10] Krishnamoorthy AS. (2004). APK2 - A commercial strain of Milky mushroom from India. *Mushworld* 18: www.mushworld.com.

[11] Krishnamoorthy AS *et al.* (2013). Commercial Prospects of Milky mushroom (*Calocybe indica* var.APK2) and its Chitosan Biopolymer for Plant and Human Health. In: Proc. of the 7th Intl. Medicinal Mushroom Conference. 26-29th Aug.2013, Beijing, China. pp 453-459.

MOLECULAR CHARACTERIZATION AND *IN VITRO* EVALUATION OF INDIGENOUS *SUILLUS* ISOLATES FOR THE PRODUCTION OF MYCORRHIZAL BLUE PINE (*PINUS WALLICHIANA*) SEEDLINGS

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ABSTRACT

Fresh basidiocarps of *Suillus* species were collected from conifer forests of the northwestern Himalayan region of India during monsoon seasons. Eight pure cultures were obtained from the basidiocarps of a range of *Suillus* species. Internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) genes of all the *Suillus* isolates obtained were amplified. Variations within the amplified ITS region of the rDNA genes of *Suillus* isolates were examined by restriction fragment length polymorphism (RFLP). Inter-specific variations in the length and number of restriction sites within the ITS region were observed. Restriction enzyme digests of the ITS–rDNA products for eight *Suillus* isolates separated the isolates into five different groups. When compared the ITS sequences with exiting database and the RFLP patterns, the *Suillus* species were reliably distinguished into five different species, namely *S. sibiricus*, *S. granulatus*, *S. triacicularis*, *S. himalayensis* and *S. indicus*. In addition, some physiological attributes of all the *Suillus* isolates, such as radial growth, biomass yield and *in vitro* mycorrhizal capacities were evaluated to select efficient native fungal inocula for the production of mycorrhizal blue pine (*Pinus wallichiana*) seedlings in nursery. Inter-specific and intra-specific variations were observed in radial growth, biomass yield and mycorrhizal capacities of different *Suillus* isolates. Furthermore, the effects of fungal isolates on growth and biomass yield of *P. wallichiana* seedlings were assessed after four months of the mycorrhizal inoculation. All the *Suillus* isolates enhanced the growth and biomass yield of *P. wallichiana* seedlings as compared to the control treatment, but at different rates. *Suillus sibiricus* isolate SNW06 showed highest improvement in plant growth, biomass and concentration of most nutrients, whereas *S. himalayensis* isolate SNW03 was found to be least effective. On the basis of physiological analysis, mycorrhizal colonization and growth response of *P. wallichiana* seedlings, *S. sibiricus* isolate SNW06 was found to be the most effective *Suillus* isolate for mycorrhizal inoculation of *P. wallichiana* seedlings in nurseries and experimental plantations, followed by *S. indicus* isolate SNW02. Thus, the present study evaluated different indigenous *Suillus* isolates that are best adapted to the local environmental conditions and led to the selection of native and efficient ectomycorrhizal strains for blue pine afforestation programmes.

Keywords: ectomycorrhizal fungi, Himalayan, ITS, *Pinus wallichiana*, *Suillus*

INTRODUCTION

Ectomycorrhizal (ECM) fungi generally improve growth and survival of host plants, increase their nutrient and water uptake, and provide them resistance against biotic (e.g. plant pathogens) and abiotic (e.g. heavy metals) stresses [1-3]. There are many studies demonstrating the positive effect of ECM fungi on growth and nutrient contents of plants, especially the pine trees [4-11]. While considering the ECM *Suillus* species, the fungi have been shown to promote plant height, root shoot biomass as well as nitrogen and phosphorus uptake in pine trees [12-16]. Furthermore, *Suillus* isolates exhibit metal tolerance to many toxic metals, such as Zn, Cu and Cd [17-19] and these metals tolerant *Suillus* isolates have been shown to protect mycorrhizal pine seedlings from metal stress [19-22]. Thus, *Suillus* isolates can be used as an excellent mean for large-scale afforestation and regeneration of pine seedlings.

The ECM fungi differs in their physiological attributes, such as morphology, growth rates and mycorrhizal ability and this is certainly true for *Suillus* species, which exhibit remarkable inter-specific as well as intra-specific variations for a wide range of physiological traits [14, 23, 24]. On the basis of these differentiating features, suitable and efficient ECM isolates can be selected for mass inoculums production for forestry purposes. *In vitro* mycorrhizal capacity of the different local *Suillus* isolates has been evaluated for the growth of a typical Mediterranean pine species (*Pinus halepensis*) with an aim to select

suitable isolates for afforestation programmes [14]. Although there are few studies reporting positive effects of different ECM fungi on growth, biomass, and nutrient contents of *Pinus wallichiana* seedlings [7, 8, 10, 25], but the studies focusing on isolation and evaluation of mycorrhizal capacity of indigenous *Suillus* species to promote growth of *P. wallichiana* seedlings are still lacking. Owing to different advantages provided by *Suillus* species to the host plant, particularly pine species, the present study aimed at isolation of local *Suillus* species and evaluation of their mycorrhizal capacities to select suitable isolates for mass inoculums production was undertaken.

MATERIALS AND METHODS

Isolation of pure cultures

Modified Melin-Norkrans (MMN) agar media [26], Malt Extract (ME) agar (2% w/v) media and Potato dextrose agar (PDA) media supplemented with streptomycin (50µg/ml) were used to isolate pure cultures. Fresh basidiocarps collected were surface sterilized with rectified spirit and cut along the pileal surface with sterile surgical blades to expose the inner pileal flesh. Two–three pieces of clean fresh pileal flesh were transferred to each agar plate and incubated at 25°C for one month. Plates were checked weekly for any contamination and sub-culturing was done, if required. The pure cultures isolated are being maintained on Malt Extract (ME) agar (2%, w/v) media in our laboratory. All the cultures isolated have been and submitted to Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and the corresponding basidiocarps have been deposited in the Herbarium of the Botany Department (PUN), Punjabi University, Patiala, India.

Molecular studies

For molecular analysis of *Suillus* species, genomic DNA was extracted from isolated cultures according to Zhou *et al*. (1999) [27]. The internal transcribed spacer (ITS) region was amplified by PCR using the universal primers [28]. For sequencing purpose, PCR products were separated by electrophoresis on 1.5% agarose gels and purified using a Gel Extraction Kit. In order to obtain restriction patterns for different *Suillus* isolates, purified ITS products were subjected to RFLP (Restriction fragment length polymorphism) analysis. Three different restriction enzymes, namely *AluI*, *HaeIII* and *MboI* (Fermentas) were used in separate digestion reactions with the amplified ITS products. The digested DNA was electrophoresed through 2.0 % (w/v) agarose gels containing ethidium bromide (0.5 ìg/ml) for 4 h at 50 V. The restriction patterns were visualized and photographed using gel documentation system (Quantum–ST4–3026/WL/26M, Vilber Lourmat).

Fungal growth

Growth of *Suillus* cultures isolated was measured on 2% malt extract media with regular sub-culturing. Briefly, fungal plugs (approximately 6 mm diameter) were grown on 90 mm Petri-dishes containing 2% malt extract agar media with three replicates for each isolate. Radial growths of fungal isolates were recorded after incubation at 25 ºC in dark for 4 weeks. Biomass yields of above eight isolates in broth media were also studied. On 90 mm malt extract agar plates, a single colony of each fungus was established until they were 2.0–3.0 cm in diameter. From these plates, single agar plug (approximately 6 mm diameter) was removed from the edges of all the colonies and placed in 250 ml screw-capped Erlenmeyer flasks (three replicates for each isolate) containing 25 ml of malt extract broth media. The loosely capped flasks were incubated for 4 weeks at 25 ºC in dark conditions. The mycelia were harvested by filtration through pre-weighed filter papers (Whatman No. 1). The mycelia were washed with 3 volumes of distilled water and recovered biomasses were dried at 70 ºC until constant weight was achieved.

In vitro mycorrhization

The growth performance of *P. wallichiana* seedlings inoculated with different *Suillus* isolates was evaluated, as described previously by Beatriz *et al*. (2006) with some modifications [14]. In brief, *P. wallichiana* seeds were washed with tap water followed by washing with distilled water and finally surface sterilized in 30% H_2O_2 (v/v) for 25 min in a sterilized flask. The seeds were again rinsed three times with sterilized distilled water, sown in 1.0% water-agar plates and incubated

in slanted position at 25 ºC for two weeks. The germination rate was 70–80% after 8-12 days. Pre-germinated seedlings (1–2 cm root length) were transferred into the tubes containing one month grown *Suillus* isolates. For this purpose, tubes (50 cm^3) were filled with peat, vermiculite (1:10, v/v) mixture and supplemented with 15 ml of liquid Malt extract (2% w/v) media. Twelve tubes were inoculated with 3–4 mycelia plugs (6 mm diameter) cut from the margin of a three weeks old fu ngal colony of each *Suillus* isolate and twelve tubes without any fungus were kept as control. Tubes were incubated at 25 °C for one month to colonize the substrate with the fungus. After transferring the pre-germinated seedlings, tube were wrapped with aluminum foil to protect the roots from direct light. Plants were kept in a growth chamber and grown at 23±2 °C with 16 h photoperiod of 250 ìmol photon m^{-2} s^{-1} light. After 4 months of growth period, shoot height, root length, seedling's fresh weight, and dry weight were measured to study the effect of different *Suillus* isolates on pine growth.

Growth, biomass and ectomycorrhizal root colonization

Shoot height, root length and fresh weights of seedlings were measured directly after washing the roots with tap water. To determine the seedlings dry weight, seedlings were oven dried at 70°C until constant weight was achieved. The percentage of ectomycorrhizal colonization (number of ECM short root/total number of short roots × 100%) in each root sample was determined visually under the stereomicroscope for each soil treatment.

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $p < 0.05$. All the analysis was performed using Graph Pad Prism 5.0 software.

RESULTS

Suillus isolates obtained from the Northwestern Himalayas

All of the *Suillus* isolates got isolated on malt extract (ME) agar (2% w/v) media. Although a few cultures were obtained also on Modified Melin-Norkrans (MMN) agar media, but the growth rate was comparatively low or otherwise poor. PDA (Potato dextrose agar) media was not found satisfactory for isolating *Suillus* cultures as we didn't get any culture isolated on PDA agar. Total of eight cultures were isolated from fresh basidiocarps (Table 1) and have been designated from "SNW01–SNW08" (SNW stands for '*Suillus* species from north western Himalayas'). The corresponding collection number and PUN number of basidiocarps from which cultures were isolated are also mentioned in Table 1.

Table 1. Different *Suillus* isolates (SNW01–SNW08) obtained from the basidiocarps collected from coniferous forests of the northwestern Himalayas, India

S. No.	Species	Collection/PUN No.	Isolate	MTCC accession No.
01	*Suillus triacicularis*	SHP27/PUN 5538	SNW01	11954
02	*Suillus indicus*	SHP07/PUN 6578	SNW02	11955
03	*Suillus himalayensis*	SHP26/PUN 5537	SNW03	11956
04	*Suillus granulates*	SJK13/PUN 5525	SNW04	11957
05	*Suillus sibiricus*	SJK01/PUN 5520	SNW05	11958
06	*Suillus sibiricus*	SHP05/PUN 6577	SNW06	11959
07	*Suillus sibiricus*	SHP12/PUN 6579	SNW07	11960
08	*Suillus sibiricus*	SUK12/PUN 5532	SNW08	11961

RFLP analysis of *Suillus* isolates

Variation within ITS region of all the *Suillus* isolates (SNW01, SNW02, SNW03, SNW04, SNW05, SNW06, SNW07 and SNW08) was examined by RFLP analysis of ITS-PCR products obtained with primers ITS1 and ITS4. PCR

products (approx. 700 bp) amplified from all the *Suillus* isolates were digested with the restriction endonucleases. Restriction digests produced using three different restriction enzymes *i.e.*, *AluI*, *HaeIII* and *MboI* are shown in Fig. 1. The restriction fragments obtained with all the three endonucleases were used to determine polymorphism among different isolates. Due to poor visibility in the gel, fragments below 61 bp were ignored during RFLP analysis. Restriction digestion with *Alu*I resulted into two, *Hae*III into four and *Mbo*I into three types of restriction patterns for all the isolates obtained in the present study. RFLP patterns of ITS region grouped the isolates into five different ITS-RFLP taxa. BlastN analysis also revealed that these isolates belonged to five different *Suillus* species.

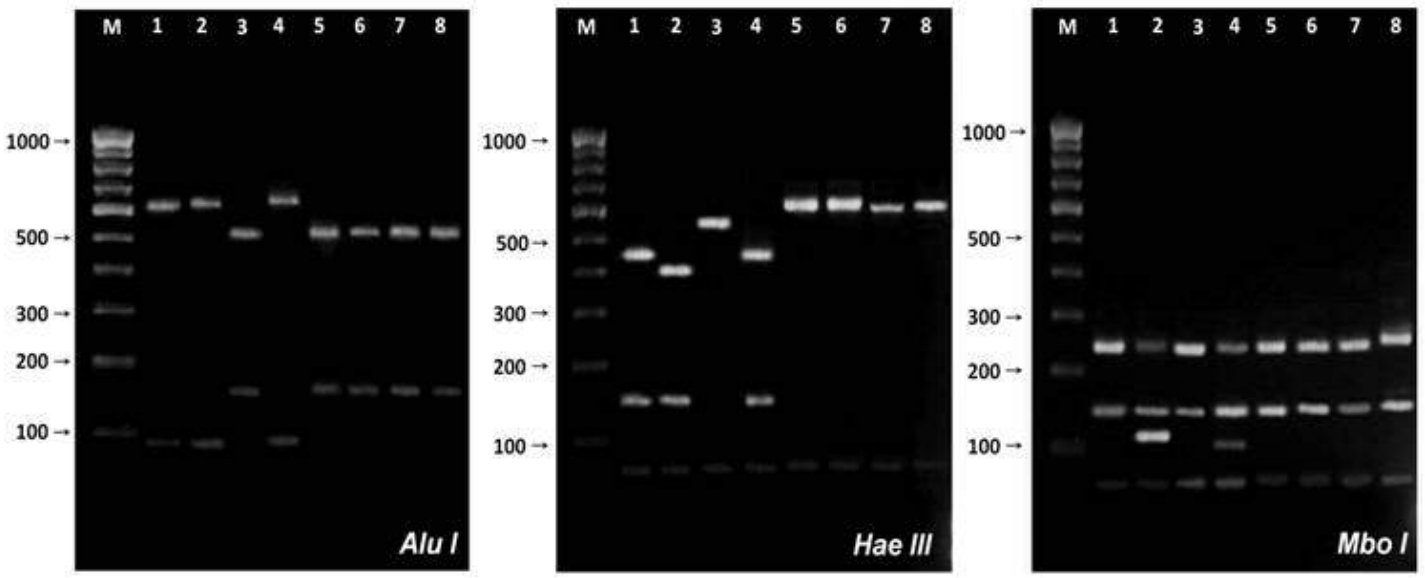

Figure 1. ITS-RFLP Analysis of *Suillus* isolates digested with three different restriction enzymes (*AluI*, *HaeIII*, and *MboI*). Lane M-DNA marker, Lane 1-8 are isolates of SHP27/SNW01, SHP07/SNW02, SHP26/SNW03, SJK13/SNW04, SJK01/SNW05, SHP05/SNW06, SHP12/SNW07 and SUK12/SNW08, respectively. The figure is a composite figure made from different gels.

Radial growth and biomass yield

Radial growth of *Suillus* isolates examined in the present study ranged from 2.2±0.20 cm in *S. granulatus* SNW04 to 5.0±0.40 cm in *S. indicus* SNW02 (Fig. 2). Biomass yield varied from 0.56±0.09 mg/ml in *S. granulatus* SNW04 to 4.16±0.31 mg/ml in *S. indicus* SNW02 (Fig. 3). *S. indicus* isolates SNW02 and *S. sibiricus* SNW06 showed comparatively higher radial growth than most of the other *Suillus* isolates and *S. granulatus* isolate SNW04 showed the lowest growth. Thus, significant inter-specific differences within the growth values of *Suillus* isolates were detected (Fig. 2, 3). On the basis of radial growth, *Suillus* isolates of present study can be divided into two types: one with high growth values (SNW01, SNW02, SNW06, SNW07 and SNW08) and other with low growth values (SNW03, SNW04 and SNW05). Also, significant intra-specific differences were detected in growth within the *S. sibiricus* isolates. *S. sibiricus* isolate SNW05 exhibited significantly lower radial growth and dry weight as compared to other *S. sibiricus* isolates, whereas *S. sibiricus* isolate SNW06 showed the highest value. Isolates of *S. sibiricus* (SNW06, SNW07 and SNW08) were more homogeneous in growth and no significant intra-specific differences in radial growth and dry weight were found within these strains. In general, *S. sibiricus* isolates can also be divided into two groups either with high growth values (SNW06, SNW07 and SNW08) or with low growth value (SNW05).

Mycorrhizal root colonization of *P. wallichiana* seedlings

All the *Suillus* isolates tested colonized the roots of *P. wallichiana* seedlings irrespective of their natural host type. Mycorrhizal root colonization of *P. wallichiana* roots by different isolates of *Suillus* species varied considerably (Fig. 4) and ranged from 23% in case of *S. triacicularis* SNW01 to 71% in *S. sibiricus* SNW06. Variations in the root colonization were also observed even within the isolates of *S. sibiricus* (isolate SNW05–SNW08) that were found to be 37, 71, 62 and 49% in

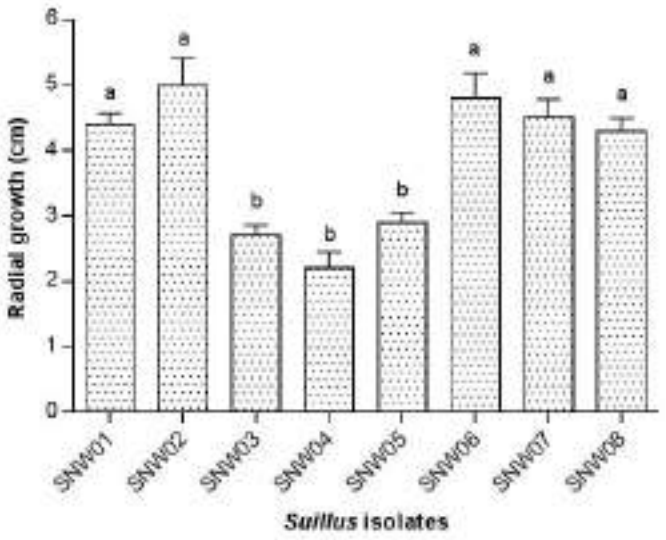

Figure 2. Radial growth (cm) of *Suillus* isolates (SNW01–SNW08) on 2% malt extract agar medium as inferred from means of colony diameter. Different letters represent significant differences among inoculation treatments according to the Tukey's test at $p<0.05$

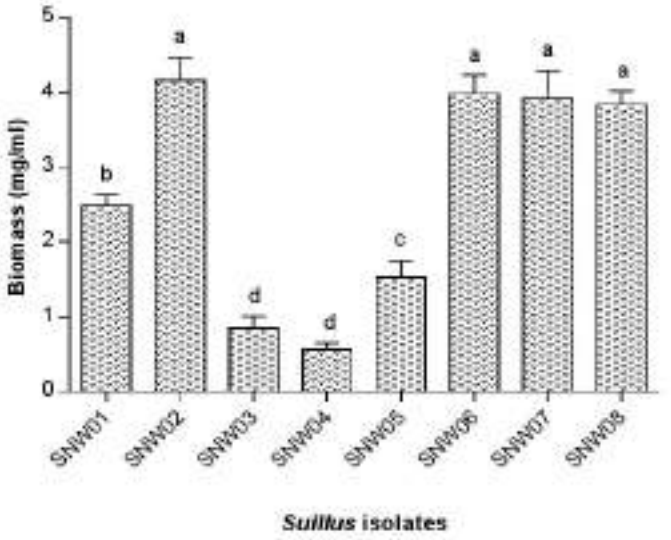

Figure 3. Biomass yield (mg/ml) of *Suillus* isolates (SNW01–SNW08) in 2% malt extract broth medium as inferred from means of mycelium dry weight. Different letters represent significant differences among inoculation treatments according to the Tukey's test at $p<0.05$

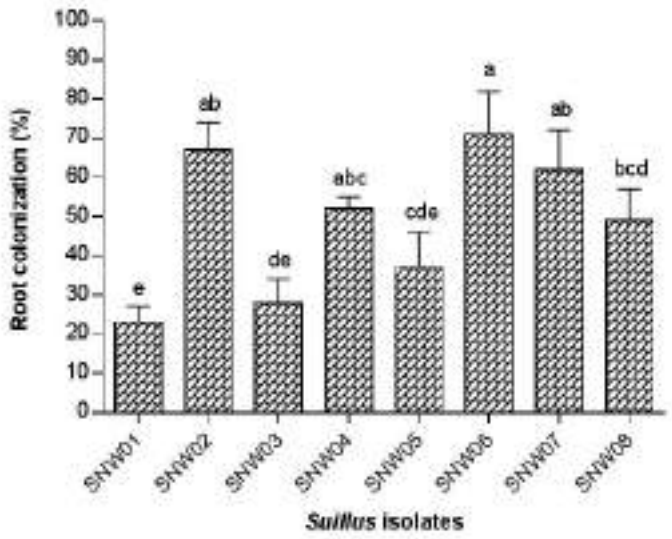

Figure 4. Effect of inoculation with different *Suillus* isolates on ectomycorrhizal root colonization of blue pine (*Pinus wallichiana*) seedlings. Different letters represent significant differences among inoculation treatments according to the Tukey's test at $p<0.05$.

S. sibiricus SNW05, *S. sibiricus* SNW06, *S. sibiricus* SNW07 and *S. sibiricus* SNW08, respectively. Among the different *Suillus* isolates, treatment with *S. sibiricus* SNW06 (71%) showed significantly higher mycorrhizal root colonization followed by *S. indica* SNW02 (67%). On the contrary, *S. triacicularis* SNW01 (23%) showed significantly lower root colonization. Treatments with other isolates showed intermediate colonization percentage values. No ECM colonization was observed on un-inoculated control seedlings.

Influence on growth and biomass of *P. wallichiana* seedlings

Impact of all the *Suillus* isolates inoculations on growth and biomass of *P. wallichiana* seedlings were studied and outcomes are illustrated in Table 2. The results revealed that all the *Suillus* inoculants generally enhanced growth of *P. wallichiana* seedlings and significantly improved the plant growth (seedling height and root length) and biomass (fresh and dry plant

weight) compared to the un-inoculated control treatment. Among the different *Suillus* inoculants tested individually, the *S. sibiricus* isolate SNW06 showed significantly higher plant growth and biomass followed by *S. indicus* SNW02, *S. sibiricus* SNW07 and *S. granulatus* SNW04 isolates. The blue pine seedlings inoculated with *S. sibiricus* SNW06 showed 51.39, 66.67, 43.57 and 52.29 percent increase in shoot height, root length, plant fresh and dry weight, respectively as compared to control treatment. The highest growth enhancement by *S. sibiricus* SNW06 may be attributed to higher mycorrhizal colonization (71%) of *P. wallichiana* roots by *S. sibiricus* SNW06 isolate in comparison to the other isolates. The minimum increases in the growth and biomass was observed in case of *S. himalayensis* SNW03, which comparatively exhibits poor mycorrhizal root colonization (28%). Thus, the present study identified *S. sibiricus* SNW06 and *S. indicus* SNW02 as efficient *Suillus* isolates for enhancement of growth and biomass of blue pine seedlings.

Table 2. Influence of inoculation with different *Suillus* isolates on growth and biomass of blue pine (*Pinus wallichiana*) seedlings after four months of the growth period

***Suillus* isolate**	**Shoot height (cm)**	**Root length (cm)**	**Plant fresh weight (mg/plant)**	**Plant dry weight (mg/plant)**
SNW01	8.2±0.6 (ab)*	8.6±1.5 (ab)	579±91 (abc)	268±28 (ab)
SNW02	10.8±1.5 (a)	11.2±2.1 (a)	727±77 (ab)	329±31 (a)
SNW03	7.7±0.8 (ab)	7.8±0.6 (ab)	557±28 (bc)	251±29 (ab)
SNW04	9.3±1.4 (ab)	9.8±1.6 (ab)	686±84 (abc)	302±44 (ab)
SNW05	8.4±1.7 (ab)	8.9±0.5 (ab)	590±43 (abc)	276±61 (ab)
SNW06	10.9±1.1 (a)	11.5±1.9 (a)	748±62 (a)	332±49 (a)
SNW07	9.9±1.9 (ab)	11.0±1.6 (a)	712±38 (ab)	315±17 (ab)
SNW08	8.7±1.0 (ab)	9.1±0.6 (ab)	652±73 (abc)	297±41 (ab)
Control	7.2±0.9 (b)	6.9±1.4 (b)	521±52 (c)	218±35 (b)

* Letters within parenthesis in a single column denote significant differences among the treatments by the Tukey's test at $p<0.05$. Mean±SD with the same small letters are not significantly different at $p=0.05$.

DISCUSSION

Molecular ecological studies on ECM fungi have mainly employed restriction analysis of the ITS region (ITS-RFLP) for identification and differentiation at the species level [29]. ITS-RFLP analysis of *Suillus* isolates of present study (Fig. 1) grouped them into five different ITS-RFLP taxa. Molecular typing using ITS-RFLP technique detected twelve ITS-RFLP taxa on non-inoculated, *S. collinitus*-inoculated, and naturally regenerated trees in a fire-disturbed *P. halepensis* plantation [30]. In naturally established *Pinus nigra* nursery, RFLP patterns of ITS region resulted in typing of four ITS-RFLP taxa [31]. Beatriz *et al*. (2006) molecularly characterized nineteen *Suillus* isolates from different pine forests of Central Spain and based on ITS-RFLP analysis, clustered the isolates into six different groups [14]. These studies discussed herein confirm the potential of ITS-RFLP for the identification, molecular characterization, species delimitation and ecological studies of *Suillus* species.

Significant inter-specific differences were found in radial growth and mycelia dry weight of *Suillus* isolates when grown under *in vitro* conditions (Fig. 2 & Fig. 3). Five *Suillus* isolates (*S. triacicularis* SNW01, *S. indicus* SNW02, *S. sibiricus* SNW06, *S. sibiricus* SNW07 and *S. sibiricus* SNW08) showed significantly higher radial growth as compared to other three *Suillus* isolates (*S. himalayensis* SNW03, *S. granulatus* SNW04 and *S. sibiricus* SNW05) that showed significantly lower radial growth. Considering the mycelia dry weight, four *Suillus* isolates (*S. indicus* SNW02, *S. sibiricus* SNW06, *S. sibiricus* SNW07 and *S. sibiricus* SNW08) exhibited highest growth values, one (*S. triacicularis* SNW01) high-intermediate value, one (*S. sibiricus* SNW05) intermediate value and rest two (*S. himalayensis* SNW03 and *S. granulatus* SNW04) showed lowest growth values. Moreover, significant intra-specific differences were also detected within radial growth and mycelia dry weight of different *S. sibiricus* isolates. Furthermore, *in vitro* mycorrhizal root

colonization of *P. wallichiana* roots by *Suillus* isolates was studied. The results revealed that *Suillus* isolates differ in their ability to colonize *P. wallichiana* roots and variations were also observed even within the isolates of *S. sibiricus* (Fig. 4). Inter-specific as well as intra-specific variations in axenic fungal growth and *in vitro* mycorrhizal root coonization of *P. halepensis* roots by different *Suillus* isolates have also been detected by Beatriz et al. (2006) from Central Spain [14].

Outcomes of the present investigation suggested that all the *Suillus* isolates tends to increase seedlings growth (seedlings height, root length) and biomass (fresh weight and dry weight) as compared to the un-inoculated control seedlings. Following ectomycorrhizal inoculations, increase in growth and nutrients content of pine seedlings have been also observed by various authors while working with a variety of pines and ECM fungi [5-10, 14]. While contemplating *in vitro* mycorrhizal capacity of different indigenous *Suillus* isolates for the growth of *P. halepensis* seedlings, Beatriz et al. 2006 noticed that *Suillus* isolates stimulated the growth of *P. halepensis* seedlings in different rates [14]. Our experiments also showed the similar results. In the present study, *S. sibiricus* isolate SNW06 showed significantly higher plant growth and biomass in comparison to other *Suillus* isolates. The enhancement in the growth of blue pine seedlings was followed by *S. indicus* SNW02, *S. sibiricus* SNW07 and *S. granulatus* SNW04 isolates. Comparatively lower increase in the growth and biomass of blue pine seedlings was detected when inoculated with *S. himalayensis* SNW03. On the basis of mycorrhizal root colonization and the effects on growth and biomass of blue pine seedlings, *S. sibiricus* SNW06 and *S. indica* SNW02 were found to be the most effective and suitable *Suillus* isolates for growth of *P. wallichiana* seedlings and therefore suggested suitable for mass inoculums production and field inoculations.

ACKNOWLEDGEMENTS

The authors wish to thank the University Grant Commission (UGC), Bahadur Shah Zafar Marg, New Delhi, India for the financial support provided during the present investigation. We also acknowledge TIFAC-Core, Thapar University, Patiala, Punjab, India for providing necessary laboratory facilities for undertaking the experimental work.

REFERENCES

[1] Garbaye J. (2000). The role of ectomycorrhizal symbiosis in the resistance of forest to water stress. *Outlook on Agriculture* 29: 63–69.

[2] Chalot M *et al*. (2002). An update on nutrient transport processes in ectomycorrhizas. *Plant and Soil* 244: 165–175.

[3] Hall JL. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* 53: 1–11.

[4] Guerin-Laguette A *et al*. (2004). The mycorrhizal fungus *Tricholoma matsutake* stimulates *Pinus densiflora* seedlings in vitro. *Mycorrhiza* 14: 397–400.

[5] Rincón A *et al*. (2005). Effects of ectomycorrhizal inoculation and the type of substrate on mycorrhization, growth and nutrition of containerised *Pinus pinea* L. seedlings produced in a commercial nursery. *Annals of Forest Science* 62: 817–822.

[6] Sim MY and Eom AH. (2006). Effects of ectomycorrhizal fungi on growth of seedlings of *Pinus densiflora*. *Mycobiology* 34: 191-195.

[7] Dar GH *et al*. (2007). Effect of source and inoculum load of ectomycorrhizae on the growth and biomass of containerized kail pine (*Pinus wallichiana*) seedlings. *Applied Biological Research* 9: 19–28.

[8] Dar GH *et al*. (2010). Influence of ectomycorrhizal inoculation on Blue pine (*Pinus wallichiana*) and Deodar (*Cedrus deodara*) seedlings. *Trends in Biosciences* 3: 60–62.

[9] Dalong M *et al*. (2011). Growth response of *pinus densiflora* seedlings inoculated with three indigenous ectomycorrhizal fungi in combination. *Brazilian Journal of Microbiology* 42: 1197–1203.

[10] Ahangar MA *et al*. (2012). Growth response and nutrient uptake of blue pine (*Pinus wallichiana*) seedlings inoculated with rhizosphere microorganisms under temperate nursery conditions. *Annals of Forest Research* 55: 217–227.

[11] Sousa NR *et al*. (2012). Ectomycorrhizal fungi as an alternative to the use of chemical fertilizers in nursery production of *Pinus pinaster*. *Journal of Environmental Management* 95: S269–S274.

[12] Timonen S *et al*. (1997). Outcome of interactions between genets of two *Suillus* spp. and different *Pinus sylvestris* L. genotype combinations: identity and distribution of ectomycorrhiza and effects on early seedling growth in N-limited nursery soil. *New Phytologist* 137: 691–702.

[13] Colpaert JV *et al*. (1999). Short-term phosphorus uptake rates in mycorrhizal and non-mycorrhizal roots of intact *Pinus sylvestris* seedlings. *New Phytologist* 143: 589–597.

[14] Beatriz R *et al*. (2006). Molecular characterization and evaluation of mycorrhizal capacity of *Suillus* isolates from Central Spain for the selection of fungal inoculants. *Mycorrhiza* 16: 465–474.

[15] El Karkouri K *et al*. (2006). Molecular markers detecting an ectomycorrhizal *Suillus collinitus* strain on *Pinus halepensis* roots suggest successful inoculation and persistence in Mediterranean nursery and plantation. *FEMS Microbiology Ecology* 55: 146–158.

[16] Liu Q *et al*. (2007). Effect of mycorrhizal inoculation on rhizosphere properties, phosphorus uptake and growth of pine seedlings treated with and without a phosphate rock fertilizer. *Journal of Plant Nutrition* 31: 137–156.

[17] Colpaert JV *et al*. (2000). Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus*. *New Phytologist* 147: 367–379.

[18] Colpaert JV *et al*. (2004). Evolutionary adaptation to zinc toxicity in populations of suilloid fungi. *New Phytologist* 162: 549–559.

[19] Adriaensen K *et al*. (2005). Copper-adapted *Suillus luteus*, a symbiotic solution for Pines colonizing Cu mine spoils. *Applied and Environmental Microbiology* 71: 7279–7284.

[20] Colpaert JV and Van Assche JA. (1993). The effects of cadmium on ectomycorrhizal *Pinus sylvestris*. *New Phytologist* 123: 325–333.

[21] Adriaensen K *et al*. (2004). A zinc-adapted fungus protects pines from zinc stress. *New Phytologist* 161:549–555.

[22] Krznaric E *et al*. (2009). Cd-tolerant *Suillus luteus*: A fungal insurance for pines exposed to Cd. *Environmental Pollution* 157: 1581–1588.

[23] Dahlberg A and Finlay RD. (1999). *Suillus*. In: Ectomycorrhizal fungi, key genera in profile-Cairney J.E.G.C., Chambers S.M. (eds). Springer, Berlin Heidelberg New York, pp 33–64.

[24] Cazzoli P. (2002). Approccio al genere *Suillus*. *Revista Iberoamericana de Micología* 1: 3–27.

[25] Itoo ZA and Reshi ZA. (2014). Influence of ectomycorrhizal inoculation on *Pinus wallichiana* and *Cedrus deodara* seedlings under nursery conditions. *Frontiers in Biology* 9: 82–88.

[26] Marx DH. (1969). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. *Phytopathology* 59: 153–163.

[27] Zhou Z *et al*. (1999). Analysis of genetic structure of a *Suillus grevillei* population in a *Larix kaempferi* stand by polymorphism of inter-simple sequence repeat (ISSR). *New Phytologist* 144: 55–63.

[28] White TJ *et al*. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: a guide to methods and applications-Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. (eds). Academic Press, New York, pp. 315–322.

[29] Horton TR and Bruns TD. (2001). The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Molecular Ecology 10: 1855–1871.

[30] El Karkouri K *et al*. (2004). Diversity of ectomycorrhizal symbionts in a disturbed *Pinus halepensis* plantation in the Mediterranean region. *Annals of Forest Science* 61: 705–710.

[31] El Karkouri K *et al*. (2005). Diversity of ectomycorrhizal fungi naturally established on containerised *Pinus* seedlings in nursery conditions. *Microbiological Research* 160: 47–52.

CROP CYCLE TIME, YIELD AND PROTEIN CONTENT OF *LENTINUS SQUARROSULUS* PRODUCED ON SOME ORGANIC WASTES

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ABSTRACT

Agricultural production and the agro-food industry furnish large volumes of solid wastes, which when unutilized could lead to environmental pollution. An attempt was made to utilize wastes from the oil palm and timber industries for the production of *Lentinuss quarrosulus*, a Nigerian edible mushroom. Mahogany sawdust (MSD), *Gmelina* sawdust (GSD), oil palm fruit fibre (OPFF) and oil palm empty fruit bunch (OPEFB), significantly influenced crop cycle time, yield and protein content of the mushroom. The shortest crop cycle time achieved (47 days) was with *Gmelina* sawdust, while oil palm fruit fibre produced mushrooms with the highest protein content (27.42%). Although, there were no significant differences ($p > 0.05$) in yield of mushrooms produced with oil palm fruit fibre and mahogany sawdust, oil palm fruit fibre gave the highest and most consistent yields. Considering the desirable characteristics of yield and protein content, oil palm fruit fibre proved the best waste for commercial production of *L. squarrosulus*.

Keywords: *Lentinus squarrosulus*, yield, protein content, crop cycle time.

INTRODUCTION

Nigeria is one of the largest timber and oil palm producing countries in the world. Unfortunately, much of the wastes from the oil palm and timber industries are either burned, shredded or used as landfill even though these wastes constitute a potentially valuable resource and can be recycled for the production of edible food for man [1]. Several reports have shown that various lignocellulosic residues from agro-industrial sector, such as oil palm and timber wastes among others, can produce the mushroom with nutrients required for spawn run and fructification, which under controlled conditions and procedures result in an optimum product yield [2].

Sawdust is the most popular basal ingredient used in the formulation of substrate for producing various types of mushroom. Christopher and Custodio [3] revealed that hardwoods like mahogany contain much higher amounts of structural carbohydrate than softwood trees and hence, have more nutrients that could be utilized by mushrooms for their growth and fructification. Oil palm fruit fibre and empty bunches are the major components of all solid waste produced from the palm oil industry. These palm oil wastes contain heterogenous water insoluble materials consisting of cellulose, hemicelluloses and lignin and to a lesser extent pectin, starch and other polysaccharides [4]. The fruit fibre has been shown to possess high potential to be used as mushroom growing substrate without any further treatment [5].

The greatest difficulty in feeding humans is to supply a sufficient quantity of the body building protein [6]. In Africa, the gap between the increasing population and supply of protein is somewhat wide since the traditional sources of protein have not kept pace with population growth. In view of the general shortage of animal protein in some developing countries, the need to explore vegetable protein as an alternative source has been duly recognized. Mushrooms are a nutritious food source being rich in protein, vitamins and minerals. The Food and Agricultural Organization (FAO) recognizes mushrooms as food contributing to the protein nutrition of the countries which depend largely on cereals, because of their high protein quantity and quality [7].

The objective of this study was to evaluate the suitability of locally available organic wastes for the production of *L. squarrosulus*. The study therefore, outlines the production protocols for *L. squarrosulus* that can be adopted by rural farmers for commercial, nutritional and conservation purposes.

MATERIALS AND METHODS

Sources of materials

Stock culture of *Lentinus squarrosulus* used for the experiment was obtained in January 2013 from the Pathology Unit of Forestry Research Institute Ibadan, Nigeria. Fresh hardwood sawdust of mahogany (*Khaya ivorensis*) and *Gmelina aborea* were collectedfrom a local sawmill in Nsukka, Nigeria while the oil palm empty fruit bunch (OPEFB) and oil palm fruit fibre (OPFF) were obtained from a local oil palm industry in Nru, Nsukka and Nigeria in February 2013.

Spawn preparation

The spawn was prepared using Sorghum (*Sorghum bicolor*) grains as substrate. Empty salad cream jars were filled with parboiled sorghum grains (75% water) to three quarter full and covered with cotton wool. The jars were autoclaved at a temperature of 121 ^{o}C and 103 KNM^{-2} pressure for 30 min and allowed to cool down overnight after which they were inoculated with the pure culture of *Lentinus squarrosulus* under aseptic conditions in the inoculation chamber. The inoculated grains were incubated at room temperature (25 ± 2 ^{o}C). After the grains had been fully colonized, they were stored in a refrigerator at 4 ^{o}C until required.

Substrate analysis

The cellulose, hemicellulose and lignin contents of the organic wastes were determined before mushroom growth by the method of Datta [8] while the nitrogen content of the wastes was determined by the method of AOAC [9].

Substrate preparation

The fresh OPEFB (chopped into small pieces of about 1-5 cm) and OPFF were soaked in distilled water overnight in order to wash out the remaining oil in the fibre and to gain 75% moisture content. The moisture content of the sawdust was adjusted to 75% with distilled water by sprinkling. Three hundred grams oven-dry-weight equivalent of the moistened substrates were each filled into ten (10) high porosity polypropylene plastic bags measuring 17.5x15 cm each. A polyvinyl chloride pipe measuring 5 cm wide and 3 cm long was passed through the top of each bag. Thereafter, the mouth of each bag was plugged with cotton wool and covered with foil paper. The bags were autoclaved at a temperature of 121 ^{o}C and 103 KNM^{-2} pressure for 30 min.

Substrate spawning and incubation

Autoclaved bags were allowed to cool down to ambient temperature. The bags were randomly picked and spawned with 25 g spawn per 500 g substrate (5% w/w) [10] under aseptic conditions and incubated at a temperature of 28-30 ^{o}C .

Fruit body induction and harvesting

Bags were transferred to a growing room once primordia had formed. Fruit body induction was achieved by reducing the temperature of the environment by spraying water into the room and opening of the mouth of the bags. Fruiting bags were sprayed daily with sterile water using a hand sprinkler and water was placed in a reservoir on the floor to maintain humidity at about 85%-90%. Fresh air was circulated in the growing room using an electric fan. The room was lit on a 12 h on/off cycle using an electronic fluorescent lamp of 85 watts. Fruiting bodies were harvested three days after primordia emergence when the lamellae were fully exposed.

Determination of crop cycle time and yield

Data were collected on the average number of days to spawn run, primordia initiation and fruiting body development on the different wastes. Stipe length and pileus diameter of harvested fruiting bodies were measured and expressed in centimetres (cm) while yield was expressed as the average fresh and dry weights of the mushrooms harvested per bag and expressed in (g). Harvested mushrooms were placed in a paper bag and oven dried at 60 ^{o}C for 48 h. Dried mushrooms were placed

on a laboratory bench for 2 h to cool down before weighing. Mushroom weights were determined using an Ohaus weighing balance (Model AR3130, 0.001 g accuracy, made in England).

Protein Analysis

The protein analysis was conducted at the National Centre for Energy Research and Development, University of Nigeria, Nsukka. The mushrooms harvested from the different wastes were analyzed for their protein content using Micro-Kjeldahl's method as described in AOAC [9].

Experimental design and data analysis

The experiment comprising four substrate types (mahogany sawdust, *Gmelina* sawdust, oil palm fruit fibre and oil palm empty fruit bunch) was laid out using a Completely Randomized Design (CRD) with ten replicates per treatment.

Data on mushroom yield and crop cycle time were analyzed by one way ANOVA using SPSS version 17.0. Mean separation was done using Duncan Multiple Range Test (DMRT). All analysis was done at 5% level of significance.

RESULTS AND DISCUSSION

Mushroom crop cycle time

Variations in the number of days to spawn run, primordia development and fruiting body formation as influenced by the organic wastes can be seen in Table 1. The shortest spawn run of the mushroom (6.4 days) was observed on oil palm fruit fibre. Analysis of variance showed that mushroom spawn run varied significantly ($p < 0.05$) in all the organic wastes

Table 1. Mean crop cycle time of *Lentinus squarrosulus* mushrooms grown on oil palm fruit fibre (OPFF), oil palm empty fruit bunch (OPEFB), *Gmelina* sawdust (GSD) and mahogany sawdust (MSD).

Substrate	Spawn run (days)	Primordia development (days)	Fruit body formation (days)
OPFF	6.4^{d}	39.4^{a}	48.8^{a}
OPEFB	9.9^{c}	34.4^{b}	47.3^{ab}
GSD	15.4^{a}	28.2^{c}	46.6^{b}
MSD	12.3^{b}	33.3^{b}	48.6^{a}

ð Means followed by the same letter (s) in the same are not significantly different at $p > 0.05$ according to Duncan multiple range test (DMRT).

evaluated. Pinheads emerged as small rounded lumps that were grouped at various parts of the substrate surfaces. The rate at which the mushroom developed mycelium on oil palm fruit fibre could probably be due to the residual oil and the appreciable nitrogen content of the waste (Table 2). Nitrogen content of substrates has been shown to affect earliness of fructification and productivity of mushrooms [11]. Schisler and Patton [12] had also reported that lipids are stimulatory to mushroom growth. Substrate structure is an important factor for the growth of fungal mycelium as it should allow for the ramification of the fungus. The shortest spawn run on oil palm fruit fibre may be attributed to the waste providing adequate

Table 2. Chemical analysis of the main constituents of oil palm fruit fibre (OPFF), oil palm empty fruit bunch (OPEFB), *Gmelina* sawdust (GSD) and mahogany sawdust (MSD) before mushroom cultivation.

Substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Nitrogen (%)
OPFF	44.29	12.00	8.50	1.36
OPEFB	42.86	11.50	10.00	0.69
GSD	27.14	8.50	17.50	0.30
MSD	51.71	10.00	19.30	0.49

aeration for the ramification of the mushroom mycelia. Tinoco *et al*. [13] observed that the larger the surface area and pore size of substrates the more the mycelium growth rate. It is worth noting that *Gmelina* sawdust showed, on the average, the slowest rate in spawn run (Table 1). However, in terms of fruiting, it had the shortest time for primordia development and fruiting body formation. This probably suggests that *Gmelina* sawdust may contain some kind of compound that induces the development of mushroom reproductive phase. The shortest crop cycle time of the mushroom on *Gmelina* sawdust could also be due to the initial composition of the waste, which may present a C:N ratio that delays spawn run but accelerates the formation of fruiting bodies as demonstrated by Stamets [14] for some mushroom species.

Mushroom yield parameters

The various organic wastes significantly influenced the fresh and dry weights, stipe height and pilei diameter of the mushroom (Table 3). The highest mushroom mean fresh weight (16.05 g) and dry weight (2.78 g) were obtained from oil palm fruit fibre; however, fresh weight was not significantly different ($p > 0.05$) from the mahogany sawdust (Table 3). Mushrooms with the highest mean stipe height (4.5 cm) were harvested from oil palm fruit fibre while mahogany sawdust produced fruiting bodies with the widest mean pilei diameter (5.76 cm) (Table 3). The considerable yield of the mushroom in this experiment on oil palm fruit fibre may be attributed to the residual oil of the waste. Reports have shown that developing sporophores require a supply of lipids and proteins (ratio 50/50), that are needed for expanding cell membranes and hence, promote higher yields [15]. Mahogany sawdust also influenced yield and pilei diameter of the mushroom. This could probably be due its appreciable cellulose content of 51.71% (Table 2) and compactness on wetting when compared to the other organic wastes evaluated. Thomas *et al*. [16] had earlier demonstrated that cellulose content of substrates is an important factor for fruit body development. The lowest fresh and dry weights recorded for fruit bodies harvested from oil palm empty fruit bunch could be due to the complex nature of the waste and/or the presence of little or no vital nutrients needed for the mushroom growth in the substrate. Schisler [15] associated the reduction in fresh weight of mushrooms to the absence of certain specific nutrients in the substrate required by the mushroom for its growth.

Table 3. Mean stipe height (cm), pilei diameter (cm), fresh and dry weights (g) of *L. squarrosulus* harvested from mahogany sawdust (MSD), *Gmelina* sawdust (GSD), oil palm fruit fibre (OPFF) and oil palm empty fruit bunch (OPEFB).

Substrate	Stipe height (cm)	Pileus diameter (cm)	Fresh weight (g) per bag	Dry weight (g)
MSD	3.17^{b}	5.76^{a}	15.10^{a}	1.31^{bc}
GSD	2.46^{c}	4.06^{c}	9.02^{b}	1.56^{b}
OPFF	4.50^{a}	4.86^{b}	16.05^{a}	2.78^{a}
OPEFB	2.10^{d}	3.01^{d}	4.12^{c}	1.06^{c}

Each value is a mean of 10 replicates. Values in the same column followed by the same letter (s) are not significantly different at $p > 0.05$ according to Duncan multiple range test (DMRT).

Mushroom protein content

The percentage protein content of harvested mushrooms varied significantly as a result of the influence of the various organic wastes. Table 4 shows that the percentage protein content ranged from 13.27% for mushrooms produced from mahogany sawdust to 27.42% for those grown on oil palm fruit fibre. In the present study, harvested mushrooms were evaluated for their nutritional status on the basis of their chemical composition. The observed variations in the protein content of the mushrooms harvested from the different organic wastes may be due to the differential availability of usable nitrogen in the wastes after spawn run which in turn influenced the amount of nitrogen available for the formation of fruit bodies. Nitrogen has been reported to be an important nutrient required for fungal growth due to its involvement in protein, chitin and nucleic acid syntheses [17]. However, other factors

Table 4. Percentage protein content of harvested mushrooms from Oil palm fruit fibre (OPFF), Oil palm empty fruit bunch (OPEFB), *Gmelina* sawdust (GSD) and Mahogany sawdust (GSD)

Organic wastes	Protein content of harvested mushrooms (%)
OPFF	27.42
OPEFB	17.57
GSD	19.79
MSD	13.27

such as the size of pileus, harvest time, stage of development and species of mushrooms have been reported to influence the protein content of the fruit bodies [18]. The crude protein content of this mushroom compared favourably with and in some instances surpassed those reported for most legumes except groundnut and soybeans grown in West Africa [19]. Using this protein content as approximate indices of nutritional quality, it would appear that this mushroom falls between most legumes and meat. However, while the protein content of the mushroom is still lower than that found in eggs, meat and fish, it is adequate to be used as a substitute in the diets of the populace in the developing countries.

CONCLUSION

In conclusion, the dwindling forests and the absence of commercial cultivation of *L. squarrosulus* has resulted to its scarcity with the few available very expensive. This study suggests that many locally available organic wastes have high potentials for utilization as substrates for growth and production of *L. squarrosulus*. Our experiment reveals the possibility of the production of adequate protein containing *L. squarrosulus* on oil palm fruit fibre (OPFF) and the waste is also recommended as a very good substrate for spawn production.

REFERENCES

[1] Chang ST and Buswell JA. (1996). Mushroom nutriceuticals. *World J. Microbiol.Biotechnol.* 12: 473-476.

[2] Fung YW *et al*. (2005). Evaluation of different Colombian agro industrial wastes as substrates for the growth and production of *Lentinula edodes* Berk. Pegler (shiitake). *Acta Edulis Fungi.* 12: 285-290.

[3] Christopher J and Custodio D. (2004). Coco lumber sawdust. In: Gush R (Ed.). Mushroom Grower's Handbook I. Mush world Inc.

[4] Thomsen MH. (2005). Complex media from processing of agricultural crops for microbial fermentation. *Appl. Microbiol. Biotechnol.* 68: 598-606.

[5] AbdRazak DL *et al*. (2012). Comparative study of mycelia growth and sporophore yield of *Auricularia polytricha* (Mont.) Sacc.on selected palm oil wastes as fruiting substrate. *Appl. Microbiol.Biotechnol.* 97: 3207-3213.

[6] Chang ST. (2008). Overview of mushroom cultivation and utilization as functional foods. In: Peter, C. and Cheung, K. (Eds.). *Mushrooms as Functional Foods.* John Wiley and Sons Inc. pp. 1-31.

[7] Kuforiji OO and Fasidi IO. (2009). Biodegradation of agro-industrial wastes by an edible mushroom *Pleurotus tuberregium* (Fr.). *Journal of Environmental Biology.* 30 (3): 355-358.

[8] Datta R. (1981). Acidogenic fermentation of lignocellulose-acid yield and conversion of components. *Biotechnol. Bioeng.* 23 (9): 2167-2170.

[9] AOAC. (2002). *Official Methods of Analysis* (17th Edition). Association of Official Analytical Chemist, Maryland, 1106pp.

[10] Quimio TH *et al*. (1990). Technical Guide for Mushroom Growing in the Tropics. Plant Production and Protection Study. FAO., Rome.

[11] Philippoussis A *et al*. (2007). Productivity of agricultural residues used for the cultivation of the medicinal fungus *Lentinula edodes*. *Int Biodeter. Biodegr.* 59 (3): 216-219.

[12] Schisler LC and Patton TG. (1971). Yield response of selected mushroom strains to vegetable oil supplementation. *Mushroom Sci.* 8: 707-712.

[13] Tinoco R *et al*. (2001). Kinetic differences of purified laccases from six *Pleurotus ostreatus* strains. *Lett. Appl. Microbiol*. 32 (5): 331-335.

[14] Stamets P. (2000). Growing Gourmet and Medicinal Mushrooms (3rd Edition). Ten Speed Press, Berkeley, California, USA.

[15] Schisler LC. (1982). Innovations for efficient mushroom growing. In: Wuest, P. J. and Bengston, G. D. (Eds.). *Pennsylvania State* Handbook for Commercial Mushroom Growers. Pennsylvania Agriculture Experiment Station, University park, Pennsylvania USA. pp. 117-118.

[16] Thomas GV *et al*. (1998). Evaluation of lignocellulosic biomass from coconut palm as substrate for cultivation of *Pleurotus sajor-caju* (Fr.) Singer. *World J. Microbiol. Biotechnol.* 14 (6): 879-882.

[17] Adebayo GJ *et al*. (2009). Evaluation of yield of oyster mushroom (*Pleurotus pulmonarius*) grown on cotton waste and cassava peel. *Afr. J. Biotech.* 8 (2): 215-218.

[18] Wani BA *et al*. (2010). Nutritional and medicinal importance of mushrooms. *Journal of Medicinal Plants Research.* 4(24): 2598-2604.

[19] Okoro IO and Achuba FI. (2012). Proximate and mineral analysis of some wild edible mushrooms. *Afr. J. Biotechnology.* 11(30): 7720-7724.

TESTING OF ENTOMOPATHOGENIC FUNGI IN BIOLOGICAL CONTROL AGAINST PINE WEEVIL

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ABSTRACT

Coniferous forests in Slovakia as well as in other European countries in recent decades are attacked by various harmful factors. The most important abiotic factor is the wind, which can in a short time destroy large complexes of spruce stands. After processing such calamities frequently create huge areas of afforestation, making very good conditions for pine weevil presence. It is a beetle causing a lot of damage by eating bark of the trunks of young seedlings, thereby, causing a weakening or dieback of them. Actual research is focused on study of reactions of the pine weevil adult (*Hylobius abietis* L.) (Coleoptera: Curculionidae) to infection caused by various entomopathogenic fungi species (*Beauveria bassiana, Metarhizium anisopliae, Isaria fumusorosea*). *Beauveria bassiana* is one of the longest known insect pathogens. The most significant effect in reducing of food intake has fungus *Beauveria bassiana. Metarhizium anisopliae* had no effect on food intake, and beetles infected with fungus *Isaria fumusorosea* had even increased food intake. Mortality of pine weevil has increased by fungus *Beauveria bassiana,* and good efficacy in this regard has also fungus *Metarhizium anisopliae. Isaria fumusorosea* had almost no effect on their mortality rate.

Keywords: entomopathogenic fungi, *Beauveria bassiana, Hylobius abietis*, biological control

INTRODUCTION

Coniferous forests in recent decades were being attacked by various biotic or abiotic factors. The most important abiotic factor is the wind that is capable to destroy large forest complexes in a short time. An example of a great calamity in Slovakia may be a calamity in Tatras in November 2004, which hit the area of 12,600 hectares of forest. Processing of this calamity created a great area for afforestation while also created very good conditions for some biotic pests, for example pine weevil. The calamity area had enough pine stumps, suitable for pine weevil females to lay eggs, which resulted in outbreak of this pest. Swarming of a new individuals is associated with feeding on the trunks of young seedlings, feeding on the bark leads to a weakening of seedlings; or to their death. The research was aimed at testing different entomopathogenic fungi with regard to their use in biological control of this pest.

Entomopathogenic fungi infect the wide range of hosts of different insect orders. They attack at different life stages from eggs to adults, while larvae or pupae are the most commonly attacked. Some species of fungi can parasitize on different developmental stages of the same host, or only at a particular stage of development of the same kind of insects.

Conidia of the fungi caught on the host's body surface grow into the host body. Conidia start to germinate under appropriate conditions. The tip of the penetrating hyphae, after a short growth, gets into the body using the assistance of mechanical pressure and the intake of nutrients contained in the cuticle. In the form of blastospores it is rapidly spreads to the various tissues and organs. Decrease in food intake is often associated with the ongoing changes in the contested host. The insects die due to the cell collapse caused by depletion of nutrients and poisoning by toxic metabolites produced by the fungus, which occurs within a few days after infection. The host's body is gradually filling the mycelium, which in sufficient humidity overgrows on the surface and subsequently develops a great number of aerial conidia.

Success rate of invasion and killing of the host is highly dependent on the spores concentration. More spores means bigger chance for elimination of host. Entomopathogenic fungi are able to survive in different environmental conditions; however their growth and development is greatly influenced by factors of the abiotic environment, especially by the humidity and temperature of the environment and UV radiation.

Until now we have described at least 90 genera, more than 700 species of fungi which are pathogenic to arthropods, but only few of them are effectively used in biological control. The best known, practically used species of entomopathogenic fungi are: *Beauveria bassiana, Entomophaga maimaga, Verticillium lecanii, Metarhizium anisopliae, Nomurea rileya*a

Hirsutella thompsonii [1, 2]. The prior research experience in this area has shown that the *Beauveria bassiana* species appears most suitable for biological control against pine weevil.

***Beauveria bassiana* (Balsamo) Vuillemin**

The first mention of the entomopathogenic fungus *Beauveria bassiana* is from the first half of the seventh century BC. At this time an unknown disease of silkworm occured in ancient China which reduced their numbers. Later, through the increasing trade contagion came to Europe and started to attack the local insects. The losses and damages caused to bee colonies were the most serious. Extinctions of entire honeybee colonies, however, were also accompanied by previously observed unnatural behavior of individuals. At that time, because of the final stage of its symptoms - overgrowth of the host with white woolly mycelium known as "white muscardine disease" [3].

Despite the relatively rapid and large-scale expansion of the fungus, its importance in terms of usefulness to mankind was not fully known. Within the scientific community it was named *Tritirachium shiotae*.In nineteenth century, an Italian entomologist, Agostino Bassi undertook more detailed research. After a number of experiments and gathering a sufficient amount of knowledge, he presented his results to the public in 1936. He concluded that manifesting symptoms are caused by parasitic fungus in the body of adults. The first successful results of applying *Beauveria bassiana* has been recorded by a Ukrainian doctor and the discoverer of phagocytosis - Ilya Mechnikov, who successfully used it against bedbugs *Blissus leucopterus*. The genus was officially described only in the 1912 by Vuillemin, then finished the final taxonomic classification scientists Petch, MacLeod and de Hoog [4].

Entomopathogenic fungus *B. bassiana* is a cosmopolitan widespread species, which a common component of the soil microbial community. It can be found mainly in the soil as a saprophyte on organic substrate, but also as a optional parasite in the form of a thick white mycelia on the surface of dead insects while it produces a number of hydrophobic conidia.

Disease of insects normally starting vital and virulent conidia, which are distributed by random mechanisms in the environment. Of the abiotic factors involved in the wide dissemination is mostly wind, rain or water movement in the soil. Dissemination also by usual mechanisms such as contact with an infected healthy individual or contamination of eggs during their laying.

Attachment of dry, strongly hydrophobic conidia, is ensured by direct interaction between two hydrophobic surfaces or by electrostatic forces, or by the molecular interaction between substances presented on the surface of conidia and substances on the surface of the host´s cuticle. Conidia are sufficiently stocked to the germination, without the need to absorb external nutrients. Their germination on the surface of the host starts in the appropriate temperature and high relative humidity.

From a certain phase of germination further, development of the fungus is dependent on external nutrient intake. The fungus begins to receive substances contained in the cuticle of the insect, then simply absorb the nutrients from the internal tissues of the insect. For this purpose, the fungus penetrates through the direct penetration or through natural openings into the body of the contested individual.

After penetrating of the individual, the fungus begins to produce secondary metabolite beauvericin, which weakens the immune system. Consequently, there is a rapid filling of tissues. This development phase is characterized by transition of filamentous fungus forms on the rapidly dividing and proliferating hyphal bodies, which by means of hemolymph are spreading throughout the body. They are propagated by gemmation, exponentially and very quickly. Individual dies after the destruction of all lymphocytes.

Inside the body fructification fibers are created, on which the blastospores arise. The new hyphae growing up out of them, consume nutrients in the hemolymph needed for their growth, and fructificate. Thus, there is an increase of the mycelial mass and rapid filling of the body of insect.

At sufficiently high relative humidity hyphae grow on the surface of the killed host through the weaker parts of the cuticle, where they form a dense white mycelium to which conidiophores develop which shall progressively become airborne conidia. Conidia in a dormant state are able to sustain life for several weeks to months. The most important environmental factors needed for the development of fungi include temperature and high humidity. Ideal temperatures are between 20°C and 30°C. For mycelial growth on the surface of the body and the formation of conidia requires usually very high humidity (above 90 per cent) but for the release of conidia the sufficient humidity is below 50 per cent [1, 5].

Pine weevil (*Hylobius abietis* L.) (Coleoptera: Curculionidae)

The adult is dark brown with pale little spots on elytra. It is 10 to 14 mm long. The head is elongated into a noticeable nose with antennae on its end. The female lays eggs onto roots of fresh pine and spruce stumps. The larva is whitish, limbless, slightly arched, 12 – 23 mm long. It pupates in the bark. Feeding under the stump bark is not harmful; but, it speeds up their decay. The maturation feeding of sexually immature adults is dangerous on conifer seedlings, especially pines. Feeding of adults on trunks is square. The adult beetle feeds on the bark just above the ground, in deep, funnel-like holes up to sapwood, which causes intense resin production. In case of severe attack, it damages the entire little trunk and the seedling dies [6].

One possible way to control pine weevil is the use of biological methods, which are an important part of the integrated forest protection. Biological control is environmentally friendly way of fighting, which has a growing importance, and in the future is expected to increase its share. This method of protection is often used in areas where the use of chemicals is not possible. There are places with a higher degree of conservation or water resource protection zones. From biological methods, which are used in forestry to protect seedlings, we can use biological control by means of nematodes and biological control by means of entomopathogenic fungi [7, 8].

Entomopathogenic fungi theoretically have the potential to control the pine weevil´s population, but their successful use in field conditions is still just starting. Some laboratory experiments in different model conditions were carried out at the laboratories of the Forest Protection Service Centre in Banska Stiavnica (Slovak Republic) [8].

MATERIALS AND METHODS

Works for the current research was carried out in the laboratories of the National Forestry Centre Zvolen, workplace the Forest Protection Service Centre in Banska Stiavnica. For the experiment we have chosen 150 viable pine weevil's imago, disaggregated by gender. The sex ratio was 1:1 in 75 males and 75 females. The adults were infected by entomopathogenic fungi *Beauveria bassiana* L., *Metarhizium anisopliae* (Metchnik off) Sorokin and *Isaria fumusorosea* Wize, also we created a control, uninfected sample:

Control - 0.05% solution of surfactant Tween 80, 30x, 15 males, 15 females

Bauveria bassiana- dry application of 30 pieces, 15 males, 15 females, spore concentration of 1.58×10^7 /ml

Bauveria bassiana - a suspension of 30 pieces, 15 males, 15 females, spore concentration of 1.58×10^7/ml

Metarhizium anisoliae - dry application 30 pieces, 15 males, 15 females, spore concentration of 1.58×10^7 /ml, *Isaria fumosorosea* - dry application 30 pieces, 15 males, 15 females, spore concentration of 1.58×10^7 /ml

Since the spores of the fungus - *Metarhizium anisopliae* are green, the main feature of the disease is the green color of the contested individual. When these asexual spores come into contact with the body of the host insect, begin to germinate and hyphae penetrate into the cuticle. The fungus then develops within the body, and ultimately kills the insects. The killing effect is very effective and helps in the production of insecticidal cyclic peptides. The skin of dead individuals often turns red. If the ambient humidity is sufficiently high, then white mould grows on dead beetles and soon changes to green color. Most insects living near the soil have developed natural defenses against entomopathogenic fungi such as *Metarhizium anisopliae.*

The last test species was *Isaria fumosorosea.* This fungus is a natural enemy of many insects belonging to the order family of Hemiptera and Homoptera, Bemisia and Trialeurodes. It also has activity against Diptera and Lepidoptera. The fungus works in contact with the insect and starts producing bacteria passing through the insect body and excreted toxins which kill the host. The mummified remains of corpses and insects are at high humidity covered with whitish-violet coating. Fungi was originated from the collections of the Arboretum Mlynany, workstation of the Slovak Academy of Sciences.

The adults were infected with dry and wet way. After infection beetles were placed in Petri dishes, where they had food - pine twigs 80 mm long with diameter around 6 mm (for maintaining humidity there were two pieces of moistened cotton wool). Petri dishes were placed into the room in which the temperature varied 18-24 °C and the humidity was about 50%.

Food in Petri dishes was supplemented at regular intervals from 7 to 10 days. This food was registered, we also measured the surface area (in mm^2) and evaluated the size of nibbled area. The results were evaluated according to the type of infection and in the second experiment by gender of pine weevils.

Figure 1. Dry infection of pine weevils (Photo: JurajŠkvarenina)

Figure 2. Wet infection of pine weevils in the liquid suspension (Photo: JurajŠkvarenina)

Figure 3. Adult of pine weevil infected by *Beauveria bassiana* (Photo: JurajŠkvarenina)

RESULTS AND DISCUSSION

When evaluating the effectiveness of different species of fungi with regard to achieving pine weevil's mortality under the given circumstances, best results are obtained with *Bauveria bassiana*. Within six days after the "dry application" of spores all male beetles died, females died after ten days. Overgrowth of imago by fungus was observed since the tenth day after infection. The male adults infected by "wet" application died within seventeen days and females within 28 days.

Number of authors dealt with fungus *Beauveria bassiana*, many of them have confirmed its pathogenic effect on various insect species. Markova, Simsiankova (1990) tested its effectiveness to the *Limanria dispar* [9]. Doberski (1981) found that the fungi species *Beauveria bassiana* and *Metarhizium anisopliae* were killing the adults of *Scolytus scolytus* very well [10]. Recently Agulló-Guerra *et al*. (2010) demonstrated that the "dry" spores of *Beauveria bassiana* had stuck to the cuticle of an adult *Rhynchophorus ferrugineus* better than 'wet' spores [11].

During the experiment, the *Metarhizium anisopliae* species killed all the pine weevils to the 45th day. The result was in agreement with Ansari and Butt (2012) who used *Metarhizium anisopliae* to fight pine weevil and achieved 100% mortality. All imago were killed by fungus within twelve days [12]. *Isaria fumosorosea* was not effective against pine weevil as it

killed only one male and only two females after one week. Efficiency of this fungus to other kinds of insects was confirmed by several works such as Pell and Vanderberg (2002) who investigated the efficacy of this fungus on aphid *Diuraphis noxia* [13]. Very encouraging results of testing *B. bassiana* in the biological control against bark beetle (*Ipstypog raphus*) under practical conditions of forestry in the Slovak Republic was obtained by Noge (2013) [14].

One of the first signs of the effect of entomopathogenic fungi was reduction of food intake.Among the different fungi tested, best efficiency to reduce the intake of food was caused by *B. bassiana* fungus. Fungus-infected beetles died after ten days, and food was changed after twenty-two days. It is assumed that if the pine weevils survive to the age fifteen days, the nibbled areas would be smaller than the control.This assumption was confirmed by the results of Lalik (2013), beetles in its experiment had the same mortality rate, but the nibbled areas were smaller compared to the control. Fungus *M. anisopliae* did not affect the reduction of nibbled areas during sexual feeding. The fungus *I. fumosorosea* did not affect feed intake, but on the contrary, it resulted in increase in food consumption[15].

The other results of testing the effectiveness of the biological products suggest that adult pine weevils are susceptible to different strains of entomopathogenic fungi depending upon the concentration used. Very effective preparations had a high concentration of spores. Fastest reaction of pine weevils was recorded by use the entomopathogenic fungus *B. bassiana* from the natural environment applied by dry method in which pine weevils died within 6-8 days of infection. Quick reaction of pine weevils was also noticed by application of wet process, using the suspensions at concentrations of 10^7 and 10^8 spores /ml.

Good mycelial growth depends mainly on very high relative humidity reaching 90% or more. Entomopathogenic fungal hyphae overgrowth was observed mostly under the microscope already on the first day after killing of beetle. Single hyphae of *Beauveria bassiana* started to overgrow among the softer parts of individual body parts of dead beetle mostly joints of the legs, the connections of the head and chest, chest and abdomen, but primarily it was an oral and anal hole.

Growing of mycelium was macroscopically visible 1-4 days after death. Mycelium was visible in the form of initially little groups of tiny fungal fibers, which began to form larger sparse clusters, filling the space between the elements of the insect body. Mycelial growth was relatively quick, and at the third to fourth day after the occurence of the fungus, snout white, thick fluffy formations were created in the limbs and joints area. At this stage its mycelium grew very quickly, fluffy formations were connected together and formed a continuous layer of dense white covering mainly the lower body beetle; fully covered in exceptional cases, in addition to its elytron. Entomopathogenic fungus *Metarhizium anisopliae* over grown essentially the same part of the body as *Beauveria bassiana*. The mycelium was initially very dense and created the white coatings that gradually changed the color to olive green. Its growth was not as intense as the kind *Beauveria bassiana*.

2nd day **3rd day** **4th day** **5th day**

Figure 4. The growth of the mycelium on the head of Beauveriabassiana recorded automatically every 24 hours from the second to the fifth day from the death. (Photo: JurajŠkvarenina)

Table 1. Pine weevil´s mortality depending on time, sex and infection

	Number of male and female pine weevils dead/ overgrown by different entomopathogenic fungi (15 insects in each treatment)									
On date	**Control**		***Beauveria bassiana* - dry**		***Beauveria bassiana* -suspenion**		***Metarhizium-anisopliae***		***Isaria fum-osorosea***	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
19.7	0	0	0	0	0	0	0	0	0	0
23.7	0	0	15//1	12//2	0	0	0	1/0	0	0
26.7	0	0	15/15	15/15	5//2	2//1	1/0	1/0	0	1/0
30.7	0	0	15/15	15/15	14//12	10//7	3//3	6/4	0	1//1
2.8	0	0	15/15	15/15	15/14	13//9	4//4	8/4	0	1//1
6.8	0	0	15/15	15/15	15/14	14//11	4//4	8/4	0	1//1
9.8	0	0	15/15	15/15	15/15	14//12	8//6	9/7	0	5//4
13.8	0	0	15/15	15/15	15/15	15/14	10/9	9/7	0	5//5
16.8	0	0	15/15	15/15	15/15	15/14	11/10	12/11	0	5/5
20.8	0	0	15/15	15/15	15/15	15/14	13/10	12/11	0	5/5
23.8	0	0	15/15	15/15	15/15	15/14	14/13	14/14	0	5/5
27.8	0	0	15/15	15/15	15/15	15/14	14/13	14/14	1//1	6/6
30.8	0	0	15/15	15/15	15/15	15/14	15/15	15/15	1//1	6/6
3.9	0	1/1	15/15	15/15	15/15	15/15	15/15	15/15	1//1	6/6
6.9	1/1	1/1	15/15	15/15	15/15	15/15	15/15	15/15	1//1	6/6

CONCLUSION

Damage caused by the activity of pine weevil in several European, but especially in the Scandinavian countries, is forcing to intensify research on possible methods of protection of seedlings and young plants in forests by control of this pest. The forest stands status after the large-scale, but also the size of the smaller disasters, creates favorable conditions for its expansion and the emergence of significant damage. Due to the limited options for avoid the emergence the appropriate conditions for its reproduction and distribution, they are searching possibilities of its active control. Therefore, the biological control appears as one of the most effective, close to the nature. The aim of our research was to evaluate the biological activity of three species of entomopathogenic fungi on adults of pine weevil and to evaluate survival and food intake by infected imago.

Entomopathogenic fungus had certain influence on feeding of pine weevil only at higher concentrations in applicated biological preparations. The results show that under laboratory conditions fungus work quite well and their research should definitely continue. It is appropriate to focus for the particular application of different entomopathogenic fungi at higher concentrations and of selecting the fungi species that are effective.

ACKNOWLEDGEMENTS

This research was supported by grants from the Grant Agency APVV No. 0744-12.

REFERENCES

[1] Landa Z. (2007). Vyu•ití houby *Beauveria bassiana* v *ochranì* proti lyko•routu smrekovému. *Lesnická práce (Forestry journal)* è. 10/2007, pp. 14-15.

[2] Medo J. (2009). Druhové spektrum entomopatogénnych húb z radu Hypocreales izolovaných z pôdnych vzoriek pochádzajúcih z rôznych lokalít Slovenska a genetická štruktúra populácií *Beauveria bassiana*: autoroferát k Dizertaènej práci. Nitra; UKF, 23 p.

[3] Kunca A. and Zubrik M. (2008). Doká•eme ju vyu•i• na obranu porastov pred podkôrnym hmyzom *Les a lesokruhy*, è. 1-2/2008, s. 38.

[4] Vega FE. and Blackwell M. (2005). Insect-fungal associations - Ecology and evolution, In: Oxford university press è 7/2005, pp. 3 – 27.

[5] Koubova D. (2009). Vyu•ití hub v biologické ochranì rostlin proti škùdcùm. UZEL, Agronavigátor 1-42.http://www.agronavigator.cz/UserFiles/File/Vyuit%20hub%20v%20biologick%20ochran%20rostlin%20proti%20kdcm.pdf

[6] Zubrik M *et al.* (2013). Insects and Diseases damaging trees and shrubs of Europe. N.A.P.Edition, ISBN:978-2-91 388-18-6, 535 p.

[7] Vakula J *et al.* (2010). Rozvoj súèasných technických mo•ností pri štúdiu niektorých spôsobov aplikácie entomopatogénnej huby Beauveria bassiana v rámci biologických metód ochrany lesa. In: Aktuálne problémy v ochrane lesa 2010. Kunca A. Ed. Národné lesnícke centrum Zvolen, ISBN 978-80-8093-108-7. pp. 63-64.

[8] Galko J *et al.* (2013). Usmernenie lesníckej ochranárskej slu•by ku kontrole a obrane sadeníc pred poškodením tvrdoòom smrekovým a lykokazmi rodu Hyastes. Lesnícky výskumný ústav, Zvolen, pp. 4-12.

[9] Markova G and Simsianakova A. (1990). Laboratory studies on the effect of the entomopathogenic fungus *Beauveria bassiana* on larvae of *Lymantria dispar*. *Nauka za Gorata* 27, pp. 81–84

[10] Doberski JW. (1981). Comparative laboratory studies on three fungal pathogens of the elm bark beetle *Scolytu sscolytus*: Pathogenicity of *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* to larvae and adults of *S. scolytus*. *Journal of Invertebrate Pathology*, 37, pp.188–194.

[11] Guerri-Agulló B *et al.*(2010): Infection of the red palm weevil (*Rhynchophorus ferrugineus*) by the entomopathogenic fungus *Beauveria bassiana*: A SEM study. *Microsc Res. Tech.* 73, pp. 714–725.

[12] Ansari MA and Butt TM. (2012). Susceptibility of different developmental stages of large pine weevil *Hylobius abietis* (Coleoptera: Curculionidae) to entomopathogenic fungi and effect of fungal infection to adult weevils by formulation and application methods. *Journal of Invertebrate Pathology*. 111(1): 33-40.

[13] Pell JK and Vandenberg JD. (2002). Interactions among the aphid *Diuraphis noxia*, the entomopathogenic fungus *Paecilomyces fumosoroseus* and the coccinellid Hippodamia convergens. *Biocontrol Sci. Technol*. 12, pp. 217–224.

[14] Noge M. (2013). Impact of the entomopathogenic fungus *Beauveria bassiana* on mortality of bark beetle (*Ips typographus*). *Acta Facultatis Forestalis Zvolen.* Roè. 55, Suppl. 1 (2013), pp. 19-26.

[15] Lalík M. (2013). Vplyv vybraných entomopatogénnych húb na mortalitu tvrdoòa smrekového (*Hylobius abietis*). Bakalárska práca. TU vo Zvolene.

USE OF PHASE II MUSHROOM COMPOST IN *AGARICUS SUBRUFESCENS* PRODUCTION

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ABSTRACT

In the last years, the cultivation of the medicinal mushroom *Agaricus subrufescens* Peck has evoked great interest worldwide. During the crop development, techniques and methods previously established for *A. bisporus* production have been adopted, trying to take advantage of existing buildings and technologies. This work assesses the production parameters and composition of the harvested fruiting bodies of *A. subrufescens* that have been produced from three commercial compost specifically prepared for the production of *A. bisporus*.Two loading densities of compost (60 to 70 kg m^{-2}) were used. A growth cycle of *A. subrufescens* under controlled conditions has been carried out.There were no significant differences among treatments for any of the considered parameters. Biological efficiency values obtained were between 46.10 and 58.52 kg dt^{-1}, which is higher than those registered in the references consulted.The fact that significant differences in behavior between different compost used have not been observed, along with the good agronomic performance of them, allows confirming its suitability for the production of *A. subrufescens* in the conditions used. As regards the loading density, the range between 60 and 70 kg m^{-2} could be applied to commercial scale. The proximate analysis of the harvested carpophores did not provide significant differences between treatments or factors. With regard to the content of other cultivated species of edible fungi, *A. subrufescens* is remarkable in general for its low content of water, crude fat, crude fiber and ash, and high content in protein and total carbohydrates.

Keywords: *Agaricus subrufescens*, *Agaricus blazei*, compost, production, proximate analysis

INTRODUCTION

The cultivation of the medicinal mushroom *A. subrufescens* Peck has generated a notable interest worldwide in the last few years, becoming increasingly popular and as a result expanding through many countries. This is mainly due to its high international market price, which is related not only to the remarkable medicinal properties contributed by high content of bioactive compounds but also to the culinary value added by its slightly nutty pleasant aroma [1]. Several studies recently reviewed by Wisitrassameewong *et al*. [2] highlight the importance of *A. subrufescens* medicinal properties. It was traditionally used to treat many common diseases like atherosclerosis, hepatitis, hyperlipidemia, diabetes, dermatitis and cancer [3]. Among the beneficial properties from *A. subrufescens* that have been published are tumor growth reduction, immune modulatory activities, immune stimulatory effects, antimicrobial and antiviral activities and anti-allergy effects [2].

Besides its medicinal interest, *A. subrufescens* is a food of high nutritional value, rich in protein, fiber and minerals (mainly potassium, phosphorus and calcium), with low lipid content [1]. Nowadays consumers concern to look for healthier food, find on the consumption of mushroom an alternative with two major advantages, in some varieties, the therapeutic properties and an important source of high value proteins [4]. Considering the special requirements of the crop, based on the high temperature needed, the production of *A. subrufescens* could be a good choice by the edible mushroom growers of different countries, particularly during the summer months. This crop allows the use of poor technology facilities with low energy costs. The well known *Agaricus bisporus* (Lange) Imbach production technology can mostly be used for *A. subrufescens*, so the growers should not have any problem in adjusting to the new species. Furthermore, the marketing conditions of this mushroom, mainly dried, facilitate to deal with the short life of edible cultivated mushroom allowing a flexible commercialization of the product throughout the year.

Along the crop development, those processes and techniques established for the production of *A. bisporus* have been considered, although the specific technology for the crop studied is still under review. To increase the yield and to get a proper adaptation of each country, it is necessary to evaluate different crop parameters. The cultivation conditions in the different stages of the crop cycle (temperature, relative humidity, carbon dioxide concentration, light cycle) as well as the aspects concerning the casing materials should be thoroughly analyzed. In this context, the aim of this work was to achieve the production of *A. subrufescens* while using substrates specifically prepared for *A. bisporus* cultivation by a traditional composting system of two phases, and making use of the existing facilities and technologies for this purpose.

MATERIALS AND METHODS

To determine the physical, chemical and biological characteristics of composts, the following measurements were taken: water content, pH, total N content, organic matter and ash, C/N ratio, crude fibre, crude fat, nitrogen-free extracts, cellulose, hemicellulose, lignin and neutral detergent-solubles, pathogenic nematodes, mites and competitor moulds. The methodology previously described by Pardo-Giménez *et al.* [5] was used. Composts characteristics are presented in Table 1.

The experimental design used was a 3×2 equilibrated factorial plan with six replicates (randomised blocks with two factorial factors). Factor 1, with three levels, corresponded to three phase II commercial composts (obtained from three different plants of composting) prepared for the production of *A. bisporus*. Factor 2, with two levels, corresponded to the loading density of compost (60 and 70 kg m^{-2}). A total of 36 trays (1450 cm^2 each one) were employed, which were placed at two levels on both sides of the growth chamber. Strain ABL99/30 (Mycotec of the Moìdulo deCogumelos, FCA-UNESP, Brazil) was selected for the experiment. This strain, collected in Piedade (São Paulo, Brazil) in 1999, is characterized by its medium to small size fruit bodies, strong texture, high yield and precociousness, reduced time to first harvest (±38 days), and slightly low fructification temperature (±25 °C).

A. subrufescens trial was carried out in a 20 m^3 experimental growth chamber equipped with a humidification system, a heating/cooling system, and internal air circulation/external ventilation. This allowed the automatic control of temperature, relative humidity and carbon dioxide. Composts were inoculated with grain spawn at a rate of 12 g kg^{-1} of fresh weight of compost. The mushroom growth cycle was carried out according to the fast cooling method: incubation with a compost temperature of 28 ºC, drop to 20 ºC for fruiting and compost temperature of 26 ºC and 700 ppm CO_2 during the cultivation. Temperatures and plan for primordial induction is presented in Fig. 1. A peat based casing was used.The total growth cycle lasted 110 days; five flushes of mushrooms were harvested.

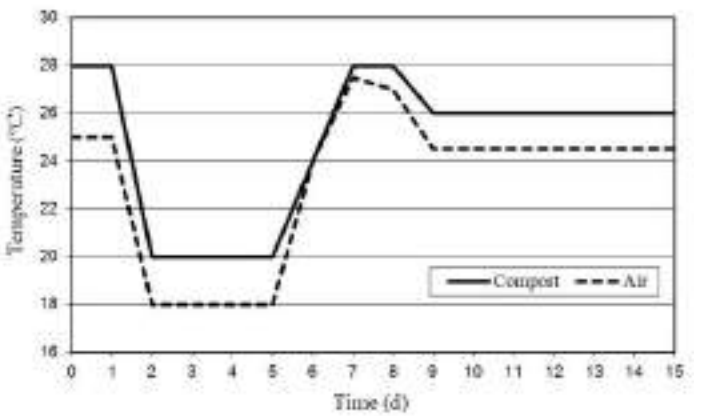

Figure. 1. Temperatures of induction for *A. subrufescens* fruiting in fast cooling process

The main production parameters (earliness, number of mushrooms, yield per unit of area, biological efficiency, unitary weight and dry matter content) were evaluated, and a proximate analysis of harvested mushrooms was carried out. Mushrooms were harvested every day at their optimal commercial development stage, with the highest possible weight before the opening of the pileus.To assess the production parameters, weight before stipe trimming and the total number of mushrooms picked from each tray (1450 cm^2) were recorded daily. Yield is expressed as kg per cultivated area; biological efficiency, an estimation of mushrooms' ability to convert substrate into fruiting bodies, was calculated by dividing the total fresh weight of mushrooms harvested from one crop (several flushes) by the total substrate dry weight, expressing the fraction as kg dt^{-1} compost.The size of the mushrooms, expressed as unit weight in g, was calculated from the yield and number of mushrooms harvested. Earliness, or days to the first harvest, was expressed as the number of days between casing and the beginning of the first flush harvest. On the day when most mushrooms were picked during the first flush, the

dry matter and proximate analysis were determined for those mushrooms of uniform size and at the same development stage.

Dry matter and water contents were determined by measuring weight loss after oven drying at 105°C for 72 h [6]. Mushrooms protein content was calculated by multiplying the total nitrogen content, obtained by the Kjeldahl method [7], by a conversion factor of 4.38. Crude fat was estimated gravimetrically by filter bag technique after petroleum ether extraction of the dried sample in an extraction system Ankom XT10 [8]. To determine the content of crude fiber, Weende technique adapted to the filter bag technique was applied. This method determines the organic residue remaining after digestion with solutions of sulfuric acid and sodium hydroxide, using an Ankom 220 Fiber Analyzer [9]. Total carbohydrate content was calculated by subtracting the sum of the crude protein, total fat, water and ash from the total weight of the mushrooms [10]. Nitrogen-free carbohydrates content is calculated by subtracting the crude fiber from the total carbohydrate content [6]. To determine ash content, mushrooms were ashed at 540°C for at least 6 h. The energy value of the mushrooms was estimated as per Lau [6]. ANOVA was used to analyze the data and the Tukey-HSD test employed to establish significant differencesbetween means (P = 0.05). All calculations were performed using Statgraphics Plus v. 4.1 software (Statistical Graphics Corp., Princeton, NJ, USA).

Table 1. Chemical characteristics of different composts used

Characteristics	Compost 1	Compost 2	Compost 3
pH (1:5, w/v)	7.64	7.61	7.40
Water (g kg^{-1})	695.0	672.3	689.3
Total nitrogen (g kg^{-1})	20.7	23.0	22.7
Protein (g kg^{-1})	129.4	143.8	142.1
Ash (g kg^{-1})	224.8	258.8	281.7
Organic matter (g kg^{-1})	775.2	741.2	718.3
C/N	21.7	18.8	18.3
Crude fiber (g kg^{-1})	333.2	334.2	310.6
Crude fat (g kg^{-1})	3.9	1.8	3.6
N-free extract (g kg^{-1})	308.7	261.6	262.0
Hemicellulose (g kg^{-1})	134.4	125.2	151.7
Cellulose (g kg^{-1})	235.9	201.5	202.1
Lignin (g kg^{-1})	185.7	214.3	166.9
Neutral-det. sol. (g kg^{-1})	219.2	200.3	197.6

RESULTS AND DISCUSSION

No significant differences were registered among the treatments for none of the considered parameters (Table 2). The biological efficiency (BE) values ranged between 46.10 and 58.52 kg dt^{-1}, with yield per unit of area between 9.06 and 11.23 kg m^{-2}, being generally higher than the obtained by the literature reviewed.

Kopytowski-Filho and Minhoni [11] evaluated the behaviour of the variety ABL 99/30, used in this work, over three different kinds of compost, registering BE values between 21.1 and 34.9 kg dt^{-1} by using a mixture of mineral soil and vegetal carbon. In Taiwan, Liang *et al*. [12] obtained a highest yield of 1.93 kg/m^2 through substrates basedon two kinds of sawdust [12]. In Slovenia, Gregori *et al*. [13] employed *A. bisporus* commercial compost achieving 700 g of mushrooms from 3 kg of compost. Also in 2008, Cavalcante *et al*. evaluated in Brazil several casing materials based on local mineral soils and sand with different additives, obtaining BE values between 17.73 and 25.97 kg dt^{-1} [14]. Siqueira *et al*. [15] reached substantial BE values, by studying compost from crushed sugarcane bagasse, Coast cross hay and corn husk

Table 2. Mean values of production parameters assessed in the mushrooms originating from the various treatments

	Number of mushrooms m^{-2}	**Yield (kg m^{-2})**	**Unitary weight (g)**	**Biological efficiency (kg dt^{-1})**	**Earliness (days from casing)**	**Dry matter (%)**
Compost 160 kg/m^2	535 a	10.61 a	19.90 a	57.99 a	34.5 a	10.70 a
Compost 170 kg/m^2	575 a	11.04 a	19.38 a	51.71 a	33.3 a	10.76 a
Compost 260 kg/m^2	534 a	9.06 a	16.97 a	46.10 a	34.1 a	10.98 a
Compost 270 kg/m^2	618 a	11.23 a	18.71 a	48.92 a	33.3 a	11.00 a
Compost 360 kg/m^2	572 a	10.91 a	19.13 a	58.52 a	34.5 a	11.61 a
Compost 370 kg/m^2	553 a	10.61 a	19.64 a	48.74 a	33.6 a	12.12 a
MEAN	565	10.58	18.96	52.00	33.9	11.20

Values followed by a different letter within a column are significantly different at 5% level according to Tukey's HSD test.

amended with wheat bran, lime, gypsum, super phosphate and urea [15]. Similarly, Zied *et al*. [16], with two kinds of compost prepared with Tifton grass and oat straw, respectively achieved BE values between 23.25 and 36.25 kg dt^{-1}. Colauto *et al*. (2010) got BE values of 27.3 kg dt^{-1} from compost based on different local materials and a Brazilian pasteurized peat casing layer [17], and 36.1 kg dt^{-1} by using pasteurized lime schist as casing layer [18]. In repeat trial with lime schist as casing material, the same group obtained BE of 46.8 kg dt^{-1} for a crop cycle of 66 days [19] and 60.4 kg dt^{-1} for crop cycle of 90 days [20]. González Matute *et al*. reported BE values between 13.1 and 34.3 kg dt^{-1} on the uncomposted substrates with a mixture of peat and calcium carbonate as casing layer [21]. Zied *et al*. studied different varieties of *A. subrufescens* using compost based on wheat straw and chicken manure; a highest yield of 18.6%, equal to a BE of 57.2 kg dt^{-1} was obtained [22]. Recently, Pardo-Giménez *et al*. analyzed the effect of different casing layers over several strains of *A. subrufescens*, using compost based on wheat straw and chicken manure; a highest BE of 27.57 kg dt^{-1} was achieved for the strain ABL99/30 with a peat-based casing material [23].

The fact of non-significant differences among the compost kinds used (Table 3), together with the good agronomic behavior of them, allows to confirm that the use of the compost studied is suitable for the production of *A. subrufescens* in the conditions employed. Concerning the loading density, though significant differences were not recorded (Table 3), it is noteworthy that with70 kg m^{-2} a higher yield was obtained per unit of area than with 60 kg m^{-2} (10.96 against 10.19 kg m^{-2}), despite the biological efficiency registered (49.81 kg dt^{-1}) was lower than that obtained with the lowest density (54.19 kg dt^{-1}). This range between 60 and 70 kg/m^2 could be applied at commercial scale.

The proximate analysis of the harvested carpophores (Tables 4 and 5) did not show any significant differences between treatments or factors, with the exception of the crude fat content between composts. The harvested sporophores were found to contain (dry wt. basis) protein 291.1 g kg^{-1}; crude fat 20.4 g kg^{-1}; total carbohydrates 615.6 g kg^{-1}; available carbohydrates 540.9 g kg^{-1}; crude fiber 74.7 g kg^{-1}; ash 73.0 g kg^{-1}; energy value 353 kcal per 100g. In general, the gross composition of mushrooms is water (90%), protein (2–40%), fat (2–8%), carbohydrates (1–55%), fiber (3–32%) and ash (8–10%) [3]. With regard to the composition of other edible species of fungi, *A. subrufescens* is, in general, low in water, crude fat, crude fiber and ash, high in protein, total carbohydrate and mean values of available carbohydrates and energy value [24].

The protein contents, between 268.8 and 307.9 g kg^{-1}, are similar to those recently reported by Siqueira *et al.* (283.3-293.5 g kg^{-1}) [25] and Zied *et al*. (262-349 g kg^{-1}) [22]. According to data collected by Eira [26] on sporophores of *A. subrufescens* grown in Brazil, the protein contents (289.4-391.8 g kg^{-1}) is higher than those recorded for *A. bisporus* (263 g kg^{-1}), *Lentinula edodes* (175 g kg^{-1}), *Pleurotus florida* (189 g kg^{-1}), *Volvariella diplasia* (285 g kg^{-1}) and *Pleurotus ostreatus* (187 g kg^{-1}) [26]. Chang and Miles (2004) describe it as one of the species with higher protein

Table 3. Mean values of production parameters assessed in mushrooms for the different factors considered in the experimental design

	Number of mushrooms m^{-2}	**Yield (kg m^{-2})**	**Unitary weight (g)**	**Biological efficiency (kg dt^{-1})**	**Earliness (days from casing)**	**Dry matter (%)**
Compost 1	555 a	10.83 a	19.64 a	54.85 a	33.9 a	10.73 a
Compost 2	576 a	10.15 a	17.84 a	47.53 a	33.7 a	10.99 a
Compost 3	562 a	10.76 a	19.38 a	53.63 a	34.1 a	11.87 a
Density 60 kg/m^2	547 a	10.19 a	18.67 a	54.19 a	34.3 a	11.10 a
Density 70 kg/m^2	582 a	10.96 a	19.24 a	49.81 a	33.4 a	11.29 a
Mean	565	10.58	18.96	52.00	33.9	11.20

For each factor within a column, values followed by a different letter are significantly different at 5% level according to Tukey's HSD test.

Table 4. Proximate analysis of mushrooms originating from the various treatments

	Water (g kg^{-1})	**Crude Protein (Nx4.38)**	**Crude Fat (g kg^{-1} d.m.)**	**Total Carboh. (g kg^{-1} d.m.)**	**Nitrogen-Free Extract**	**Crude Fiber (g kg^{-1} d.m.)**	**Ash (g kg^{-1} d.m.)**	**Energy Value (kcal/100g d.m.)**
Compost 160 kg/m^2	893.0 a	290.2 a	19.1 a	613.6 a	537.7 a	75.9 a	77.1 a	350 a
Compost 170 kg/m^2	892.5 a	291.5 a	21.6 a	611.4 a	534.4 a	77.0 a	75.6 a	351 a
Compost 260 kg/m^2	890.2 a	297.8 a	24.2 a	604.5 a	525.9 a	78.6 a	73.6 a	352 a
Compost 270 kg/m^2	890.1 a	307.9 a	23.9 a	594.6 a	516.6 a	78.0 a	73.6 a	352 a
Compost 360 kg/m^2	883.9 a	290.1 a	18.2 a	620.6 a	549.0 a	71.6 a	71.1 a	354 a
Compost 370 kg/m^2	878.8 a	268.8 a	15.3 a	648.9 a	581.9 a	67.0 a	67.0 a	358 a
Mean	888.0	291.1	20.4	615.6	540.9	74.7	73.0	353

Values followed by a different letter within a column are significantly different at 5% level according to Tukey's HSD test.
d.m.= dry matter

Table 5. Proximate analysis of mushrooms for the different factors considered in the experimental design

	Water (g kg^{-1})	**Crude Protein (Nx4.38)**	**Crude Fat (g kg^{-1} d.m.)**	**Total Carboh. (g kg^{-1} d.m.)**	**Nitrogen-Free Extract**	**Crude Fiber (g kg^{-1} d.m.)**	**Ash (g kg^{-1} d.m.)**	**Energy Value (kcal/100g d.m.)**
Compost 1	892.7 a	290.8 a	20.3 ab	612.5 a	536.1 a	76.5 a	76.3 a	351 a
Compost 2	890.1 a	302.8 a	24.07 a	599.5 a	521.3 a	78.3 a	73.6 a	352 a
Compost 3	881.3 a	279.5 a	16.8 b	634.7 a	565.4 a	69.3 a	69.0 a	356 a
Density 60 kg/m^2	889.0 a	292.7 a	20.5 a	612.9 a	537.5 a	75.3 a	73.9 a	352 a
Density 70 kg/m^2	887.1 a	289.4 a	20.2 a	618.3 a	544.3 a	74.0 a	72.1 a	354 a
Mean	880.0	291.1	20.4	615.6	540.9	74.7	73.0	353

For each factor within a column, values followed by a different letter are significantly different at 5% level according to Tukey's HSD test.d.m.= dry matter

content among all the cultivated edible mushrooms, over two times higher than *L. edodes* [24]. Fat content reported in this paper (15.3-24.2 g kg^{-1}) is, in general, of the same order as those reported in the literature. According to Chang and Miles [24], the fat content of different species of mushrooms varies between 11 and 83 g kg^{-1}on dry weight basis, with a mean content of 40 g kg^{-1} [24]. Recently, Pardo-Giménez *et al*. recorded mean values of 15.9 g kg^{-1} in carpophores of *A. subrufescens*cultivated in Spain [27].

The harvested carpophores had an average of 74.7 g kg^{-1} of crude fiber content. The data collected by Eira [26] in sporophores of *A. subrufescens* cultivated in Brazil set out fiber contents (55.6-118.1 g kg^{-1}) in the same range as *A. bisporus* (104 g kg^{-1}), *Lentinula edodes* (80 g kg^{-1}) and *Pleurotus florida* (115 g kg^{-1}), although lower than those of *Volvariella diplasia* (174 g kg^{-1}) and *Pleurotus ostreatus* (156 g kg^{-1}) [26]. The mineral content ranged between 55.6-118.1 g kg^{-1}, with an average of 73.0 g kg^{-1}, as reviewed by Pardo-Giménez *et al*. [27]. These values are, in general, lower than the found for other cultivated mushrooms [24, 26]. Finally, the energy value, with an average of 353 kcal per 100g of dry matteris low, though similar to those values found within the reviewed literature (344 and 362 g kg^{-1}) [27].

CONCLUSION

After the investigation and pursuant there to, compost prepared for *A. bisporus* cultivation, based on cereals straw (wheat, barley) and chicken manure, and produced by a classical composting two-phase system, are valid for the commercial growing of *A. subrufescens*. This opens the possibility to take advantage of existing technologies and infrastructures to facilitate the diversification of the edible fungi production. As regards the loading density, the range between 60 and 70 kg m^{-2} could be applied to commercial scale. The carpophores gathered had a composition similar to that recorded by other authors under different conditions.

ACKNOWLEDGEMENTS

We would like to thank the "Diputación Provincial de Cuenca" and the "Consejería de Agricultura de Castilla-La Mancha" (Spain) for the financial support.

REFERENCES

[1] Largeteau ML *et al*. (2011).The medicinal *Agaricus* mushroom cultivated in Brazil: biology, cultivation and non-medicinal valorisation. *Appl. Microbiol. Biotechnol.* 92: 897-907.

[2] Wisitrassameewong K. *et al*. (2012). *Agaricus subrufescens*: A review. *Saudi J. Biol. Sci.*19: 131-146.

[3] Firenzouli F *et al*. (2008). The medicinal mushroom *Agaricus blazei* Murrill: review of literature and pharmaco-toxicological problems. *Evid. Based Complement. Alternat. Med.* 5(1): 3-15.

[4] Henriques GS *et al*. (2008). Avaliação *in vivo* da qualidadeprotéica do champignon do Brasil (*Agaricus brasiliensis* Wasser *et al.*). *Rev. Nutr.* 21: 535-543.

[5] Pardo-Giménez A *et al*. (2012). Effect of supplementing compost with grapeseed meal on *Agaricus bisporus* production. *J. Sci. Food Agric.* 92: 1665-1671.

[6] Lau O. (1982). Methods of chemical analysis of mushrooms. In: Tropical Mushrooms. Biological Nature and Cultivation Methods. Chang ST and Quimio TH Eds. The Chinese University Press, Hong Kong. 87-116.

[7] FOSS. (2003). The determination of nitrogen according to Kjeldahl using block digestion and steam distillation. Foss Application Note AN 300. FOSS Tecator AB Höganäs, Sweden.

[8] ANKOM. (2009). Rapid Determination of Oil/Fat Utilizing High Temperature Solvent Extraction. ANKOM Technology Method 2, AOCS Official Procedure Am 5-04. ANKOM Technology. Macedon (NY), USA.

[9] ANKOM. (2008). Crude Fiber Analysis in Feeds By Filter Bag Technique. ANKOM Technology Method 7, AOCS Approved Procedure Ba 6a-05. ANKOM Technology. Macedon (NY), USA.

[10] Sullivan DM. (1993). Proximate and mineral analysis. In: Methods of Analysis for Nutrition Labeling. Sullivan DM and Carpenter DE Eds. AOAC International. Arlington (VA), USA. pp. 105-109.

[11] Kopytowsky-Filho and Minhoni MTA. (2007). Produtividade e eficiênciabiológica da linhagemABL 99/30 de *Agaricus blazei* emtrêstipos de compostos e emdoisambientes de cultivo. *Energ. Agric. (Botucatu)*. 22: 65-78.

[12] Liang ZC *et al*. (2008). Heavy metal contents of the cultivated culinary-medicinal Royal Sun Agaricus (the himematsutake mushroom) *Agaricus brasiliensis* S. Wasser *et al*. (agaricomycetideae) using different casing materials. *Int. J. Med. Mushrooms* 10: 181-188.

[13] Gregori A *et al*. (2008). Influence of carbon dioxide, inoculum rate, amount and mixing of casing soil on *Agaricus blazei* fruiting bodies yield. *Acta Agriculturae Slovenica*. 91: 371-378.

[14] Cavalcante JLR *et al*. (2008). Cultivation of *Agaricus blazei* in the environmental protection area of the Baturité region under three types of casing soils. *Acta. Sci. Agron.* 30: 513-517.

[15] Siqueira FG *et al*. (2009). Cultivation of *Agaricus blazei* ss. Heinemann using different soils as source of casing materials. *Sci. Agric.(Piracicaba, Braz.)*. 66: 827-830.

[16] Zied DC *et al*. (2009). Produção de *Agaricus blazei* ss. Heinemann (*A. brasiliensis*) emfunção de diferentescamadas de cobertura e substratos de cultivo. *Interciencia*. 34: 437-442.

[17] Colauto NB *et al*. (2010). Pasteurização de turfabrasileirapara o cultivo de *Agaricus brasiliensis*. *Semina: Ciências Agrárias, Londrina* 31(Supl. 1): 1331-1336.

[18] Colauto NB *et al*. (2010).Tratamentostérmicos do calxistoparausocomocamada de cobertura no cultivo de *Agaricus brasiliensis*. *Ciência Rural*. 40: 1660-1663.

[19] Colauto NB *et al.* (2010). Alternative to peat for *Agaricus brasiliensis* yield. *Bioresource Technol.* 101: 712-716.

[20] Colauto NB *et al*. (2011). Production flush of *Agaricus blazei* on Brazilian casing layers. *Braz. J. Microbiol.* 42: 616-623.

[21] González Matute R *et al*. (2011). *Agaricus blazei* production on non-composted substrate based on sunflower seed hulls and spent oyster mushroom substrate. *World J. Microbiol. Biotechnol.* 27: 1331-1339.

[22] Zied DC *et al*. (2011).”Indoor” method of composting and genetic breeding of the strains to improve yield and quality of the almond mushroom *Agaricus subrufescens*. In: *Mush. Biol. Mush. Prod.* Savoie JM, Foulongne-Oriol M., Largeteau M., and Barroso G. Eds. 7: 424-432.

[23] Pardo-Giménez A *et al*. (2014). Adaptabilidad de cepasbrasileñas de *Agaricus subrufescens* Peck a la fructificaciónsobrediferentescapas de cobertura en cultivocomercial. *Rev. Iberoam. Micol.* 31: 125-130.

[24] Chang ST and Miles PG. (2004).Mushrooms. Cultivation, Nutritional Value, Medicinal Effect, and Environmental Impact (2nd ed.). CRC Press. Boca Raton (FL), USA.

[25] Siqueira FG *et al*. (2011). Biological efficiency of *Agaricus brasiliensis* cultivated in compost with nitrogen concentrations. *Hortic.Bras.* 29: 157-161.

[26] Eira AF. (2003). Cultivo do Cogumelo Medicinal *Agaricus blazei* (Murrill) ss. Heinemann o *Agaricus brasiliensis* (Wasser *et al*.). Aprenda Fácil Editora.Viçosa (MG) Brasil.

[27] Pardo-Giménez A *et al*. (2013). Análisis proximal de carpóforos de *Agaricus subrufescens* Peck producidos sobre diferentes capas de cobertura. *ITEA-Inf. Tec. Econ. Agrar.* 109: 290- 302.

CLADOBOTRYUM MYCOPHILUM, CAUSAL AGENT OF COBWEB DISEASE ON COMMERCIAL *AGARICUS BISPORUS* AND *PLEUROTUS ERYNGII* CROPS IN SPAIN

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ABSTRACT

Between 2008 and 2011, outbreaks of cobweb were observed in commercial white button and king oyster mushroom crops in Castilla-La Mancha (Spain) based on morphological and genetic analysis the casual agent was identified as *Cladobotryum mycophilum*. Two *Agaricus bisporus* mushroom cropping trials inoculated with *C. mycophilum* were performed. The total area of the crop affected by cobweb was 30% in the inoculated blocks of trial A and 45% in trial B. The non-inoculated blocks remained healthy. Compared with the uninoculated controls, a decrease in the yield of 10.7% was observed in trial A and 9.1% in trial B. Pathogenicity trial was also performed using blocks containing sterilized, spawned and incubated *P. eryngii* substrate. The first cobweb symptoms developed 23 days after inoculation and *C. mycophilum* was consistently re-isolated from nine (37.5%) of the inoculated blocks. Non-inoculated blocks remained healthy. In a second test, conidial suspensions of three isolates of *C. mycophilum* were inoculated onto *P. eryngii* fruit bodies. *C. mycophilum* grew between 80 and 85% of the inoculated fruit bodies, while the control fruit bodies remained symptomless.

Keywords: mushroom disease, pathogenicity, white button mushroom, king oyster mushroom

INTRODUCTION

Several fungal diseases can affect *A. bisporus* and *P. eryngii* commercial crops [1, 2]. Among them, cobweb is a common disease of mushroom in every country that causes yield reduction and subsequently economic losses [3]. Different taxa from the genus *Cladobotryum* Nees can generate the pathology in *A. bisporus* and *P. eryngii*. *Cladobotryum dendroides* (Bull.: Fr.) W. Gams & Hoozem. (conidial state of *Hypomycesrosellus*) has hitherto been considered to be the most common cause of cobweb in *A. bisporus* crops. However, the most common causal agent is now *Cladobotryum mycophilum* (Oudem.) W. Gams & Hoozem.(conidial state of *Hypomycesodoratus*). This cobweb pathogen varies somewhat from published descriptions of *C. mycophilum* in conidial septation and the cultures lack the characteristic camphor-odour [1, 4, 5]. Other species like *C. multiseptatum*, *C. varium* and *C. verticillatum* from the same genus have been reported causing the disease in white button mushroom crops [6-8]. *C. dendroides* has been reported as pathogen to *Pleurotus eryngii* [9] and recently, *C. mycophilum* has also been identified as the causal agent of cobweb in cultivated king oyster mushroom in Spain and Korea [10-13].

Between 2008 and 2011 a strong presence of the cobweb disease were observed in *A. bisporus* mushroom crops in Castilla-La Mancha (Spain). A fluffy white mycelium grows over the casing or carpophores that quickly decay and become rotten. This feathery mycelium becomes denser due to a profuse sporulation occasionally acquiring yellow and pink hues when aged. Brown or grey spotting can also affect the mushroom caps. The first kind of spots, with a fuzzy outline, appear over the infected caps when a harmful airborne spore lands over a button mushroom and germinates into its surface, while the grey spots are due to a infection by the pathogenic mycelium (Fig. 1 a-d).

In the same way, symptoms of cobweb were also observed in cultivated *P. eryngii* during 2010. A soft-light cobweb-like mycelium appeared at the end of the crop cycle affecting substrate cultivated. Small white patches on the casing soil at first, subsequently spreading to the nearest king oyster mushroom by means of a fine gray-white mycelium, and eventually sporulating to produce masses of dry spores. The mycelium can quickly cover pinheads, stalks, pileus, and gills, eventually resulting in decomposition of the entire fruit body (Fig. 1 e, f).

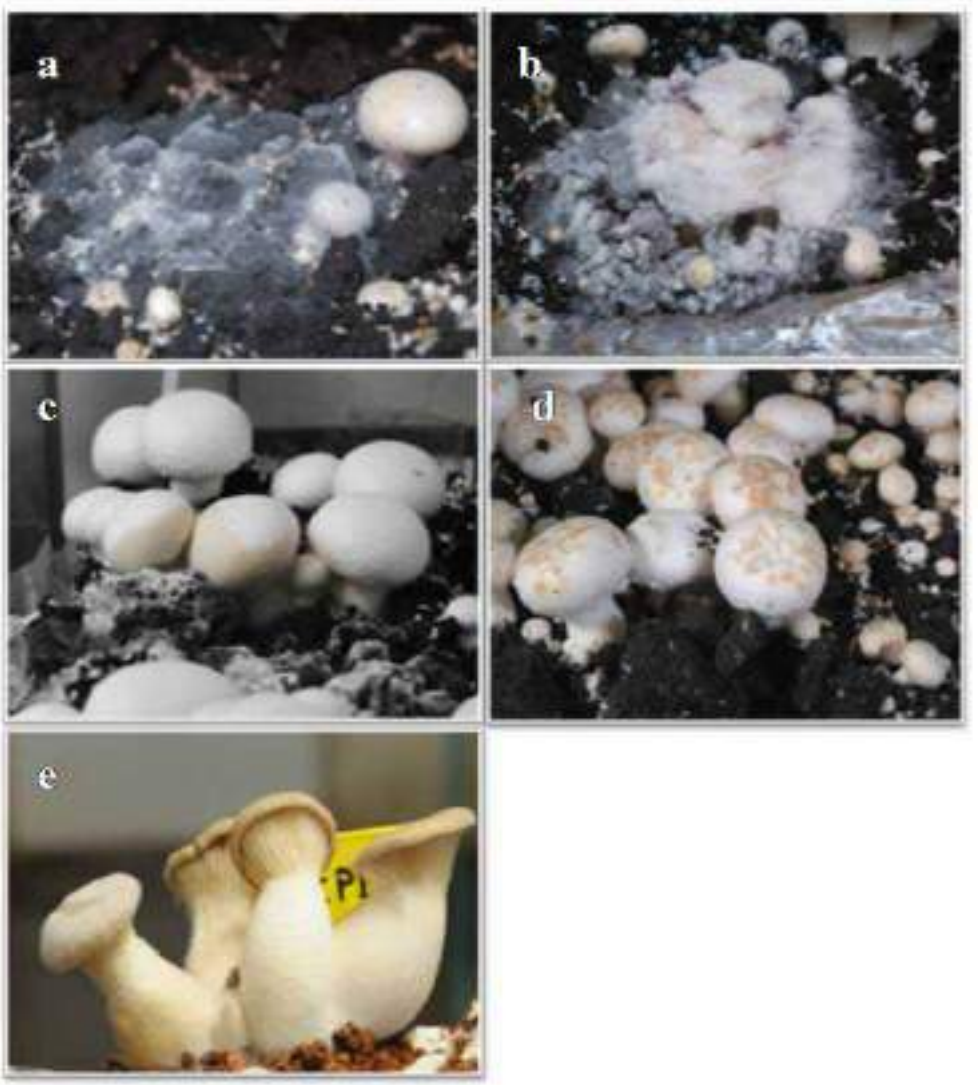

Figure 1. Cobweb symptoms on diseased crops of *A. bisporus* and *P. eryngii*. **a)** Fluffy mycelium over the casing. **b)** Dense mass of sporulation over the casing engulfing diseased fruit bodies. **c)** Regular grey spots due to a infection *via* mycelium. **d)** Irregular brown spots over the caps. **e)** Fluffy mycelium growing over *P. eryngii*

The objectives of the current work were to identify and characterize the pathogen responsible for cobweb disease in *A. bisporus* and *P. eryngii* Spanish crops. The pathogenicity of the cobweb causal agent has been also studied.

MATERIALS AND METHODS

Several isolates of *Cladobotryum* were recovered from *A. bisporus* and *P. eryngii* crops showing disease symptoms (Table 1). Samples were collected between 2009 and 2010 from mushroom farms located in Castilla-La Mancha (Spain), plated onto potato dextrose agar (PDA; Oxoid, Basingstoke, England) culture medium and maintained in growth chamber at 22 ºC in darkness.

Morphological characterization

Fungal structures were mounted on glass slides with lactic acid for microscope examination. Measurements of all taxonomically relevant characters (conidia and conidiogenous cells size, number of septa per conidium) were performed using Nikon software NIS-Elements Advanced Research (Nikon, Japan). Two hundred conidia and one hundred conidiogenous cells were measured from each isolate. The presence or absence of a conspicuous basal hilum on the conidia and phialide extensions or rachises was observed. Chlamydospore and/or microsclerotium production, the colony reverse colour side

Table 1. Strains collected from mushroom crops

Strain	Host	Isolation date	Casing	Flush
CL80	*A. bisporus*	28-1-2010	Mineral	3rd
CL55	*A. bisporus*	28-12-2009	Mineral	2nd
CL30	*A. bisporus*	4-2-2009	Mineral	3rd
PE26	*P. eryngii*	4-10-2010	Mineral	1st
PE40	*P. eryngii*	29-4-2010	Peat	1st
PE72	*P. eryngii*	29-4-2010	Mineral	1st

and the odour detectable upon lifting the lid of the Petri dish was also noted. The isolates were then identified according to the descriptions from Gams and Hoozemans [14].

Genetic characterization

DNA from two *Cladobotryum* isolates, CL80 and PE72, collected from *A. bisporus* and *P. eryngii* infected crops respectively, was extracted using the E.Z.N.A. fungal DNA commercial kit (Omega Bio-Tek, Doraville, USA) without mercaptoethanol. A 0.5 cm^{-2} fragment of mycelium growing on PDA medium was taken from the edge of the colony for identification of each isolate. Polymerase chain reaction (PCR) was performed using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) with primers ITS1F and ITS4 [15, 16] following cycling conditions proposed by Martín and Winka [17]. Amplicons were visualized in 2% agarose gels stained with SYBR® safe (Invitrogen, Eugene, OR, USA) under visible light. PCR bands were excised from the agarose gels, cleaned by the QIA quick DNA gel extraction (Qiagen, CA, USA) and sequenced in an ABI Prism 3130 Analyzer (Applied Biosystems). The DNA sequences were edited in Sequencher v.4.2 (Gene Codes Inc., Ann Arbor, MI, USA) and identification was achieved by conducting BLASTn searchs on GenBank (NCBI).

Pathogenicity trials in *Agaricus bisporus* mushroom growing rooms

Two cropping trials (A and B) were placed in experimental mushroom growing rooms, according to the standard practices used in mushroom farms in Spain. *A.bisporus* was cultivated in experimental trays (0.15 m^2 in area) filled with 10 kg of commercial mushroom phase III compost spawned at 1% (Gurelan 45 strain, Gurelan S. Coop., Huarte, Pamplona, Spain). On day 0 of the cropping cycle, trays of spawn-run compost were cased with a 30 mm of a peat based casing layer (5.5 l per block) (EuroveenB.V., BVB Substrates, Grubbenvorst, Limburg, Holland). Proper culture practices of peat cased crops were set up during the cropping cycle. A randomized block design of 24 blocks per trial was used and the assay was carried out in duplicate (A and B). Nine days after casing, a spore suspension of *C. mycophilum* ($7.5x10^3$spores ml^{-1}) was sprayed onto the surface of the casing layer (20 ml per block) of 12 blocks at 10^6 conidia m^{-2}. 12 blocks were sprayed with sterile distilled water as a control treatment. Blocks were maintained at 17.5 °C and 90% relative humidity. The effect of cobweb on mushroom productivity during three flushes was evaluated by the biological efficiency of the cultures inoculated with the pathogen [calculated as the ratio of the fresh weight of total yield of harvested mushrooms (healthy and diseased) to the weight of dry substrate at spawning and expressing the fraction as kg 100 kg^{-1} compost], as well as by the number of infected blocks, earliness of infection and final crop area affected.

Pathogenicity trials in *Pleurotus eryngii* mushroom growing rooms

A pathogenicity test was performed using 24 blocks containing sterilized, spawned (M2600, Mycelia bvba, Nevele, Belgium) and incubated *P. eryngii* substrate (3.6 kg, 352 cm^2 in area). Twelve blocks were cased with a 20 mm layer of a casing soil (0.7 l/block) made with mineral soil + sphagnum peat 4:1 (v/v) and the other twelve blocks were cased with a 20 mm layer of Topterra® (0.7 l/block). Five days after casing, six blocks from each casing type were inoculated with a conidial suspension (7×10^3 conidia ml^{-1}) of a *C. mycophilum* strain. Each block was sprayed (5 ml per block) onto the

surface of the casing layer at a rate of 10^6 conidia/m^2. Twenty-two blocks were sprayed with sterile distilled water as a control. A temperature of 16-17 ºC, 90% relative humidity, 900 ppm de CO_2 and cool-white fluorescent light were maintained throughout cropping. *P. eryngii* fruit bodies were harvested daily for each treatment over a period of eight days. Blocks were also prospected daily for cobweb symptoms.

Pathogenicity test on *A. bisporus* and *P. eryngii* fruit bodies

Three selected isolates (PE26, PE40 and PE72) were used for the pathogenicity test. Conidial suspensions (ca. 10^5 conidia/ml) of each isolate were inoculated onto 20 *A. bisporus* and 20 *P. eryngii* fruit bodies. *A. bisporus* carpophores were externally inoculated over the mushroom cap with 50 µl of conidial suspension, a scalpel incision of 0.5 cm in a cross shape was previously made in the inoculation point. Ten fruit bodies of *P. eryngii* were inoculated externally, while the other ten fruit bodies were cut in half and inoculated internally with 50 µl of conidial suspension per fruit body. Sterilized distilled water was used as a control. All the fruit bodies were then incubated in a moist chamber at 22 ºC in darkness. Assays were conducted twice and the results were recorded after seven days. Sterilized distilled water was used as a control. Koch´s postulates were verified by re-isolating the pathogenon PDA from the mushrooms artificially inoculated.

RESULTS AND DISCUSSION

Morphological characterization

On PDA, whitish to buff mycelium develops fast after inoculation. The mycelium sporulates profusely in a few days, mainly in the edge of the colony. Usually in 3-4 days, the colonies turn into pink hues that evolves toward strong red-blood when the isolate gets old (Fig. 2). This change in shade is more remarkable in the lower side of the plate because this pigment is copiously secreted by the hyphae submerged in the media while the aerial mycelium remains white [19]. These isolates produce microsclerotia and chlamydospores (Fig. 3b)[5] and the cultures lack characteristic camphor odour, normally associated with *C. mycophilum* [4].

Conidia hyaline, ellipsoidal, globose to subglobose, sometimes slightly curved, with a central hilum. Conidia measurements (Table 2) are 17.6-23.1 x 9.1-9.3 µm in isolates obtained from *A. bisporus* crops, and 20.9-22.2 x 8.8-10.1 µm in isolates from *P. eryngii* crops. Conidia septated (0-3 septa), majority of the spores were 2 celled. Conidiogenous cells hyalines, subulatedto almost cylindrical (Fig. 3a). Conidiogenous cells measurements (Table 2) in isolates obtained from *A. bisporus* crops are 25.4-34.8 µm long, 5.3 µm wide near base, attenuating gradually to 2.6-3.0 µm at the tip; and 29.0-30.5 µm long, 5.6-6.0 µm wide near base, and 2.7-3.1 µm at the tip, in isolates from *P. eryngii* crops.

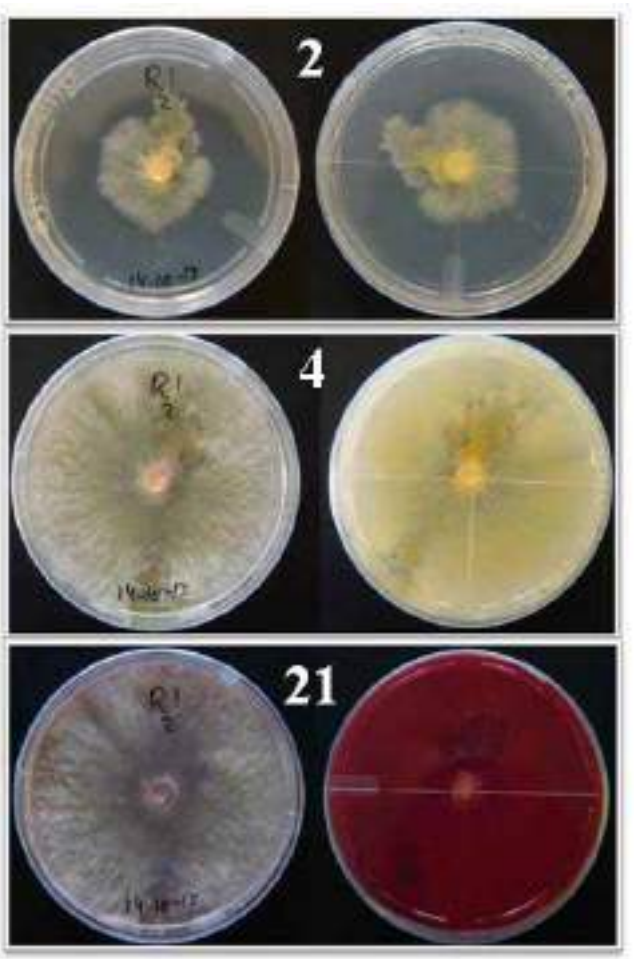

Figure 2. *C. mycophilum* on PDA, temporary evolution. Front and back side of the plates. *2, 4, 21: Age in days from inoculation

Table 2. Fungal structures measurement (µm) of studied strains

Strain	Conidia measurements (n=200)			Phyalide measurements (n=100)		
	Length	Width	Septa	Length	Base width	Apex width
CL80	23.1±0.3	9.1±0.1	1 (51%)	33.1±0.7	5.3±0.1	2.6±0.1
CL55	19.6±0.3	9.1±0.1	1 (62%)	25.4±0.7	5.3±0.1	3.0±0.1
CL30	21.7±0.3	9.3±0.1	1 (72%)	34.8±0.7	5.4±0.1	2.7±0.1
PE26	22.1±0.3	10.1±0.1	1 (68%)	30.5±0.7	6.0±0.1	3.1±0.1
PE40	21.6±0.3	9.3±0.1	1 (60%)	30.0±0.7	5.8±0.1	2.8±0.1
PE72	20.9±0.3	8.8±0.1	1 (81%)	29.0±0.7	5.6±0.1	2.7±0.1

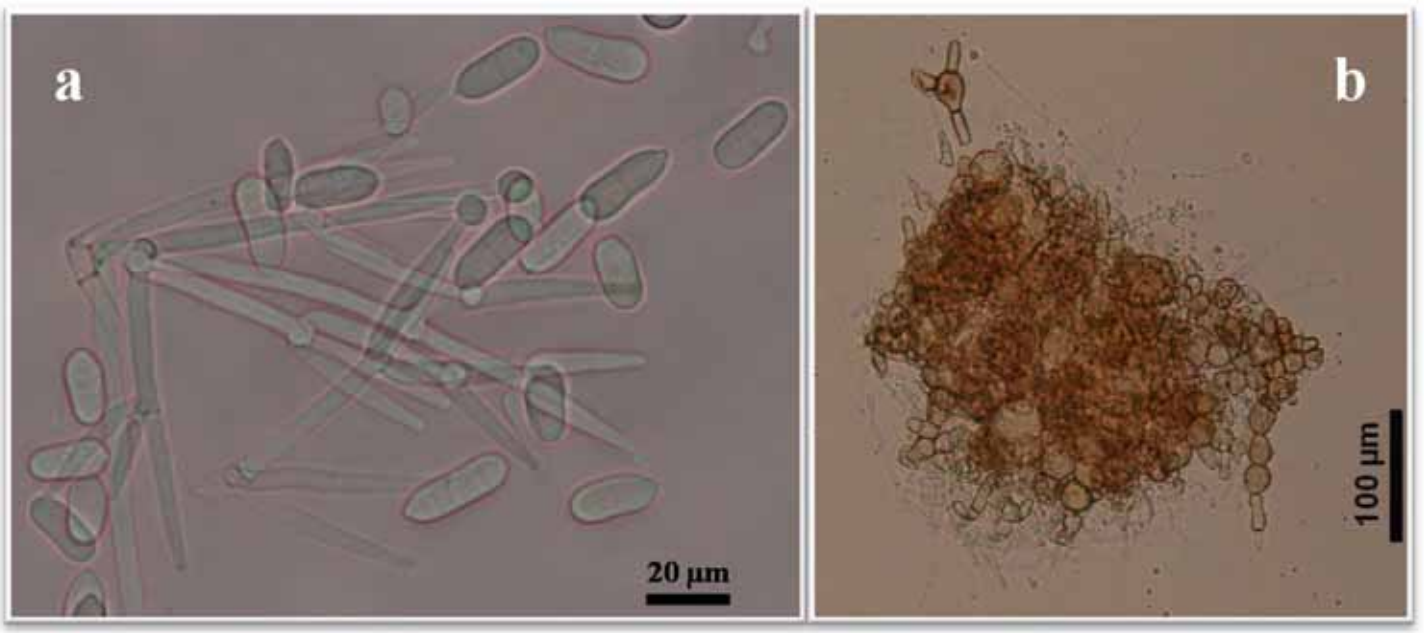

Figure 3. Morphological characteristics of *C. mycophilum* isolated from infected fruit bodies. **a)** Conidia and phyalides. **b)** Microsclerotium

Genetic characterization

The amplicon of the PE72 isolate (GenBank Accession Nº JF505112), showed 100% similarity of the obtained ITS sequence with two sequences of *Cladobotryum mycophilum* (teleomorph *H. odoratus*) (GenBank Accession Nos. Y17096 and Y17095) [5]. The amplicon of the CL80 isolate (GenBank Accession Nº JQ004732) also revealed highest similarity (99 and 100%) of the ITS sequence to four ITS sequences of *C. mycophilum* (teleomorph *H. odoratus*) (GenBank Accession Nos. AB527074, JF505112, Y17095, and Y17096) [10, 18].

Pathogenicity trials in *Agaricus bisporus* mushroom growing rooms

In the *A. bisporus* crop assays, the first cobweb symptoms developed 25 days after inoculation, between the second and third breaks in trial A; and after 11 days, between the first and second breaks in trial B (Fig. 3). *C. mycophilum* was consistently reisolated from eight inoculated blocks (67%) in trial A, and 11 inoculated blocks (92%) in trial B. As the growing cycle progressed the area colonized by the pathogen increased; by the end of the cycle, the total area of the crop affected by cobweb was 30% in inoculated blocks in trial A and 45% in trial B. As a result it is remarkable that the sooner the infection appears the heavier is the dispersion through the crop. The noninoculated blocks remained healthy. Compared with the noninoculated control blocks, a 10.7% decrease in yield of mushrooms was observed in trial A and 9.1% in trial

B. Production was lower in the second trial than in the first; that could be as well conditioned in some degree for the widespread of the outbreak.

Pathogenicity trials in *Pleurotus eryngii* mushroom growing rooms

The first cobweb symptoms developed 23 days after inoculation of *C. mycophilum* in the *P. eryngii* crop trial (Fig. 3). The pathogen was consistently reisolated from nine (37.5%) of the inoculated blocks. Noninoculated blocks remained healthy.

Pathogenicity test on *A. bisporus* and *P. eryngii* fruit bodies

The isolates tested were able to infect *P. eryngii* and *A. bisporus* carpophores. The strains revealed higher pathogenicity against *A. bisporus*, infecting 100 % of inoculated mushrooms. A radial mycelium grew from the point of inoculation and quickly evolved to a profuse sporulation mass within few days. The characteristics brown spots appeared over some of the infected mushrooms, which may suggest conidia dispersion through the moist chamber. In addition, they also resulted pathogenic against *P. eryngii* sporophores, 85% of the internally inoculated king oyster mushrooms and the 40% of those externally treated showed disease symptoms. So it is noticeable that external tissue of *P. eryngii* is less sensitive to the pathogen, and thereby it should be advisable to remove stipes wastes from the crop after harvest. A white fluffy mycelium grew over the *P. eryngii* infected fruit bodies from the inoculation point that started to sporulate within one or two days, although this sporulation was lighter than for the *A. bisporus* infected carpohores. The control mushroom remained healthy. These results satisfied Koch´s postulates, because the pathogen was re-isolated from the diseased tissues.

In light of the report findings, the same pathogenic specie is able to produce the cobweb disease over different kind of edible mushroom cultivated in Spain.

CONCLUSION

On the basis of the results, the causal agent of cobweb disease in *A. bisporus* and *P. eryngii* Spanish crops is *Cladobotyum mycophilum* (Oudem.) W. Gams & Hoozem. Pathogen isolated from diseased *P. eryngii* fruit bodies was able to infect *A. bisporus* and *P. eryngii* carpophores. Cobweb disease provokes yield decrease and reduces the crop area by colonizing the casing surface. Punctual outbreaks are adequately removed by covering the patches with a damp paper to avoid conidia dissemination and applying salt generously over it [3].

ACKNOWLEDGEMENTS

Funding for this research was provided by the Ministerio de Economía y Competitividad (INIA) and FEDER (Project RTA2010-00011-C02-01).

REFERENCES

[1] Fletcher JT and Gaze RH. (2008). Fungal diseases. In: Mushroom Pest and Diseases Control, pp 63-92, ISBN: 978-84076-083-5.

[2] Gea FJ. (2001). Plagas y enfermedadesdelgénero *Pleurotus* spp. In: La biología y el cultivo de *Pleurotus* spp., pp 205-224, ISBN 968-18-6357-7.

[3] Adie B *et al*. (2006). Temporal and spatial dispersal of *Cladobotryum* conidia in the controlled environment of a mushroom growing room. *Appl Environ Microbiol*. 72 (11): 7212-7217.

[4] Grogan HM. (2005). Cobweb confusion-Cobweb isolates explained. *Mushroom J*. 660: 21-33.

[5] McKay GJ *et al.* (1999). Genetic and morphological characterization of *Cladobotryum* species causing cobweb disease of mushrooms. *Appl. Environ. Microbiol*. 65: 606-610.

[6] Sinden JW. (1971). Ecological control of pathogens and weed moulds in mushroom culture. *Annu. Rev. Phytopathol*. 9: 411-432.

[7] De Hoog GS. (1978). Notes on some fungicolus hyphomycetes and their relatives. *Persoonia.*10: 33-81.

[8] Sharma SR *et al.* (2007). Fungal diseases and competitor moulds. In: Diseases and competitor moulds of mushrooms and their management. Technical Bulletin.National Research Centre for Mushroom. Indian Council of Agricultural Research.Chambaghat, Solan. pp. 2-43.

[9] Gioia T *et al.* (2007). Morpho-productive compariso among some wild and commercial strains of *Pleurotus eryngii* (D.C.:Fr.) Quél, in protected cultivation. *Micologia Italiana*. 36 (1): 35-46.

[10] Gea FJ and Navarro MJ. (2011). First report of *Cladobotryum mycophilum* causing cobweb on cultivated king oyster mushroom in Spain. *Plant Dis.* 95(8): 1030.

[11] Back CG *et al.* (2012). Characterization of species of *Cladobotryum* which cause cobweb disease in edible mushrooms grown in Korea. *Mycobiology*. 40(3): 189-194.

[12] Kim MK *et al*. (2012). First report of cobweb disease caused by *Cladobotryum mycophilum* on the edible mushroom *Pleurotuseryngii* in Korea. *Plant Dis*. 96(9): 1374.

[13] Kim MK *et al.* (2014). Fungicide sensitivity and characterization of cobweb disease on a *Pleurotuseryngii* mushroom crop caused by *Cladobotryum mycophilum*. *Plant Pathol. J.* 30 (1): 82-89.

[14] Gams W and Hoozemans ACM. (1970). *Cladobotryum*-konidienformen von *Hypomyces*-arten. *Persoonia*. 6(1): 95-110.

[15] Gardes M and Bruns TD. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Mol. Ecol*. 2: 113-118.

[16] White TJ *et al.* (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, a Guide to Methods and Applications*, pp 315-322, ISBN: 978-0123721815.

[17] Martín MP and Winka K. (2000). Alternative methods of extracting and amplifying DNA from lichens. *Lichenologist*. 32: 189-196.

[18] Back CG *et al.*(2010). Cobweb disease on *Agaricusbisporus* caused by *Cladobotryum mycophilum* in Korea. *J. Gen. Plant Pathol.* 76: 232-235.

[19] Põldmaa K. (2011). Tropical species of *Cladobotryum* and *Hypomyces* producing red pigments. *Stud Mycol*. 68: 1–34.

EFFECT OF ESSENTIAL OILS ON MYCOPATHOGENS OF *AGARICUS BISPORUS*

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ABSTRACT

The control of mycopathogens in mushroom cultivation is based on the usage of very limited active substances, cultural protection practices and sanitation precautions. The lack of registered chemicals is a problem in the practice. Integrated disease management have been used in mushroom cultivation but there is a need for novel selective fungicides and cheap reliable disinfectants.The main target of the present work is to find natural products which can be tested in cultivation against the major *Agaricus bisporus* fungal diseases with effect of prevention and/or curative methods. In the trial essential oils of aromatic plants; *Bacillus subtilis* and active substance prochloraz-Mn were tested *in vitro.* The examined pathogens were *Clabobotryum dendroides, Mycogone perniciosa, Lecanicillium fungicola* and *Trichoderma aggressivum* f. *aggressivum.* The effect of essential oils on the growth of a cultivated hybrid variety of *A. bisporus* was also tested: (1) hole-test on agar plates; (2) contact test on agar plates; (3) volatile fraction test on agar plates and (4) test on mushroom fruit bodies. Among the tested oils *Cinnamonium, Mentha* and *Pelargonium* proved to be the most effective which completely inhibited the growth of all four mycopathogens. It can be concluded that *Citrus* and *Tyhmus* were not effective in this case. *Bacillus subtilis* were effective against all tested pathogens in contact test. Further research is necessary to determine correct application and concentrations, because almost all tested oils inhibited the growth of the cultivated mushroom hyphae as well. From that point of view, only *B. subtilis* looks prospective in cultivation.

Keywords: disease, integrated, *Mycogone*, *Lecanicillium*, mushroom

INTRODUCTION

A number of pests and diseases cause problems in white button mushroom (*Agaricus bisporus*) production, as they significantly reduce both the quality and quantity of mushrooms. Due to the short growing period, the special cultivation technology and the lack of available synthetic substances, the scope of applying any chemical against pests and pathogens is very limited [1].

There is a growing tendency of using biological plant protection products in vegetable production (e.g. tomato, pepper and cucumber), where high efficacy can be ensured with advanced climate control. This should serve as an example for mushroom production as well. Resistance against registered products can be observed in more and more cases [2, 3], thus widening the range of active substances is an urgent task. The diseases regularly present and causing loss of revenue in button mushroom cultivation are the following [1]: cobweb disease (*Clabobotryum dendroides*), wet bubble disease (*Mycogone perniciosa*), dry bubble disease (*Lecanicillium fungicola*) and compost green mould (*Trichoderma aggressivum* f. *aggressivum*).

In each case the first step of mushroom protection is prevention. Some producers apply chemicals regularly, instead of using them only when pests and pathogens are really present in the culture. To provide proper hygienic conditions from the beginning and to treat already infected mushroom cultures, we are seeking active agents that are selective, easy to license, inexpensive and easy to use in mushroom production. One of the possible solutions is to try essential oils, about which some data are already available [4-6]. The effect of essential oils of herbs on mushroom pathogens has been examined in Serbia. Their results showed that thymol, carvacrol and cimol, which are the main essential oil components of thyme, significantly inhibit the growth of *Lecanicillium fungicola* and *Trichoderma harzianum* [7]. The oils of peppermint

(*Mentha piperita*) and spearmint (*Mentha spicta*) could also block the growth of *Trichoderma harzianum*, *Verticillium fungicola* and *Pseudomonas tolaasii* [7]. The same effect can be observed in case of wet bubble disease [8-10]. In another study [11] the toxicity of lavender, anise, parsley, chamomile, fennel, geranium, oregano and common sage was tested against bubble diseases and cobweb disease. The main component of pelargonium (geraniol and citronellol) and oregano (carvacrol and thymol) had the strongest effect on pathogens [11]. Initial studies on the post-harvest application of essential oils on fruitbodies are being conducted [12]. However, no recommendations on components, technologies or dosage exist or available yet. It is especially important to come up with solutions since neither the selectivity of these oils, nor their possible antifungal effect on the mycelia of the cultivated mushrooms has been tested so far.

The main target of the present work is to find natural products which can be tested in cultivation against the major *A. bisporus* fungal diseases, with effect of prevention and/or curative methods. In the trial essential oils from aromatic plants; *Bacillus subtilis* and active substance prochloraz-Mn were tested *in vitro* to compare the commonly used active substances with novel ingredients.

MATERIALS AND METHODS

1. Materials

In this study the pure essential oils of an essential oil producing company (*Aromax*) were used. To compare efficiency a licensed active substance (prochloraz-Mn complex) and a bacterial product were applied parallel to the essential oils (Table 1).

Table 1. Essential oils and other active substances used for *in vitro* tests

Complex essential oils of	Other active substances
Cinnamonium zeylandicum (cinnamon)	*Bacillus subtilis* QST 713 strain ('Virtuoso')
Citrus aurantium (bitter orange)	Prochloraz-Mn, 50% ('Sporgon 50WP')
Matricaria chamomilla (chamomile)	
Mentha spicata (spearmint)	
Pelargonium graveolens (geranium)	
Salvia officinalis (common sage)	
Thymus vulgaris (common thyme)	

The pure pathogen cultures of the culture collection of the Corvinus University of Budapest, Department of Vegetable and Mushroom Growing were used for the purpose of this study. The tested pathogens were the following: *Cladobotryum dendroides; Mycogone perniciosa; Lecanicillium fungicola; Trichoderma aggressivum* f. *aggressivum. Agaricus bisporus* 'A15' was used as control for testing selectivity.

2. Methods

Hole-test

PDA media (Biolab, 39 g/l) were poured into petri-dishes (90 mm diameter), than with a corkborer (5 mm) 3 holes were cutin media. One hole was filled with 150 µl essential oil (0.01% concentration) while rest two holes with 150 µl sterilized DW. Other petri-dishes were filled with 150 µl prochloraz-Mn (0.5 g/l) solution or 150 µl *Bacillus* suspension (2 g/l). Each treatment had 4 replicates and were placed on 25 °C. After 10 days incubation a picture was taken with the characteristics of the developed mycelia.

Contact test

Into the PDA media (Biolab, 39 g/l) after autoclaving (121 °C, 20 min) chemicals were mixed still in liquid phase:

- Control: 1 drop Tween-20
- Essential oils: cc. 150 µl/l + 1 drop Tween-20
- Prochloraz-Mn: 0.5 g/l
- *Bacillus*: 2 g/l

After solidification in petri-dishes, 5 days old pure mycelial culture (diameter 7.5 mm) was placed into the center.Every treatment had four replicates. The petri-dishes were incubated on 25 °C for 10 days than colony diameters were measured.

Volatile fraction test

PDA media (Biolab, 39 g/l) were poured into petri-dishes than inoculated with 10 days old pure mycelia cultureand turned down. Into the lid a sterilized paper was laid and sprayed with 500 µl essential oil in 0.01% concentration. As a negative control 500 µl of prochloraz-Mn (0.5g/l) solution was also tested, sterilized distilled water was used as positive control. 500 µl of *Bacillus* (2 g/l) was also tested. Every treatment had four replicate. The petri-dishes were incubated at 25 °C for 10 days than colony diameters were measured.

Test on mushroom fruit bodies

The effect of the tested materials was also studied on fruitbodies. 100 µl of essential oils in two concentrations (cc., 1000× dilution); *Bacillus* (2 g/l) and prochloraz-Mn (0.5 g/l) as well in two concentrations (cc., 1000× dilution) were pipetted onto mushrooms than incubated 24 hours on room temperature and lesions were noticed.

RESULTS AND DISCUSSION

Results of Hole-test

The developed mycelia did not show the typical concentric shape, but it was distorted opposite the essential oil (figure not shown). In this type of test no differences in efficacy were registered, since all the essential oils diffused in the media and inhibited mycelial growth. The same effect was seen not only by each pathogen, but in case of the button mushroom as well. This result could mean that in practice an even more intensive translocation of the oils can be expected, since the cultivating media is less compact.

Results of Contact test

Fig. 1 shows the diameters of the mycelia growth 10 days after inoculation on poison agar plates. It can clearly be seen that cinnamon, spearmint and geranium essential oils were to be most effective in mycelial growth inhibition. Due to these oils, each pathogen stopped developing. Unfortunately the tested essential oils are not selective enough, since they blocked the button mushroom mycelia as well. Amongst the tested oils, thyme was the only one with selectivity: it did not affect the mycelial growth of button mushroom significantly, and in the meantime blocked the growth of *Cladobotryum*, *Mycogone* and *Trichoderma*.The figure shows a slight but not significant ($p<0.05$) positive effect in case of *Lecanicillium*, *B. subtilis* also proved to be effective. Results prove the contact effect of essential oils. They all have a certain level of strong inhibition. Spearmint can be characterised with partial selectivity: it blocked mycelia growth in all except one (*Lecanicillium*) case.

Results of Volatile fraction test

This type of setting is most comparable to a possible large scale application. The essential oils evaporate from the paper stripe placed into the Petri-dish. The inhibiting effect is just like in case of contact application of oils mentioned above.

Cinnamon, spearmint and geranium oils proved to be most effective inhibitors in case of each pathogen. Chamomile completely blocked the mycelial growth of the button mushroom too (Fig 2).

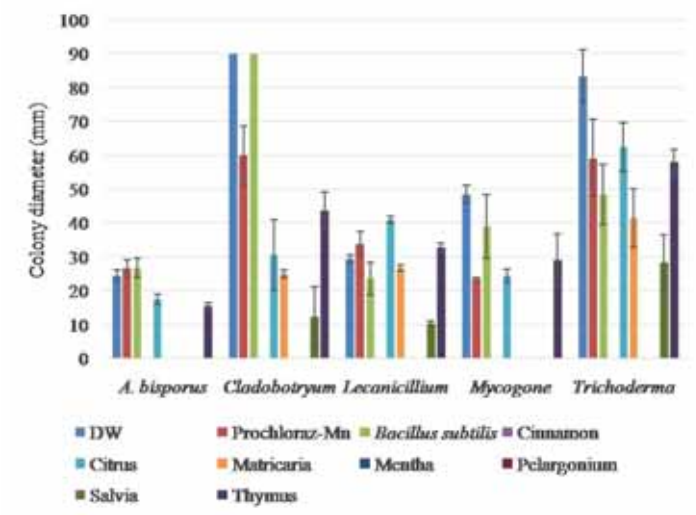

Figure 1. Influence of essential oils with poison agar technique on mycelial colony diameter

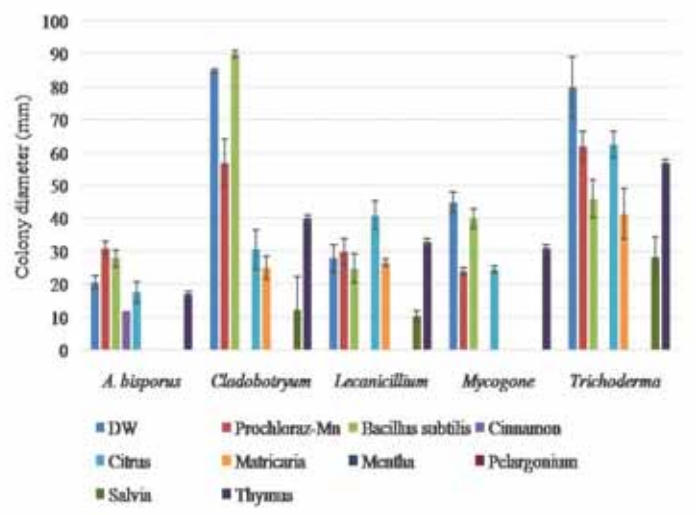

Figure 2. Influence of volatile fraction of essential oils on mycelial colony diameter

Based on the results it can be concluded that the selectivity of essential oils is not satisfying. Those oils that do not have a inhibiting effect on button mushroom mycelium do not inhibit the pathogens growth as well. The essential oils (cinnamon, mentha, geranium) with significant inhibition on pathogens block button mushroom growth too. The bacterium *B. subtilis* only affected *Trichoderma*, but the contact effect is acceptable.

Results of test on mushroom fruit bodies

Fig. 3 and 4 show the effect of essential oils in the fruitbodies. As it can be seen on Fig. 3, each undiluted oil cause similar deep, dark lesions. Essential oils at this concentration damage fruit bodies so much that they cannot be used or consumed anymore. The undiluted bacterial product caused brown lesions, while the active agent (prochloraz-Mn) of a licensed product caused lighter, but distinctive lesions on the mushrooms.

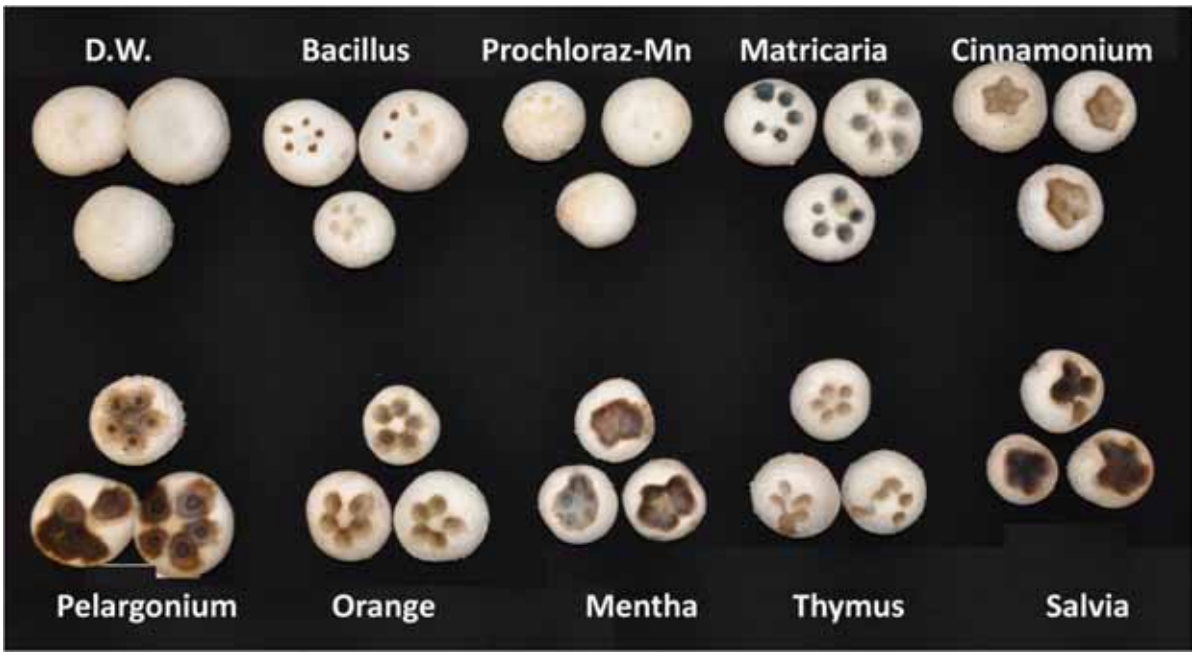

Figure 3. Effect of concentrated essential oils and active substances on to mushroom fruit bodies after 24 hour incubation

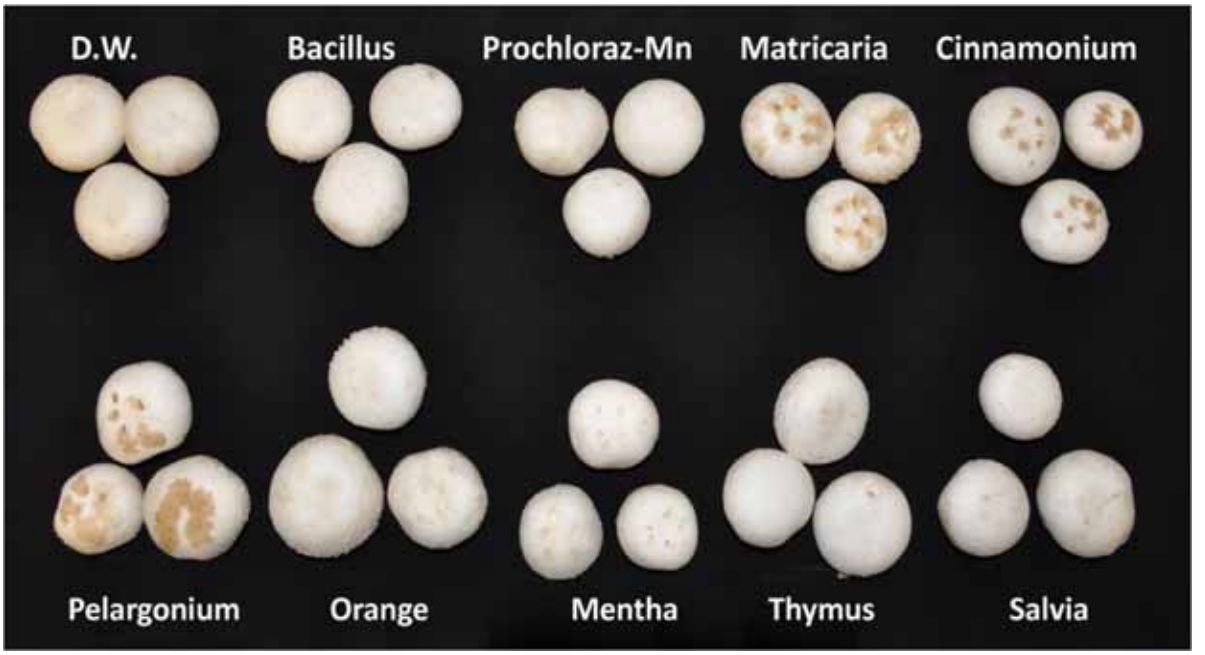

Figure 4. Effect of diluted essential oils (1000×) and active substances onto mushroom fruit bodies after 24 hour incubation

Fig. 4 shows the results of the tests conducted with 1000 times diluted oils. At this concentration spots were recorded in case of cinnamon, chamomile and geranium. The lesions caused by spearmint and sage oils were smaller and not as deep. The diluted bacteria product did not induce any spots, thus from this point of view it can be applied during the growing period.

CONCLUSION

A number of studies have examined the effect of essential oils on the pathogens of button mushroom. In some cases, the positive effects were proven and even the components responsible for inhibition were identified. In this study 7 essential oils were included, on which previously no or not complete tests have been conducted. Results of the *in vitro* tests show that cinnamon, geranium and spearmint oils have a strong inhibiting effect on the pathogens *Cladobotryum*, *Mycogone*, *Lecanicillium* and *Trichoderma.* It has been proven that essential oils not only have a contact effect, but also by vaporizing, the active agents can block the vegetative growth of pathogens. Some previous studies [8, 9] have not included button mushroom mycelia in the tested species. Our experiment shows that since essential oils are not selective enough and damage the mycelia and the fruit body of the cultivated mushroom as well, further *in vivo* studies of the above mentioned three species are recommended. Although a certain level of yield loss is predictable, it should be noted that already licensed and applied products have the same effect too. Since essential oils proved to be fungistatic, alternative application method (e.g. preventive treatment) should be considered. Based on the price and difficulty in application (rooted in the non water soluble property of oils), essential oils probably will not be competitive with traditional disinfectant products.

ACKNOWLEDGEMENTS

Present research was supported by post-doc research program of the Research Institute of Organic Agriculture (ÖMKI, Hungary).

REFERENCES

[1] Fletcher JT and Gaze RH. (2008). Mushroom Pest and Disease Control, a ColourHandbook. Manson Publishing, Boston.

[2] Romaine CP *et al.* (2008). Emergence of benzimidazole-resistant green mold, *Trichoderma aggressivum*, on cultivated *Agaricus bisporus* in North America. *Mushr. Sci.* 17: 510-523.

[3] Bonnen AM and Hopkins C. (1997). Fungicide resistance and population variation in *Verticillium fungicola*, a pathogen of the button mushroom, *Agaricus bisporus*. *Mycol. Res.* 1: 89-96.

[4] Bakkali F *et al.* (2008). Biological effects of essential oils - A review. *Food Chem. Toxicol*. 2: 446-475.

[5] Shah S and Nasreen S. (2011). Evaluation of Bioagents against the Infection of Green Mould (*Trichoderma* spp.) in *Pleurotus sajor-caju* Cultivation. *Intl. J. of Plant Pathol.* 2: 81-88.

[6] Górski R *et al.* (2010). Effect of selected natural essential oils on *in vitro* development of fungus *Trichoderma harzianum* found in common mushroom (*Agaricus bisporus*) cultivation. *Ecol. Chem. Eng*. 17(2): 177-185.

[7] Sokovic MD *et al.* (2009). Chemical composition of essential oils of *Thymus* and *Mentha* species and their antifungal activities. *Molecules* 14: 238-249.

[8] Glamoclija J *et al.* (2008). Anti fungal activities of mint and thyme essential oils against mycopathogen *Mycogone perniciosa*. Abstract of the Sixth International Conference on Mushroom Biology and Mushroom Products. Bonn, Germany: 76.

[9] Potoènik I *et al.* (2010). Sensitivity of *Mycogone perniciosa*, pathogen of culinary-medicinal button mushroom *Agaricus bisporus* (J. Lge) imbach (*Agaricomycetideae*), to selected fungicides and essential oils. *Int. J. Med. Mushr*. 12(1): 91-98.

[10] Regnier T and Combrinck S. (2010). *In vitro* and *in vivo* screening of essential oils forthe control of wet bubble disease of *Agaricus bisporus*. *S.Afr. J. of Bot.* 76(4): 681-685.

[11] Tanovic B *et al*. (2009). *In vitro* effect of essential oils from aromatic and medicinal plants on mushroom pathogens: *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa*, and *Cladobotryum* sp. *Arch. Biol. Sci*. 61(2): 231-237.

[12] Gao M *et al.* (2014). Browning inhibition and quality preservation of button mushroom (*Agaricus bisporus*) by essential oils fumigation treatment. *Food Chem*. 149: 107-113.

INTEGRATED APPROACHES FOR THE MANAGEMENT OF *MYCOGONE PERNICIOSA* CAUSING WET BUBBLE DISEASE

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ABSTRACT

Wet bubble in white button mushroom (*Agaricus bisporus*) incited by *Mycogone perniciosa* Magn. has been reported as one of the serious diseases from almost all the major mushroom growing countries of the world. Bubble or mole, first described from Paris in 1888, was reported in India for the first time in 1978. As the pathogen inflicts serious damage to the crop, various attempts have been made to manage the disease through various means. Interaction between *A. bisporus* and *M. perniciosa* studies conducted in dual, half plate and paired cultures. The average growth of *A. bisporus* and *M. perniciosa* in either dual culture was 16.13 and 28.86 mm, respectively. Growth of *A. bisporus* remain unaffected (16.02 mm) and *Mycogone* enhanced to 36.91mm (21.80% increase) when both grown in dual culture. Pre spawning of casing soil 5-20 days prior to pasteurization resulted in reduced incidence of wet bubble disease. Thermal death point of *Mycogone* observed to be at 44-45 °C. Moisture contents of casing soil less than 60% at the time of pasteurization favour the survival of *M. perniciosa*. *Mycogone* failed to survive in casing soil having moisture contents 60% or above at 60 °C or above temperature. Two bacterial isolates B-9 (*Bacillus*) and B-18 (*Alcaligenes*) proved to be very promising bio control agents for the management of wet bubble disease both under laboratory and mushroom house conditions. Out of five fungicides and two other chemicals tried, carbendazim proved most effective in managing wet bubble disease among all the fungicides/ chemicals tested.

Keywords: *Mycogone perniciosa*, *Agaricus bisporus*, wet bubble, fungicides, control

INTRODUCTION

Wet bubble in white button mushroom incited by *Mycogone perniciosa* Magn. has been reported as one of the serious diseases from almost all the major mushroom growing countries of the world. Bubble or mole (*M. perniciosa*), first described from Paris in 1888, is stated to be responsible for the heaviest losses in mushroom beds in France, England and United States [1]. The disease has also been reported to assume serious proportion in other major mushroom growing countries of the world such as United Kingdom, Netherlands, USA, China, Taiwan, South Africa, Brazil, Hungary, Australia and Poland from time to time. In India, this disease was reported for the first time in 1978 from mushroom farms in Jammu and Kashmir [2]. Later this disease was reported from Himachal Pradesh, Haryana and Maharashtra. Symptoms of wet bubble at different stages of mushroom development have been described by many workers. It has been estimated [3] that qualitative and quantitative losses caused by wet bubble and dry bubble in 70s in Pennsylvania (USA) were 2.2 million lbs and 19.7 million lbs, respectively. Natural incidence of wet bubble disease of button mushroom ranged from 1 to 100 % in North India [4].

MATERIALS AND METHODS

Interaction studies

Mycogone and *A. bisporus* were grown together in paired culture on PDA, mushroom extract agar (MEA) and mushroom extract dextrose agar (MDA) in 90 mm Petri plates to view their interaction *in vitro*. Five mm diameter plugs from the edge of actively growing *A.bisporus* and *M. perniciosa* were inoculated opposite of each other in 90 mm Petri plates. Control consisted of plates of either *A.bisporus* or *M. perniciosa* at both sides of the Petri plates.

Five mm diameter plugs from actively growing *A.bisporus* and *M. perniciosa* were also inoculated in the centre of the bottom of 90 mm, MA Petri plates, to make dual culture. After 48 h the bottom of the Petri plates containing plugs of *A. bisporus* were joined with bottom from Petri plates containing plugs of *M. perniciosa* by taping the respective bottom plates together with insulation tapes. In some cases plates containing *M. perniciosa* plugs were the bottoms of the dual culture and others *A.bisporus* plates were the bottom plates. Control consisted of taped cultures with both top and bottom plates of the dual culture containing *M. perniciosa* or *A. bisporus*, respectively.

In third set of experiment both *M. perniciosa* and *A. bisporus* were grown together on MEA, MDA and PDA, in 90 mm half Petri plates to examine the effect of volatiles on fungal growth and interaction *in vitro.* Five mm diameter plugs from the edge of actively growing *A. bisporus* and *M. perniciosa* were inoculated in separate compartments of 90 mm half Petri plates. Control consisted of half plates of either *M. perniciosa* or *A. bisporus* in both compartments of the plate.

Thermal death point of mycelium under dry conditions

Each culture was grown in Petri plates on malt extract medium. A bit of 4 mm actively growing mycelium of *Mycogone perniciosa* was transferred to empty 200 µl PCR tubes (dry condition) as well as PCR tubes containing 100µl sterile water (wet condition) in such a way so that the culture bit got submerged in the water under aseptic conditions in laminar flow. The culture containing PCR tubes were loaded in the gradient PCR (ASTEC) machine where different temperature starting from 38.0 to 47.9 °C in the gap 0.9 °C was maintained for 10 minutes. After giving the heat shock treatment the culture bits were transferred to fresh slants of malt extract agar (MEA) medium under aseptic conditions and incubated at 25 °C to observe the viability of the culture.

Management studies

Compost prepared by short method was used to raise button mushroom crop. Strain (U-3) was used. Ten kg ready compost was filled in each polythene bag in a way to give the surface area of 0.1 m² and thorough spawning was done. The bags were incubated in cropping rooms at 25±1 °C. After the colonization of compost by the mushroom mycelium after 18 days, 2 cm layer of casing mixture (FYM + spent compost, 50:50 w/w) was applied. The temperature was gradually reduced from 25 °C to 16 °C in the next 8-10 days. Watering was done daily and aeration was provided as per the requirement of the crop. The fruit bodies were harvested and data on yield was recorded.

Prespawning studies

Prespawning of casing mixture was done with *A.bisporus* spawn @ 0.5%. and incubated at 25±1 °C for 20, 15, 10 and 5 days. Control consisted of unspawned casing mixture. After incubation the casing mixture was pasteurized at 65-67 °C for 8h and used as casing material for the experiment.

In one set of experiment various moisture levels viz., 50, 55, 60, 65 and 67% of the casing mixture were maintained. Casing mixture was inoculated with 0.1% of *M. perniciosa* wheat grain based inoculum.Three sets of casing mixture with different moisture levels were maintained. After incubation at 25±1 °C for 5 days, one set was pasteurized at 60-65 °C for 8h, another at 65-67 °C for 8h and third at 67-70 °C for 6h.

Management studies

Two bacterial spp (*Bacillus* sp and *Alcaligenes* sp.) and an actinomycete, *Streptomyces* sp isolated from casing mixture were multiplied on nutrient broth at 28 °C for 24h. One ml bacterial culture containing 10^6-10^7cfu/ml diluted in 50 ml of water was sprayed on each bag after two days of casing application.

Different fungicides viz., Prochloraz manganese (1-N-propyl-N-2(2,4,6- trichlorophenoxy) ethyl) carbomoyl imidazole + Manganese), Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile), mancozeb (Manganese ethylene bisdithiocarbamte), formalin (formaldehyde),carbendazim (Methyl-2 benzimidazolecarbamate), K_2HPO_4 and $CaCl_2$ were sprayed at three different concentration concentrations one day after casing. A fungicidal solution of 50 ml / 0.1 m² was sprayed at each bag.

RESULTS AND DISCUSSION

Interaction studies

In Dual culture: The average growth of *A. bisporus* and *M. perniciosa* in either dual culture was 16.13 and 28.86 mm, respectively (Table 1a&b). The growth of *A. bisporus* remain unaffected (16.02 mm) and *Mycogone* enhanced to 36.91mm (21.80% increase) when both grown in dual culture.

Table 1a. Interaction of *Mycogone* and *Agaricus* in paired and half plate culture

Paired culture	**Growth of mycelium in petriplates (in nm) in pairs**					
	***Agaricus* Control (mm)**		***Mycogone* Control (mm)**		**Interaction(mm)**	
Medium	**A with A**		**M with M**		**A with M**	
MA	10.75	10.85	21.2	21.5	11.07	22.15
MDA	9.62	10.1	23.7	24.2	11.6	24.07
PDA	10.45	10.47	23.72	23.95	12.02	24.02
Mean	**10.37**		**23.84**		**11.56**	**23.41**
Half Plate Culture						
MA	10.46	11	29.23	29.76	10.9	30.06
MDA	10.2	10.46	28.53	29.4	9.9	28.8
PDA	9.8	10.06	20.96	20.83	9.33	19.26
Mean	10.33		26.45		10.04	26.04

Table 1b: Interaction of *Mycogone* and *Agaricus* in dual plate technique

	***Agaricus* Control (mm)**		***Mycogone* Control (mm)**		**Interaction(mm)**			
Medium	**A**	**A**	**M**	**M**	**A (bottom)**	**A (Top)**	**M (Top)**	**M (Bottom)**
MA	15.96	16.30	28.70	29.03	15.88	16.16	36.63	37.20
Mean	16.13		28.86		16.02		39.91	
CD(0.05)	0.7		0.8		0.8		1.2	

A = *Agaricus*; M = *Mycogone*

Interaction between *Mycogone* and *A. bisporus* in paired culture on three media (MEA, MDA and PDA) was studied. On all the three media *A. bisporus* grew more (111.56 mm, mean) in paired culture as compared to its alone culture (10.37 mm, mean) Whereas the growth of *M. perniciosa* was at par in paired (23.84 mm, mean) and alone culture (23.41 mm, mean) in all the three media.

In half Petridish culture the mean growth of *A. bisporus* was 10.33 mm in alone culture against its 10.04 mm growth in paired culture. Growth of *M. perniciosa* was 26.45 mm in alone culture against its 26.04 mm growth in paired half plate culture.

Thermal death point

Thermal death point of mycelium of *Mycogone perniciosa* was observed to be at 45.2 °C under dry conditions whereas mycelium failed to recover at 44.3 °C under wet conditions clearly indicating that the thermal death point of *M. perniciosa* is between 44-45 °C (Table 2).

Table 2. Thermal death point of mycelium of *Mycogone*

Temperature of Incubation	Under Dry conditions (10 min)	Under wet conditions (10 min)
38.0	+	+
38.9	+	+
39.8	+	+
40.7	+	+
41.6	+	+
42.5	+	+
43.4	+	+
44.3	+	-
45.2	-	-
46.1	-	-
47.0	-	-
47.9	-	-

Under dry conditions thermal death point of *M. perniciosa* was 44.3 °C and under wet conditions the thermal death point was 43.4 °C. Since the culture consisted mainly of mycelium and condia, so the thermal death point observed in the present case may not hold true for cultures having chlmydospoes. In that case thermal death point might be on higher side. Wuest and Moore [5] suggested that aerated steam at 54.4 °C for 15 minutes can eliminate *M. perniciosa* from casing soil.

Effect of pre spawning of casing soil on the survival of *Mycogone perniciosa*

Interaction studies between *A. bisporus* and *M. perniciosa* indicated that *A. bisporus* enhances the growth of *M. perniciosa*. Since the pathogen attack host only in the casing layer it might be possible that the volatiles released by *A.bisporus* stimulate germination/ growth of *Mycogone* spores/ mycelia in the casing layer. Pre spawning experiment aimed to study such effect revealed that pre spawning of casing soil 5-20 days prior to pasteurization resulted in reduced incidence of wet

Table 3. Effect of pre spawning on *Mycogone* inhibition

Duration (Days)	Treatments	Yield
20 Days prior	Spawned +Inoculated	13.06
	Inoculated (Only *Mycogone*)	11.08
	Unspawned + Uninoculated	13.98
15 Days prior	Spawned +Inoculated	12.56
	Inoculated (Only *Mycogone*)	10.14
	Unspawned + Uninoculated	10.80
10 Days prior	Spawned +Inoculated	13.74
	Inoculated (Only *Mycogone*)	10.41
	Unspawned + Uninoculated	12.53
5 Days prior	Spawned +Inoculated	11.65
	Inoculated (Only *Mycogone*)	8.46
	Unspawned + Uninoculated	13.57
CD (0.05)		0.78

bubble disease (Table 3). Pre spawning of casing soil with *A. bisporus* stimulated the germination of *M. perniciosa* inoculmn in the casing soil and after pasteurization resulted in death of active inoculmn i.e. mycelial form.

Effect of casing soil moisture contents and pasteurization temperature on the survival of *M. perciosa*

Wet conditions leads to coagulation of microbial proteins at lower temperature. With this intention the present experiment concerning effect of different moisture levels and temperature on the survival of *M. perniciosa* was laid which reveals that lower moisture contents of casing soil at the time of pasteurization favours the survival of *M. perniciosa. Mycogone* failed to survive in casing soil having moisture contents 60% or above at 60 °C or above temperatures (Table 4). Results explicitly indicated the role of moisture contents in the survival of inocula in relation to moisture contents in the casing soil at the time of pasteurization.

Table 4. Effect of casing soil moisture contents and pasteurization temperature on the survival of *Mycogone perniciosa*

Moisture (%)	Pasteurization temp. (°C) and duration	Uninoculated	Inoculated
50	60-65 /8h	11.39	6.82
55		12.97	7.66
60		10.52	8.41
65		12.81	10.17
67		12.60	13.82
70		13.69	13.40
50	65-67/8h	11.38	6.70
55		9.10	8.45
60		12.19	8.74
65		12.63	11.70
67		12.99	12.09
70		13.10	12.80
50	67-70/6h	12.55	7.42
55		10.70	7.26
60		12.94	9.30
65		11.93	10.80
67		12.60	12.20
70		12.71	12.06
CD (0.5)		0.38	0.43

Mycogone management through micro-organisms

Spraying of two bacterial spp (*Bacillus* sp and *Alcaligenes* sp) and an actinomycete *Streptomyces* sp @one ml bacterial culture containing 10^6-10^7cfu/ml diluted in 50ml of water on each bag (0.1m^2area) after two days of casing application revealed that *Bacillus* and *Alcaligenes* sp are capable of controlling wet bubble disease. In control-II which was inoculated with *Mycogone* and was not sprayed with any microbial solution yielded 6.75 kg/dt compost whereas after spraying with *Bacillus s*p and *Alcaligenes* sp the yield increased to 14.12 and 17.75 kg/dt compost, respectively (Table 5).

Management of wet bubble through chemicals

Evaluation of different fungicides (carbendazim, chlorothalonil, sporgon, Indofil Z-78, Indofil M-45) against *Mycogone perniciosa* and *A. bisporus* revealed that carbendazim and sporgon gave 100 per cent inhibition of *M. perniciosa* at all the concentrations tried 20-200 ppm whereas it resulted just 17 and 10% inhibition of *A. bisporus*, respectively (Table 6). Chlorothalonil also resulted in 100 per cent inhibition at 100 and 200ppm of *M. perniciosa* and it gave 31 % inhibition of *A. bisporus* at this concentration. Both Indofil Z-78, Indofil M-45 were more toxic to *A.bisporus* at 500ppm as compared to *M. perniciosa*.

Table 5. *Mycogone* management through micro-organisms

	Treatments	Inoculated / uninoculated with *Mycogone*	Yield kg dt^{-1}	No. of No. of Fruit bodies of *Agaricus*	Sclerodermoid	
					Small	Large
1.	B9	Inoculated	14.12	1307	74	0
2.	B9	uninoculated	16.87	1075	0	0
3.	B18	Inoculated	17.75	1623	43	13
4.	B18	uninoculated	18.62	1455	0	0
5.	B20	Inoculated	9.30	604	32	17
6.	B20	uninoculated	14.52	1355	0	0
7.	Control-I (No *Mycogone)*	Uninoculated	18.77	1375	0	0
8.	Control-II (with *Mycogone*)	Inoculated	6.75	507	77	32
CD (0.05)			0.87			

B9=*Bacillus* sp; B18=*Alcaligenes;*B20= *Streptomyces*

Table 6. *In vitro* efficacy of some fungicides against *Mycogone perniciosa*

Fungicide	Concentration	Growth of *Agaricus bisporus* (mm)	Growth of of *Mycogone perniciosa* (mm)
Carbendazim	20	24 (17)	0 (100)
	50	22 (24)	0 (100)
	100	18 (37)	0 (100)
	200	18 (37)	0 (100)
Chlorothalonil	20	22 (24)	11 (82)
	50	22 (24)	10 (84)
	100	20 (31)	0 (100)
	200	20 (31)	0 (100)
Sporgon	20	26 (10)	0 (100)
	50	26 (10)	0 (100)
	100	9 (69)	0 (100)
	200	9 (69)	0 (100)
Indofil Z-78	50	29 (0)	32 (50)
	100	26 (10)	26 (59)
	200	8 (72)	16 (75)
	500	0 (100)	12 (81)
Indofil M-45	50	22 (24)	26 (49)
	100	16 (44)	22 (65)
	200	10 (65)	16 (75)
	500	0 (100)	10 (84)
Control		29	63

Figures in parentheses represent % inhibition

Jhune *et al*. [6] screened 12 isolates of bacteria and 71 isolates of actinomyectes isolated from mushroom compost and casing mixture and observed AJ-117, AJ-136 and AJ-139 as promising bioagents. Though, almost negligible attempts

have been made to control *M. perniciosa* through botanicals but the inhibition of fungal growth by plant extracts is not uncommon and has been reported earlier by a number of workers [7,8]. Gandy [9] made an interesting observation that *Acremonium strictum* produces a heat stable antibiotic compound possibly a cephalosporin, which is inhibitory to *M. perniciosa* but no attempts have been made to explore this approach as both fungi are pathogenic to mushrooms. Bhatt *et al.* [10] claimed good success with a siderophore producing volatile (C116) of fluroscent pseudomonas against *M. perniciosa.*

Out of five fungicides (carbendazim, mancozeb, formalin, chlorothalonil, sporgon) and two other chemicals (K_2HPO_4, $CaCl_2$) tried, carbendazim proved most effective in managing wet bubble disease among all the fungicides/ chemical tested at two concentration (Table 7). Sporgon also proved equally good at all the three concentrations tried mancozeb was as effective as the two other chemicals K_2HPO_4 and $CaCl_2$. In control the yield was as low as 2.03 kg per quintal of compost.

Table 7. Management of wet bubble through chemicals

Fungicide	**Concentration**	**Yield ($kgdt^{-1}$)**	
		Inoculated	**Uninoculated**
Carbendazim	0.1	10.87	12.35
	0.15	10.75	11.04
	0.2	8.91	10.59
Mancozeb	0.1	8.70	7.35
	0.15	6.33	6.85
	0.2	6.52	5.90
Formalin	0.1	7.63	10.24
	0.15	9.70	9.39
	0.2	9.39	9.93
Chlorothalonil	0.1	9.82	10.77
	0.15	9.03	9.63
	0.2	9.92	11.80
Sporgon	0.1	10.54	11.95
	0.15	10.37	11.36
	0.2	10.90	11.89
K_2HPO_4	0.1	8.36	12.60
	0.15	5.37	12.62
	0.2	6.39	12.60
$Cacl_2$	0.1	8.24	12.62
	0.15	7.54	11.84
	0.2	7.43	10.11
Control		2.03	11.66
	CD (0.05)	0.93	1.01

Bubble diseases infect the reproductive mycelia and are unable to infect the vegetative mycelia. The infection takes place through specific and a specific interaction between the surface molecules of both the fungi. Fungal hydrophobins self assemble at hydrophobic and hydrophilic interfaces into surface active amphipathic membranes. The pathogen and host attach to each other by hydrobic interactions between the hydrobhobin layers. After the initial attachment *Mycogone* can grow inter and intra cellular in *A. bisporus* fruiting body hyphae. Since bubble disease infect the reproductive hyphae only, it is logically appropriate that effective concentration of fungicide should available at that point of time in the casing to inhibit the infection. *M. perniciosa* appeared to be quite sensitive against sporgon, carbendazim and chlorothalonil. All these fungicides resulted in effective control of the pathogen. Application of carbendazim, benomyl, chlorothalonil, TBZ, prochloraz manganese complex (Sportak 50 WP) into casing mixture have been reported very effective for the management of wet bubble by several workers [6,10-18].

REFERENCES

[1] Nielsen O. (1932). Mushroom diseases. Reprinted from Gartnertidende, 2p.

[2] Kaul TN *et al.* (1978). Diseases and competitors *of* mushroom farmsin Kashmir valley. *Indian Mush. Sci.* 1: 193-203.

[3] Forer LB *et al*. (1974). Occurrence and economic impact of fungal diseases of mushrooms in Pennsylvania. *Pl. Dis. Reptr.* 58: 987-991.

[4] Sharma SR and Kumar S. (2000). Studies on wet bubble disease of white button mushrooms *(Agaricus bisporus)* caused by *Mycogone perniciosa. Mushroom Sci.* 15: 569-575.

[5] Wuest PJ and Moore RK. (1972). Additional data on the thermal sensitivity of the selected fungi associated with *Agaricus bisporus. Phytopathology.* 62(12): 1470-1472.

[6] Jhune *et al*. (1990). The antifungal micro-organisms to *Mycogone perniciosa* Magn. In cultivated mushroom *Agaricus bisporus* Lange) Imbach. *Korean J. Mycology*. 18 (2):96-101.

[7] Flierman CB. (1973). Inhibition of *Histoplasma capsulatum* by garlic. *Mycopathologia* & *Mycologia Applicata.* 50: 227-231.

[8] Michael RT and Judith AA. (1975). Inhibition of fungal growth by garlic extract. *Mycologia.* 67: 409-413.

[9] Gandy DG. (1979). Inhibition of *Mycogone perniciosa* growth by *Acremoniums trrictum*. *Tran. Br. Mycol. Soc*. 72(1): 151-154.

[10] Bhatt Nand and Singh RP. (2000). Chemical and biological management of major fungal pathogens *Agaricus bisporus* Lange) Imbach. *Mush. Sci*. XV :587-593.

[11] Hsu Hoang-Kao and Han You-Hsin (1981). Physiological and ecological properties and chemical control of *Mycogone perniciosa* Magn causing wet bubble in cultivated mushroom, *Agaricus bisporus*. *Mushroom Science* 11(2): 403-425.

[12] Fletcher JT *et al*. (1983). The control of bubble disease and cobweb disease of mushroom with Prochloraz. *Pl. Pathol.* 32(2): 123-131.

[13] Zaayen, AV and Van Adrichem JCJ. (1982). Prochloraz for control of fungal pathogens of cultivated mushrooms. *Netherlands J. Pl.Pathol.* 88(5): 203-213.

[14] Eicker A. (1984). A report on the use of thiabendazone for the control of fungal pathogen of cultivated mushrooms. *South African J. Bot.* 3: 179-183.

[15] Eicker A. (1987). A report on the use of Prochloraz Manganese complex for controlling of major fungal pathogens of the cultivated mushroom (*Agaricus bisporus*) in South Africa. *South African J. Bot.* 53(5): 345-348.

[16] Sharma VP and Singh Chirag. (2003). Biology and control of *Mycogone perniciosa* Magn. causing wet bubble disease of white button mushroom. *J.Mycol. Pl.Pathol.* 33: 257-264.

[17] Sharma VP and Jandaik CL. (2003). Management of bubble diseases through chemicals. *Indian J. Mush.* 21: 21-24.

[18] Francisco J Gea *et al*. (2010). Efficacy and effects on yield of different Fungicides for control of wet bubble disease of mushroom caused by the mycoparasite *Mycogone perniciosa*. *Crop Protection.* 29: 1021-1025.

INVESTIGATION ON MUSHROOM DISEASES IN CHINA

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ABSTRACT

Mushroom diseases were investigated in over 22 provinces in China on 15 different kinds of mushrooms and pathogenecity of disease isolates was tested. In total 9 diseases of mycelium and 20 of fruit bodies were evaluated and these included green, brown, white moulds, scars, cancer, spots, blotches of various types in addition to wet bubble, bacterial diseases, etc.

Keywords: wet bubble, brown blotch, slippery scar, black spot, bacterial rot

INTRODUCTION

China is a major country of edible mushroom cultivation in the wrold, and the commonly cultivated mushrooms are *Lentinula edodes*, *Pleurotus ostreatus*, *Auricularia polytricha*, *P. eryngii*, *Coprinus comatus*, *Agaricus bisporus*, *Flammulina velutipes, Auricularia auricula-judae*, *Ganoderma lucidum*, *P. nebrodensis*. The occurrence of diseases has impeded the development of mushroom industry in China. Diseases like stipe canker on *Coprinus*, slipper scar on *Auricularia* have been reported in the country [1, 2]. Systematic investigation on the edible mushroom diseases was carried out in more than 22 provinces in China that have a wide range of mushrooms under cultivation since 2009.

MATERIALS AND METHODS

Mushroom diseases were continuously investigated on more than 15 kinds of mushrooms. The disease occurring area, occurring period, severity, occurrence condition, symptoms, transmission route, pathogen source, variety resistance and cultivation method were studied. Most pathogens were isolated and identified by morphology and its analysis. The pathogenicity of isolates was confirmed by the Koch·s rule.

RESULTS AND DISCUSSION

Fourteen competitors were found in the wood log, bagged sawdust and compost during the cultivation including *L. edodes*, *A. auricula-judae, A. bisporus*, *P.ostreatus* and *F. velutipes.* Nine mycelial mycoparasites and 20 fruit body diseases were found and identified successively (Table 1). Five most serious mushroom diseases were selected as the research focus.

Wet bubble disease of *Agaricus bisporus*

The surface of morbid mushroom casing generated white fluffy hyphae. Fruit body presented symptoms of puffball-like or distortion or brown blotch (Fig.1). Brown liquid also occurred sometimes. Its pathogen is *Hypomyces perniciosa* Magnus is a common soil microorganism. Casing soil and old wooden bed were the most important pathogen sources. The severity of wet bubble disease was more when compost was fermented incompletely. High temperature and high humidity favour the disease. The disease spread was mostly through water spray.

Brown blotch disease of *Pleurotus ostreatus*

When oyster mushroom was infected by *Pseudomonas tolaasii* on the surface of the fruit body appear brown blotch or patches of yellow spot. There was always a yellow or red circle pattern around the spot (Fig.2). Diseased fruit body stop developing. Incomplete sterilized compost and surrounding rubbish were the main pathogen sources. Spread of the bacteria pathogen relied principally on the action of spray water. A temperature18-22 ^{o}C, poor ventilation, and water films on the mushroom surface appeared to promote the occurrence of the disease.

Table 1. The mushroom infectious diseases list in China

	Disease	**Pathogen**	**Hosts**	**Pathogenicsite**
1	Black spot	*Hypoxylon annulatum*	*A. auricula*	Mycelia
2	*Trichoderma* disease	*Trichoderma viride, T. koningii*	*A. auriculae*	Mycelia
3	Black spot	*Hypoxylon annulatum*	*Grifolafrondosa*	Mycelia
4	brown blotch	*Pseudomonas* sp.	*G. lucidum*	Mycelia
5	Slippery scar	*Scytalidium lignicola*	*A. polytricha*	Mycelia
6	Green mold	*Trichoderma viride, T. koningii*	*P. ostreatus*	Mycelia
7	White mould	uncertain	*L. edodes*	Mycelia
8	Brown slime moulds	Mycetozoa	*L. edodes*	Mycelia
9	Yellow slime moulds	Mycetozoa	*L. edodes*	Mycelia
10	Bacterial rotten	*Pseudomonas* sp.	*P. nebrodensis*	Fruit body
11	Black spot	*Verticillium fungicola*	*C. comatus*	Fruit body
12	Grey mold	*Cladobotryum protrusum*	*C. comatus*	Fruit body
13	Stipe canker	*Trichothecium roseum* [1]	*C. comatus*	Fruit body
14	Bacterial rotten	*Pseudomonas*	*F. velutipes*	Fruit body
15	Brown plotch	*Pseudomonas tolaasii*	*F. velutipes*	Fruit body
16	Pythium rot	*Cladobotryum vairum*	*F. velutipes*	Fruit body
17	Black spot	uncertain	*G. lucidum*	Fruit body
18	Green mold	*Trichoderma viride, T. koningii*	*G. lucidum*	Fruit body
19	Slime moulds	mycetozoa	*A. polytricha*	Fruit body
20	Brown blotch	*Pseudomonas tolaasii*	*P. ostreatus*	Fruit body
21	Dry bubble	*Verticillium fungicola*	*A. bisporus*	Fruit body
22	Brown blotch	*Pseudomonas putida*	*A. bisporus*	Fruit body
23	Wet blotch	uncertain	*A. bisporus*	Fruit body
24	Wet bubble	*Hypomyces perniciosa*	*A. bisporus*	Fruit body
25	Fruit body wilting	mycovirus	*L. edodes*	Fruit body
26	Nematode disease	*Ditylenchus myceliophagus* *Aphelenchoides composticola* *Rhabditis* sp.	*L.edodes*	Fruit body
27	Bacterial rotten	*Pseudomonas putida*	*P. eryngii*	Fruit body
28	Bacterial rotten	*Pseudomonas*	*P. geesteranus*	Fruit body
29	White mildew	uncertain	*Cordyceps militaris*	Fruit body

Figure 1. Symptom of wet bubble disease in *Agaricus bisporus*

Slippery scar disease in *Auricularia polytricha*

The pathogen, *Scytalidium lignicola*, infected the mycelia only of *A. polytricha* [2]. Slippery, glossy, and dark-brown scars were formed on the surface of infected mycelia in cultivated bag. There was a red-brown antagonistic line between the infected and healthy mycelia, and the margin of this line was irregular (Fig. 3). Grey white and thin mycelia grew on the PDA plates within 2-3 days. After 3-4 days, the colony became dark brown and flat, and the aerial mycelia started to flourish. The colony surface was glossy and honeycomb shaped, and produced catenulate chlamydospores. The most important reason of its occurrence could be attributed to the incomplete sterilization of the compost or the breakage of cultivated bags in the process of cultivation. Chlamydospores of the pathogen infect healthy bags from the puncture after pricking and harvesting via airflow and frequent spraying or pouring of water.

Figure 2. Symptom of brown blotch disease in *Pleurotus ostreatus*

Figure 3. Symptom of slippery scar disease in *Auricularia polytricha*

Black spot disease in *Coprinus comatus*

The cap of fruitbody formed black or brown patches, and the patch might crack along the scale when the humidity of shed was low, or generate a layer of white mildew if the humidity was high (Fig. 4). The initial inoculum sources of this disease were mainly from covering material and later spraying. The pathogen (*Verticillium fungicola*) produced large number of spores, then diffused by flow of air, water, or diseased mushroom debris. The temperature during 22-28 °C and water films on mushroom surface were found to favour the disease development.

Figure 4. Symptom of black spot disease in *Coprinus comatus*

Bacterial rot disease in *Pleurotus eryngii*

The infected mushrooms have watery spots on the stipe and cap, even the whole fruitbody covered by disease spots if the invasion of *Pseudomonas* was particularly serious. Finally fruit bodies rotted, and appeared rough yellow-tan fetid bacterial ooze on the surface (Fig. 5). Bacteria mainly originated from contaminated composts and unclean water, then spread by spraying. Hydrops in the opening site of bag, flooded primordium, water films on mushroom surface all easily result in the occurrence of this disease, especially for those mushrooms growing in high temperature, high humidity and poor ventilation.

Figure 5. Bacterial rotten disease in *Pleurotus eryngii*

Besides these five important mushroom diseases, mushroom-stick rot of *L. edodes* caused by *Trichoderma* spp. occurred seriously too. The high temperature in mushroom shed resulted in decrease of disease resistance of mushroom mycelia. If the bag has a breakage simultaneously, *Trichoderma* spp. or other wood-rotting pathogens would invade, then occupy the substrates. The mushroom-stick would rot after the compost decomposed due to pathogens (Fig. 6).

The main factors for disease occurrence appeared to be improper pasteurization of compost, imperfect disinfection

Figure 6. Mushroom-stick rot of *Lentinus edodes* caused by *Trichoderma* spp.

of cropping, high temperature and humidity in the cropping rooms/environment breakage of cultural bags and reduced disease resistance of mushroom mycelia. Furthermore, improper management would result in abnormal fruit body, such as excessive ventilation, high levels of carbon dioxide, low temperature and heavy use of pesticides, all of which need the attention of mushroom growers. Mushroom virus disease caused by mycovirus are also worth researching. Some symptoms of mycelia decline and fruit body wilting have been suspected to be connected with virus. The virus diseases are latent and spread through infected spores.

General control measures of mushroom disease in China

According to the pathogenesis of these diseases, the corresponding preventive and control measures were made in order to reduce the economic losses as much as possible that are caused by the diseases. On the basis of production model and level of edible mushroom in China, integrated control measures for the mushroom diseases have been put forward. These includes:

1. Selecting strains with high resistance, ensure spawn purity and vigour.

2. Choose fresh compost with low water contents and addition of 2-3 per cent lime. Thorough sterilization of substrates and use of high quality cultivation substrates.

3. Proper disinfection and maintenance of hygienic conditions in the inoculation/spawning area.

4. Use solarized fluviatile mud and sub-soil below 20 cm depth from paddy field.

5. Maintain appropriate temperature, humidity and ventilation in the cropping rooms.

6. Spray appropriate quality water during cropping and avoid long wetness of mushroom fruit bodies by introducing ventilation.

7. Take timely measures in case of disease outbreak. When disease occurs on fruit body or mushroom bed, the infected fruit bodies should be removed. Then cover the infection site with lime. Remove infected substrate along with mushrooms from the adjoining area immediately and spray fungicides like sporgon. If necessary, the infected bags should be discarded and burned.

8. Clean up the waste bags and covering material after the harvest. In the idle period remember to solarize shed and shelves by means of disclosing the outer mulch.

REFERENCES

[1] WH Dong and YB Bian. (2013). Stipe canker caused by *Trichothecium roseum* on the edible shaggy mane *Coprinus comatus* in China. *Plant Disease*. 97(11): 1507

[2] Jie Sun and Yin bing Bian. (2012). Slippery Scar: A new mushroom disease in *Auricularia polytricha*. *Mycobiology*. 40(2): 129-133

CHEMICAL AND BIOLOGICAL CONTROL OF DIPTERA IN SPANISH MUSHROOM CROPS

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ABSTRACT

The phorid *Megaselia halterata* and the sciarid *Lycoriella auripila* are common pests in Spanish mushroom farms. *M. halterata* has been seen to be the dominant species (ratio 4:1), with the highest number in spring and autumn. The objective of this study was to look for alternatives to control mushroom flies, especially phorids. Several assays were carried out using entomopathogenic nematodes [106 IJ m^{-2} *Steinernema feltiae* and 0.5 x 10^6 IJ m^{-2} *S. feltiae* + 0.5 x 10^6 IJ m^{-2} *S. carpocapsae*], and the insecticides diflubenzuron [1 g m^{-2}] and triflumuron [0.5 and 1 g m^{-2}]. The effectiveness against flies and effect on yield were evaluated. Effectiveness trials were set up in an experimental growing room, after spawn running trays were exposed to diptera infestation in a commercial crop. On days 16-17, after casing, treatments were applied. The experiments terminated after the emergence of the first generation of adults. The captured flies were identified and the percentage reduction in adult emergence was calculated. Phytotoxic trials were set up as described above, but without infestation. The effect of treatments on productivity was evaluated from the yield, the biological efficiency of the crop and the earliness of the harvest. No decrease in the population of *M. halterata* was detected with either nematode or insecticide treatment. Population of *L. auripila* fell with both nematode treatments applied, particularly with the *S. feltiae* treatment. Diflubenzuron and triflumuron were highly effective against sciarids, regardless of the application dose. The production parameters were not affected by any treatment.

Keywords: *Megaselia halterata*, *Lycoriella auripila*, diflubenzuron, triflumuron, entomopathogenic nematodes

INTRODUCTION

The phorid *Megaselia halterata* (Wood) and the sciarid *Lycoriella auripila* Winnertz are common pests in mushroom farms of Castilla-La Mancha, Spain [1]. In their larval stage, these flies feed on mushroom mycelia and even burrow into mushrooms once they are formed [2]; as adults, they act as vectors of other pests and diseases [2-5]. Unlike in other European countries, where sciarids are present throughout the year and phorids undergo a period of hibernation [6], in Spain *M. halterata* populations are present in high numbers throughout the year, whereas *L. auripila* is detected in much lower numbers (four times less than *M. halterata*) and mainly in spring and autumn [1]. Traditionally, flies in mushroom crops have been controlled by insecticides. Other methods for controlling flies are based on the use of physical barriers to exclude adult individuals from growing in farms [7], by biocontrol organisms, such as mites, bacteria and entomopathogenic nematodes [8-11]; and, more recently, by plant extracts [12-16].

In the last years of twenty century, the list of insecticides allowed for using in European mushroom farms was very large: malathion, diazinon, methoprene, permetrin, dichlorvos, chlorpyrifos, deltametrin, carbofuran, bendiocarb, cyromazine, diflubenzuron, triflumuron and azadirachtin. Literature shows varying results with regards to the efficacy against phorids and/or sciarids [15,17-21]. However, the application of phytosanitary products may give rise to two further problems: detrimental effects on the mushroom mycelium, leading to a loss of yield or quality [2,15,19, 22-25], and the presence of residues in the mushrooms once harvested [26]. Furthermore, resistance to certain products has been recorded [9, 27-28]. It must also be borne in mind that the evaluation of active substances set in motion by the EU (Directive 91/414/ECC) has considerably reduced the number of insecticides authorized for use in mushroom cultivation: at this moment, just malathion, chlorpyrifos, deltametrin, cyromazine, diflubenzuron, triflumuron and azadirachtin are allowed in Europe mushroom farms. In Spain, deltametrin (2.5%, [EC] P/V, 1.5% [EW] P/V), azadirachtin (3.2%, [EC] P/V), diflubenzuron (25%, WP) and cyromazine (75%, [WP] P/P) are the only insecticides allowed for use in mushroom crops. Deltametrin and azadirachtin are effective against adult flies, whereas diflubenzuron and cyromazine are effective against sciarid larvaes

[2,14, 29-31], but not against phorids [18,32]. Spanish mushroom growers have not any tool for controlling phorid populations [30]. Triflumuron has been shown effective against sciarids [5, 31], but information on the effectiveness against phorid has not been found.

As regards the biocontrol organisms, nematodes are often applied to control mushroom flies. Richardson [33] was the first to use entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* to control mushroom flies [33]. *Steinernema* spp. locate and invade fly larvae via anus, mouth or spiracles, and release bacteria associated with them (*Xenorhabdus* sp. or *Photorhabdus* sp.), which provokes the death of the infested flies [34]. However, apart from their compatibility with the insecticides [35], the effectiveness of nematodes in mushroom culture depend on other factors such as temperature, moisture and CO_2 levels, which may also affect them [34]. Information about phorid control by nematodes is scarce in the literature. The application of *S. feltiae* against phorid flies has shown varied results [9,16,32], whereas *S. carpocapsae* treatments have got favourable results [11]. For the control of sciarids, many authors recommend the use of *S. feltiae* [9,14]. In the case of *S.carpocapsae*, Gouge and Hague [36] reported the relative inefficacy of this species against sciarids, whereas, other authors defend its efficacy, but with a lower infectivity than that of *S. feltiae* [37]. Furthermore, some authors observed a detrimental effect of treatments involving entomophathogenic nematodes on mushroom mycelia, such as reduced yield in early flushes, depending on the nematode dosage rate [24].

The objective of this study was to look for alternatives to control mushroom flies, especially phorids, in Spanish mushroom farms checking insecticides as triflumuron, and entomopathogenic nematodes as *S. feltiae* and *S. carpocapsae*.

MATERIALS AND METHODS

Effectiveness assays

Effectiveness trials were set up in an experimental mushroom growing room. At the end of the spawn running, some trays (10 kg of compost, 870 cm^2 per tray) were exposed to infestation in a commercial mushroom farm, where they were left for 48 hours. Afterwards, the trays were placed in the experimental room again, where they were covered with a structure made of anti-trips mesh. A yellow sticky trap was placed inside each of them to capture the flies. On days 16-17, after casing, treatments were applied.

Two nematode effectiveness trials were set up. A randomised complete block design with four treatments and ten replicates was used in the nematode trials: Sf, *S. feltiae* treatment, fly-infested trays and subsequent application of 10^6 IJ m^{-2} *S. feltiae*, Biorend R champiñones, (Idebio S.L., Salamanca, Spain) together with 1 ml of chitosan in 150 ml water per tray; Sf+Sc, *S. feltiae*+*S. carpocapsae* treatment, infested trays and subsequent application of $0.5x10^6$ IJ m^{-2} *S. feltiae*+ $0.5x10^6$ IJ m^{-2} *S. carpocapsae*, Biorend R champiñones + Biorend Rpalmeras, (Idebio S.L., Salamanca, Spain) together with 1 ml of chitosan in 150 ml water per tray. Ten trays free of fly infestation and with no nematode application were used as non-infested controls (C), and ten fly-infested trays with no nematode application were used as infested controls (IC). In the first trial, trays were exposed to fly infection pressure of 800 phorids and 52 sciarids per day on day 5 of spawn running. In the second trial, on day 11 of spawn running trays were exposed to infection of 1,237 phorids and 82 sciarids per day.

Two insecticide effectiveness trials were set up. A randomised complete block design with four treatments and six replicates was used in the insecticide trials: IC, fly-infested trays; *D*, fly-infested trays and subsequent application of diflubenzuron (Dimilin, UniRoyal Chemical) (1 g m^{-2} a.i.); *A1*, fly-infested trays and subsequent application of triflumuron (Alsystin, Bayer Crop Science SL) (0.5 g m^{-2}a.i.); *A2*, fly-infested trays and subsequent application of triflumuron (1 g m^{-2}a.i.).Trays were exposed tofly infection on day 11 of spawn running. The infection fly pressure was 584 phorids and 139 sciarids per day in the first trial, and 388 phorids and 29 sciarids per day in the second trial. The experiments finished after the emergence of the first generation of adults. The captured flies were counted and identified and the percentage reduction in adult emergence was calculated.

Effect on mushroom production

Nematode trial was set up in the same way as described before, but there was no infestation. A randomised complete block design with three treatments and twelve replicates (10 kg of compost, 870 cm^2 per replicate) was used in the trials. Treatments were C, Sf and Sf + Sc. Mushrooms were harvested daily for two flushes. The number and total weight of the fruit bodies were recorded for each treatment. The effect of entomopathogenic nematodes on mushroom productivity was evaluated from the total yield of harvested mushrooms, and from the number and unitary weight of mushrooms. The effect of treatments on mushroom productivity was also evaluated from the biological efficiency of the crop, calculated as the ratio of the fresh weight of the total yield of harvested mushrooms to the weight of dry substrate at spawning, expressing the fraction as kg $100kg^{-1}$ compost. In addition, the earliness of each treatment was expressed as the number of days between casing and harvesting of the first flush.

A randomised complete block design with four treatments and six replicates (84 kg of compost, 1.5 m^2 per replicate) was used in the insecticide trial. Treatments were: *C*, control trays, and *D*, *A1* and *A2* treatments. Mushrooms were harvested daily for three flushes.The effect of insecticides on mushroom productivity was evaluated from the total yield of harvested mushrooms. Biological efficiency and the earliness were also calculated.

Statistical Analysis

Analysis of variance, with logarithmic or square root transformation for count data and angular transformation of the percentage data to stabilize variances when necessary, were done using the software package Statgraphics Plus, version 4.1 (StatisticalGraphics Corp., Princeton, NJ, USA). Tukey's test, at 5% probability, was used to establish significant differences between means.

RESULTS AND DISCUSSION

Nematode effectiveness assays

The total number of flies captured in the first trial was lower than in the second one, reflecting the lower degree of initial infection pressure in the first trial. Although fly captures were registered in the non-infested trays C (Table 1), in both trials and for both flies, the data obtained for treatment IC were significantly higher than those for C, indicating the good progress of the infection process. In the first trial, the mean number of emerging *M. halterata* was 233 for the infested control trays (IC), and 186.3 and 221.8 for the nematode treatments Sf and Sf+Sc, respectively, with no significant differences between them ($F_{2,29} = 0.63$; $p = 0.5415$). No significant differences ($F_{2,23} = 1.21$; $p = 0.3168$) were observed between the numbers of phorid flies captured in the treatments IC, Sf, and Sf+Sc in the second trial (Table 1). There was also no effect of the treatments Sf and Sf+Sc in the emergence of *M. halterata* adults in both trials, and no phorid percentage reduction was verified for any treatment.

Table 1. Total number (mean and standard deviation) of phorids and sciarids captured for each of the nematode treatments in both assays. In the case of *L. auripila* the percentage reduction obtained for each treatment in both trials is also included[(1)].

Treatments	*M. halterata*		*L. auripila*		*L. auripila* reduction (%)[(2)]	
	First trial	Second trial	First trial	Second trial	First trial	Second trial
C*	14.1±6.9a	22.3±18.0a	2.0±2.2a	40.7±13.2a	—	—
IC	233.0±97.6b	462.7±166.2b	136.2±60.7c	184.4±46.7c	—	—
Sf	186.3±57.3b	366.1±80.3b	50.3±26.2b	116.2±35.4b	63.1B	37.0A
Sf+Sc	221.8±121.6b	406.3±133.2b	68.1±47.4b	180.0±53.2c	50.0B	2.4A

[(1)]For each trial, means followed by equal letters, lowercase in the columns, do not differ significantly by Tukey's test, at 5% probability.
[(2)]For reduction (%), means followed by equal uppercase letters in the files do not differ significantly by Tukey's test, at 5% probability.
*C, non-infested control; IC, infested control without nematode treatments; Sf, nematode application of 10^6IJ m^{-2}*Steinernema feltiae*; Sf+Sc, nematode application of $0.5x10^6$ IJ m^{-2} *S. feltiae* + $0.5x10^6$IJ m^{-2} *S. carpocapsae.*

The lack of effectiveness of *S. feltiae* for controlling phorids agrees with most of the consulted literature [32,38]. However, it was not in agreement the findings of Long *et al*. [39] in laboratory bioassays. These authors reported that *S. feltiae*is effective in the control of *M. halterata*, probably because nematodes applied on growing substrates show different behaviour. The results of the present work also differed with the findings of other authors, who observed a reduction of more than 70% in the emergence of *M. halterata* adults after the application of *S. feltiae* [9,16]. This divergence in results was probably due to the fact that in the present study only the first generation of adults after infestation was considered, whereas the above authors also considered subsequent generations. The ineffectiveness of the Sf+Sc treatment on *M. halterata* was observed regardless of the trials, which differed with the results obtained by Jess and Bingham [11], who confirmed the use of *S. carpocapsae* to control phorids. This discrepancy might be due to the lower application doses used or to the application of both nematode species. In trial conditions, the inefficacy of both treatments against *M. halterata* was independent of the time elapsing between infestation and nematode application. For optimum efficacy, nematodes should be applied when a majority of hosts are susceptibleto nematode infection. In the first trial, considering the environmental conditions and the time elapsing between infestation and treatment (9–10 days), the larvae were presumed to be in a sufficiently advanced stage (third larval instar) to allow infestation by nematodes [11, 38]. However, not even in these favorable conditions did the applications lead to an acceptable level of reduction when the nematode formulations were used. Further efforts to develop entomopathogenic nematodes against *M. halterata* are necessary.

As regards the sciarid flies, in the first trial, the mean number of emerging *L. auripila* was 136 for the infested control trays (IC), and significantly lower ($F_{2,29}$= 9.42; p = 0.0007) for both nematode treatments. There was no significant difference between the Sf and Sf+Sc treatments (Table 1). In the second trial, 184 sciarid flies were captured emerging from the infested control treatment, a value statistically similar to that recorded for the Sf+Sc treatment; however, this number was significantly reduced ($F_{2,23}$ = 6.53; p = 0.0057) in the Sf treatment (116 sciarid flies). In this trial, only the Sf treatment reduced the adult emergence of *L. auripila* when compared to the infested control. Gouge and Hague [3] established that *S. carpocapsae* was relatively ineffective against different sciarid species. The results obtained in the present work are in agreement with this, but contradict those of Kim *et al*. [37], who found that *S. carpocapsae* can be an effective tool for the management of the fungus gnat larvae [37]. Both nematode species were applied together so that each could combat its own target pest. However, there was no improvement in the efficacy of the combined treatment against either fly species targeted. For the control of sciarids (Table 1), the reduction percentage (63%) obtained with the Sf treatment in the first trial can be considered satisfactory; however, it was less than the 80–95% reduction in sciarids obtained by other authors [9,14, 40]. In the second trial, the reduction percentage (37%) was significantly lower than that in the first trial ($F_{1,18}$ = 9.15; p = 0.0073). In the case of the Sf+Sc treatment, the reduction percentage obtained in first trial (50%) was also satisfactory and significantly higher than the one in the second trial ($F_{1,13}$ = 19.41; p = 0.0007). Efficacy was lower for each treatment in the second trial. Considering the environmental conditions and the time elapsing between infestation and treatment (3–4 days), larvae were probably not large enough to be parasitized. This agrees with the recommendation of Scheepmaker *et al*. [9] to postpone treatment to a week after infestation. A longer time between infestation and nematode application would favour fly control because the nematodes would find larger larvae (third and fourth stage), which are more vulnerable to *S. feltiae* attack [11,38].

Insecticide effectiveness assays

In the first trial, the mean number of emerging *M. halterata* was 512.7 for the infested control trays (*IC*), and 577.8, 503.8 and 388.5 for the insecticide treatments *D, A1* and *A2*, respectively, with no significant differences between them ($F_{3,20}$ = 0.31; p = 0.8213) (Table 2). In the second trial, no significant differences between the mean number of emerging *M. halterata* captured in all of the treatments ($F_{3,20}$ = 0.91; p = 0.4525) (Table 2) were registered. No phorid percentage reduction was verified for any treatment.

As regards, *L. auripila*, in the first trial, the mean value of captures for the infested control trays (*IC*) was significantly higher ($F_{3,20}$ = 87.99; p = 0.0000) than those for the insecticide treatments (*A1, A2* and *D*), which noted a 99% sciarid emergence reduction (Table 2). In the second trial, significant differences between number of sciarid captured for *IC* and insecticide treatment *D, A1* and *A2* were also noted ($F_{3,19}$ = 92.23; p = 0.0000), with 97% of emergence reduction (Table 2).

Table 2. Total number (mean and standard deviation) of phorids and sciarids captured for each of the insecticide treatments in both assays[(1)].

Treatments	*M. halterata*		*L. auripila*	
	First trial	Second trial	First trial	Second trial
*IC**	512.7 ± 219.3	666.7 ± 365.7	649.8 ± 137.5b	143.5 ± 91.0b
D	577.8 ± 437.8	915.2 ± 986.3	4.0 ± 5.0a	2.4 ± 1.3a
A1	503.8 ± 476.6	846.7 ± 571.5	5.0 ± 4.1a	2.3 ± 2.0a
A2	388.5 ± 143.2	1,110.0 ± 439.2	2.3 ± 1.5a	2.7 ± 0.8a

[(1)]For each trial, means followed by equal letters, lower case in the columns, do not differ significantly by Tukey's test, at 5% probability. **IC*, infested control without insecticide treatments; *D*, fly-infested trays and subsequent application of diflubenzuron (1 g m^{-2}a.i.); *A1*, fly-infested trays and subsequent application of triflumuron (0.5 g m^{-2}a.i.); *A2*, fly-infested trays and subsequent application of triflumuron (1 g m^{-2}a.i.).

As well as diflubenzuron [30,32], the application of triflumuron had not any effect on the *M. halterata* populations, regardless of the infestation pressure and/or the rate. Up to now, authors are ignorant of any reference about the effectiveness of triflumuron on mushroom phorids. On the other hand, the application of triflumuron was very effective against *L. auripila* populations, regardless of the infestation pressure and/or the rate. This result agrees with Shamshad *et al.* [5], who described an excellent effectiveness of triflumuron 25% WP (application rate: 20 mg kg^{-1}) on *Bradysia ocellaris* (Comstock) populations in Australia. Erler *et al.* [31] also described 78% of *L. ingenue* (Dufour) population reduction as results of triflumuron 48% application (1g m^{-2}) in Turkey.

Effect of nematode treatments on mushroom production

The mean values of the mushroom yields, per flush and total yield, for each of the treatments (C, Sf, and Sf+Sc) were statistically similar for the first flush ($F_{2,31}$ = 1.60; p = 0.2188), the second flush ($F_{2,31}$ = 2.98; p = 0.0657), and for total harvest ($F_{2,31}$ = 0.31; p = 0.7346); a total yield of around 20 kg m^{-2} was obtained in all three cases (Table 3). This result reflects the statistically similar biological efficiency ($F_{2,31}$ = 0.31; p = 0.7343) of all the treatments, with values close to 85 kg of mushrooms per 100 kg of compost (dry weight) collected over two flushes (Table 3).

Regarding the number of mushrooms harvested, both treatments showed lower numbers than the control in the first flush. In the Sf+Sc treatment this decrease was compensated in the second flush since the total results for total yield only showed a significant drop ($F_{2,31}$ = 5.28; p = 0.0107) for the Sf treatment (Table 3). Moreover, the unitary weight of the mushrooms was significantly higher ($F_{2,31}$= 10.27; p = 0.0001) for the Sf treatment than for the control and for Sf+Sc (Table 3). Lastly, earliness, defined as the time between applying the casing mixture and the first flush, varied between 21.5 and 21.7 days, with no significant differences ($F_{2,31}$ = 0.28; p = 0.7577) between treatments.

Table 3. Mushroom production (kg m^{-2} and number m^{-2}), Biological Efficiency (BE: kg of mushroom 100 kg^{-1} dried compost), Unitary weight (g) and Earliness (days to first flush harvest) in each of the treatments[(1)].

Treatments	First flush		Second Flush		Total harvest		BE	Unitary weight	Earliness
	Yield	Nº	Yield	Nº	Yield	Nº			
C*	12.1	963b	7.6	639ab	19.7	1602b	84.8	12.5a	21.5
Sf	11.1	708a	8.7	588a	19.8	1296a	85.3	15.4b	21.6
Sf + Sc	11.6	776a	7.7	783b	19.3	1559b	83.0	12.7a	21.7

[(1)]Means within a column followed by the same letter are not significantly different according to Tukey's test (p<0.05).
*C: control; Sf: trays with nematode application (10^6 IJ m^{-2} of *S. feltiae*); Sf+Sc: trays with nematode application (0.5 x 10^6 + 0.5 x 10^6) IJ m^{-2} of (*S. feltiae* + *S. carpocapsae*).

Some authors maintain that the use of entomopathogenic nematodes affects mycelial growth, depending on the nematode dosage rate [24], leading to decreased yields for the first flush,although subsequent flushes typically compensate for this early yield loss [38]. The results found in the present work contradict this affirmation since the overall yields were similar in both flushes for all treatments. For the number of carpophores collected, Grewal *et al.* [24] obtained 20% more mushrooms after the application of *S. feltiae*, which was attributed to the increased dispersion of the bacterium *Pseudomonas putida*, responsible for the formation of primordia in the casing material. In the present study,the application of *S. feltiae* alone reduced the number of mushrooms harvested, but the unitary weight was higher, which might be considered advantageous for post-harvest quality management, especially in the first flush when there are normally too many mushrooms to pick [25].

Effect of insecticide treatments on mushroom production

There were no statistical differences of yield values between the treatments (Table 4) for first flush ($F_{3,20}$= 0.26; p = 0.8520), second ($F_{3,20}$ = 0.15; p = 0.9266), and third flush ($F_{3,20}$= 1.03; p = 0.3996), neither for total harvest ($F_{3,20}$= 0.11; p = 0.9512). Insecticide application had not any effect on biological efficiency ($F_{3,20}$= 0.11; p = 0.9512) neither on earliness ($F_{3,19}$= 0.76; p = 0.5304), with values of 83.43-85 kg mushroom per 100 kg dried compost and 23.7-23.8 days, respectively for all treatments (Tabla 4).

Table 4. Yield (kg m^{-2}) of mushroom crop, biological efficiency and earliness (days)[(1)]

Treatments	First Flush	Second Flush	Third Flush	Total Harvest	BE	Earliness
*C**	6.5	10.8	3.5	20.9	83.4	23.7
D	6.3	10.4	4.2	20.9	83.7	23.8
A1	6.4	10.7	4.1	21.2	85.0	23.7
A2	6.2	10.5	4.1	20.8	83.3	23.8

[(1)]Means within a column followed by the same letter are not significantly different according to Tukey's test (p<0.05).
**C*: control without insecticide treatments; *D*, application of diflubenzuron (1 g m^{-2}a.i.); *A1*, application of triflumuron (0.5 g m^{-2}a.i.); *A2*, application of triflumuron (1 g m^{-2}a.i.).

The results shown that triflumuron has not any phytotoxic effect on mushroom production. This affirmation agrees with Shamshad *et al.* [5] and Erler *et al.* [31] neither of whom described production reduction attached application of triflumuron on casing.

CONCLUSION

The application of *Steinernema feltiae* ten days after the foreseen time of infestation is beneficial for the control of sciarids.Triflumuron and diflubenzuron are also effective for the control of mushroom sciarids.The application of diflubenzuron, triflumuron or entomopathogenic nematodes has no effect on the phorids found in mushroom crops.The application of insecticides diflubenzuron or triflumuron, or the application of entomopathogenic nematodes has no adverse effect on mushroom production.

ACKNOWLEDGEMENTS

The authors wish to thank Idebio S.L. and Bayer Crop Science S.L. for providing the products used in this study.

REFERENCES

[1] Navarro MJ *et al*. (2002). Evolution and seasonal abundance of phorid and sciarid flies in Spanish mushroom crops. In: Mush. Biol. Mush. Prod. Sánchez JE, Huerta G. and Montiel E. Eds., 189-195.

[2] Shamshad A. (2010). The development of integrated pest management for the control of mushroom sciarid flies, *Lycoriella ingenua* (Dufour) and *Bradysia ocellaris* (Comstock), in cultivated mushrooms. *Pest Manag. Sci.* 66: 1063-1074.

[3] Clift A *et al*. (2004). Flies and dry bubble on cultivated mushrooms. In: *Science and cultivation of the edible and medicinal fungi*. Romaine CP., Keil CB., Rinker DL and Royse DJ. Eds. s459-464. ISBN 1-883956-01-13

[4] Navarro Lozano MJ *et al*. (2004). *Biología y control del ácaromiceliófago Brennandanialambi (Krczal) en los cultivos de champiñón de Castilla-La Mancha*. Ministerio de Agricultura, Pesca y Alimentación Eds., 203p. ISBN 84-491-0632-X

[5] Shamshad A *et al*. (2009). Effect of compost and casing treatments of insecticides against the sciarid *Bradysia ocellaris* (Diptera: Sciaridae) and on the total yield of cultivated mushrooms, *Agaricus bisporus*. *Pest.Manag. Sci.* 65: 375-380.

[6] Jess S *et al*. (2007). Potential sources of sciarid and phorid infestations and implications for centralised phases I and II mushroom compost production. *Crop Protection.* 26: 455-464.

[7] Coles PS. (2002). Specific control techniques. In: *Exclusion in Pennsylvania mushroom integrated pest management.*The Pennsylvania State University Eds., 92p. CAT AGRS-83.

[8] Scheepmaker JWA *et al*. (1995). Control of the mushroom sciarid (*Lycoriella auripila*) and the mushroom phorid (*Megaselia halterata*) by entomopathogenic nematodes. In: Science and Cultivation of Edible Fungi. Elliot T. Eds., 491-498.

[9] Scheepmaker JWA *et al*. (1997). Control of the mushroom pests, *Lycoriella auripila* (Diptera: Sciaridae) and *Megaselia halterata* (Diptera: Phoridae) by *Steinernema feltiae* (Nematoda: Steinernematidae) in field experiments. *Ann. Appl. Biol.* 131: 359-368.

[10] Keil C. (2002). Field trials of the Gnatrol WDG formulation of *Bacillus thuringiensis* var. *israelensis* for control of mushroom flies in Pennsylvania and California. *Mush. News* 50: 12-19.

[11] Jess S and Bingham JFW. (2004). Biological control of sciarid and phorid pests of mushroom with predatory mites from the genus *Hypoaspis* (Acari: Hypoaspidae) and the entomopathogenic nematode *Steinernema feltiae*. *Bull. Entomol. Research.* 94: 159-167.

[12] Park IK *et al*. (2006). Fumigant activity of plant essential oils and components from horseradish (*Armoracia rusticana*), anise (*Pimpinella anisum*) and garlic (*Allium sativum*) oils against *Lycoriella ingenua* (Diptera: Sciaridae). *Pest Manag. Sci.* 62: 723-728.

[13] Park IK *et al*. (2008). Toxicity of plant essential oils and their Components against *Lycoriell aingenua* (Diptera: Sciaridae). *J. Econ. Entomol.* 101: 139-144.

[14] Shamshad A *et al*. (2008). Toxicity of six commercially formulated insecticides and biopesticides to third instar larvae of mushroom sciarid, *Lycoriell aingenua* Dufour (Diptera: Sciaridae), in New South Wales, Australia. *Austr. J. Entomol.* 47: 256-260.

[15] Erler F *et al*. (2009a). Control of the mushroom phorid fly, *Megaselia halterata* (Wood), with plant extracts. *Pest Manag. Sci.* 65: 144-149.

[16] Erler F *et al*. (2009b). Evaluation of microbial products for the control of the mushroom phorid fly, *Megaselia halterata* (Wood). *J. Entomol. Sci.* 44: 89-97.

[17] Cantelo WW. (1983). Control of a mushroom-infesting fly (Diptera: Sciaridae) with insecticides applied to the casing layer. *J. Econ. Entomol.* 76: 1433-1436.

[18] Cantelo WW. (1985). Control of *Megaselia halterata*, a phorid fly pest of commercial mushroom production, by insecticidal treatment of the compost or casing material. *J. Entomol. Sci.* 20: 50-54.

[19] Geels FP and Rutjens AJ. (1992). Bendiocarb and diflubenzuron as substitute insecticides for endosulfan in commercial mushroom growing. *Ann. appl. Biol.* 120: 215-224.

[20] Keil CBO and Bartlett GR. (1995). Azatin for control of *Lycoriella mali* in *Agaricus* mushroom production. *Mush. News.* 43: 10-13.

[21] Baba ZA and Sandhu GS. (1996). Efficacy of diflubenzuron and neem seed powder and cake for the control of *Bradysia tritici* (Coq.) infesting button mushrooms. *J. Insect Sci.* 9: 133-136

[22] Cantelo WW *et al*. (1982). Variation in sensitivity ofmushroom strains to diazinon compost treatment. *J. Econ. Entomol*. 75:123–125.

[23] Richardson PN and Grewal PS. (1991). Comparative assesment of biological (Nematoda: *Steinernema feltiae*) and chemical methods of control for the mushroom fly *Lycoriella mali* (Diptera: Sciariidae). *Biocon. Sci. Technol.* 1: 217-228.

[24] Grewal PS *et al*. (1992). Comparative effects os *Steinernema feltiae* (Nematoda: Steinernematidae) and insecticides on yield and cropping of the mushroom *Agaricus bisporus*. *Ann. appl. Biol.* 121: 511-520.

[25] Scheepmaker JWA *et al.* (1998). Influence of *Steinernema feltiae* and diflubenzuron on yield and economics of the cultivated mushroom *Agaricus bisporus* in Dutch mushroom culture. *Bioc. Sc. And Technol.* 8: 269-275.

[26] Navarro MJ and Gea FJ. (2006). Estudio de la fitotoxicidad del insecticida diflubenzuron en el cultivo de champiñón. Estudiodel nivel de residuos. *Boletín de la Asociación Española de Cultivadores de Champiñón.* 48: 32-34.

[27] Bartlett GR and Keil BOC. (1997). Identification and characterization of a permethrine resistence mechanism in populations of the fungus gnat *Lycoriella mali* (Fitch) (Diptera: Sciaridae). *Pestic.Biochem. Physiol.* 58: 173-181.

[28] Smith JE. (2002). Dimilin resistance in mushroom sciarids. *Mush. J.* 626: 15.

[29] Gea FJ and Navarro MJ. (2002). Valoración de la fitotoxicidad y de la actividad biológica del Align® contra dípteros en el cultivo de champiñón. *Phytoma.* 138: 50-52.

[30] Gea FJ and Navarro MJ. (2008). Insecticidasquímicos, biológicos y nematodosentomopatógenos aplicados para el control de dípteros en el cultivo de champiñón: efectofitotóxico y actividadbiológica. In: *Avances en la Tecnología de la Producción comercial del champiñón y otroshongos cultivados*. Patronato de Desarrollo Provincial Eds., 237-246. ISBN 978-84-96890-90-9.

[31] Erler F *et al.* (2011). Control of mushroom sciarid fly *Lycoriella ingenua* populations with insect growth regulators applied by soil drench. *J. Econ. Entomol.* 104: 839-844.

[32] Grewal PS *et al*. (1993). Evaluation of a genetically selected strain of *Steinernema feltiae* against the mushroom sciarid *Lycoriella mali*. *Ann. appl. Biol*. 123: 695-702.

[33] Richardson PN. (1987). Susceptibility of mushroom pests to the insect-parasitic nematode *Steinernema feltiae* and *Heterorhabditis heliothidis*. *Ann. appl. Biol.* 111: 433-438.

[34] Kirk DJ and Keil CB. (2001). Factors influencing efficacy of two- entomopathogenic nematodes used for fly control in commercial mushroom crops. *Mush. News* 49: 4-17.

[35] Koppenhöfer AM and Grewal PS. (2005). Compatibility and interactions with agrochemicals and other biocontrol agents. In: *Nematodes as Biocontrol Agents*, CABI Eds.,363-381. ISBN 978-0851990170.

[36] Gouge DH and Hague NGM. (1995). The susceptibility of different species of sciarid flies to entomopathogenic nematodes. *Journal of Helminthology.* 69: 313-318.

[37] Kim HH *et al.* (2004). *Steinernema carpocapsae* (Rhabditida:Steinernematidae) as a biological control agent against the fungus gnat *Bradysia agrestis* (Diptera: Sciaridae) in propagation houses. *Biocon. Sci. Technol.* 14: 171-183

[38] Jess S *et al.* (2005). Mushroom applications. In: Nematodes as Biocontrolagents.CABI Eds., 191-213. ISBN978-0851990170.

[39] Long SJ *et al*. (2000). Infectivity of entomopathogenic nematodes (Steinernematidae, Heterorhabditidae) to mushroom phorid fly (*Megaselia halterata*) larvae. *Nematology.* 2: 451-459.

[40] Jess S and Schweizer H. (2009). Biological control of *Lycoriella ingenua* (Diptera: Sciaridae) in commercial mushroom (*Agaricus bisporus*) cultivation: a comparison between *Hypoaspis miles* and *Steinernema feltiae*. *Pest Manag. Sci.* 65: 1195-1200.

IN VITRO LETHAL CAPABILITY OF TEN STRAINS OF EDIBLE MUSHROOMS AGAINST *HAEMONCHUS CONTORTUS* (NEMATODA) INFECTIVE LARVAE

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ABSTRACT

Haemonchus contortus is considered to be the most pathogenic haematophagous gastro-intestinal parasitic nematode in small ruminants. The most common control method for this and other nematodes is the continuous administration of chemical anthelmintic (AH) drugs in the host animals; however, the indiscriminate use of these products has triggered some problems including anthelmintic resistance. New alternatives of control are necessary to diminish the use of AH drugs. This study was conducted to evaluate the *in vitro* nematicidal activity of mycelia from ten strains of edible mushrooms against *Haemonchus contortus* infective larvae. This study was carried out using water agar plates. Eleven groups of plates were established: One control (without mycelium) and ten groups each containing mycelia of each selected fungi. Five hundred *H. contortus* L_3 were deposited on each plate (n=10) and incubated at 18-25 °C, for 5 days. The highest lethal effects (82-99%) were recorded with *P. ostreatus* ECS-1123 and ECS-0152, *P. eryngii* ECS-1292, *P. cornucopiae* ECS-1328 and ECS-1330 and *L. edodes* ECS-0401.

Keywords: *Haemonchus contortus*, edible mushrooms, biological control

INTRODUCTION

Gastrointestinal parasitic nematodes (GIN) severely affect animal health and diminish the productive potential of cattle and small ruminants leading to severe economic losses worldwide [1]. Global sales of anti-parasitic compounds are estimated at approximately tens of billions of dollars [2].Worldwide, the most common GIN genera spread around the world are *Haemonchus*, *Trichostrongylus*, *Teladorsagia*, *Nematodirus* and *Cooperia* [3]. The main method of control of these parasites is chemotherapy, which consists of the regular administration of chemical anthelmintic drugs to the animals to diminish the parasitic burden and its health consequences; however, the indiscriminate and continuous use of these products has resulted in anthelmintic resistance (AR) by GIN throughout the world [4,5].

Furthermore, other problems derived from the use of chemical anthelmintic drugs have been observed; for example, animal products and sub-products (meat, milk and wool) for human consumption can contain residues of such drugs, thus constituting a public health risk [6]. Additionally, some active anthelmintic drug molecules are eliminated through feces of treated animals to the soil, causing harmful effects on beneficial organisms and putting the environment at risk [7]. Therefore, there is a need for the development of alternative and complementary methods that reduce the use of chemical antiparasitic drugs.

Researchers throughout the world have become increasingly interested in alternative methods of control in order to diminish the use of chemical strategies against parasites. These include the use of plants with anthelmintic activity [8], vaccines [9] and the use of natural nematode antagonists such as nematophagous fungi. Edible mushrooms have been used by ancient cultures as natural medicines such as antioxidants [10], antitumoral [11]. Recent studies have demonstrated that some species of edible mushrooms such as the *Pleurotus* genus possess nematicidal activity through the production of a nematoxin which is able to inhibit the nematode movement allowing hyphal penetration and finally digesting the body by enzymatic action [12]. Such biological activity could be the result of a self-defense mechanism in mushrooms which acts against the attack of myceliophagous nematodes. Research into this mechanism could lead to the obtention of elite molecules which could be developed into natural antiparasitic products [13,14]. This research was aimed to evaluate the *in vitro* nematicidal

activity of mycelia of ten strains of edible mushrooms against the infective larvae of the sheep parasitic nematode *Haemonchus contortus*.

MATERIALS AND METHODS

The biological material used included mycelia of the following fungi: *Pleurotus ostreatus* ECS-0152 and ECS-1123, *P. eryngii* ECS-1290 and ECS-1292, *P. cornucopiae* ECS-1328 and ECS-1330, *Coprinus comatus* ECS-1103, *Panus* sp. ECS-801, *Lentinula boryana* ECS-0402 and *L. edodes* ECS-0401 from the Tropical Mushrooms Laboratory of El Colegio de la Frontera Sur (ECOSUR), in Tapachula, Chiapas, Mexico. The *in vitro* confrontation of the different fungal strains was carried out in Petri dishes (60x15 mm) containg 2% water-agar. Eleven series of 10 water agar plates each were implemented. The age of selected fungal cultures was 20 days. The first series was considered the negative control containing only nematode larvae without any fungi. Five hundred *H. contortus* infective larvae (L_3) were added to each dish in each series, and incubated at 18-25 °C for 5 days.

Recovery of total larvae (live or dead) from the plates was performed by placing the content of each plate on the Baermann funnel for 12 h. Larvae were quantified and means of recovered larvae were compared with the mean of recovered larvae from control. The mean number of total alive larvae recovered from the control series was considered as 100% viability. The lethal activity of fungi was expressed as percentage mortality. Data were square root transformed $\sqrt{x+0.5}$ and a completely randomized design was used. An ANOVA analysis followed by the Tukey test ($\alpha = 0.05$) was used. The mean number of dead larvae recovered in each treatment was considered as the dependent variable. SAS statistic software was used for the analysis. Percentage mortality was estimated using the following formula:

$$\% \text{ Mortality} = \frac{\bar{x} \text{ Control} - \bar{x} \text{ Treated}}{\bar{x} \text{ Control}} \text{x } 100$$

Where: X Control = Mean of *H. contortus* larvae recovered from control group;

X Treated= Mean of *H. contortus* larvae recovered from treated group.

RESULTS

Results showing the mean numbers and standard deviations of *Haemonchus contortus* (L_3) recovered larvae after confrontation with mycelia of the different edible mushrooms, in addition to the variant coefficient (%) and larval mortality percentage are shown in Table 1.

Note: Same small letters in the mortality column indicate that the values were not statistically different, according to Tukey's test ($\alpha = 0.05$), $\bar{x}$ =Average L_3 alive; SD= Standard deviation.

The nematicidal activity of the fungal mycelia against *H. contortus* L_3, ranged from 4.8% to 99.6% mortality. The strains *L. boryanus* ECS-0402 and *C. comatus* ECS-1103 presented 61 and 56.3% mortality; respectively. The highest nematocidal activity corresponded to *L. edodes* ECS-0401 (99.6%), *P. eryngii* ECS-1292 (98.1%), *P. ostreatus* ECS-1123 (97.2%), *P. cornucopiae* ECS-1328 (92.9%), *P. ostreatus* ECS-0152 (92.1%), *P. cornucopiae* ECS-1330 (85.9%) and *P. eryngii* ECS-1290 (82.2%). It is worth mentioning that once the plates were revised at the end of the incubation period some larvae were observed to be motionless with their bodies experiencing no apparent changes and maintaining their normal integrity.

In some cases, after adding some water drops or applying a physical stimulus with a fine needle, some larvae were observed to be actively moving on the agar surface. The bodies of other motionless larvae were observed to have been invaded by some of the assessed mushroom strains; this finding was most evident particularly in three species of the evaluated mushroom mycelia: *Pleurotus cornucopiae* (strain 1328), *P. cornucopiae* (strain 1330) and *Lentinul aboryanus* (strain 402). A set of selected microphotographs showing some larvae invaded by mushroom mycelia of these species are shown in Fig. 1.

Table 1. Mean number and standard deviation of *Haemonchus contortus* (L_3) recovered larvae after 5 days *in vitro* confrontation with mycelia of different edible mushrooms, and larval mortality percentage.

Strain	$\bar{x}$± (SD)	Mortality (%)
Pleurotus ostreatus ECS-1123	14±(18.4)	97.2[c]
Pleurotus ostreatus ECS-0152	38±(39.3)	92.1[c]
Pleurotus eryngii ECS-1292	6±(9.6)	98.1[c]
Pleurotus eryngii ECS-1290	68±(45.4)	82.2[c]
Pleurotus cornucopiae ECS-1328	34±(28.3)	92.9[c]
Pleurotus cornucopiae ECS-1330	78±(41.5)	85.9[c]
Coprinus comatus ECS-1103	144±(63.8)	56.3[b]
Panus sp. ECS-801	314±(58.1)	4.8[a]
Lentinula boryanus ECS-0402	184±(123)	61.8[b]
Lentinula edodes ECS-0401	2 ±(6.2)	99.6[c]

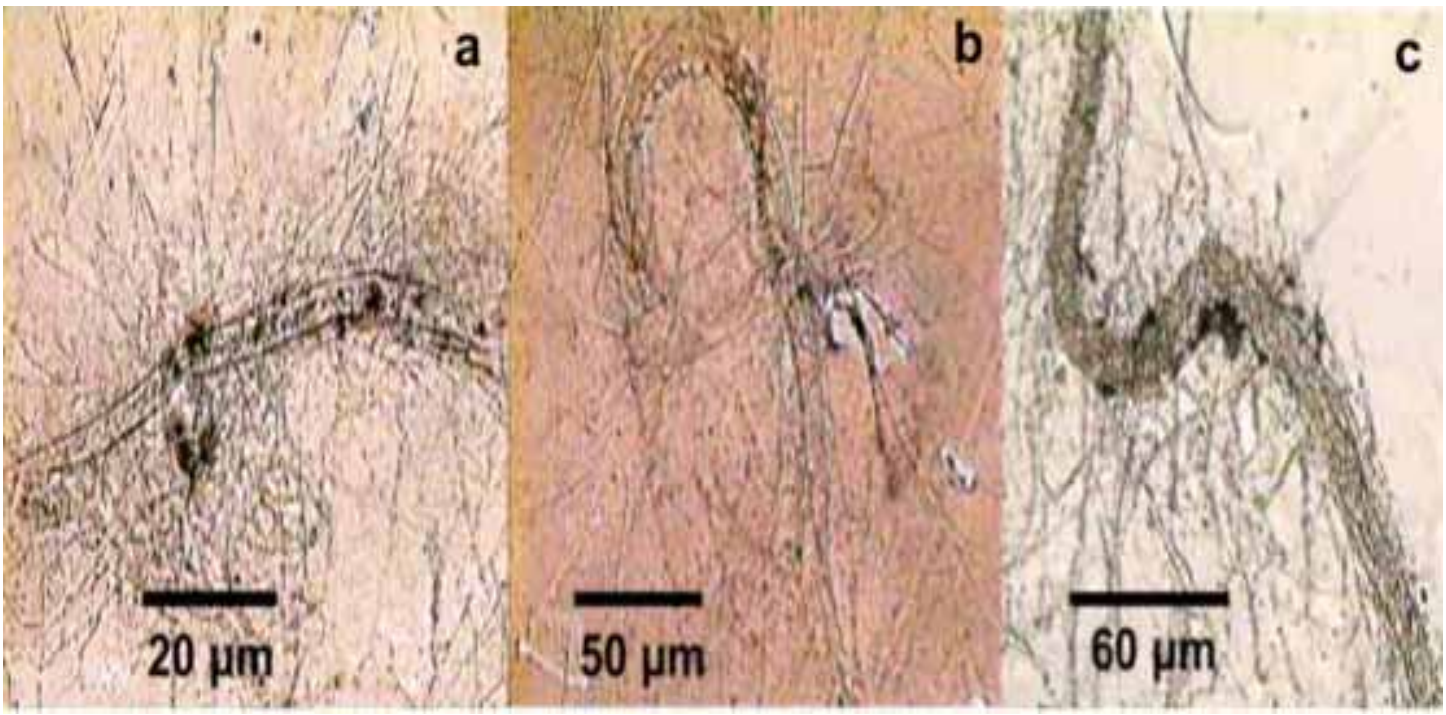

Figure 1. Microphotographs showing the aspect of *Haemonchus contortus* infective larvae (L_3) invaded and degraded by mycelia of 3 edible mushroom, after 5 days *in vitro* confrontation on water agar plates at 25 ºC. a) *Pleurotus cornucopiae* (strain 1328); b) *P. cornucopiae* (strain 1330) and c) *Lentinula boryanus* (strain 402).

DISCUSSION

Edible mushrooms have demonstrated many medicinal properties; therefore, are strong candidates for potential nutraceutic agents. This study analyses *in vitro* nematocidal activity of mycelia from a group of edible mushrooms against *H. contortus* infective larvae. Other studies have shown that *P. ostreatus* produces a nematotoxin similar to peroxidases which inhibit the movement of nematodes and subsequently degrade them, reaching a mortality of 95% in the free-living nematode *Panagrellus redivivus* (adults) and the phytopathogen nematode *Bursaphelenchus xylophilus* [15].

The information obtained in the present study is similar to the data reported by these authors, since the mortality values obtained with *P. ostreatus* ECS-0152 and ECS-1123 were 92.1% and 97.2%, respectively. Another study conducted by [16], shows that *P. eryngii* attains 50% mortality against the phytopathogenic nematode *Heteroderas chantii* which causes wilting in sugarcane and other crops. Similar to our findings, these authors reported movement inhibition and nematode body invation and degradation. Interestingly, the mortality percentage obtained in the present study against *H. contortus* L_3 with *P. eryngii* ECS-1292 and ECS-1290 strains was higher (98.1 and 82.2%, respectively).

Table 2. Nematode-toxic edible mushrooms, nematicidal compounds, general conditions and efficacy.

Genus/specie of mushroom	Nematicidal compounds	Test nematodes	Efficacy	References
Pleurotus ostreatus	————	*Heterodera schachtii*	%	Palizi*et al*. [16]
Pleurotus ferulae	Cheimonophyllon E.5-hydroxymethyl-furancarbaldehyde.	*Bursaphelenchus xilophilus, Panagrellus redivivus*	—	Li *et al.* [17,18]
Pleurotus ostreatus	Trans-2-decenedioic acid.	*Panagrellus redivivus*	95	Kwok *et al.* [19]
Pleurotus pulmonarius	*p*-anisaldehydep-anisyl alcohol1-(4-methoxyphenyl) -1,2-propanediol.2-hydroxy-(4´-methoxy)-propiophenone. Fatty acid S-coriolic acid.	*Caenorhabditis elegans*	—	Stadler *et al*. [20] Koitabashi *et al*. [21]
Pleurotus ostreatus	————	*Meloiodgyne arenaria*	87-94	Xiand and Feng, [22]
Coprinus comatus	5-Methylfuran-3-carboxylic acid.5-hydroxy-3.5-dimethylfuran -2(5H)-one.5-hidroxy-3-(hydroxymethyl)-5-methylfuran -2(5H)-one4,6-dihydroxyiso benzufuran-1,3-dione4,6-dihydroxyisobenzufuran-3 (2H)-one4,6-dihydroxyiso benzufuran-1(3H)-one3-formyl-2.5-dihydroxybenzyl acetate	*Panagrellus redivivus, Meloidogyne javanica*	90	Luo *et al.* [13]

A list of nematode-toxic mushrooms, their nematocidal compounds and their nematocidal efficacy against genera/specie of different taxonomic groups of nematodes, are shown on Table 2. In general, nematocidal activity shown by the different genera/specie of assessed mushrooms ranged between 4.8-99.6% mortality.

The nematode lethal effect of specific edible mushroom, could be influenced by a number of factors, for example: temperature and incubation time of the confrontation or even inner genetic characteristics of each of the strains and/or differences between nematode species used. The fact that dead larvae that had not been invaded by any fungal mycelium were found suggests that as with *Coprinus comatus* strains, a nematotoxin can be produced by these mushroom species, resulting in nematode death. Alternatively, the fact that some dead larvae were observed with mycelium inside their bodies could

suggest that the larvae death was a consequence of body rupture and invasion by fungal mycelia. However, another possibility is that larvae could have been invaded by fungal mycelia after death. Currently, we do not know if the nematotoxin produced by these strains is sufficiently active to kill larvae before invading their bodies. However, Mamiya [13] found that a strain of *C. comatus* immobilizes, kills and consumes free-living nematodes *Panagrellus redivivus* and the root-knot nematode *Meloidogyne arenaria*. Authors of the present study, did not find any data regarding the lethal activity of *C. comatus* against *H. contortus* larvae; thus this study could be the first regarding this.

With respect to the strain *C. comatus*, Mamiya [13] reported mechanical damage in the free-living nematode *P. redivivus*, 8 h post-confrontation with *C. comatus* mycelia, resulting in 90% nematode immobilization and subsequent degradation, at 24 °C incubation. Data obtained in the present study differs from the results found by these authors, since *C. comatus* ECS-1103 caused 58.3% mortality at 5 days post-confrontation. These differences may be associated with the previously described factors; however, in both studies mechanical damage was observed in nematodes. In relation to *L. edodes*, Dong *et a*l. (2006), reported that the mortality against the phytonematode *B. xylophilus* was 57.6% after 72 h of incubation at 26 °C, while in the present study we found that *L. edodes* ECS-0401 presented 99.6% mortality against *H. contortus* (L_3) after 5 days of incubation at a temperature range of 18-25 °C.

Iijima *et al*. [7], isolated and cloned nematode degrading molecules (PCL-F) from the fungus *P. cornucopiae*, composed of two homodimers and a heterodimer combined with two subunits with 16 and 15 k Da molecular weight. These molecules PCL-F showed similarities with lectins from traps formed by the nematophagous fungus *Arthrobotrys oligospora*. Lectins are carbohydrate-binding proteins and their function is the recognition of molecules in the interaction of a variety of organisms, such as fungus-nematode. However, no information was found on the confrontation of edible mushroom mycelia against any nematodes at such level of complexity.

Regarding the strains *Panus* sp. ECS-801 and *Lentinula boryana* ECS-0402, no report about nematocidal activity of mycelia of these genera/specie was found. Thus, according to the results of the present research, both of these genera/specie of edible mushrooms have the potential for controlling sheep haemonchosis. To achieve this goal future research will be required.

CONCLUSIONS

The strains of the edible mushrooms *P. ostreatus* ECS-1123 and ECS-0152, *P. eryngii* ECS-1290 and ECS -1291, *P. cornucopiae* ECS-1328 and ECS-1330 and *L. edodes* ECS-0401 displayed high nematocidal activity, presenting a range of 82 to 99% mortality, while *Coprinus comatus* ECS-1103, *Panus* sp. ECS-801 and *L. boryana* ECS-0402 showed a low *in vitro* nematocidal activity against *H. contortus*, considered as the most economically and pathogenic parasite affecting the sheep industry worldwide.

ACKNOWLEDGEMENTS

Study funded by the National Institute of Investigations on Forestry, Agriculture and Husbandry of Mexico (INIFAP), Project No. 9442232005.

REFERENCES

[1] Qamar FM *et al*. (2011). Economic losses due to haemonchosis in sheep and goats. *Sci.Int*. (Lahore). 23(4): 321-324.

[2] Roeber F *et al*. (2013). Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance - an Australian perspective. *Parasites & Vectors*. doi: 10.1186/1756-3305-6-153.

[3] Rehbein S *et al*. (1996). The helminth fauna of slaughtered sheep from upper Bavaria: 1. Species composition, prevalence and worm counts. *Berl MuenchTieraerztl Wschr*. 109: 161-167.

[4] Torres Acosta *et al*. (2012). Research and implementation of novel approaches for the control of nematode parasites in Latin America and the Caribbean: is there sufficient incentive for a greater extension effort? *Vet Parasitol*. 186(1-2): 132-142.

[5] Areskog M *et al*. (2014). A controlled study on gastrointestinal nematodes from two Swedish cattle farms showing field evidence of ivermectin resistance. *Parasites & Vectors. doi:* 10.1186/1756-3305-7-13.

[6] Fitzpatrick JL. (2013). Global food security: the impact of veterinary parasites and parasitologists. *Vet Parasitol*. 1, 195(3-4): 233-48.

[7] Iijima N *et al*. (2002). Two genes encoding fruit body lectins of *Pleurotus cornucopiae*: sequence similarity with the lectin of a nematode-trapping fungus. Department of Bioresources Chemistry, Faculty of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan. 66 (10): 2083-2089.

[8] Lumaret JP *et al.* (2012). A Review on the Toxicity and Non-Target Effects of Macrocyclic Lactones in Terrestrial and Aquatic Environments. *Curr Pharm Biotechno*.13(6): 1004-1060.

[9] Acharya J *et al.* (2014). *In vitro* screening of forty medicinal plant extracts from the United States Northern Great Plains for anthelmintic activity against *Haemonchus contortus*. *Vet Parasitol*. 201(1-2): 75-81.

[10] Fawzi EM *et al.* (2014). Vaccination of lambs against *Haemonchus contortus* infection with a somatic protein (Hc23) from adult helminths. *International Journal for Parasitology*. doi: 10.1016/j.ijpara.2014.02.009.

[11] Tan Yee-Shin *et al.* (2014). Influence of customized cooking methods on the phenolic contents and antioxidant activities of selected species of oyster mushrooms (*Pleurotus* spp.). *J Foof Sci. doi*: 10.1007/s13197-014-1332-8.

[12] De Silva *et al.* (2012). Medicinal mushrooms in supportive cancer therapies: an approach to anti-cancer effects and putative mechanisms of action. *Fungal Diversity*. 55:1-35.

[13] Mamiya Y. (2006). Attraction of the pinewood nematode to mycelium of some Wood-decay fungi. *Japanese Journal of Nematology*. 36(1): 1-9.

[14] Luo H *et al.* (2007). *Coprinus comatus* damages nematode cuticles mechanically with spiny balls and produces potent toxins to inmobilize nematodes. *Applied and Environmental Microbiology*. 73(12): 3916-3923.

[15] Bleuler-Martinez S *et al.* (2014). Biotin-binding-proteins in the defense of mushrooms against predators and parasites. *Journal ASM*. 23: 8845-8487.

[16] Palizi P *et al*. (2009). Potential of oyster Mushrooms for the Biocontrol of Sugar beet nematode (*Heterodera schantii*). *Journal of Plant Protection Research*. 49(1): 27-34.

[17] Li GH *et al.* (2001). Nematicidal activity of nematophagous *Pleurotus* and allied fungi to *Panagrellus redivivus*. *Chinese Journal of Biological Control*: 17: 26-29.

[18] Li GH *et al.* (2007). Nematicidal metabolites from the fungus *Pleurotus ferulae* Lenzi. *Annals of Microbiology*. 57: 527-529.

[19] Kwok OC *et al.* (1992). A nematicidal toxin from *Pleurotus ostreatus* NRRL 3526. *Journal of Chemical Ecology*. 18(2): 127-135.

[20] Satou T *et al*. (2008). The Toxin Produced by *Pleurotus ostreatus* reduces the head size of nematodes. *Biology Pharm. Bull*. 31(4): 574-576.

[21] Stadler M *et al.* (1994). Fatty acids and other compounds with nematicidal activity from cultures of basidiomycetes. *Planta Medica*. 60: 128-132.

[22] Koitabashi M *et al.* (2004). Antifungal substances produced by fungal strain Kyu-W63 from wheat leaf and its taxonomic position. *Journal of General Plant Pathology*. 70: 124-130.

[23] Xiang HQ and FengZX. (2001). The nematicidal toxicity of the fruits bodies of *Pleurotus ostreatus*. *Journal of Shenyang Agricultural University*. 32: 173-175.

PREVALENCE OF COMPETITOR MOULDS AND DISEASES IN STRAW MUSHROOM (*VOLVARIELLA VOLVACEA*) BEDS AND THEIR MANAGEMENT

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ABSTRACT

Volvariella volvacea, commonly known as paddy straw mushroom, is the most popular and palatable mushroom in South-East Asian countries. It ranks sixth among the edible mushrooms accounting for 3% of the global production. Odisha is the leading state in terms of straw mushroom production in India. However, a number of competitor moulds infest the beds at different stages of crop growth and bring down the productivity substantially. Data from a comprehensive survey conducted in the leading mushroom growing districts of Khurda and Puri at 10 locations both in outdoor and indoor conditions revealed as many as eight competitor moulds contaminating the straw mushroom beds during the fruiting stage. *Coprinus* spp. was predominant of all in both outdoor and indoor farming situations. However, outdoor farming recorded more bed contamination (46.8%) compared to the indoor one (27%). Bacterial button rot disease was recorded to the tune of 9 and 13% in outdoor and indoor situations, respectively. Pre-soaking of the substrate with 2% calcium carbonate solution for six hours was significantly superior among the treatments in giving a fruit body yield of 1016.67 g/bed with a corresponding biological efficiency of 14.52%. Application of 2% neem leaf extract was next in order in respect of yield (946.67 g/bed) and biological efficiency (13.52%). Further, the intensity of *Coprinus* spp. was low in the above mentioned treatments as compared to the untreated control.

Keywords: straw mushroom, competitor moulds, diseases, management

INTRODUCTION

Paddy straw mushroom (*Volvariella volvacea*) has become the nutritional and economic main stay of the predominantly agrarian population of the state. At present the state is producing approximately 8000 tonnes of paddy straw mushroom annually contributing to 66% of the total mushroom production of the state [1]. It is largely cultivated outdoor, in open fields and in low cost thatched houses. In most of the cases, cultivation is done in non-pasteurized paddy straw with incorporation of wheat bran as the additive. Large number of competitor moulds namely *Coprinus* spp., *Aspergillus flavus, A.niger, Penicillium* spp., *Mucor* sp. and *Rhizopus oryzae* have been recorded in the beds because of non-pasteurization or improper pasteurization of the straw and raising of beds in non-congenial environmental conditions. Hence, the biological efficiency varies from 8-10%, which is unusually low even though factors like hot and humid coastal agro-ecological situation of Odisha, abundance of agricultural waste and manpower are most favourable for raising paddy straw mushroom crop. There is ample scope for improving yield standards through effective substrate management. In this context, an investigation has been planned for evaluating management practices of competitor moulds with physical, chemical and biological agents.

MATERIALS AND METHODS

An exhaustive survey was conducted in the leading mushroom growing districts of Khurda and Puri at 10 locations both in indoor and outdoor situations to observe and record the contamination of paddy straw mushroom beds with competitor moulds. A minimum number of 100 beds per location were observed for the contaminants and diseases. Per cent contamination of beds by moulds and incidence of diseases of all the locations were recorded. Samples having contaminant moulds and bacterial button rot were collected, pure cultured on appropriate nutrient medium, identified in the laboratory of the Department of Plant Pathology, OUAT, Bhubaneswar and preserved for further studies.

For effective management of competitor fungus like *Coprinus* spp. and bacterial bud rot pathogen, a trial was designed with 10 treatments including the untreated control with three replications for each treatment. Paddy straw mushroom beds of recommended size (1.5' x 1.5' x 1.5') were raised after soaking the straw in clean and cold water for six hours, followed

by draining off excess water. Beds were spawned at the rate of 3% of the dry weight of the substrate in three layers at 1:1:2 proportions. The beds were supplemented with wheat bran @ 200 g/bed. After raising beds, they were sprayed treatment wise with benomyl (0.2%), bleaching powder (0.02%), streptocycline (0.01%), benomyl (0.2%) + streptocycline (0.01%), benomyl (0.2%) + bleaching powder (0.02%) and 4% tamarind leaf extract. Besides, three treatments received pre-treatments namely, soaking of straw in solution of formalin and carbendazim (each 90 litres of water mixed with 125 ml of formalin and 7.5 g of bavistin), soaking of straw in 2% calcium carbonate powder and treatment of straw in boiled water at 70-80 ^{o}C for one hour. The untreated control received no specific treatment.

Observations on time taken (days) to pin head emergence, first harvest, number of sporophores/bed, average weight of sporophore, fresh weight of sporophores and incidence of competitor fungi/bacterial bud rot were recorded. Biological efficiency in respect of individual treatments was calculated.

RESULTS AND DISCUSSION

Data on survey conducted in the leading mushroom growing districts of Khurda and Puri at 10 locations both in outdoor and indoor situations revealed as many as eight competitor moulds contaminating the straw mushroom beds during the fruiting stage (Table 1). *Coprinus* spp. was predominant of all both in outdoor and indoor farming situations. However, outdoor farming recorded more bed contamination (46.8%) in comparison to the indoor one (27 %). The incidence of other contaminants namely, *Aspergillus* spp., *Mucor* sp., *Penicillium* sp., *Rhizopus* sp., *Sclerotium rolfsii* and *Trichoderma* sp. was in the range of 2 to 17.8% in both situations. Bacterial button rot was recorded to the tune of 13 and 9% in indoor and outdoor situations, respectively. It was observed that appearance of competitor moulds was more (14.17%) in outdoor farming in comparison to indoor one (10.98%). Mean per cent incidence of bed contamination of both the situations was recorded at 12.58% in the investigation. Prevalence of *Coprinus* spp., *S. rolfsii* and *Aspergillus* spp. in higher proportions in non-pasteurized paddy straw under conventional method of cultivation has been reported by many workers [2-5].

Table 1. Prevalence of competitor moulds and diseases in straw mushroom beds

Sl.No.	Competitor moulds/ disease	Per cent incidence (Indoor)	Per cent incidence (Outdoor)	Mean per cent incidence
1	*Aspergillus flavus*	6.00	11.00	8.50
2	*A. niger*	13.90	17.80	15.85
3	*Coprinus* sp.	27.00	46.80	36.90
4	*Mucor*sp.	2.90	2.00	2.45
5	*Penicillium* sp.	6.60	12.00	9.30
6	*Rhizopus* sp.	11.70	8.00	9.85
7	*Sclerotium rolfsii*	12.00	16.00	14.00
8	*Trichoderma* sp.	5.80	5.00	5.40
9	Bacterial button rot (*Pseudomonas* spp.)	13.00	9.00	11.00
	Mean	10.98	14.17	12.58

The data depicted in Table 2 revealed that pre-soaking of substrate with 2% calcium carbonate for six hours was superior among the treatments in giving a yield of 1016.67 g/bed with the corresponding biological efficiency of 14.52%, which was followed by treatment of the bed with 4% tamarind leaf extract and benomyl (0.2%) + streptocycline (0.01%) with yields of 946.67 g and 643.33 g/bed, respectively. Pre-soaking of substrate with formalin (37.5 ppm) and bavistin (500 ppm) for the same period was found inferior giving a yield of 650 g/bed in the investigation. Further, pre-treatment of substrate with

boiled water at 70-80 °C for one hour after six hours of soaking yielded only 790 g/bed with 11.28% biological efficiency. The difference in days to first harvest was found non-significant among the treatments. Beds without any treatment (control) gave a mean yield of 630 g/bed accounting 9% biological efficiency. Overall intensity of *Coprinus* was found to be low in all the treatments as compared to untreated control, which recorded high incidence in the trial.

Table 2. Effect of different physical, chemical and biological agents on the incidence of *Coprinus* and yield of *V. volvacea*

Sl. No.	Treatment	Days to emergence of pin head	Days to first harvest	No. of fruit bodies	Av. weight of fruit bodies (g)	Wt. of fruit bodies (g)	Biological efficiency (%)	Intensity of *Coprinus**	% yield increase over control
1	Control	8.0	13.00	33.33	20.33	630.00	9.00	+++	-
2	Benomyl (0.2%)	10.0	15.00	41.33	18.00	726.67	10.38	+	15.34
3	Bleaching powder (0.02%)	10.0	15.00	32.33	20.66	637.00	9.10	++	1.11
4	Streptocycline (0.01%)	8.0	13.00	33.00	25.00	820.00	11.71	++	30.15
5	Benomyl (0.2%) + Streptocycline (0.01%)	9.0	15.00	51.67	20.00	943.33	13.47	+	49.73
6	Benomyl (0.2%) + Bleaching powder (0.02%)	9.0	14.33	42.33	18.00	743.33	10.61	+	17.98
7	Calcium carbonate (2.0%)	9.0	13.66	40.67	25.00	1016.67	14.52	+	61.37
8	Formalin + Bavistin (125 ml of formalin and 7.5 g bavistin / 90 litres of water)	10.0	15.00	34.33	19.00	650.00	9.28	+	3.17
9	Boiled water	7.0	13.00	44.67	148.00	790.00	11.28	+	25.39
10	Tamarind leaf extract (4.0%)	8.0	14.00	37.00	22.33	646.67	15.32	+	50.26
	CD (0.05)	0.85	NS	3.92	3.06	56.64	-	-	-
	C.V. (%)	5.22	7.76	7.04	8.66	6.65	-	-	-

A number of harmful fungi are encountered in the beds during straw mushroom cultivation, as majority of the growers lack proper substrate processing and pasteurization facilities. Use of non-pasteurized substrate coupled with unhygienic conditions help in the perpetuation of various moulds, besides resulting in reduced level of production. At times, there is complete crop failure depending upon the stage of infection, quality of substrate and environmental conditions. The improvement of pH of the substrate through calcium carbonate supplementation could be the sole factor in suppression of *Coprinus* and consequently yield improvement. Application of anti-bacterial agents like bleaching powder (0.02%) and streptocycline (0.01%) had little role on yield improvement, which suggested that *Coprinus* infestation of beds was the most important reason for yield deterioration. Workers observed that alkaline pH helped in suppressing the growth of weed fungi including *Coprinus* [6]. Likewise, role of phytoextracts in suppressing weed fungi in mushroom beds has earlier been demonstrated by Rivera-Vargas and Hepporly [7]. Further, positive role of fungicides like carbendazim and benomyl has been observed by several researchers [7, 8].

CONCLUSION

Paddy straw mushroom (*V. volvacea*) largely grown as an outdoor crop in the hot and humid coastal agro-ecological situation is infested with number of competitor moulds during fruiting stage. *Coprinus* spp. is the most encountered one as recorded in both the situations. At time, it results in substantial crop yield loss or complete crop failure. Pre-soaking of the straw with calcium carbonate for a period of six hours proved to be useful in suppression of contaminating moulds as well as improvement of yield standards. Being a low cost technology, this could well be adopted by the resource poor mushroom growers of the state.

REFERENCES

[1] Thakur MP and Mohapatra KB. (2013). Tropical mushrooms: present status, constraints and success story. Proceedings of Indian Mushroom Conference, 16-17 April, 2013, PAU, Ludhiana. pp. 42-43.

[2] Pani BK and Patra AK. (1997). Utilization of some phytoextracts for the control of *Sclerotium rolfsii* during paddy straw mushroom (*Volvariella volvacea*) cultivation; a new approach. *Mushroom Research.* 6(1): 37-41.

[3] Ahlawat OP and Tewari RP. (2007). Cultivation technology of paddy straw mushroom (*Volvariella volvacea*). National Research Centre for Mushroom (ICAR), Chambaghat, Solan (HP), 36 p.

[4] Mohapatra KB *et al.* (2007). Management of competitor fungi in paddy straw mushroom. Proceedings of National Symposium on sustainable pest management for safer environment, 6-7 December, 2007, OUAT, Bhubaneswar, pp.122-123.

[5] Sahoo AK *et al.* (2012). Effect of substrate processing and bed dimension on production of straw mushroom (*V. volvacea*) following conventional method of cultivation. *Environment and Ecology.* 30 (4A): 1413-1415.

[6] Yee NT and Yung CH. (1980). Interaction between *Volvariella volvacea* and some weed fungi. *Transactions of British Mycological Society.* 75(3): 498-501.

[7] Rivera-Vargas LI and Hepperly PR. (1987). Fungicides to control fungal competitors in Chinese straw mushrooms. *Journal of Agriculture of the University of Puerto Rico.* 71(2): 165-176.

[8] Vijay B and Sohi HS. (1987). Cultivation of oyster mushroom, *Pleurotus sajor-caju* (Fr.) Singer on chemically sterilized wheat straw. *Mushroom Journal of Tropics.* 7: 67-75.

MICROBIAL CONTAMINANTS IN OYSTER MUSHROOM (*PLEUROTUS OSTREATUS*) CULTIVATION THEIR MANAGEMENT AND ROLE OF METEOROLOGICAL FACTORS

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ABSTRACT

Survey revealed the occurrence of seven contaminants in mushroom beds and out of which *Trichoderma harzianum, Penicillium notatum, Sclerotium rolfsii* and *Coprinus* spp. were found to be most dominant fungal contaminants and occurrence was high during June and July (28.4 & 35.8 %) causing maximum loss to mushroom yield. The incidence of contaminants were minimum during January (2.87%) and maximum during the month of June (32.8 %). A good harvest of mushroom (105% BE) was obtained during the month of October. A range of average maximum temperature (24.63 – 33.18 ^{o}C), minimum temperature (9.40 -25.51 ^{o}C) and average relative humidity (68.90 -85.27%) was found most appropriate for the cultivation of oyster mushroom in this region. Among the botanicals tested for management of competitors moulds, *Azadirachta indica* (neem) showed its supremacy and exhibited maximum inhibitory effect (54.1 to 71.6 %) against *Aspergillus* spp., *Trichoderma* spp., *Coprinus* spp., and *Penicillium* spp. and was found to be less effective against *Sclerotium rolfsii in vitro* followed by extracts of *Pongamia pinnata* (42.4 to 61.3%). A range of 35.3 to 62.4% reduction in inky caps (*Coprinus sp.*) and 26.3 to 68.4% in green moulds (*Trichoderma* spp) were recorded with different phyto-extracts. The botanicals except *Acacia nilotica* reduced the incidence of competitor moulds (18.18 to 70.91%) in mushroom beds which increase the yield up to 21.3 %. The study will provide the idea of appropriate cultivation time as well as provide an alternative method of surface sterilization.

Keywords: oyster mushroom, environmental factors, contaminants, plant extract, botanicals

INTRODUCTION

Oyster mushroom (*Pleurotus* spp.) belonging to class Basidiomycetes and family Agaricaceae is popularly known as 'dhingri' in India. The popularity of oyster mushroom has been increasing due to its ease of cultivation, high yield potential and high nutritional value [1, 2]. Oyster mushroom help to remove the toxicity produces by the agro wastes [3-5]. The use of fungicides for controlling the competitor moulds and diseases in oyster mushroom cultivation is very common in India. The hazardous effects of chemicals in human health and environmental aspect are known. Apart from these problems continuous usage of same chemicals may lead towards pest's resistance. Studies on various aspects of fungal contaminants and diseases of *Pleurotus* spp. were undertaken by various workers [6-8] and they reported *Trichoderma harzianum*, *Aspergillus* spp., *Penicillium* spp., *Monilia sitophila, Stemonitis* spp. and *Coprinus* spp. were the major contaminants of *Pleurotus* spp. These species become prevalent in *Pleurotus* cultures if the substrate has not been uniformly or properly pasteurized. Among these contaminants, *Trichoderma harzianum* was reported to be the most damaging one, competing aggressively with the mycelium of *P. pulmonarius* and *P. ostreatus in- vitro* and reducing the production surface from 30 to 50% [9]. While, *Aspergillus niger*, *Coprinus* sp, *Penicillium* sp and *Sclerotium rolfsii* were the most predominant fungal contaminant of mushroom beds of *P. florida* [10]. Spilman [11] recognized *Trichoderma* as green mould on the production bed of oyster mushroom. *Trichoderma, Aspergillus* and *Rhizopus* on oyster mushroom bed were predominant microorganisms and especially occurrence of these was severe in summer and spring seasons than autumn and winter [12]. Considering the above, an experiment was conducted to develop a suitable management practice against the competitor moulds of *Pleurotus ostreatus* in an eco- friendly manner under the agro-ecological condition of lateritic belt of West Bengal.

MATERIALS AND METHODS

Survey for Natural Incidence of Competitor Moulds: Five home scale mushroom farms of Bolpur and its adjacent areas were surveyed every month from January 2010 to December 2011 for the occurrence of contamination in mushroom beds of oyster mushroom (*P. ostreatus)*. The incidence of different competitor moulds were recorded. Infected mushroom bags were tagged and the contaminated microflora were identified. Total number of infected beds were counted from each farm. In addition, five mushroom beds were raised in Ballabpur, Bolpur farmar's cropping room in first week of every month and allowed for spawning and yield. Month wise average data on three important weather factors viz., temperature, relative humidity and rainfall were recorded. Spawn of oyster mushroom (*P. ostreatus)* was supplied from the laboratory to the progressive farmers for cultivation. Paddy straw was taken as substrate and chemical sterilization technique [13] was followed in cultivation. Container system of cultivation (polythene bags) was followed in all experiments. Samples of various diseases and competitor fungi were collected on a regular basis from the cropping room and subsequently, *in vitro* studies have been carried out in laboratory.

Preparation of Beds: Chopped paddy straw was soaked into a solution containing the requisite amount of sterilizing agent for 16-18 hours. Thorough spawning @ 4% by wet weight basis was followed. The spawned substrate was filled in polypropylene bags (45x30 cm). A unit of 2 kg of dry straw was used for each treatment, which was equally distributed in four bags representing each as a replication. The moisture content of the straw at the time of spawning was kept around 72-75%. The filled bags were incubated in a dark room at a temperature ranging between 24-30 ^{o}C, where 90% relative humidity was maintained till the spawn run was complete. When the straw is fully covered with milky white mycelium in the bag, it is regarded as complete spawn run, then the bags were cut open and compacted mass of aggregated straw called, as "bed" was ready for cropping. The beds were hanged by nylon string at a distance of 60 cm. Harvesting was done when the small primordial converted into a full grown sporophore.

Isolation and Purification of Competitor Moulds: Competitor moulds fungi were collected from the damaged beds in sterilized petriplates with the help of a sterile forceps and thereafter transferred into PDA plates under *in vitro* conditions. Inoculated PDA plates were incubated at 27 ^{o}C ($\pm$ 2 ^{o}C) for 3 to 4 days. A single colony was isolated from the PDA plate and again transferred to PDA plates for obtaining the pure culture. All the pure cultures were kept in refrigerator at 4 ^{o}C for preservation.

Preparation of Photo-Extracts: For preparation of phyto-extracts, 100 gram plant products were collected, washed in tap water, air dried and homogenized with equal amount of distilled water (100 ml) by crashing them with electric grinder machine. The extract was filtered through double-layered muslin cloth and centrifuged at 4000 rpm, for 10 minutes. The supernatant was collected and filtered through Whatman No.1 filter paper which was considered as standard solution.

***In vitro* study:** Different eco-friendly botanicals i.e. *Azadirachta indica,Lantana camera, Pongamia pinnata,Acacia nilotica,Clerodendron indicum,Eucalyptus hybrid,Datura metal, Cassia tora* and chemicals (carbendazim 75 ppm + formalin 500 ppm) were evaluated individually against competitor moulds and oyster mushroom (*P. ostreatus*). Poisoned food technique [14] (Grove and Moore, 1962) was carried out to evaluate the inhibitory effect of fungal mycelia. For one competitor mould, 4 ml of each plant extract (standard solution) was incorporated in 100 ml of potato dextrose agar medium (PDA) and autoclaved for 20 minutes at 1.41 kg/ cm^2 pressure. The molten media were poured into four sterilized glass petriplates (90 mm) considering each as a replication. After solidification of the agar plates were inoculated with 5 mm diameter mycelial cut from 6 day old culture of competitor moulds i.e. *Aspergillus* spp., *Trichoderma* spp., *Coprinus* spp., *Penicillium* spp and *Sclerotium rolfsii* and *P. ostreatus*. The petriplate without any treatment served as control. The plates were incubated at room temperature till the complete growth observed in control plates. The mycelial inhibition was calculated as [(dc-dt)/dc] x 100 where dc was dia of control and dt is dia in the treatment.

***In vivo* study:** Phyto-extracts and chemicals were further evaluated *in vivo* to see their inhibitory effect against the major contaminants, i.e. *Trichoderma* sp., *Aspergillus* sp., *Coprinus* sp., *Sclerotium rolfsii* and *Penicillium* spp. Paddy straw was dipped into the solution containing appropriate concentration of phyto-extracts and chemicals separately for 16-18

h and spawning was done @ 4% wet weight basis. The polythene bags were cut open when colonization was completed. Untreated paddy straw was used as control. Data on different parameters were collected on a regular basis during the cropping. Percent contamination index was calculated for the test and control beds depend upon the following scale

Grade 0: 0% – incidence of contaminants
Grade 1: >0 – 20% coverage by the contaminants
Grade 2: >20 – 40% coverage by the contaminants
Grade 3: >40 – 60% coverage by the contaminants
Grade 4: >60 – 80% coverage by the contaminants
Grade 5: >80 – 100% coverage by the contaminants

Per cent contamination index = (sum of the total scores/maximum rating) x total number of observations x 100

RESULTS

Role of Meteorological Factors: The effect of fluctuations in climatic factors on the incidence of competitor moulds and yield of oyster mushroom was studied and the data obtained are presented in Table 1. Survey revealed the occurrence of seven contaminants i.e. *T. harzianum*, *P. notatum*, *A. niger*, *Coprinus* spp., *Mucor* sp., *Rhizopus* sp., and *S. rolfsii* in mushroom beds and out of which *T. harzianum*, *P. notatum*, *S. rolfsii* and *Coprinus* spp. were found to be the most dominant fungal contaminants. The incidence of the contaminants were minimum during the month of January (2.875 %) and it increased considerably with the fluctuating climatic conditions and reached its peak during the month of June (32.8 %). Thereafter, a decline trend in contamination % was noticed in this region. Minimum range of contamination (2.95 to 5.2%) was observed during the period from January to February and again from October to December, when maximum biological efficiency 105% was obtained. A range of average maximum temperature (24.63–33.18 °C), minimum temperature (9.40 -25.51 °C) and average relative humidity (68.90 -85.27%) was found most appropriate for the cultivation of oyster mushroom in this region (Fig. 1). Significant positive correlations were obtained between average rainfall (1.00**) and minimum temperature (0.695*) and incidence of microbial contaminants in mushroom beds. However, relative humidity in cropping room was the most deciding factor of yield of mushroom (0.779**). High R^2 values of (1.00**) and (0.916**)

Table 1. Relationship between meteorological factors,incidence of microbial contaminants and yield of oyster mushroom

Cropping month	Mean temperature (°C)		Mean RH % m.m.	Total Rainfall	Contamination %			Yield (Kg/bed) in Ballabpur farm	Biological Efficiency%
	Max.	Min.			Five Farmer's farm (avg.)	Ballabpur farm	Average		
January	24.63	9.40	68.90	3.1	2.80	2.95	2.87	0.815	81.5
Febuary	29.62	14.72	82.65	16.1	6.19	5.2	5.69	0.895	89.5
March	36.88	20.80	65.34	6.9	8.10	6.4	7.25	0.776	77.6
April	40.28	25.55	52.9	54.9	16.31	14.9	15.60	0.598	59.8
May	36.0	25.41	46.1	109.5	21.30	18.5	19.9	0.420	42
June	36.29	25.86	49.4	172.8	35.8	32.8	34.3	0.371	37.1
July	33.91	26.33	81.94	267.2	30.2	28.4	29.3	0.595	59.5
August	33.72	26.38	85.73	111.0	24.2	21.4	22.8	0.786	78.6
September	33.18	25.51	85.27	177.8	13.4	12.2	12.8	0.883	88.3
October	33.04	23.27	82.80	57.3	5.60	4.7	5.15	0.985	98.5
November	31.24	18.79	78.21	11.9	4.30	3.8	4.05	1.050	105
December	25.28	12.67	77.13	33.1	3.50	3.90	3.7	0.930	93

indicated 100 % contribution of all meteorological factors on the incidence of contaminants and about 91.6% contribution of meteorological factors and microbial contaminants respectively towards the yield of *P. ostreatus*, Table 2. and 3.

Table 2. Correlation between meteorological factors, incidence of microbial contaminants and biological efficiency of oyster mushroom

Sl.No	Meteorological factors	"r" Values	
		Incidence of microbial contaminants (%)	Biological Efficiency (%)
1	Maximum Temperature	0.349	-0.568
2	Minimum Temperature	0.695*	-0.5120
3	Average Relative Humidity	0.028	0.779**
4	Rainfall	1.000**	0-.530

* Significant at 5% level ** Significant at 5% level

Sl.No.	Multiple correlation between	R^2 Value
1.	Y^1 and X^1, X^2, X^3, X^4	+1.00**
2.	Y^2 and X^1, X^2, X^3, X^4, X^5	+.0.916**

**** = Significant at 1% level * = Significant at 5% level**

Where : X^1 = Maximum temperature X^2 = Minimum temperature
X^3 = Average relative humidity X^4 = Rainfall
X^5 = Average contamination %
Y^1 = Average contamination %
Y^2 = Biological efficiency%

***In vitro* study:** The extent of inhibition of mycelium growth of *P. ostreatus* and different competitor moulds varied considerably with different botanicals and chemicals used (Table 4). Significant differences were obtained among all the treatments. Chemical treatment (bavistin 75 ppm + formalin 500 ppm) proved its superiority among all the treatments and found to be most effective in inhibiting the mycelial growth of five contaminants (65.4 to 86.6 %). Among the botanicals, *A. indica* (neem) showed maximum inhibitory effect (54.1 to 71.6 %) against the growth of four competitor moulds fungi i.e. *Aspergillus niger*, *Trichoderma viride*, *Coprinus* spp. and *Penicillium* sp., and was found less effective against the mycelium growth of *P. ostreatus* (4.4%). This was followed by extracts of *Pongamia pinnata* (karanja) 42.4 to 61.3% (mould fungi) and 6.7% (*P. ostreatus*) and *Clerodendron indicum* (clerodendron) which inhibited 40.0 to 53.8% and 8.9% mycelium growth of mould fungi and *P. ostreatus* respectively. Among the five contaminants, *Sclerotium rolfsii* was reported to be more resistant against most of the botanicals used. However, the growth of the same fungi was reduced considerably with the extract of *P. pinnata* (53.3%) and *Eucalyptus hybrida* (43.3%).The extracts of *Acacia nilotica* showed minimum inhibitory effect (18.9 to 35.3%) against the contaminants tested and also exhibited adverse effect on the growth of mushroom mycelium (25.6%) as given in Fig. 2.

***In vivo* study:** The response of eight plant extracts and chemicals used as surface sterilizing agent for cultivation of *P. ostreatus* are presented in Table 4. Data indicated the supremacy of chemical treatment over the botanicals used. An increase of 35.20 % in biological efficiency and 81.36% reduction in the incidence of competitor moulds were observed from chemically treated substrate. Similar trends were also noticed with the botanicals *in vivo* ,where maximum yield of mushroom (95.10 % B.E.) was obtained from the substrate treated with *A. indica* which checked 70.91% incidence of competitor moulds in beds. This was followed by *P. pinnata* 92.20% (B.E.) and 61.36% (reduction in mould incidence) and *Clerodendron indicum* treated substrate 89.00% and 56.82% respectively (Fig. 3).

The substrate treated with chemicals (bavistin 75 ppm + formalin 500 ppm) has taken minimum period (14 days) for completing the spawn run where, no moulds attack has been noticed. The extract of *A. indica* showed excellent response towards the growth of mushroom mycelia and completed the spawn run within 16 days. It also reduced the incidence level of competitor moulds (4.1% PCI). However, no correlation exist between the treatments in terms of average weight of sporophores.

Table 3. Multiple correlation between meteorological factors, incidence of microbial contaminants and biological efficiency of oyster mushroom

S.N.	Treatments	Dose	Radial growth of mycelium and percentage growth inhibition 8 days after inoculation{mycelium growth in (mm) and growth inhibition in (%)}												Sem± Treatment mean	CD at 5% (mycelium growth)
			Pleurotus ostreatus		*Coprinus sp.*		Aspergillus niger		*Penicillium sp.*		*Sclerotium rolfsii*		*Trichoderma* spp			
			mm	%	mm	%	mm	%	mm	%	mm	%	mm	%		
1	*Azadirachta indica*	4%	86.0	4.4	29.3	62.4	31.2	54.1	22.4	71.6	68.0	24.4	27.2	68.4	0.899	2.671
2	*Lantana camera*	4%	73.0	18.9	54.0	30.8	*45.0*	33.8	50.0	36.7	62.0	31.1	61.0	29.1	1.067	3.170
3	*Pongamia pinnata*	4%	84.0	6.7	30.2	61.3	35.0	48.5	43.0	45.6	42.0	53.3	49.5	42.4	0.853	2.536
4	*Acacia nilotica*	4%	67.0	25.6	50.5	35.3	52.0	23.5	54.0	31.6	73.0	18.9	63.4	26.3	1.106	3.286
5	*Clerodendron indicum*	4%	82.0	8.9	36.0	53.8	36.5	46.3	38.0	51.9	54.0	40.0	51.0	40.7	1.02	3.032
6	*Eucalyptus hybrida*	4%	80.0	11.1	39.0	50.0	42.0	38.2	33.0	58.2	51.0	43.3	46.0	46.5	1.027	3.052
7	*Datura metal*		71.0	21.1	37.0	52.6	35.0	48.5	46.0	41.8	57.0	36.7	55.0	36.0	1.130	3.358
8	*Cassia tora*	4%	75.0	16.7	41.0	47.4	49.0	27.9	58.0	26.6	64.0	28.9	59.0	31.4	0.971	2.887
9	Bavistin + Formalin l	75ppm+ 500ppm	83.0	7.8	15.0	80.8	23.5	65.4	15.5	80.4	25.0	72.2	11.5	86.6	0.824	2.450
10	Control		90.0	0.0	78.0	0.0	68.0	0.0	79.0	0.0	90.0	0.0	86.0	0.0	0.726	2.158
	Sem± Treatment mean		1.125		0.887		0.965		1.037		0.953		0.826			
	CD at 5%		3.250		2.563		2.787		2.997		2.754		2.387			

Table 4. *In vivo*evaluation of different phyto-extracts and chemicals against major competitor moulds of oyster mushroom

Sl No.	Treatment	Dose	Average yield from 500 g substrate (g)	Biological Efficiency %	Average weight of sporophore(g)	Days to emergence of Pinhead	% increase/ decrease in B.E.	% incidence of CM	% (+) /(-) in incidence of CM	Remarks
1	*Azadirachta indica*	4.0%	475.5	95.10	6.9	16	21.30	4.1	-70.91	-
2	*Lantana camera*	4.0%	412	82.40	6.4	26	5.10	7	-31.82	*Cs, An, Sr.*
3	*Pongamia pinnata*	4.0%	461	92.20	6.85	17	17.60	5	-61.36	-
4	*Acacia nilotica*	4.0%	354	70.80	5.32	26	-9.69	24	+9.09	*Cs, An, Ps, Sr*
5	*Clerodendron indicum*	4.0%	445	89.00	6.75	19	13.52	6	-56.82	_
6	*Eucalyptus hybrida*	4.0%	425	85.00	6.2	21	8.42	7.8	-36.36	*Sr, Cs*
7	*Datura metal*	4.0%	436	87.20	6.1	20	11.22	6.5	-45.45	_
8	*Cassia tora*	4.0%	402	80.40	5.9	23	2.55	8.5	-18.18	*Sr, Tr*
9	Bavistin +Formalin	75 ppm+ 500 ppm	530	106.00	7.3	14	35.20	0	-81.36	
10	Control		392	78.40	5.8	24	0.00	22	0.00	*Cs, Ps, Sr,Tr*
	SE (treatment mean)			4.251	0.850	0.235	0.695	0.683		
	CD at 5%			12.279	2.455	0.679	2.007	1.973*		

Cs = Coprinus sp., *As = Aspergillus niger, Ps = Penicillium* sp., *Sr = Sclerotium rolfsii, Tr = Trichoderma spp., CM= Competitor moulds*

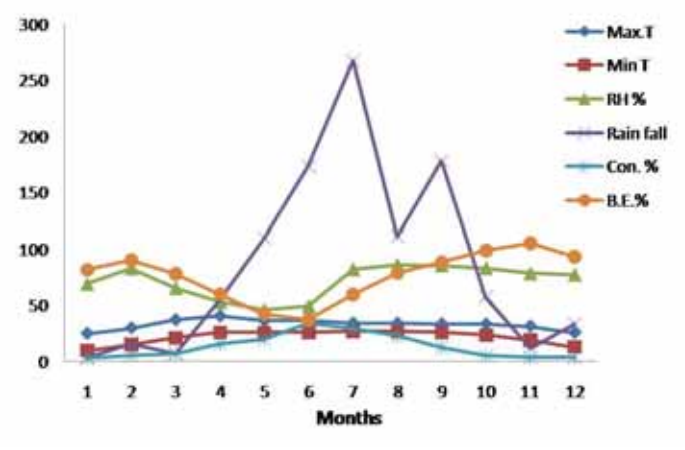

Figure 1. Relationship between meteorological factors, incidence of microbial contaminants and yield of oyster mushroom

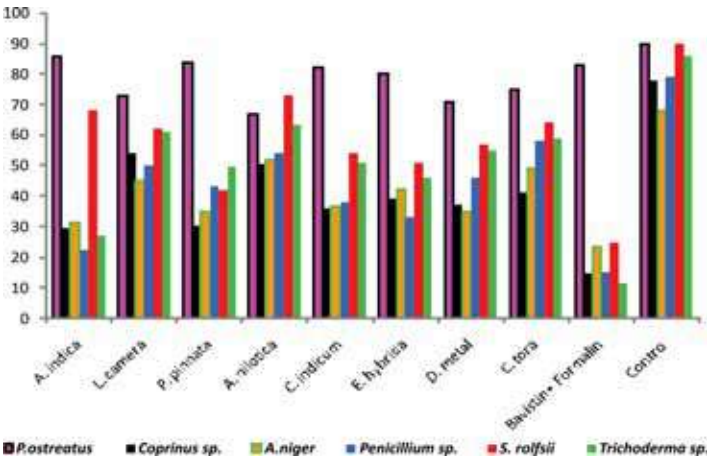

Figure 2. *In vitro* evaluation of botanicals against competitor mould fungi

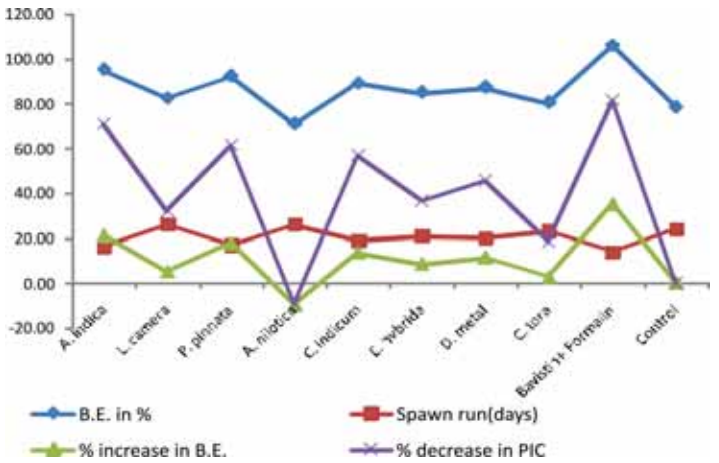

Figure 3. *In vivo* evaluation of different botanicals against competitor mould fungi and B.E.

DISCUSSION

The optimal temperature for the spread of the mycelium or vegetative growth of *Pleurotus* spp. is around 25-28 °C [15] and for fruiting body formation the temperature requirement is nearly 2 to 4 °C less than that. If there is too cold then the mycelial growth is to be arrested and at more than optimum temperature there is the risk of mould and bacterial contamination on the production beds which leads to destruction of the mushroom mycelia. In present investigation, heavy rain coupled with elevated minimum temperature (>25 °C) increased the contamination in beds was probably due to the decreasing of oxygen supply and increase in CO_2 concentration in the mushroom house or growing bags which may reduce the growth rate of oyster mushroom mycelia. Different concentration of carbendazim (bavistin) and it's combination with formaldehyde (formalin) were evaluated against the major contaminants of *P. sajorcaju*, *P. flabellatus* and *P. citrinipileatus* [13,16] and they reported complete inhibition of the mould fungi under in vitro and/ or in vivo. Complete inhibition of most of the competitor moulds of oyster mushroom was obtained with the application of 50 ppm benomyl + 100 ppm thiram [17,18]. In the present study the inhibition patterns of carbendazim 37.5 ppm + formalin 500 ppm against the competitor moulds *in vitro* further support the findings of Jain & Vyas [19]. Carbendazim show high affinity for tubulin protein in fungi, a heterodimetric protein with subunits as alternating helices of α and β tubulin, which forms an essential part of fungal cytoskeleton as well as are active in spindle formation. Thus it primarily acted upon cell and nuclear division of fungi which inhibited the mould growth completely on the beds and hence increased the biological efficiency of mushroom (106 %). Phyto-extract of *A. indica* (neem) showed maximum inhibition against the competitor moulds was due to the presence of

antifungal and antibacterial molecules azadirachtin, limonoids and terpinoids [20,21]. Leaf extract of *A. indica* having antifungal properties against *A. parasiticus* an aflatoxin producer [22] it's azadirachtin, and meliantriol etc. interfered with the release of ammonium during the early stages of fruiting body formation of *Coprinus* spp [23]. The above mentioned facts can be attributed to the higher biological efficiency of *P. ostreatus*. Extract of *P. pinnata* (karanj) leaf and oil contain karanjin, oleic acid, karanjic acid and their three esters, karanj ketone and its oxime derivaties. All these compounds have outstanding antifungal activity [24]. The karanj based products exhibited outstanding antifungal activity against the soil-borne phytophagous fungus like *S. rolfsii* (Sacc.) [25]. Excellent response of *P. pinnata* leaf extract against *S. rolfsii* was probably due to the detrimental effect of one or more above mentioned antifungal molecules, which not only minimize the growth of fungal contaminants but also contributed to spawn run period (17 days) and biological efficiency (92.20%). Good response of leaf extractof *E. hybrida* against *S. rolfsii* was probably due to the effect of 2', 6'-dihydroxy-3'-methyl-4'-methoxy-dihydrochalcone, eucalyptin and 8-desmethyl-eucalyptin which have strong antifungal and antibacterial activity [26, 27].

CONCLUSION

Microbial contamination of oyster mushroom bed is one of the major hindrance in increased yield of *Pleurotus* spp. under the agro-ecological condition of undulating red and lateritic belt of West Bengal, which was maximum during the rainy season. Plant extract have shown considerable promises as an effective alternatives for minimizing the infection of competitor moulds and diseases of oyster mushroom under the agro-ecological condition of lateritic belt of West Bengal,India. Leaf extract of *A. indica* (neem) showed maximum inhibitory effect against the growth competitor moulds which increased the biological efficiency of oyster mushroom (*P. ostreatus*) up to the tune of 95.10 % as against 106 0%, obtained through popular chemical method (carbendazim 37.5 ppm + formalin 500 ppm) commonly practiced in this region. *A. indica* (neem) and *P. pinnata* (karanj) are the two important perennial trees having tremendous medicinal values and found abundantly in this area. Leaf extract of these plants can be used successfully for surface sterilization of substrate during oyster mushroom cultivation which not only protect the environment and human health from hazardous effects of chemicals but also minimize the cost of cultivation.

REFERENCES

[1] Banik S and Nandi R. (2004). Effect of supplementation of rice straw with biogas residual slurry manure on the yield, protein and mineral con tents of oyster mushroom. *Ind Crops Prod*. 20: 311-319.

[2] Gregori A *et al*. (2007). Cultivation techniques and medicinal properties of *Pleurotus* spp. *Food Technol Biotechnol*. 45(3): 238-249.

[3] Fan L *et al*. (2000a). Solid state culturing-an efficient technique to utilize toxic agro-industrial residue. *J Basic Microbiol*. 40: 177-187.

[4] Fan L *et al*. (2000b). Comparison of coffee industry residues for production of *Pleurotus ostreatus* solid state fermentation. *Acta Biotechnol*. 20: 41-52.

[5] Murthy PS and Manonmani HK. (2008). Bioconversion of coffee industry wastes with white rot fungus *Pleurotus florida*. *Res. J. Environ. Sci*. 2(2): 145-150.

[6] Castle A *et al*. (1998). Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. *Appl. Environ. Microbiol*. 64(1): 133-137.

[7] Hermosa MR *et al*. (1999). Isolation of *Trichoderma harzianum* Th2 from commercial mushroom compost in Spain. *Plant Disease*. 83: 591.

[8] Mamoun ML *et al*. (2000). Interactions between the pathogen *Trichoderma harzianum* Th2 and Agaricus bisporus in mushroom compost. *Mycologia*. 92: 233-240.

[9] Shin GC. (1987). Harmful fungi associated with rice straw media for growing of oyster mushroom, *Pleurotus ostreatus*. *Kor J Mycol*. 15: 92-98.

[10] Biswas MK and Kuiry Sanjeeb. (2013). Yield performance of different species of oyster mushroom (*Pleurotus* spp.) under the agro-ecological condition of lateritic zone of West Bengal, India. *Int. J. of Bio-res. Str. Mgt.* 4(1): 043-046.

[11] Spillman A. (2002). What'skilling the mushrooms of Pennsylvania? – A mushroom mystery. *Agricultural Res*.

[12] Jaivel N and Marimuthu P. (2010). Strain improvement of *Aspergillus terrus* for increased lovastatin production. *Int. J. Eng. Sci. Technol*. 2(7): 2612-2615.

[13] Upadhyay RC *et al*. (1987). *Pleurotus* species (oyster or dhingri mushroom) Annual report of National Center of Mushroom Research and Training. pp 20-25.

[14] Grove and Moore (1962). Toximetric studies of fungicides against brown rot organisms *Sclerotia fruveticola* and *S. laxa*. *Phytopath*. 52: 876-880.

[15] Balaza S and Szabo I. (1979). Temperature requirement studies of some new mushroom species in culture. *Mush. Sci*. 10(2): 421-427.

[16] Vijay B and Sohi HS. (1987). Effect of different sterilants and farm wastes on yield of *Pleurotus citrinopileatus. Mush. J. Tropics*. 7: 67-75.

[17] Doshi A and Singh RD. (1985). Control of weed fungi and their effect on yield of oyster mushroom *Pleurotus sajor-caju. Indian J. Mycol. Plant Pathol.* 15:269-273.

[18] Sharma AD and Jandaik CL. (1983). Some preliminary observation on the occurrence of Sibrina rot of cultivated mushrooms in India and its control. *Tai. Mush*. 6 (1,2): 38-42.

[19] Jain AK and Vyas D. (2002). Yield response of *Pleurotus florida* on wheat straw in combination with other substrates. *Mush. Res*. 11(1): 19-20.

[20] Nathan SS *et al*. (2005). Effect of limonoids on the malaria vector Anopheles stephensi Liston (Ditera: Culicidae). *Acta. Trop*. 96: 47-55.

[21] Jarvis APE and Morgan D. (2000). Analysis of small samples of limonoids of neem (Azadirachta indica) using solid phase extraction from tissue culture. Photochem Anal, 11: 184-189.

[22] Allameh R *et al*. (2002). Effect of neem leaf extract on production of aflatoxin and activities of fatty acid synthetase, isocitrate dehydrogenase and glutathione S-transferase in *Aspergillus parasiticus. Mycopathologia*. 154:79-84.

[23] Morimoto N *et al*. (1981). Effect of ammonia on fruiting body induction of *Coprinus cinerius* in darkness. *Plant Cell Physiol*. 22: 247-254.

[24] Tirkey A. (2006). Some ethnomedicinal plants of family-Fabaceae of Chhattisgarh state, *Indian Journal of Traditional Knowledge*. 5(4): 551-553.

[25] Kesari Vigya *et al*. (2010). Physico-chemical characterization and antimicrobial activity from seed oil of *Pongamia pinnata*, a potential biofuel crop, *Biomass and Bioenergy.* 34, (1):108-115.

[26] Takahashi T *et al*. (2004). Antimicrobial activities of eucalyptus leaf extracts and flavonoids from *Eucalyptus maculata*. *Let. Appl. Microbiol*. 39: 60-64.

[27] Ranaware A *et al*. (2010). In vitro antifungal study of the efficacy of some plant extracts for inhibition of *Alternaria carthami* fungus. *Ind. J. Natural. Products Res*. 1(3): 384-386.

EFFECT OF COOKING ON ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT OF VARIOUS SPECIES OF EDIBLE MUSHROOMS OF INDIA

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ABSTRACT

Mushrooms are well known as medicinal foods and various health benefits are associated with dietary intake of mushrooms. Mushrooms are known for their anti-carcinogenic properties which are attributed to its antioxidant activity due to various bio-molecular components. Cooking of mushrooms has an effect on the antioxidant activity to various extents. In the present study, effect of various cooking methods on antioxidant activity by DPPH inhibition, thiobarbituric acid (TBA) reactive compounds and total phenols on common edible mushrooms of India viz., *Agaricus bisporus, Calocybe indica, Volvariella volvacea, Lentinula edodes* and *Pluerotus ostreatus* was done. It was found that antioxidant activity as DPPH inhibition in fresh mushrooms was found to be in the decreasing order as *A.bisporus, V. volvacea, C. indica, L. edodes* and *P. ostreatus.* TBA reactives were found to be in the decreasing order as *A.bisporus, V. volvacea, C.indica, P.ostreatus* and *L.edodes.* Total phenols as estimated by Folin ciocalteu assay was found to be in decreasing order as *P. ostreatus, C. indica, V. volvacea, A. bisporus* and *L. edodes*. These mushrooms were also analyzed after cooking by various methods as microwaving for 2 min, boiling in water for 5 min and stir frying in sunflower oil for 2 min. The prepared samples were analyzed for antioxidant activity, TBA reactives and total phenols. Trials were done in triplicates and results were compared statistically using t-test, it was found that antioxidant activity as DPPH inhibition in case of all the mushrooms decreased in microwave treatment as well as in boiling which can be attributed to leaching of biomolecules whereas increased slightly in stir frying of mushrooms that can be attributed to concentration of biomolecules due to frying. Similar results were observed in case of total phenols with high content measured in fried mushrooms because of higher rate of conversion to quinones during stir frying. TBA reactives decreased in all the mushrooms by all forms of cooking that indicates less of carbonyl compounds which are measured by TBA assay.

Keywords: DPPH inhibition, TBA reactives, total phenols

INTRODUCTION

Free radicals formation is a natural process associated with metabolism of cells. These free radicals can interact with macromolecules in cells like proteins, DNA and lipids, generating more reactive molecules as new radicals and lipid peroxides [1-3]. These free radicals can be damaging for the living system and are causative of many diseases such as cancer, coronary heart diseases and aging [4,5]. Antioxidants are free radical scavengers and thus limit action of free radicals on cellular biomolecules [6,7]. In food antioxidants are found as phenols, flavonoids, vitamins and certain enzymes. Their antioxidant activity is based on redox properties [8-10].

Mushrooms are known to have effective anti-cancerous, antibacterial, antiviral and immune-modulating activities [11-14]. Mushrooms, due to its inherent constituents such as phenolics, organic acids and alkaloids are used as functional foods [15-17]. Mau *et al.* [18] and Hirano *et al.* [19] have attributed the protective roles of mushroom consumption to their ability to capture metals, inhibit oxidative enzymes and scavenging free radicals.

Mushrooms are generally not consumed raw but are either cooked or processed to various culinary dishes industrially or at home. Cooking processes bring about a number of changes in physical characteristics and chemical composition of vegetables [20]. There are various studies on quantification of antioxidants in mushrooms but less work has been done on effect of cooking on antioxidant properties. The main objective of this study was to evaluate different edible mushrooms of India for antioxidant activity, TBA reactives and total phenols. *A. bisporus, P. ostreatus, C. indica, V. volvacea* and *L. edodes* were evaluated. Also effect of cooking by boiling, stir frying and microwaving on these properties was done to understand the carryover of antioxidants in mushrooms.

MATERIALS AND METHODS

Mushrooms

A. bisporus, P. ostreatus, C. indica, V. volvacea and *L. edodes* were obtained from ICAR - Directorate of Mushroom Research, Chambaghat, Solan, HP, India. The mushrooms were washed and used for analysis on the same day as harvested.

Cooking Processes

Boiling: 250 ml of water was put to boil in 500 ml beaker. 50 g of mushroom sample was added and boiled for 5 min. The sample was drained off and cooled immediately to prevent further heat damage.

Microwave cooking: 50 g mushroom sample was placed in 250 ml beaker and 100 ml water was added to it. The material was cooked in commercial microwave (Samsung) of 1400 W for 2 min. Samples were drained off and cooled immediately.

Stir frying: Mushroom sample (50 g) was fried in 10 ml of sunflower oil in a non-stick frying pan on a commercial induction cook top (Prestige) set at 180 ^{o}C for 2 min. Sample were then put on chilled stainless steel plate for rapid cooling.

Analytical methods

Raw and cooked mushroom samples were crushed in a blendor (maxTUFF) for 1 min and analyzed for antioxidant activity (as % DPPH inhibition), TBA reactives and total phenols.

Determination of antioxidant activity

Determination of antioxidant activity of sample was done by 2,2-diphenyl-2-picryl-hydrazyl (DPPH) inhibition method [21]. Sample (1 g) was taken in 10 ml ethanol and was kept overnight for extraction. This eluted extract was taken (0.2 ml) and to it 1ml of DPPH solution (80 µg/ml ethanol) was added. A control was set up with 0.2 ml distilled water as blank and left at room temperature for 30 min. The sample sets were centrifuged at 3000 rpm for 15 min (Sigma laboratory centrifuge 3 K 18, Germany). In cuvette 0.5 ml of centrifuged solution was taken and to it 1ml of ethanol was added. Absorbance was taken at 517 nm separately for blank and samples with pure ethanol as reference using PerkinElmer UV/VIS spectrometer Lambda 25, Germany.

$$\% \text{ DPPH inhibition} = (A_B - A_S / A_B) \times 100$$

Where A_B = OD for blank

A_S = OD for sample

Determination of total phenols

The total phenolic content was determined using Folin-Ciocalteu (FC) reagent, as given by Singleton and Rossi [22] with some modifications. 1 g sample was kept overnight for extraction with 10 ml of 50% aqueous methanol. The mixture was centrifuged at 10000 rpm for 15 min. 0.5 ml of centrifuged supernatant was added to test tube containing 5 ml FC reagent (10% aqueous solution) and 4 ml aq. sodium carbonate. The tubes were held for 15 min and were then analyzed by spectrometry for absorbance at 765 nm. Results were expressed as mg gallic acid equivalents/ L extract.

Determination of TBAreactives

The method of Ottolenghi [23] was referred. 2 ml of 20% trichloroacetic acid and 2 ml of .67% 2-thiobarbituric acid was added to 1 ml of ethanolic extract of sample solution, as prepared for DPPH method. The mixture was placed in boiling water bath for 30 min and was then cooled and centrifuged at 3000 rpm for 20 min. Absorbance of supernatant was measured at 552 nm.

Statistical analysis

The results are presented as average values and standard error of triplicate readings. The data was analyzed for significance differences between raw and cooked samples by *t* test for means ($p<0.05$).

RESULTS AND DISCUSSION

Five cultivated mushroom species (*A. bisporus, P. ostreatus, C. indica, V. volvacea* and *L. edodes)* were evaluated for their antioxidant activity, TBA reactives and total phenolic content. The analysis was carried out using entire mushroom fruit body. The data is presented in Table 1. Figure 1 shows the effect of cooking on antioxidant ativity, TBA reactives and total phenols in mushrooms.

Effect of cooking methods on antioxidant activity of mushrooms

Anioxidant activity as % DPPH inhibition in mushroom samples was found to be in decreasing order as *A. bisporus*> *V. volvacea*>*C. indica*>*L. edodes*>*P. ostreatus*. The corresponding values are mentioned in Table 1. Amongst these *A. bisporus* displayed maximum radical scavenging activity with DPPH inhibition at 59.33% whereas least activity was found to be associated with *P. ostreatus* at 21.36%. . The antioxidant activity in different varieties of mushrooms before and after cooking is shown in Fig. 1a. Though antioxidant activity declined in microwaved and boiled samples, difference was not statistically significant ($p> 0.05$). It was only found to be significant decrease in boiled and microwaved sample of *C. indica*, where the retention was 71.11 and 73% respectively. This indicates leaching of antioxidant constituents of mushrooms during boiling and microwave cooking treatments. Also cell structure damage during heating might cause release of potent radical scavengers. Zhang and Hamauzu [20] also demonstrated decrease in antioxidant activity of broccoli after aqua-thermal treatment. Puupponen-Pimia *et al*., [24] also demonstrated that DPPH inhibition by cauliflower decreased by 23% during blanching. Conversely Turkmen *et al.*, [25] reported that boiling, microwave heating and steaming increases antioxidant activity in pepper, spinach and green beans. It was interesting to note that antioxidant activity increased in case of fried samples though not significantly except in case of *P. ostreatus* and *L. edodes* where increase was found to be 157.25 and 144.03%, respectively. This increase can be attributed to moisture loss during frying leading to concentration on biomolecules. Also heating inactivates oxidative enzymes thereby increasing antioxidant activity in fried samples. It was also observed that either no change or slight improvement in antioxidant activity was found in stir fried samples of colored pepper and paprika [26].

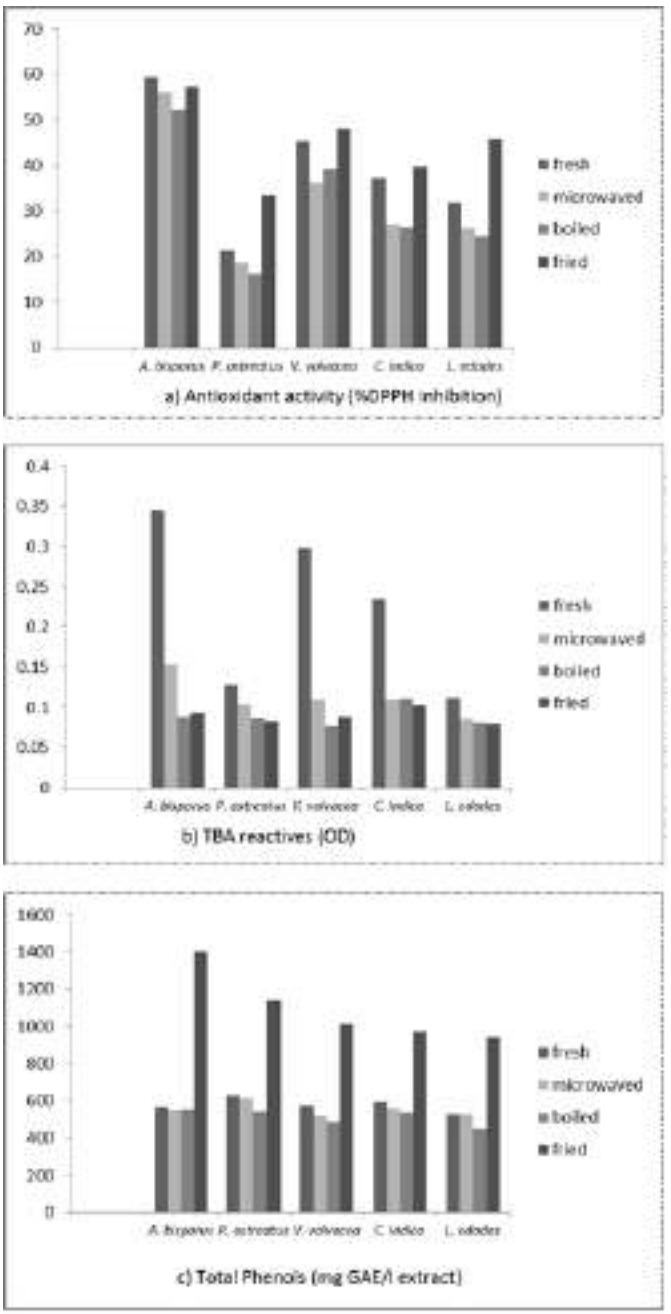

Figure 1. a) Antioxidant activity (% DPPH inhibition), b) TBA reactives, c) Total phenols (mg GAE/L extract) of *A. bisporus, C. indica, V. volvacea, L. edodes* and *P. ostreatus* in fresh and cooked samples

Effect of cooking methods on TBA reactives of mushrooms

At later stages of oxidation peroxides decomposes to carbonyl compounds that are measured by TBA method and is principally used to measure lipid oxidation. The TBA activity in mushrooms on day 1 were found to be in decreasing order as *A. bisporus*>*V. volvacea*>*C. indica*>*P. ostreatus*>*L. edodes*. The TBA reactives in different varieties of mushrooms before and after cooking is shown in Fig. 1b. Cooking of all these mushroom species lead to decrease in TBAreactives significantly (p>0.05) with maximum decrease in fried samples followed by boiled and least depreciation in microwaved samples that shows decreased level of oxidation in fried samples. This pattern can be attributed to inactivation of oxidative enzymes due to heat treatment in cooking processes and also to initial low lipid content of mushrooms. In *A .bisporus* microwave treatment, boiling and frying demonstrated 55.53, 74.42 and 73.26% decreased TBA reactives in mushroom samples. This result is inverted from TBA activity of cooked meat samples that demonstrate increase in TBA reactives accumulation on cooking [27]. Conversely, Du and Li [28] concluded that when cooking increases proteins can react with TBA solution, reducing TBA values. The TBAreactives values and corresponding percentage retention on cooking by various methods is mentioned in Table 1.

Table 1. Mean values of antioxidant activity, TBA reactives and total phenols of common cultivated mushrooms of India (*A. bisporus, C. indica, V. volvacea, L. edodes* and *P. ostreatus*) in fresh and cooked samples

S.No	Mushroom	Processing	Antioxidant activity		TBAreactives		Total phenols	
			(% DPPH inhibition)	% retention	(OD)	% retention	(mg GAE/ L extract.)	% retention
1	*A.bisporus*	Fresh	59.33	100	0.344	100	567.90	100
2		Microwaved	56.15	94.64	0.153	44.47	548.82	96.64
3		Boiled	52.28	88.11	0.088	25.58	554.43	97.62
4		Fried	57.33	96.62	0.092	26.74	1399.55	246.44
5	*P.ostreatus*	Fresh	21.36	100	0.128	100	626.26	100
6		Microwaved	18.6	87.07	0.103	80.46	612.99	97.88
7		Boiled	16.23	75.98	0.086	67.18	542.08	86.55
8		Fried	33.59	157.25	0.082	64.06	1140.29	182.07
9	*V. volvacea*	Fresh	45.33	100	0.297	100	572.89	100
10		Microwaved	36.31	80.10	0.109	36.70	520.67	90.88
11		Boiled	39.2	86.47	0.076	25.58	490.24	85.57
12		Fried	47.94	105.75	0.088	29.62	1011.56	176.47
13	*C. indica*	Fresh	37.08	100	0.234	100	596.07	100
14		Microwaved	27.07	73.00	0.108	46.15	560.04	93.95
15		Boiled	26.37	71.11	0.11	47.00	537.59	90.18
16		Fried	39.86	107.49	0.102	43.58	970.03	162.73
17	*L. edodes*	Fresh	31.86	100	0.111	100	531.98	100
18		Microwaved	26.19	82.20	0.085	76.57	528.6	99.36
19		Boiled	24.43	76.67	0.081	72.97	451.06	84.78
20		Fried	45.89	144.03	0.079	71.17	943.09	177.27

Effect of cooking methods on total phenols of mushrooms

Total phenolic content of mushrooms was measured as mg equivalents of gallic acid, which was found to decrease in order as *P. ostreatus*>*C. indica*>*V. volvacea*>*A. bisporus*>*L. edodes*. Total phenols in fresh mushrooms were found to be ranging from 626.26 to 531.98 mg GAE/ L extract The total phenolic content in different varieties of mushrooms before and after cooking is shown in Fig 1c. Results show that, after cooking phenolic content decreased very slightly in microwave heated and boiled samples of all the mushrooms and the decline is not statistically significant (p>0.05). This can be attributed to inactivation of polyphenoloxidases due to heat treatment thereby hindering phenol degradation. It has been reported that boiling, microwaving or further warm holding did not affect the level of polyphenols in green beans and onions. On the other hand frying lead to significant increase in phenolic content of all the mushroom samples. Stewart *et al.* [29] reported that heat treatment increased the level of free flavanols. Turkmen *et al*. [25] also concluded that cooking leads to increase in phenolic content in vegetables.

CONCLUSION

In general, it can be concluded that frying does not affect antioxidant activity but boiling and microwave cooking depletes radical scavenging ability of mushrooms. Thus it is also vital to consume water used for cooking mushrooms so that leaching losses can be minimized from diet. All the cooking methods demonstrated decrease in TBA reactives thereby indicating inactivation of lipid oxidizing enzymes by heat. Phenolic content was not much affected by boiling or microwaving but showed exemplary increase in fried mushroom samples indicating stir frying as a cooking option might be useful in improving health properties of mushrooms.

REFERENCES

[1] Torel *et al.* (1986). Antioxidant activity of flavonoids and reactivity with peroxy radical. *Phytochemistry*. 2: 383-385.

[2] Pryor *et al.* (1982). The production of free radicals *in vivo* from the action of xenobiotics: the initiation of autooxidation of polyunsaturated fatty acids by nitrogen dioxide and ozone. In: Lipid peroxides in Biology and Medicine, pp 1-22, New York: Academic Press.

[3] Gyamfi *et al.* (2002). Free radical scavenging action of medicinal herbs from Ghana Thonningia sanguinea on experimentally induced liver injuries. *Pharamacol*. 32: 661-667

[4] Keys A. (1995). Mediterranean diet and public health personal reflection. *Amer. J. Clin. Nutr.* 61: 1321S-1323S.

[5] Lipworth *et al.* (1997). Olive oil and human cancer: an assessment of evidence. *Prev. Medicine*. 26: 181-190.

[6] Halliwell B. (1997). Antioxidants in human health and disease. *Ann. Rev. Nutr.*16: 33-50.

[7] Hu FB. (2000). Dietary pattern analysis: a new direction in nutritional epidemiology. *Curr. Opin. Lipid.* 13: 3-9.

[8] Bors W and Saran M. (1987). Radical scavenging by flavonoid antioxidants. *Free Radical Res. Communications*. 2: 289-294.

[9] Visioli *et al.* (1998). Free radical scavenging properties of olive oil polyphenols. *Biochem. Biophys. Res. Communications*. 247: 60-64.

[10] Nakayama *et al.* (1993). Suppression of active oxygen-induced cytotoxicity by flavonoids. *Biochem. Pharmacol*. 45: 265-267.

[11] Wasser SP and Weis AL. (1999). Medicinal properties of substances occurring in higher Basidomycetes mushrooms: current perspective (review). *Intern. J. Medicinal Mushrooms*. 1: 31-62.

[12] Yang *et al.* (2002). Antioxidant properties of several commercial mushrooms. *Food Chem.* 77: 229-235.

[13] Ribeiro *et al.* (2007). Phenolic compounds, organic acids profiles and antioxidative properties beefsteak fungus (*Fistulina hepatica*). *Food and Chemical Toxicol*. Doi:10.1016/j.fct.2007.03.015.

[14] Ribeiro *et al.* (2006). Contents of carboxylic acids and two phenolics and antioxidant activity of dried Portuguese wild edible mushrooms. *J. Agric. Food Chem.* 54: 8530-8537.

[15] Bendini *et al.* (2006). Phenol content related to antioxidant and antimicrobial activities of *Passiflora* spp. extracts. *Euro. Food Res. Technol.* 223: 102-109.

[16] Mato *et al.* (2003). Significance of nonaromatic organic acids in honey. *J. Food Protect.* 66: 2371-2376.

[17] Quezada. *et al*. (2004). Antioxidant activity of crude extract, alkaloid fraction and flavonoid fraction from boldo (Peumus boldus Molina) leaves. *J. Food Sci*. 69: 371-376.

[18] Mau *et al*. (2004). Antioxidant properties of methanolic extracts from *Grifola frondosa*, *Morchella esculenta* and *Termitomyces albuminosus* mycelia. *Food Chem*. 87: 111-118.

[19] Hirano *et al*. (2001). Antioxidant ability of various flavonoids against DPPH radicals and LDL oxidation. *J. Nutr. Sci. Vitaminol. (Tokyo)*.47: 357-362.

[20] Zhang D and Hamauzu Y. (2004). Phenolics, ascorbic acid, carotenoids and antioxidant activity of broccoli and their changes during conventional and microwave cooking. *Food Chem*. 88: 503-509.

[21] Nishino *et al*. (2000). Transit of radical scavenging activity of milk products prepared by Millard reaction and *Lactobacillus* caseiistrain shirota fermentation through the hamster intestine. *J. Dairy Sci*. 83: 915-922.

[22] Singleton VL and Rossi JA Jr. (1965). Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Amer. J. Enol. Viticul*. 16: 144-158.

[23] Ottolenghi A. (1959). Interaction of ascorbic acid and mitochondria lipids. *Arch. Biochem. Biophy*. 79: 355-363.

[24] Puupponen-Pimea *et al*. (2003). Blanching and long term freezing affect various bioactive compounds of vegetables in different ways. *J. Sci. Food Agric*. 83: 1839-1402.

[25] Turkmen *et al*. (2005). The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chem*. 93: 713-718.

[26] Chuah *et al*. (2008). Effect of cooking on the antioxidant properties of coloured peppers. *Food Chem*. 111: 20-28.

[27] Fasseas *et al*. (2007). Antioxidant activity in meat treated with oregano and sage essential oils. *Food Chem*. 106: 1188–1194.

[28] Du H and Li H. (2008). Antioxidant effect of Cassia essential oil on deep-fried beef during the frying process. *Meat Sci*. 78: 461–468.

[29] Stewart *et al*. (2000). Occurrence of flavanols in tomatoes and tomato based products. *J. Agric. Food Chem*. 48: 2663-2669.

IMPROVEMENT IN NUTRITIONAL AND THERAPEUTIC PROPERTIES OF DAILY MEAL ITEMS THROUGH ADDITION OF OYSTER MUSHROOM

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ABSTRACT

Mushroom is the choicest food of nutritionists because of its hypolipidemic, hypocholesterolemic, hypoglycaemic, antitumor properties. Keeping in view unique chemical composition of mushroom, it was incorporated in daily food items. Fresh as well as dry mushrooms were incorporated at 50 per cent and 10 per cent level, respectively. Ingredients, their proportion and procedure of each control and experimental food items were standardized and the items were studied for their acceptance on a nine point Hedonic Scale by a panel of ten judges on a three consecutive days. Nutritive value was calculated and therapeutic use was reviewed. The study showed that mushroom is a suitable food for incorporation in breakfast, lunch, dinner and snacks food items. It can be used in sweet as well as salty and spicy foods. All the mushroom added foods were acceptable. Addition of mushrooms improves the nutritional quality and therapeutic properties as well.

Keywords: oyster mushroom, therapeutic foods, daily meals

INTRODUCTION

A large segment of our population requires attention and assistance to formulate health promoting recipes and diets, which are region and culture specific. Number of attempt have been made to develop nutritious recipes, especially out of Protein Concentrates like fish, leaf, milk, whey milk, soy, etc [1-6]. Still planners and nutritionist are thinking about the alternate source of protein in India because traditional sources of protein foods have not been keeping pace with the population growth. Next to protein, our diets are grossly deficient in micronutrients. Therefore, protein and micronutrients fortification is an important area of interest. For this purpose nutritionists are looking for ingredients of value addition especially, which impart specific nutritional properties. Mushrooms have been recognised as the alternate source of protein since they contain 25-35 per cent protein (dry weight basis) which is quite higher than vegetable and animal sources and the quality of protein is nearly as good as animal protein because of presence of all essential amino acids [7-8]. Mushrooms are good source of B vitamins i.e. thiamine, riboflavin and niacin. They are good source of potassium, phosphorus, magnesium and contain low but available form of iron.

Keeping in view the nutritional properties of oyster mushrooms, and increasing awareness for nutrition, health and quality of food consciousness, and because the demand of dietetic or therapeutic foods are increasing for the prevention and management of disease, mushroom was substituted with suitable ingredient of traditional Indian daily meal recipes in order to get optimal acceptability. Care was taken to maintain the original nutritional composition of mushrooms while formulating of mushroom recipes.

MATERIALS AND METHODS

Series of experiments were carried out for the development of food products. Developed products out of mushroom were then compared with the control (traditional) ones to find out the difference in the sensory and nutritional characters of experimental food products.

Selection of daily meal items and their standardization

From the Indian cuisine, twelve lunch and twelve breakfast and snacks items were considered. Fresh as well as dry oyster mushroom (*Pleurotus sajor caju)* was used in the food preparations as shown in Table 1A and 1B. In the experimental products fresh (50 g) and dry mushroom powder (10 g) was incorporated since this level was found acceptable in earlier studies [9-11]. The proportion of other ingredients and procedure of food preparations selected was standardized to get uniform product.

Sensory evaluation of standardized daily meal items

Human sensory perception has been the only basis of assessment of the product quality. Therefore, after recipe standardization and process standardization, the sensory attributes of the standardise food were studied using a score card of nine point Hedonic Scale with scale ranging from like-extremely to dislike-extremely [12]. The scores were allotted in ascending order that the start from like extremely (1) and end to dislike extremely. Necessary characters of individual recipe were included in the score card. All the 24 recipes, control (traditional) and experimental were prepared for a three consecutive days and subjected to sensory evaluation. Then overall acceptability mean score was calculated by considering the scores of sensory attributes evaluated (appearance, texture, taste, etc.) along with the standard deviation. Further statistical test (*t*-test) was not applied because visually, there was very minor difference in the mean scores of control and experimental preparations and the result was on the acceptable or favourable side of the scale.

Nutritional contribution of daily meal items

Nutritive values of food ingredients were calculated with the help of food value Table [13]. Since the nutritive value of oyster mushroom is not given in food tables, other sources were use [8, 14]. The available values of macronutrients (protein, fat, carbohydrate), micronutrients thiamine, riboflavin and niacin minerals (potassium, phosphorous, magnesium, sodium, calcium and iron) and essential amino acids (leucine, isoleucine, lysine, tryptophan, methionine, valine and phenylalanine) were taken for the study.

RESULT AND DISCUSSION

Sensory evaluation of daily meal items

Sensory perception assessment of preparations under study was done in quantitative terms by rating of a panel of judges with the use of Hedonic Scale. The results obtained are given in Table 1A and 1B.

Table 1A. Mean scores of overall acceptability of main meal food items

Sr. No.	Food Items		Control Food M ± S.D.	Experimental Food M ± S.D.	Remark
	English Name	Traditional Name			
1.	Indian Wheat Bread	*Phulka*	2.0(± 0.03)	2.3(± 0.09)	Like very much
2.	Indian Wheat Bread	*Chapati*	2.2(± 0.02)	2.4(± 0.08)	Like very much
3.	Indian Mix Grain and Vegetable Bread	*Missi Roti*	1.9(± 0.04)	2.4(± 0.02)	Like very much
4.	Indian Mix Grain Preserved Bread	*Khakara*	2.5(± 0.04)	2.8(± 0.03)	Like very much to like moderately
5.	Indian Pearl Millet Bread	*Bajara Roti*	3.3(± 0.08)	2.8(± 0.07)	Like moderately
6.	Indian Sorghum millet Bread	*Jawar Roti*	3.0(± 0.08)	3.3(± 0.08)	Like moderately
7.	Indian Maize Bread	*Makai Roti*	3.6(± 0.08)	4.0(± 0.08)	Like slightly
8.	Tomato Soup	*Sar/ Kadhi*	2.1(± 0.07)	2.0(± 0.08)	Like very much
9.	Green Salad	*Koshimbir/ Kachumbar*	2.0(± 0.02)	2.1(± 0.01)	Like very much
10.	Curd with Vegetables	*Raita*	2.4(± 0.06)	2.2(± 0.09)	like very much
11.	Capsicum fry	*Shimla Mirch Subji*	1.5(± 0.02)	1.2(± 0.07)	Like extremely
12.	Ladies Finger fry	*Bhendi Sabji*	1.2(± 0.07)	1.0(± 0.09)	Like extremely

Table 1B. Mean scores of overall acceptability of breakfast and snacks food items

Sr. No.	Food Items		Control Food M ± S.D.	Experimental Food M ± S.D.	Remark
	English Name	**Traditional Name**			
1.	Wheat broken porridge	*Suji Upma*	2.1 (± 0.07)	2.3 (± 0.09)	Like very much
2.	Rice pan cake (flat round)	*Uttappa*	1.5 (± 0.06)	1.2 (± 0.08)	Like extremely
3.	Rice pan cake (ball shape)	*Appe*	1.3 (± 0.07)	1.1 (± 0.07)	Like extremely
4.	Tomato Omlete	*Chilla*	2.0 (± 0.04)	1.8 (± 0.06)	Like very much
5.	Sandwich	*Sandwich*	2.8 (± 0.03)	2.5 (± 0.02)	Like very much to like moderate range
6.	Indian Pasta with vegetables	*Sevai Upama*	1.9 (± 0.04)	2.0 (± 0.03)	Like very much
7.	Wheat Gruel	*Ambil/Pej*	2.8 (± 0.04)	2.4 (± 0.03)	Like very much to like moderate range
8.	Vegetable pan cakes	Cutlet	1.2 (± 0.07)	1.0 (± 0.04)	Like very much
9.	Indian Pasta dessert	*Sewai Kheer*	2.3 (± 0.01)	2.7 (± 0.07)	Like very much to like moderate range
10.	Mix grain ready to eat flour	*Sattu*	2.7 (± 0.03)	2.7 (± 0.07)	like moderate
11.	Wheat biscuit	Biscuit	2.1 (± 0.04)	2.4 (± 0.04)	Like very much
12.	Puffed amaranth seeds dessert	*Rajgeera Chikki*	2.3 (± 0.07)	2.0 (± 0.06)	Like very much

Table 1A and 1B reveal the mean scores of overall acceptability of control and mushroom incorporated experimental daily meal items. The scores of control and experimental preparations range between 1 to 4 indicating that all the preparations are on the positive side of the scale (like extremely to like slightly). The reason behind getting the acceptability was that the daily meal items were familiar ones and there was little variation in the organoleptic attributes in control and experimental preparations.

Table 1A indicates that experimental food like, *bhindi* fry and capsicum fry were liked extremely. Whereas, tomato soup, green salad, curd with vegetables and Indian mix grain vegetable bread, wheat breads were liked very much by the judges. Indian preserve bread, Indian sorghum bread and Indian pearl millet breads fall in liked moderately and Indian maize bread in like slightly category. Mean scores of breakfast and snacks food items indicated in Table 1B ranges between liked moderately and liked extremely category. This shows that all the experimental daily meal items are acceptable. Rice pan cake (*Uttapa* and *appe*) were liked extremely by the judges followed by broken wheat with vegetables, tomato omlet, vegetable pan cake, Indian pasta dessert, *Amaranthus* dessert and wheat biscuit. Ready to eat flour (*Sattu*), gruel (*ambil*), sandwich were rated in liked moderately category by the judges. It was found that mushrooms are best suited to the recipes which require less time for cooking e.g. pan cakes. Evaluation of sensory attributes shows that addition of mushroom does not have much effect on acceptability of food preparations. Similar results were found by the investigator in previous studies [9-11]. Moreover, inclusion of mushroom gives variety to the diet.

Nutritional composition of daily meal items

Nutritional composition of food is an important criterion in determining quality of the product. The composition depends on the ingredients used in the preparations. In this study most of the food preparations were made out of cereals and mushroom was incorporated in them.

Macronutrient composition of daily meal items: Macronutrient composition is a recognized system of the assessment of the nutritional value of food items. The calculated protein, carbohydrate and fat values of food preparations under study are given along with fibre, energy values in Table 2A and 2B. The values presented are derived out of 100 g of total ingredients. Substitution of 10 g of mushroom powder provides 2.66 g protein, 5.07 g carbohydrates, 0.2 g fat, 1.33 g fibre. Ten gram dry mushrooms were obtained from 100 g fresh mushroom (as they contain 90% moisture). In some preparation 50 g fresh mushrooms were replaced by main ingredient. About 50 g fresh mushrooms provide half part of the protein, fat, carbohydrate, fibre derived from 10 g of dry mushroom. Substitution of mushroom changes the macro and micro nutrient composition of recipes as shown in Table 2A and 2B.

Table 2A. Macronutrient in daily meal items with addition of 10g dry mushroom

S. No.	Meal Item	Sample	Protein(g)	Fat(g)	Carbohydrates(g)	Fibre(g)
1.& 2.	Indian Wheat Bread	C	12.10	01.70	69.40	1.90
		E	13.46(+1.36)*	01.70(0.0)	67.47(-1.93)	**3**.03(+1.13)
3.	Indian Mix Grain and	C	10.44	06.85	49.10	2.53
	Vegetable Bread	E	11.89(+1.45)	06.70(-0.15)	47.27(-1.83)	2.97(+0.44)
4.	Indian Mix Grain	C	11.39	06.97	51.90	1.76
	Preserved Bread	E	12.84(+1.45)	06.92(-0.05)	50.07(-1.83)	2.90(+1.14)
5.	Indian Pearl Millet Bread	C	11.60	05.00	67.50	1.20
		E	13.06(+1.46)	04.70(-1.36)	66.32(-1.18)	2.41(+1.21)
6.	Indian Sorghum millet Bread	C	10.40	01.90	72.60	1.44
		E	12.06(+1.66)	01.90(0.0)	70.30(-2.30)	2.77(+1.33)
7.	Indian Maize Bread	C	11.10	03.60	66.20	2.40
		E	12.34(+1.24)	03.40(-0.20)	64.67(-1.53)	3.73(+1.33)
8.	Mix grain ready to eat flour	C	09.70	00.90	78.00	0.70
		E	11.16(+1.46)	00.90(0.0)	76.07(-1.03)	1.93(+1.23)
9.	Puff Amaranth seeds Dessert	C	12.10	09.00	74.00	1.50
		E	13.16(+1.06)	08.80(-0.20)	72.00(-2.00)	3.13(+1.63)
10.	Wheat Biscuit	C	04.50	30.48	58.80	0.09
		E	05.83(+1.33)	30.36(-0.12)	54.90(-3.80)	0.57(+0.48)

C- Control, E- Experimental
*Values in parenthesis indicated the change in value over control

Tables 2A and 2B reveal that in general there is increase in protein and fibre value and decrease in carbohydrate and fat in the experimental foods. Substitution of 10 per cent dry mushroom in breads increases 1.06 to 1.66 per cent protein. In vegetable preparation (Tomato soup, green salad, curd with vegetable, capsicum fry and ladies finger fry) 50 g fresh mushroom was substituted with basic vegetable used. Addition of mushroom certainly gives variety in flavour, texture and appearance but there was very little change in the protein value. The difference in fat values of control and experimental preparation is very minute because mushrooms contain very little fat (0.2% in fresh mushroom). Dry oyster mushrooms contain 52 per cent and cereals have 67 per cent to 77 per cent carbohydrates. Cereal carbohydrates are mostly made up of starch but mushroom carbohydrates are starch free. They comprise fungus cellulose trehalose (the mushroom sugar), sugar alcohol, mannitol. Inclusion of 10 per cent dry mushroom supplies 5 per cent carbohydrates which are not nutritionally important so far as calories are concerned. Therefore, carbohydrates contributed by mushrooms give a small relief of calories to the experimental recipes. The fibre content of mushrooms is good on dry weight basis. Incorporation of mushroom even though in small amount supplies 1.3 per cent additional fibre. Remaining preparations were vegetable based in which fresh mushrooms were included. Fresh mushrooms have 1.3 per cent fibre therefore inclusion of 50 g mushroom supplied 0.65 per cent fibre. This amount is similar to the amount of fibre supplied by the vegetables (1.2%). Therefore, there is negligible difference in the values of fibre of control and experimental food items.

Table 2B. Macronutrient in daily meal items with addition of 50 g fresh mushroom

S. No.	Meal Item	Sample	Protein(g)	Fat(g)	Carbohydrates(g)	Fibre(g)
11.	Tomato Soup	C	01.40	10.58	06.60	0.61
		E	02.30(+0.9)*	10.58(0.0)	07.40(-1.03)	0.82(+0.21)
12.	Green Salad	C	01.80	01.10	05.80	1.13
		E	02.05(+0.25)	01.10(0.0)	05.20(-0.60)	1.11(-0.02)
13.	Curds with Vegetables	C	02.60	03.10	05.00	0.46
		E	03.90(+1.30)	03.04(-0.06)	03.64(-1.36)	0.24(-0.22)
14.	Capsicum Fry	C	01.90	11.18	05.88	2.32
		E	02.54(+0.64)	10.60(-0.58)	04.34(-1.54)	2.43(+0.11)
15.	Ladies Finger Fry	C	01.49	10.17	05.16	0.98
		E	01.73(+0.24)	10.17(0.0)	04.86(-0.30)	0.98(0.0)
16.	Wheat Broken Vegetable Porridge	C	10.00	11.40	50.00	5.90
		E	10.08(+0.08)	11.30(-0.10)	49.60(-0.40)	6.30(+0.40)
17.	Rice Pan Cake (Flat Round)	C	12.80	10.75	38.30	0.76
		E	13.19(+0.39)	10.70(-0.05)	36.68(-1.62)	1.06(+0.30)
18.	Rice Pan Cake (Ball Shape)	C	07.40	10.45	47.10	0.50
		E	07.79(+0.39)	10.40(-0.05)	45.40(-1.70)	0.80(+0.30)
19.	Tomato Omlet	C	13.00	13.46	37.30	1.36
		E	14.12(+1.12)	13.45(-0.05)	37.70(+0.40)	1.36(0.0)
20.	Sandwich	C	05.00	16.40	33.50	0.10
		E	06.30(+1.30)	12.50(-3.90)	31.00(-2.50)	0.76(+0.66)
21.	Indian Pasta Dessert	C	07.50	10.60	49.60	1.03
		E	06.80(-0.7)	10.54(-0.06)	44.30(-5.30)	0.70(-0.33)
22.	Wheat Gruel	C	04.30	17.80	26.90	1.40
		E	04.87(+0.57)	15.90(-1.90)	24.30(-2.60)	1.50(+0.10)
23.	Vegetables Pan Cake	C	04.60	10.33	58.00	1.93
		E	04.87(+0.27)	10.23(-0.10)	58.00(0.0)	1.03(-0.90)
24.	Indian Pasta with Vegetables	C	07.50	10.06	49.60	1.03
		E	08.20(+0.7)	10.00(-0.06)	44.30(-5.30)	0.70(-0.33)

C- Control, E- Experimental

*Values in parenthesis indicated the change in value over control

Essential amino acids content of daily food items: In general animal protein are considered superior and vegetable protein are considered inferior since it is deficient in one or the other essential amino acids. Lysine is a limiting amino acid of cereals and methionine is limiting amino acid of pulses. Therefore, vegetarian diet needs a good combination or supplementation of food to improve protein quality. As mushrooms are recognized for the essential amino acids, therefore, these are suitable food for supplementation or rather a good food for the combination with vegetable food items. In the present investigation, therefore, attempts were made to combine mushroom with other vegetable foods and essential amino acids were calculated. Calculated values are given in Table 3A and 3B. It is revealed from the Table 3A and 3B that all the values of essential amino acids of experimental food items are higher than control samples. It shows that food items 1 to 10 are rich in amino acids as dry mushroom was incorporated in them. Remaining preparation based on vegetable are low because vegetables have 90 per cent moisture. Lysine is a limiting amino acid of cereal and mushrooms are good source of it. Therefore, all the lysine values of experimental food items were more than the control samples. All these experimental items provide half to three forth of the daily requirement of lysine of adults. Methionine, a sulphur containing amino acid also plays important role in maintaining the health of liver. This is a limiting amino acid of pulses. Incorporation of mushroom in food item shows increase in methionine in liberal amount.

Table 3A. Essential amino acids (mg) in daily meal items with addition of 10 g dry mushroom

Sr. No.	Meal Items	Sample	Leucine	Isoleucine	Valine	Tryptophan	Lysine	Threonine	Phenylalanine	Methionine
1.&2.	Indian Wheat Bread	C	0775	416	529	132	321	340	529	170
		E	0873 (+98)*	463 (+45)	609 (+80)	149 (+17)	431 (+110)	431 (+91)	602 (+83)	198 (+28)
3.	Indian Mix Grain and Vegetable Bread	C	0682	395	458	107	367	335	469	140
		E	0788(+106)	464 (+69)	538 (+80)	124 (+17)	477 (+110)	390 (+55)	542 (+73)	168 (+28)
4.	Indian Mix Grain Preserved Bread	C	0651	360	424	099	335	314	437	130
		E	0747 (+96)	429 (+69)	504 (+80)	116 (+17)	450 (+125)	369 (+55)	510 (+73)	158 (+28)
5.	Indian Pearl Millet Bread	C	1395	484	614	205	353	446	539	279
		E	1430 (+35)	546 (+62)	685 (+71)	215 (+10)	460 (+107)	526 (+80)	610 (+71)	296 (+17)
6.	Indian Sorghum millet Bread	C	1460	448	564	116	249	349	498	166
		E	1489 (+29)	513 (+65)	640 (+76)	134 (+18)	366 (+117)	439 (+90)	573 (+75)	194 (+28)
7.	Indian Maize Bread	C	1282	427	534	071	356	498	516	214
		E	1329 (+47)	494 (+64)	612 (+78)	094 (+23)	462 (+106)	573 (+75)	589 (+73)	238 (+24)
8.	Mix grain ready to eat flour	C	0599	364	393	095	145	191	393	198
		E	0697 (+98)	432 (+68)	409 (+16)	119 (+24)	253 (+108)	282 (+91)	465 (+72)	226 (+28)
9.	Puff Amaranth seeds Dessert	C	0524	378	321	100	470	336	481	157
		E	0659 (+135)	467 (+84)	430 (+119)	125 (+25)	580 (+110)	434 (+88)	586 (+105)	192 (+35)
10.	Wheat Biscuit	C	0315	171	189	048	087	117	144	072
		E	0332 (+17)	186 (+15)	213 (+24)	052 (+04)	138 (+51)	159 (+42)	174 (+30)	078 (+06)

C- Control, E- Experimental

*Values in parenthesis indicated the change in value over control

Table 3B. Essential amino acids (mg) in daily meal items with addition of 50 g fresh mushroom

Sr. No.	Meal Items	Sample	Leucine	Isoleucine	Valine	Tryptophan	Lysine	Threonine	Phenylalanine	Methionine
11.	Tomato Soup	C	110	124	098	014	070	067	074	030
		E	167 (+57)*	110 (-14)	123 (+25)	026 (+12)	117 (+47)	101 (+34)	123 (+49)	044 (+14)
12.	Green Salad	C	106	115	099	021	083	067	081	026
		E	135 (+29)	126 (+11)	101 (+02)	024 (+03)	098 (+15)	093 (+36)	099 (+18)	035 (+09)
13.	Curds with Vegetables	C	223	130	189	032	190	125	133	029
		E	363 (+140)	158 (+28)	221 (+32)	040 (+08)	230 (+40)	156 (+31)	177 (+44)	043 (+14)
14.	Capsicum Fry**	C	-	-	-	-	-	-	-	-
		E	-	-	-	-	-	-	-	-
15.	Ladies Finger Fry	C	054	034	043	007	047	032	034	018
		E	106 (+52)	066 (+32)	081 (+38)	019 (+12)	087 (+40)	072 (+40)	074 (+40)	028 (+10)
16.	Wheat Broken Vegetable Porridge	C	596	346	397	096	304	236	401	122
		E	626 (+30)	360 (+14)	413 (+16)	098 (+02)	366 (+62)	252 (+16)	469 (+68)	132 (+10)
17.	Rice Pan Cake (Flat Round)	C	949	634	596	138	737	418	598	176
		E	1030 (+81)	682 (+48)	648 (+52)	144 (+06)	779 (+42)	472 (+54)	642 (+44)	192 (+16)
18.	Rice Pan Cake (Ball Shape)	C	323	347	207	083	142	169	225	204
		E	407 (+84)	393 (+56)	261 (+54)	090 (+07)	184 (+42)	402 (+233)	369 (+144)	220 (+16)
19.	Tomato Omlet	C	968	548	527	093	733	375	602	136
		E	1006 (+48)	560 (+02)	557 (+30)	105 (+12)	783 (+50)	412 (+37)	644 (+42)	150 (+14)
20.	Sandwich	C	-	-	-	-	-	-	-	-
		E	-	-	-	-	-	-	-	-
21.	Indian Pasta Dessert	C	442	248	308	075	217	204	301	095
		E	462 (+20)	252 (+04)	320 (+12)	123 (+48)	221 (+04)	224 (+20)	510 (+209)	105 (+10)
22.	Wheat Gruel	C	415	225	287	073	182	180	283	091
		E	477 (+62)	265 (+40)	313 (+26)	081 (+08)	232 (+50)	214 (+34)	329 (+46)	103 (+12)
23.	Vegetable Pan Cake	C	189	129	135	031	095	089	108	034
		E	203 (+24)	139 (+10)	145 (+10)	037 (+06)	105 (+10)	095 (+06)	118 (+10)	119 (+85)
24.	Indian Pasta with Vegetables	C	442	248	308	075	217	204	301	095
		E	462 (+20)	252 (+02)	320 (+12)	113 (+38)	221 (+04)	224 (+10)	510 (+209)	105 (+10)

C- Control, E- Experimental

*Values in parenthesis indicated the change in value over control

** Values are not available in food table

Micronutrient content of daily meal items

In general, mushrooms are good source of many vitamins, especially those of B-complex group. Among B complex vitamins, mushrooms are especially rich in thiamine, riboflavin, niacin and biotin as well. *Pleurotus* species contain thiamine 1.16 to 4.8 mg, riboflavin 0.40 mg and niacin 46 to 109 mg. Folic acid and vitamin B_{12} (which is absent in plant food) are present in mushrooms, although in small quantities. Like most vegetables, mushrooms are rich in potassium, followed by phosphorus, magnesium and calcium. Iron is present in appreciable amount in *Pleurotus* species. Besides, mushroom contains copper, zinc, manganese, cadmium and lead. Taking into consideration these facts, mushrooms were substituted in daily meal food preparations and change in selected micronutrient composition are shown in Table 4A and 4B.

It is revealed from the Table 4A and 4B that the most of the micronutrient values of experiment food items are higher than the traditional food items. Especially there is remarkable change in values of potassium, niacin, riboflavin and thiamine. Requirement of niacin is 6.6 mg/ 1000 Kcal. Experimental food preparations under this present study provide a good amount of niacin. Riboflavin and thiamine play important role in the several oxidation processes inside the cell and concerned with energy and protein metabolism. Indian diets are generally deficient in these vitamins. It is evident from Table 4A and 4B that all the values of thiamine and niacin of experimental food items are higher than traditional food items. The difference in values are because dry mushroom (a concentrated source of nutrient) was incorporated in 1-10 items and fresh mushroom was added in remaining experimental food items. Rest of the micronutrient values indicated in Table 4A and 4B reveal a difference which is not very significant. Addition of mushrooms does not affect much on sodium values since mushrooms are low sodium food. Mushrooms are good source of phosphorus after potassium but the change in experimental food values is not remarkable because cereals and vegetables are also good sources of phosphorus. Similar observations are seen in case of magnesium, iron and calcium. Negative difference was observed in sodium, phosphorus, iron and calcium of experimental recipes since vegetables are good source of these nutrients.

Therapeutic properties of daily meal items

Every food preparation has therapeutic properties due to presence of various nutrients. Therapeutic properties depend on the macro and micro nutrients present in food ingredients. These nutrients assist the patient to recover fast from the illness. But the recovery depends on the quantity and proportion of nutrients present in foods. Therapeutic properties of food are generally determined by feeding experiments. But initially it is necessary to find out the quantity and proportion of nutrients present in food preparations. In the present investigation presence of therapeutic properties were assessed by using food value table and their suitability was then decided. Recipes from routine diet were selected for the incorporation of mushrooms in order to get the acceptability. Care was taken to maintain the original nutritional composition of mushrooms. Mushrooms are low calorie, high protein, starch and sugar free, cholesterol free, low fat, high potassium and low sodium, good fibre, good vitamins B complex with vitamins B_{12} and good in trace elements. Mushrooms require less time for cooking and also need low temperature for cooking. Thus prevent losses during cooking. The recipes which require less time for cooking are suitable for mushrooms. Mushrooms are in general used for garnishing purpose or just to enrich aesthetic, sensory and nutritional quality of food preparations. Above said properties of mushroom are well suited to normal as well diseased individuals. Plain wheat preparations became more nutritious after substitution of ten per cent mushroom powder. Wheat is a good source of starch (69%) and have moderate amount of protein (12%). But the quality of protein is not good because it is deficient in lysine, tryptophan, threonine and methionine as well. Besides it contains moderate amount of riboflavin, thiamine and niacin. Inclusion of mushroom made this wheat preparation better in respect of protein quality, vitamin B and minerals content, hence improves the therapeutic properties. This improved food item of lunch and dinner of Indian diet can be recommended to normal, vulnerable and in therapeutic diets. Reduction in carbohydrate and increase in fibre become good for diabetes and obesity. High potassium content in mushrooms is good for heart patient. Millets are rich in carbohydrate and moderate in protein (10-11%). *Bajara* (pearl millet) is good in mineral content especially iron. Maize in better in lysine but poor in tryptophan and methionine. *Jowar* (sorghum) is poor in tryptophan, methionine and rich in leucine, potassium, calcium and maize is deficient in calcium and iron. Addition of mushroom powder has improved the quantity and quality of protein, increased thiamine, riboflavin and niacin and potassium in good amount and phosphorus, iron in moderate amount.

Table 4A. Micronutrients (mg) in daily meal items with addition of 10 g dry mushroom

Sr. No.	Meal Item	Sample	Thiamine	Riboflavin	Niacin	Potassium	Sodium §	Phosphorus	Magnesium	Iron	Calcium
1&2.	Indian Wheat Bread	C	0.49	0.17	43.00	315	20	355	132	04.90	029
		E	0.84 (+0.35)*	0.55 (+0.38)	49.50 (+6.50)	610 (+305)	24 (+04)	396 (+41)	142 (+10)	05.64 (+0.74)	028 (-01)
3.	Indian Mix Grain and	C	0.58	0.31	04.24	269	38	259	100	03.90	123
	Vegetable Bread	E	0.89 (+0.31)	0.64 (+0.33)	14.40 (+10.16)	564 (+305)	42 (+04)	300 (+41)	109 (+09)	04.74 (+0.84)	131 (+08)
4.	Indian Mix Grain Preserved Bread	C	0.60	0.27	04.19	304	28	286	118	03.98	083
		E	0.95 (+0.35)	0.65 (+0.38)	14.59 (+10.40)	599 (+295)	31 (+03)	327 (+41)	127 (+09)	04.82 (+0.84)	081 (-02)
5.	Indian Pearl Millet Bread	C	0.33	0.25	02.30	307	11	296	137	08.00	042
		E	0.95 (+0.62)	0.65 (+0.40)	14.59 (+12.29)	599 (+292)	31 (+20)	327 (+31)	127 (-10)	04.82 (-3.18)	081 (+39)
6.	Indian Sorghum millet Bread	C	0.37	0.13	03.10	131	07	222	171	04.10	025
		E	0.73 (+0.36)	0.51 (+0.38)	18.60 (+15.50)	444 (+313)	13 (+06)	275 (+52)	176 (+06)	04.95 (+0.85)	025 (00)
7.	Indian Maize Bread	C	0.42	0.10	01.80	286	16	348	139	02.30	010
		E	0.78 (+0.36)	0.49 (+0.39)	12.42 (+10.62)	583 (+297)	20 (+04)	389 (+41)	147 (+08)	03.31 (+1.01)	011 (+01)
8.	Mix grain ready to eat flour	C	0.18	0.07	02.60	221	15	182	046	02.80	040
		E	0.53 (+0.35)	0.45 (+0.38)	12.90 (+10.30)	533 (+312)	19 (+04)	228 (+46)	084 (+38)	03.50 (+0.70)	038 (-02)
9.	Puff Amaranth seeds Dessert	C	-	-	-	-	-	485	-	10.60	174
		E	-	-	-	-	-	505 (+20)	-	11.30 (+0.70)	254 (+80)
10.	Wheat Biscuit	C	0.70	-	00.90	045	04	054	027	00.90	009
		E	0.80 (+0.10)	0.20 (+0.20)	06.10 (+5.20)	166 (+121)	06 (+02)	080 (+26)	032 (+05)	00.70 (-0.20)	007 (-02)

C- Control, E- Experimental

§ Salt sodium is not included

- Values are not available

*Values in parenthesis indicated the change in value over control

Table 4B. Micronutrients (mg) in daily meal items with addition of 50 g fresh mushroom

Sr. No.	Meal Item	Sample	Thiamine	Riboflavin	Niacin	Potassium	Sodium §	Phosphorus	Magnesium	Iron	Calcium
11.	Tomato Soup	C	0.16	0.05	00.41	117	017	020	003	0.54	035
		E	0.30 (+0.14)*	0.22 (+0.16)	05.59 (+5.18)	206 (+89)	013 (-04)	048 (+28)	014 (+11)	0.89 (+0.35)	011 (-24)
12.	Green Salad	C	0.07	0.22	00.66	093	036	180	015	2.02	142
		E	0.23 (+0.16)	0.30 (+0.12)	05.73 (+5.07)	204 (+111)	019 (-17)	118 (-62)	018 (+03)	1.62 (-0.40)	083 (-59)
13.	Curds with Vegetables	C	0.01	0.12	00.27	130	035	229	005	0.45	136
		E	0.29 (+0.28)	0.44 (+0.32)	02.89 (+2.62)	284 (+154)	028 (-07)	447 (+218)	009 (+04)	0.63 (+0.18)	154 (+18)
14.	Capsicum Fry	C	0.43	0.05	00.58	048	010	032	002	1.08	049
		E	0.48 (+0.05)	0.41 (+0.36)	05.93 (+5.35)	354 (+306)	005 (-05)	078 (+46)	034 (+32)	1.76 (+0.68)	119 (+30)
15.	Ladies Finger Fry	C	0.06	0.06	00.49	092	006	044	040	0.32	054
		E	0.22 (+0.16)	0.26 (+0.20)	01.40 (+0.91)	197 (+105)	006 (00)	094 (+50)	044 (+04)	0.76 (+0.44)	056 (+02)
16.	Broken Wheat Vegetable Porridge	C	0.34	0.13	03.28	722	025	294	100	4.30	089
		E	0.54 (+0.20)	0.33 (+0.20)	07.68 (+4.40)	821 (+99)	024 (-01)	297 (+03)	102 (+02)	4.69 (+0.39)	092 (+03)
17.	Rice Pan Cake (Flat Round)	C	0.19	0.10	01.40	433	020	198	078	2.12	134
		E	0.39 (+0.20)	0.30 (+0.20)	05.00 (+3.60)	532 (+99)	018 (-02)	210 (+12)	080 (+02)	2.44 (+0.32)	132 (-02)
18.	Rice Pan Cake (Ball Shape)	C	0.08	0.06	01.30	152	007	097	092	1.27	042
		E	0.28 (+0.20)	0.26 (+0.20)	04.50 (+3.20)	253 (+101)	005 (-02)	116 (+19)	095 (+03)	1.52 (+0.25)	045 (+03)
19.	Tomato Omlet	C	0.31	0.12	01.69	462	047	211	119	3.52	088
		E	0.45 (+0.14)	0.28 (+0.16)	04.30 (+2.61)	613 (+171)	045 (-02)	325 (+114)	129 (+10)	3.82 (+0.30)	089 (+01)
20.	Sandwich	C	0.04	-	00.40	-	-	-	-	0.70	007
		E	0.24 (+0.20)	-	05.80 (+5.40)	-	-	-	-	1.00 (+0.20)	008 (+01)
21.	Indian Pasta Dessert	C	0.03	0.14	00.26	105	055	085	006	0.22	091
		E	0.83 (+0.80)	0.90 (+0.76)	20.17 (+19.91)	531 (+426)	046 (-09)	203 (+118)	036 (+30)	2.39 (+2.17)	092 (+01)
22.	Wheat Gruel	C	1.86	0.04	01.15	088	047	143	031	3.22	037
		E	2.00 (+0.14)	0.24 (+0.20)	07.45 (+6.30)	223 (+135)	042 (-05)	194 (+51)	033 (+02)	3.50 (+0.32)	035 (-02)
23.	Vegetable Pan Cake	C	0.20	0.02	00.88	123	024	129	024	0.83	026
		E	0.41 (+0.21)	0.20 (+0.18)	05.10 (+4.12)	208 (+85)	021 (-03)	149 (+20)	026 (+02)	1.05 (+0.22)	028 (+02)
24.	Indian Pasta with Vegetables	C	1.03	0.02	01.13	110	149	039	044	2.06	032
		E	1.13 (+0.10)	0.20 (+0.18)	05.68 (+4.55)	222 (+112)	147 (-02)	049 (+10)	048 (+04)	2.71 (+0.65)	029 (-03)

C- Control, E- Experimental

§ Salt sodium is not included

- Values are not available

* Values in parenthesis indicated the change in value over control

Therefore, wheat breads, millet breads can be recommended in daily diet of normal as well as diseased individual. Mix grain and vegetable bread (*Missi roti*) and preserved bread (*khakara*) are basically considered nutritious as these recipes are combination of cereal, pulse and green. Addition of mushroom powder has improved especially the lysine, niacin with good amount of thiamine and riboflavin and potassium content. Therefore, these foods can be recommended as supplementary food for vulnerable and in low carbohydrate diet like obesity, diabetes and heart disease. While preparing these recipes, table salt can be avoided in low sodium diet. Tomato mushroom soup and green mushroom salad are recommended in low calorie and low sodium diet. Increase of essential amino acid has improved the quality of whatever meagre amount of protein is supplied. These contain good amount of niacin, riboflavin and thiamine. Mushroom capsicum fry and mushroom ladyfinger fry are novel vegetable preparations which can be added in the low sodium diet. Breakfast and snacks items (mushroom *dalia*, mushroom *uttapa*, mushroom *appe*, mushroom *sattu* and mushroom *rajgeera chikki*) are high protein foods, hence good for vulnerable. Mix grain ready to eat flour (mushroom *sattu*) and Indian pasta dessert (mushroom *kheer*) are better in fibre therefore good for high fibre diet. All the mushroom recipes are better in niacin, riboflavin, thiamine content hence suitable for all the therapeutic diets. In general the mushroom recipes are good in protein, low fat (saturated fat), moderate carbohydrates, low calorie, high thiamine, riboflavin and niacin, high potassium, moderate fibre, hence are suited in most of the therapeutic diets. In general due to the unique chemical composition, mushrooms are suitable in therapeutic diets. Low calorie, sugar free mushroom are delight of the diabetes. Its high potassium, low sodium ratio, few calories and good fat (rich in linoleic and free in cholesterol), mushroom is the choice of the dietician for atherosclerosis, hypertension and obesity. Soft fibre content makes them suitable for constipating individual as well. Moreover, it is well known for their precious medicinal factors i.e. antifungal, antibacterial, antiprotozoal, antiviral, anti-hypertension, anti-tumor, hypo-lipidemic, hypocholestermic etc. Their most significant active principle, immuno stimulating polysaccharides strengthen the immunity. *Pleurotus sajor caju* has hypotensive action which reduce the rate of nephron deterioration which may extend life span of chronic renal failure patents [15].

CONCLUSION

Keeping in view the improved sensory properties of developed mushroom daily meal items, they are suitable for incorporation in diet of all categories of people. The fresh and dry mushroom/ mushroom powder can be incorporated in a food item to enhance its nutritive and therapeutic value without affecting its acceptability.

ACKNOWLEDGMENTS

I offer my sincere gratitude to University Grants Commission, New Delhi and authority of Sant Gadge Baba Amravati University for giving financial support under Group D of unassigned grant for minor research project. This research paper is based on the report of the said project.

REFERENCES

[1] Jaya Prakasha HM and Brueker H. (1999). Whey protein concentrate. A potential functional ingredient for food industry. *J. Food Sci. Technol*. 5: 465-471.

[2] Patra Beatriz Navas and Lusine Garcia. (2000). Nutritional evaluation of sorghum flour on supplementation with whey protein. *J. Food Sci. Technol.* 2: 144-48.

[3] Ranjana Singh *et al*. (2000). Nutritional evaluation of soy fortified biscuits. *J. Food Sci. Technol*. 1: 33-38

[4] Pratima Awasthi and Yadav MC. (2000). Effect of incorporation of liquid dairy by products on chemical characteristics of soy fortified biscuits. *J. Food Sci. Technol*. 2:1 58-161.

[5] Mir MA and Nath N. (2000). Protein fortification of mango bar using soy protein concentrates and coconut powder. *J. Food Sci. Technol*. 5: 525-528.

[6] Lakshami B and Vimal V. (2000). Nutritive value of dehydrated green leafy vegetable powders. *J. Food Sci. Technol*. 5: 465-471.

[7] Chang ST and Hayes WA. (1978). The Biology and Cultivation of Edible Mushrooms, New York, Academic Press, pp 137-68.

[8] Chang ST and Miles PG. (1990). Edible Mushrooms and their cultivation, New Delhi, CBS Publication and Distributors, pp 27-40.

[9] Asha Mane *et al*. (2000). Acceptability and protein quality of supplementary foods developed out of oyster mushroom powder. Science and Cultivation of Edible Fungi. LJLD Van Griensven Eds, pp 753-55.

[10] Asha Mane *et al*. (2004). Effect of incorporation of mushroom powder on gluten formation in wheat dough used for bread making. Recent Trends in Biotechnology. Jodhpur India, Scientific Publisher, pp 102-105.

[11] Asha Mane *et al*. (2012). Acceptability and nutritional quality of oyster mushroom powder supplementary foods. Proceeding of the 18th Congress of the International Society for Mushroom Science, China Agriculture Press, pp 619-622.

[12] Ranganna S. (1990). Handbook of analysis and quality control for fruit and vegetable products. New Delhi, McGraw Hill 624 p.

[13] Indian Council of Medical Research. (1989). Nutritive value of Indian foods. Gopalan *et al.* Eds, Hyderabad, NIN, pp. 47-85.

[14] Chadha KL and Sharma SR. (1997). Advances in Horticulture: Mushroom. New Delhi Malhotra Publication House, 548 p.

[15] Tam SC *et al*. (1986). Hypertensive and Renal Effects of extract of the Edible Mushroom. *Life Science*. 38:1155.

INDIA ON THE THRESHOLD OF A NON-GREEN REVOLUTION

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ABSTRACT

India was a late starter as far as mushroom farming is concerned. White button mushroom was grown in the later part of 20th century under National and International Research Projects by FAO, ICAR, CSIR and Agriculture Universities/ Depts. of Horticulture etc. To follow up mushroom research in the country, the ICAR established the National Centre for Mushroom Research & Training (NCMRT) as well as an All India Coordinated Mushroom Improvement Project (AICMIP) in the year 1983 at Solan (HP) now upgraded as the Directorate of Mushroom Research (DMR), and AICRPM with 14 Coordinated and 2 Cooperating Centers in 15 States of the country. Govt. of India, during 1993-97, created 30 Composting Units and 29 Spawn Laboratories in 21 States and spent Rs. 1.36 crores (ca.US $ 0.27 millions) for training 27,300 potential mushroom growers. Mushroom farming during the last 3 decades or so, has made considerable growth both in terms of seasonal as well as climate controlled growing. The climate controlled farming of button mushroom got a real fillip, under the Govt. EOU scheme to encourage business houses to set-up organized Hi-tech Mushroom Farms as Industrial ventures which together with smaller climate control Farms and the seasonal units have raised the annual production of button mushroom to >0.11 million tons. Among the specialty mushrooms, oyster is leading the group by reaching almost the entire country. Some organized oyster Farms, and a large scale cooperative Farming in NEH States producing over 600 tons of oyster mushrooms p.a. under the auspices of Mushroom Development Foundation, Guwahati, seem to open new vistas in the growth of this mushroom in India. *Volvariella volvacea,* has attained commercial status in Odisha with a low cost cultivation method and spawn made locally on paddy grains in make shift village labs. This way the rural farmers produce over 10,000 tons of paddy straw mushroom in 9 months. *Calocybe indica*, the milky mushroom is a new entrant, but its snow-white color as well as stout & fleshy appearance have helped its fast acceptance and has crossed 10000 tons yearly production figure.

The large variety of indigenous edible species also comprise the mushroom wealth of India, many of which have high export potential. *Morchella* spp. abounding in states of J&K, Himachal and Uttrakhand; *Cordyceps sinensis/C. militaris,* the medicinal mushrooms from Himalayas in Uttarakhand and Arunachal Pradesh; *Phellorina inquinans*, *P. herculae* and *Podaxis pistllaris* from the desert state of Rajasthan, shiitake mushrooms *Lentinula edodes* and *L. lateratia*, along with *Auricularia* spp collected and sold in dried form in large quantities in NE region. Edible puffballs (*Lycoperdon / Scleroderma* spp), *Termitomyces* spp and *Macrolapiota* sp., are quite common in India. All these species need to be conserved, characterized and utilized for people's welfare and make India's non-green revolution a reality in near future.

Keywords: non-green revolution, climate control, mushroom growth

INTRODUCTION

India is primarily an agricultural country and nearly 70% of its population, including landless and marginal farmers, still depend on farming and allied activities. It is the 7th largest and 2nd most populous nation of the world and has to sustain more than 16% of world's human heads and almost half its size of livestock population, but only with 2.8% of the global land. Possibly, such an enormous pressure on land led some people to list India as a nation which can never feed itself. However, the great nobel laureate Dr. Norman E Borlaug planted the 'seeds' of India's green revolution in early 60s, the first impact of which was felt in 1967-68 when India recorded over 33% rise over its highest production of wheat recorded in 1963-64. The country celebrated its achievement by releasing a postal stamp "Wheat Revolution 1968" as a mark of the beginning of India's green revolution. Soon after, India emerged as the 3rd biggest food grain producer and the 2nd biggest producer both of rice and wheat in the world. However, in view of its population still rising @1.6%, the country is seeking the second Green Revolution sooner than later, with an ultimate aim to build-up a sustainable production system capable to

provide not only the staple food but also the functional foods to the toiling masses suffering from hunger and malnutrition, due to the decreasing land-holdings, declining soil-fertility, poverty, lack of employment & opportunities for income generation, and the failing rural economy.

Efforts to tackle such a complex problem of India's agrarian sector are under way for last several decades through a variety of farming/cropping systems, including mushroom farming, which required dovetailing the age-old traditional crop-based technologies with a frontier technology based on microbial conversion of hemi-cellulosic wastes into a nutritious and high-value functional food. Of course, mushroom farming has several additional advantages which make it an ideal secondary occupation capable to solve most of the current problems of Indian agriculture as well as peasants. Mushroom growing is a recycling process done indoors, which does not require arable land or fertile soil, and the waste land unfit for farming can be used to raise a potent cash crop from farm/forest and industrial wastes. Moreover, it gives increased productivity per unit of land, water, energy, labor and time and that too with much less investment. Besides being a fast spinning cash crop, it is also an ideal health food capable to fight malnutrition in general and protein-deficiency in particular. Yet, being a new cropping system for the rural peasants as well as for the un-employed and under-employed people of semi-urban areas of India, it took long time, spread over 5 decades or so, for the adoption and spread across the country. Yet, with efforts of all concerned, it is consoling today that India has slowly but steadily reached today the threshold of a mushroom revolution, which because of its biological capability may better be called as the beginning of a non-green revolution-2014, with a cherished hope that very soon India would be among the front runners of mushroom producing as well as consuming country.

Mushrooms in India

Mushrooms were known well in ancient India. Their habitats were described in olden epics as under: "**mDrsLonts ’kkdHkkfePN= f’kyhU/kdeAf{kfrxke; dk"B"k o{kkfn"kqrnnHkonAA**"

meaning thereby that, "Mushroom growth appears in moist places on soil, cow-dung, wood, stone-crevices and trees" Also, their references appear in the most ancient scriptures like "*The Sam-Veda*" and the ayurvedic materia medica "*The Charak-Samhita*". Two Sanskrit verses of the referred ancient epics are reproduced below:

1. "**v/;okvfnfHk lrlkeifo= vku;**" meaning thereby, that " Let the divine intoxicating juice flow here." This verse has been interpreted by various ancient scholars and contemporary scientists that the sacred nectar mentioned here probably meant "The Soma" or *soma-ras*a used by the Hindu Gods, prepared from a mushroom species, *Amanita muscaria*.

And

2. **liN=d oT;kLr cg;k ·U;k’N=tkr;%A’khrkihul d=;Jp e/kjkx0; ,o pAA**

"explaining thereby that except for toadstools which are forbidden, and those causing sinus diseases, several other species of mushrooms are high quality nutrient, which on eating give sweet and soothing effect."

Yet, mushroom remained a restricted item for the Vaishnavites (a Hindu religious sect) and some sects of Muslims, because there were some taboos prevalent among the orthodox people against mushroom eating, considering them dirty and unfit for human consumption. Also, there was some fear complex about mushrooms, particularly about the wild mushrooms due to which people avoided them out of disbelief. However, once the cultivated mushrooms became available along with some scientific facts, mushrooms acceptance among educated and elites started increasing slowly but steadily. Yet, their limited availability and high prices versus the low purchasing power of the common man also prevented their popularization among the Indian consumers for quite a long time. As such, the per capita consumption of mushrooms in India remained as low as 20-25 g [1], which in turn restricted the pace of the growth of mushroom producing industry in the country.

Artificial cultivation of mushrooms had a very humble beginning in India by some individual pioneers of the field [2-5], followed by launching of planned scientific programs by various organizations gradually opened the avenues for mushroom

farming as an enterprise suiting both to seasonal as well as commercial growers. Rest is a history of over 50 long years during which the mushroom farming spread across India, and its long journey has already been well traced [6-10], and need not be elaborated here. It would rather be of interest and concern to comprehend the current scenario of 'Mushroom Growth' in India and its expected impact over its national and international trade and also on the nutritional and food security and poverty of its rural and urban populace in coming years.

'Mushroom growth' in India

India is blessed with a varied agro-climate ranging from tropical to sub-tropical and to temperate conditions prevailing in different parts of the country. The prevalent conducive climate together with abundance of farm residues, cheaper labor and demand of protein-rich mushrooms for a primarily vegetarian population, provide ideal situation for mushroom farming in India, with shifting emphasis over temperate, sub-tropical and tropical species in different regions of the country. India currently produces, the temperate mushroom *Agaricus bisporus,* several sub-tropical species of *Pleurotus, Hypsizygus ulmarius, Lentinula edodes,* and high temperature loving tropical mushrooms *Volvariella volvacea* and *Calocybe indica*. There are several other tropical species of edible and medicinal mushrooms, the growing techniques for which have already been fine-tuned and are ready to be adopted sooner than later [11,12]. This makes a mosaic of produce in the mushroom-basket of Indian entrepreneurs enabling them to meet the varying demands both of national and international consumers at reasonable cost. Button mushroom occupies the largest share of over 3/4th quantity of India's total production, most of which is exported, although there is still ample of scope to further increase it. Mushrooms particularly for the international market, are required to strictly maintain the quality standard, which at least in the case of white button mushrooms of India, is adequately met by the Export Oriented Units (EOU) across the country. Several such units are HAACA compliant as well as FDA approved and a few of them have even started providing organically produced button mushrooms also to foreign buyers. The bulk of the demand of button mushrooms in the domestic market is, however, primarily met by seasonal growers and small commercial farms operating in different regions of the country, which are steadily increasing in number synchronizing with the rising demand and per capita mushroom consumption in the country from 25g to 40g.

As regard to the specialty mushrooms, they are mostly grown by seasonal growers in improvised growing rooms and hence their production capacity is too small to cater to the needs of export market. Moreover, they generally lack in the required hygiene and cleanliness, and hence their produce seldom meets the stringent export quality standards. Moreover, they usually pasteurize their substrate with chemicals which leave residual toxicity in their produce and make them unfit for export. However, during the last few years, commercial units growing oyster as well as milky mushrooms (*Calocybe indica*) have also come up, which are raising good export-level crops and getting favorable response from abroad, and it is hoped that they would soon obtain quality approval from the importing nations, or they would also resort to organic farming of oysters [13]. Similar is the situation with shiitake (*Lentinula edodes*), which is still in its infancy as far as its commercial cultivation is concerned, although its demand exists both inside and outside the country, particularly in Japan. Most of the domestic demands of shiitake are met from its large scale wild collections from Meghalaya, Manipur, Nagaland and adjoining areas [14,15] of NEH India.

The fastest growing and one of the tastiest mushroom is paddy straw mushroom or the chinese mushroom *Volvariella* spp, represented in India by *V. volvacea, V. diplasia* and *V. esculenta,* among which *V.volvacea* is the most commonly cultivated species in tropical areas of the country. Although it is cultivated in States like Odisha, Tamil Nadu, West Bengal, Kerala, Manipur, Chhatisgarh and Punjab, yet its farming in Odisha has attained the same status as that of button mushroom in North-Western States of Haryana, Punjab, J&K, Uttrakhand, Himachal, etc. In both the cases, the growing techniques adopted by the growers are based on seasonal as well as low cost methods. Yet, in view of their large scale adoption both by growers and domestic consumers alike, there is an urgent need to introduce at least some cost-effective and simple improvements in their methods to make their farming more profitable and sustainable. Simultaneously, some export oriented *Volvariella* farms adopting modern technology may be set up as Model Farms in Odisha to help and motivate the aspiring growers of the state to adopt EOU for paddy straw mushroom also, so as to capture the market of the countries of South East Asia and others. As far as milky mushroom (*Calocybe indica*) is concerned, it is heartening to note that some commercial units have already come up in peninsular India, particularly in Tamil Nadu and Andhra Pradesh, which are poised to capture the export market for this new Indian delicacy very soon.

Mushroom map in India

In earlier years, mushroom farming in India was almost confined to hills or the foot-hills, since the species then in use was the European mushroom *Agaricus bisporus* which only needed temperate conditions. However, as the climate control became feasible, and also when some subtropical and tropical mushroom species appeared on the scene, mushroom farming activities moved to plain areas also, where availability of raw materials, water, labor and means of communication were better for the entrepreneurs. Yet, for small growers, the local climate dictated the terms and the species got distributed on the pattern of the climatic conditions of the area. However, the climate controlled button mushroom farms were established randomly based on other considerations, like infrastructure, raw materials, marketing and financial support and incentives. Yet, the current overall scenario shows that by the end of the first decade of the 21st century, mushroom farming has spread far and wide across India along with strong R&D infrastructure and basic support services.

R&D infrastructure & services

Soon after the projects launched by FAO, IGADA, CSIR, SAUs and some State Departments of Horticulture, ICAR in the year 1983 established country's nodal research agency viz., the National Centre for Mushroom Research & Training (NCMRT) as well as an All India Coordinated Mushroom Improvement Project (AICMIP) at Solan (H.P.). Again, in the year 2008, the Solan Centre was upgraded as the Directorate of Mushroom Research (DMR), while the AICMIP was enlarged and renamed as All India Coordinated Research Project on Mushroom (AICRPM) with 14 coordinated and 2 cooperating centers covering different climatic zones in 15 States of the country. Earlier, the Govt. of India, during the VIII 5-year Plan (1993-97), launched a massive drive to create 30 composting units and 29 spawn laboratories in 21 States of the country, besides an allocation of Rs. 1.36 crores (ca.US$ 0.27 millions) for training 27,300 potential mushroom growers. This was in addition to the modern facilities created to produce pasteurized short method compost (SMC), casing soil and quality spawn under an International Cooperation for Indo-Dutch Mushroom Project in the year 1988 at 4 places located at Palampur (HP), Srinagar (J&K), Jeolikote (UP) and Bangalore (Karnataka). Further, an integrated Extension Unit "The HAIC Agro R&D Centre" was started in Sonipat district by Govt. of Haryana to serve the mushroom farmers of the area by providing compost, spawn and casing besides hand on training and advisory services. Besides these public sector units, many private spawn units have come up throughout India. A few featured companies listed by Trade India are shown in Table 1 to exhibit their geographical distribution. The number of labs is much higher. The large units mostly produce their own spawn whereas the small/seasonal growers procure spawn from government/private labs.

Button mushroom farms

There are 3 different farm types of button mushroom currently existing in India, viz., seasonal farms, small commercial farms and export oriented farms. The seasonal button mushroom farms are mostly located in North Western India in the plains of Haryana, Punjab, Delhi-NCR, Western U.P. and their adjoining areas; as also in the hills and valleys of Himachal Pradesh, J&K, Uttarakhand, north Bengal, Maharashtra and Tamil Nadu. These farms are operational only during cooler part of the year and their produce serve mostly the local/regional buyers. Over the years, a good number of small commercial button mushroom farms have come up with less than 500 TPA capacity which operate most part of the year and cater to the needs of the domestic market of cities and metros. The third category of button mushroom farms are the (EOU) which are large (over 2000 TPA), most mechanized and computerized units meant to serve the export market. All the EOUs of India are integrated farms, having their own units for composting/casing, growing and processing operations with imported machineries. Some of them prefer to buy their spawn requirements from multinational spawn companies as per the likings of their foreign customers. Although, the number of EOUs in India have been fluctuating due to closure of some old ones and opening of some new ones in the past, but the total yearly mushroom production by the sustaining farms is rising steadily. Verma [8] has listed only five such sustaining EOUs with their annual production figure showing about 0.70 lac tons of fresh mushrooms per year. However, as per current information, 7 EOUs are presently producing 0.75 lac tons of button mushroom per year. The number of domestic commercial units have also risen, but more rapidly and their contribution to the national kitty as well as India's per capita consumption over the last few years are much more. More notable is the fact that it has spread almost to the entire country (Fig.1), and is now well poised to achieve demand-driven vertical growth in the coming years.

Table 1. Some Featured Spawn Lab Companies of India 2014

1.	Microfungi, Kolkota 700046.
2.	Vee Nee Agro, 9, Green Park, Kolkota 700084.
3.	PCR Industries, Cuddalore (TN) 608001.
4.	Jupiter Biotech, Tiruvannamala (TN) 606601.
5.	MycoGroups, ManddaiyurSalai, Thirucharpalli (TN) 620020.
6.	Udhayan Mushroom Farm, Aladhar Kovil Road, Madurai 625107 (TN).
7.	Anand Mushrooms, Palvakkam, Chennai 600041.
8.	ABRM Export, Thirumudi Nagar, Pondicherry 605001.
9.	Daily Mushrooms, Gangpuram P.O., Erode,(T.N.) 638102.
10.	Wonder Mushroom, Ponda Commerce Centre, Ponda, Goa 403105
11.	Konkan Mushrooms, 76,Ponda Commercial Centre, Ponda, Goa 403105.
12.	Arora Mushroom Agency, Mohan Singh Place, C.P., New Delhi 110001.
13.	Swadeshi Enterprises, Model Town, Delhi-110009.
14.	Helin Bio Genesis, Sector-2, NOIDA, (U.P.) 201301.
15.	Jai Agro Industries, Gandhi Dham, Lilashati Nagar, Gandhi Dham, Gujarat 370201.
16.	Ever Green Mushrooms, Opposite J.K.Tower, Ring Road, Surat,Gujarat 394210.
17.	Omark Enterprises, Maruti Nagar, Hyderabad 502032, A.P.
18.	Vinayak Herbal Nutrients Pvt. Ltd. PrasantVihar, Bhubaneshwar (Odisha) 751024.
19.	Katyayni Creation, Cd-314/li P.O. Dhurva, Ranchi 834004 (Jharkhand).
20.	Renaiscence Biotech Pvt. Ltd. 88 Police Colony, Anishabad, Patna 800002 (Bihar)

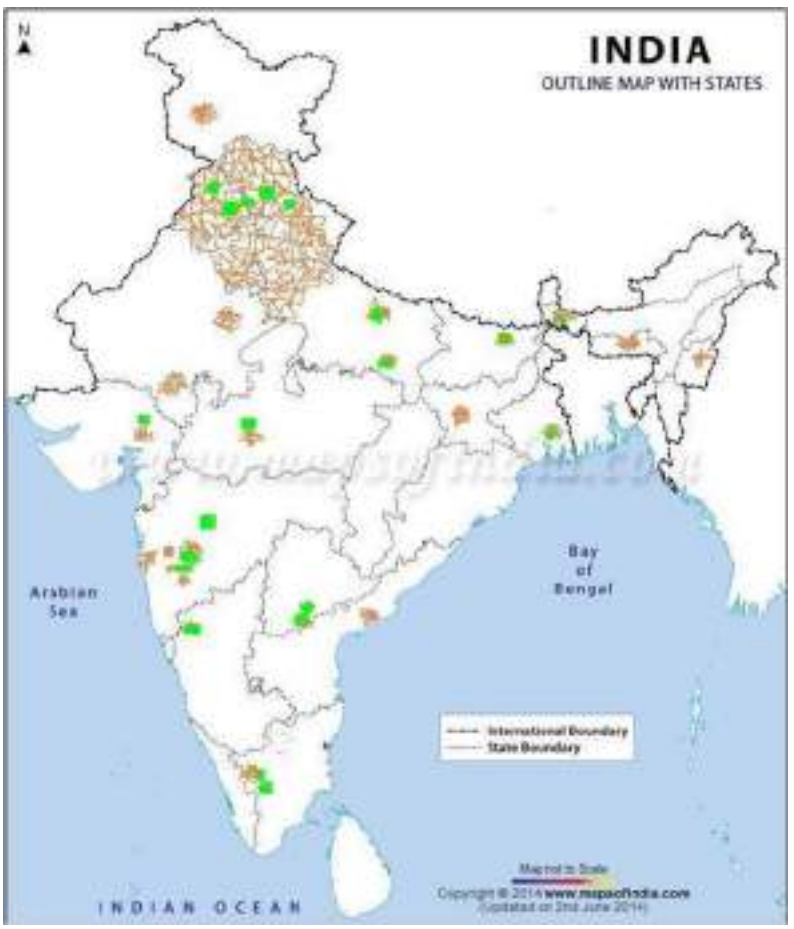

Figure 1. Button Mushroom Seasonal Δ and Commercial Φ farms in India

Specialty mushrooms: Oyster mushrooms including about a dozen species of *Pleurotus* and the Blue oyster *Hypsizygus ulmarius* have been adopted in all States and Union Territories of India and are popularly grown on a variety of substrates. In fact, the largest number of mushroom growers including women-folk belonging to self-help groups (SHGs) and members forming co-operatives, produce this mushroom as a source of livelihood or as profitable enterprises. Yet, taking advantage of the wider adaptability of this mushroom, people are growing its various species in improvised growing houses under seasonal conditions making a modest investment. However, due to increasing consumer demand, recently a few commercial units in southern states, Chhatisgarh and Goa have come up. Also, under the guidance of NGOs like BAIF, Pune; MDF, Gauhati; Divyayan, (RK Mission), Ranchi; Ram Krishna Ashram, Neem Pith, West Bengal; and Pradan, M.P., etc. large scale group farming of oyster mushroom has been adopted in rural & tribal areas .under poverty alleviation program. Obviously, covering newer areas, oyster's production is rising fast and are estimated to have crossed 20,000 tons.

The other two specialty mushroom species, which have made considerable impact, are the milky mushroom (*Calocybe indica*) and paddy straw mushroom (*Volvariella* spp). Incidently, both of these have been promoted by respective State Agricultural Universities, viz., TNAU, Coimbatore and OUAT, Bhubaneswar. While OUAT established a "Centre of Tropical Mushroom Research and Training (CTMRT)" to fulfil the task of popularising tropical mushrooms, particularly the Paddy straw mushroom among the rural youths, the TNAU introduced Milky mushroom, a high temperature loving species *Calocybe indica*. Both these tropical mushrooms have been well accepted and have already exceeded their annual production figures of 10,000 tons each, which are still increasing. Such success of these two mushrooms is obviously due to their tropical nature, but an important factor in their fast spread has been easy availability of their spawn made in improvised rural laboratories "mushrooming" in those areas. Several other mushrooms are in line for adoption by the growers in coming years, the most important one being *Lentinula edodes*, a highly tasteful and potentially medicinal mushroom with great demand abroad, especially in Japan.

Table 2. Some export –oriented and commercial button mushroom farms in India

Name	Approx. Production Capacity (TPA)	Status
Agro-Dutch Foods, Lalru (Punjab)	40,000 (?)	EOU
Flex Foods, Dehradun (UK)	2,500	EOU
Inventa Foods, Hyderabad (AP)	4000	EOU
Premier Mushroom Farms, Medchal (AP)	NA	EOU
Himalayan International, Idar, Gujarat	10000	EOU
Himalayan International, Paonta Sahib (HP)	2000	EOU
Balaji Mushrooms, Baramati (MS)	4000	Domestic
Cambium Biotech Pvt. Ltd., Nasik (MS)	3000	EOU/Domestic
Weikfield Mushrooms, Pune (MS)	3000	Domestic
Inka Foods, Nalagarh (HP)	1500	Domestic
SR Mushroom Industries, Allahabad (UP)	1500	Domestic
Kulkarni Farm Fresh, Belgaum, Karnataka	1000	Domestic
Kodai's West Hill Farms, Kodaikanal (TN)	NA	Domestic
Mushroom Cultivation, Chennai (TN)	NA	Domestic
Vikas Mushroom, Solan (HP)	NA	Domestic
Thakur Mushroom, Solan (HP)	NA	Domestic
Pankaj Internationals, Purnea (Bihar)	200	Domestic
Others (Haryana-3, UP-3, Punjab-5, Maharashtra-5, etc.)	18000	Domestic

Mushroom biodiversity

The production and productivity of a crop can be sustained and enhanced by conserving, improving and utilizing the available genetic resources. India has a rich wild mushroom flora with many of them being edible. Purkayastha and Chandra [14] had listed 261 edible macro-fungi of India. Since then many more edible genera and species have been added [13-16], based on information from local people of the region, but limited efforts for their conservation and utilization at the national level have been made so far. Yet, establishment of a Gene Bank at NRCM, Solan for mushroom germplasm conservation is a welcome effort, which might prove a step in the right direction. Earlier, work on genetic improvement at NRCM led to the release of NCH102, a hybrid variety of *Agaricus bisporus* besides a few single spore isolates. Efforts to domesticate some wild mushrooms are also expected to yield dividends in near future. Till then, ,attempts may be directed to conserve them *in situ*, as also to train the mushroom collectors for protective harvesting, processing and packaging for export purposes. Among the variety of wild edible species, many have high export potential. *Morchella* spp abounding in States of J&K, Himachal Pradesh and Uttrakhand are considered as most delicious mushrooms, and till a decade ago, they comprised the highest quantity of dried mushrooms exported from India. Though reported from Shillong also, the high Hills of NEH states of Meghalaya, Arunachal, Nagaland, Manipur and Sikkim are yet to be explored for morels. Same is the status of wild Kabul-Dhingri (*Pleurotus eryngii*) common in border-areas of Kashmir with Afghanistan. In recent years, *Cordyceps sinensis/ C. militaris*, the medicinal mushrooms collected from Himalayan ranges in Uttarakhand and Arunachal Pradesh have gained much importance and they also need to be protected for survival and sustained growth. Rajasthan, a desert state, has also two excellent wild mushrooms occurring in large quantities in sand dunes. These species are *Phellornia inquinans*, *P. herculae* and *Podaxis pistillaris* , which are still being consumed in nearby States and are yet to be exported. The NEH states are also rich in wild edible fungi, the most important being *Lentinula edodes* and *L. lateratia,* which along with *Auricularia* spp are collected in quintals and sold in dried form in big Indian cities, and probably outside also. Other delicious mushrooms native to NE region are *Cantherellus cibarius*, *Tricholoma giganteum,Termitomyces* spp, *Laccaria amethystea*, *Gomphus floccosus* and *Lactarius queticolor*. In fact, *Termitomyces* spp. are very widely distributed in India and sold in large quantities in local markets of Chhatisgarh, Jharkhand, Odisha, West Bengal and Kerala , and if processed well can be a good export commodity. Two very popular and common edible mushrooms sold in Jharkhand are the i) Puffballs (*Lycoperdon/ Scleroderma* spp) and ii) *Macrolapiota* sp., which fetch very high prices in local market due to their taste and excellent aroma. Their occurrence in large quantities make them suitable for export, if properly preserved and packed. Among the medicinal mushrooms, *Ganoderma lucidum* is the lone species, which is ready for commercial farming in India. Its farming method and potent strains, both are available within the country. What is needed is its extension and adoption by the farmers, which may not be far away.

CONCLUSION

From the above review, it is apparent that cultivated and wild edible mushrooms both have received adequate R&D support of national and international agencies as well as good scientific attention during the last 5-6 decades. These have created awareness and interest both amongst the mushroom farmers and the corporate. Also, the consumers have lately shown extra liking for mushroom eating, which has enhanced the mushroom demand and supply in India. In fact, mushroom farming in India today is at the door-step of a "Non-Green Revolution" poised to augment the gains of the green revolution by ensuring nutritional security also in addition to food-security to the toiling masses of this country. What is required is a persistent and focused effort by all dealing with this curious but responsive creature of the third kingdom.

REFERENCES

[1] Verma RN. (1999). National and International Scenario of Mushroom Production and Trade. Conference on the Mushroom Industry in India - A Decade of Achievements and Future Prospective, Dec. 3, p.5 8, Solan (HP), India.

[2] Bose SR. (1921). Possibilities of Mushroom Industry in India by its cultivation. *Agric. J.* 16: 643-647.

[3] Thomas KM *et al*. (1943). Paddy Straw Mushroom. *Indian Farming*. 4**:** 332-33.

[4] Bano Z and Srivastava HC. (1962). Studies on the cultivation of *Pleurotus* spp on paddy straw. *J. Food Sci*. 12: 363-365.

[5] Dhar BL. (1997). Mushroom Industry in India - A View. In: Advances in Mushroom Biology and Production, eds. Rai, Dhar and Verma, p.369-378, MSI, Solan (HP), India.

[6] Verma RN. (1996). Mushroom Industry in India: The Future Prospects. *In: Strategy for Horticulture Development in India*, (eds. Kaul and Dadlani), p. 133-141, DAOC, Government of India, New Delhi, India.

[7] Verma RN. (2004). Indian Industry: Contribution and Potential of Himachal Pradesh. *In: Advances in Horticulture* (ed. Sharma), p. 139-142, Deep and Deep Publications, Pvt. Ltd., New Delhi, India.

[8] Verma RN. (2013). Indian mushroom industry - past and present. *Bulletin* 8, World Society for Mushroom Biology and Mushroom Products. p16.

[9] Thakur MP. (2014). Present status and future prospects of tropical mushroom cultivation in India: A review. *Indian Phytopath*. 67 (2): 113-125.

[10] Thakur MP and Singh HK. (2014). Advances in the cultivation technology of tropical mushrooms in India. *JNKVV Res J* 48(2): 120-135.

[11] Mahto Y and Verma RN. (2008). Organic production of oyster mushroom in India. In: Mus. Biol. Mush Prod. (eds. Lelley JL and Buswell JA). 6, 212-219.

[12] Verma RN *et al*. (1995a). Mushroom Flora of North-Eastern Hills. In : Advances in Horticulture (eds. KL Chadha and SR Sharma) 13: 329-349, Malhotra Publishing House, New Delhi, India.

[13] Verma RN and Upadhyay RC. (1995b). Mushroom Genetic Resources in India. In: Mushroom Genetic Resources for Food and Agriculture (eds. Labarere and Menini) Global Network on Mushrooms under the aegis of F.A.O. p 153-157.

[14] Purkayastha RP and Chandra A. (1985). Manual of Indian Edible Mushrooms. Today &Tomorrow's Printers and Publishers, New Delhi, pp. 266.

[15] Bhawani Devi S. (1995). Mushroom Flora of Kerala. In : Advances in Horticulture (eds. KL Chadha and SR Sharma) 13: 277-316, Malhotra Publishing House, New Delhi, India.

[16] Patil BD *et al*.(1995). Mushroom Flora of Maharashtra. In : Advances in Horticulture (eds. KL Chadha and SR Sharma) 13: 317-328. Malhotra Publishing House, New Delhi, India.

CHANGING GLOBAL SCENERIO IN MUSHROOM INDUSTRY

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ABSTRACT

Mushroom industry globally has expanded both horizontally and vertically, meaning that the expansion has been in production and addition of newer types of mushrooms for commercial cultivation, both edible and non edible mushrooms. Today China is leading in global mushroom production both in cultivation of edible and non edible types. China produces approximately 70 percent of world mushroom production and mushroom is their sixth economically important crop as far as country's revenue generation is concerned. The second highest mushroom producing country is USA, followed by some European countries. European production is confined to France, Germany, Holland, Italy and other countries in western-Europe. There is a matching contribution in mushroom production in Eastern European countries like Hungary and Poland where mushroom production has received a boost as can be seen from the production figures available and mushroom activity in these countries.

Keywords: global mushroom production, mushroom consumption

WORLD SCENARIO AT A GLANCE

Mushroom farming has become popular all over the world with advancement and dissemination of information on its cultivation technology. Mushroom is an indoor crop and the production can be controlled suiting a particular market condition. Global mushroom industry has expanded horizontally and vertically, meaning that the expansion has been in production and addition of newer types of edible mushroom varieties for commercial cultivation. Today China is leading in global mushroom production. China produces approximately 70 per cent of world mushroom production (2008), and mushroom is their sixth economically important crop in terms of country's revenue generation. The second highest mushroom producing country is USA, followed by European Union Countries. European Union production is confined to France, Germany, The Netherlands, Italy, Poland, Spain, Hungary and others. Lately there is increased contribution in mushroom production from Eastern European countries like Poland and Hungary where mushroom production has received a boost as evident from the production figures of these countries. I will discuss the production, consumption and trade volume of different mushroom importing and exporting countries in this presentation.

United States: United States of America is the largest consumer of mushrooms in the world today. US trade flows for canned mushrooms dominated by imports, especially those from China, India, and Indonesia. The trade deficit trended irregularly downward for China, India, and Indonesia from 2003-06 following the continuation of high antidumping duties on imports of canned mushrooms from these countries. By 2007-08, however, imports from China, India, and Indonesia had risen to a five-year high, in spite of the antidumping duties on US imports. China, India, and Indonesia have principle suppliers of canned mushrooms to the US market since 2003-04, are global exporters of canned mushrooms and have been very successful in selling lower priced products into the US market. Imports of canned mushrooms into US amounted in 64867 tons in 2007-08, down by 2 per cent from 66212 tons in 2003-04. Imports were down by 23 per cent in volume from 2007-08 to 2008-09. Imports from China were down in 2005-06, not only for antidumping duties already mentioned, but also because of a drop in Chinese production. U.S. exports of fresh and canned mushrooms are small relative to imports, amounting to 7212 tons and 709 tons in 2007-08, and 8119 tons and 1281 tons 2008-09, respectively. Canada is the principal market for US exports of fresh mushrooms, accounted for nearly 90 per cent of export volume annually since 2003-04. US mushroom exports to Japan and France, by comparison, are principally specialty mushrooms. There are no US non tariff measures applicable to imports of canned or fresh mushrooms, nor are mushrooms subjected to any domestic content laws, guaranteed minimum prices, or requirements that import be entered through certain ports. US imports of fresh and canned mushrooms must be packaged and labelled correctly and should have all the necessary

customs entry paperwork, according to existing regulations of US. There are no sanitary / phytosanitary measures affecting the entry of any mushrooms in the US market .

Global Market: Fresh and processed button mushrooms and fresh specialty mushrooms are produced and consumed in many countries. Fresh mushrooms are perishable, so their global movement often has been restricted to transactions mainly between neighbouring countries. The movement of fresh mushrooms on a global scale increased lately but canned mushrooms are shelf stable, with a shelf life of two to three years and thus, are the major mushroom product traded globally. Global mushroom production amounted to 3.4 million tons in 2007, trending steadily upward from 2003. China remained the leading global producer of mushrooms for all uses and has been for the past five years. Since 2005-06, the Chinese national government increasingly encouraged to shift their agricultural production out of traditional crops to value-added crops like mushrooms for processing. The United States and the EU countries were the second and third largest global producers, respectively, in 2007, other important global producers included Canada, Japan, India, Australia, and Indonesia. Countries showing noticeable increases in production included China, Spain, Poland, and Ireland. The production in most of the remaining countries decreased slightly or remained almost the same.

Global Consumption: In 2007, global consumption amounted to 3.3 million tons and China, the EU countries and the United States were the leading global consumers of mushrooms. Other major consumers included Canada, Japan, Russia, Australia, and India. Virtually all consumption in China, the EU, and India was supplied from domestic production. On the other hand, virtually all Russian consumption was supplied by imports. Finally, consumption in the United States, Canada, Japan, and Australia met mostly by domestic production but also by significant quantity of imports.

Global Trade: Global exports of canned mushrooms amounted to 458,137 tons in 2008, up by 25 per cent from 365,967 tons in 2004, with China accounting for 87 per cent of total export volume in 2008 and for nearly all the rise in global exports during the report period. The increase in exports from China through 2008 resulted from a fall in freight rates from China to most global markets in 2007–08. Other major global exporters in 2008 included Indonesia and India, although export levels from Indonesia remained almost the same throughout the 2004–08 period and exports from India during the same period were down because of intense competition from Chinese exports. Global exports of fresh mushrooms averaged around 43,730 tons during 2004–07 before falling to 34802 tons in 2008. Canada and the United States were the largest global exporters of fresh mushrooms in 2008, together accounting for nearly 80 per cent of the total, with most exports from both countries shipped to each other. Other major exporters in 2008 were Malaysia and Mexico. Most of the fall in exports of fresh mushrooms from 2007 to 2008 was accounted for by a drop in exports from China, where a greater share of fresh mushroom production was processed and mushroom growers in China switched into production of other crops. Exports from traditional supplier Canada also fell following a decision by Canadian shippers to concentrate in their home market as a result of an unfavourable change in the US-Canada exchange rate. Global imports of canned mushrooms amounted to 292,267 tons in 2008, up by 12 per cent from 260,944 tons in 2004, with the United States and Russia accounting for the largest individual shares of total import volume in 2008. Global imports of fresh mushrooms amounted to 90,879 tons in 2008, up by 42 per cent from 63,618 tons in 2004. Russia and the United States together were the most important global import markets in 2008. Canada, Norway, Malaysia, and Ukraine were other major markets. The rise in imports from 2004 to 2008 was due to a rise in Russian imports, with Russia becoming the primary market for Chinese mushrooms in 2007 and 2008.

Production and exports from Asia: China, India, and Indonesia are the three most important global mushroom exporting countries in Asia. All three countries became the major suppliers of canned mushrooms to the US market since 2003-04, together accounting for 86 per cent of total US canned-mushroom import volume in 2007-08. All three Countries have mushroom canning industries that are export oriented, with US market a primary destination for their production, and all face barriers to their exports in certain third-country markets.

China: China has become the leading global producer of mushrooms and exporter of canned mushrooms since 2004. China's dominance has occurred even though most Chinese growers are using growing methods considered rather primitive and low technology relative to those used in the United States and other major producing countries. China's common-

mushroom industry consists of thousands of small-volume family-run growing operations nationwide, employing mainly family labour. Common and specialty mushrooms are grown in sheds made of bamboo, straw, and clay, or in caves, without any mechanical climate control which limits production to the cooler months of October–December and March–May. Since 2003-04, mushroom canning in China is being done in number of very modern facilities. The Bluefield Industrial Food Company started production in 2004 and is one of only a few canneries that export products which do not have to pay any antidumping duties. This cannery, with an estimated production capacity of 80,000 tons of canned foods including mushrooms, is reported to have received FDA registration for processing low-acid canned foods and Hazard Analysis and Critical Control Point (HAACP), Quality Control Certification. COFCO Industrial Food import & Export Co., a subsidiary of COFCO Corporation, also operates a modern cannery and is reported to account for 10 per cent of Chinese canned mushroom exports in recent years. Dujiangyan Xingda Foodstuff Co., another exporter of canned mushrooms to the United States, also operates 56 modern mushroom-growing houses.

India: India produces about 250 thousand tons of edible mushrooms annually (author's assessment on spawn consumption). The Indian mushroom industry is made up of a few very modern growing and processing facilities. Agro Dutch estimated production capacity of 50000-60000 metric tons of fresh mushrooms for canning annually. In 2003-04, has accounted for an estimated 25 per cent of all U.S. imports of canned mushrooms to Canada, Mexico, Israel, and Russia. A second Indian mushroom grower and processor, Himalaya International also has a state-of-the-art, ISO certified, and HAACP compliant mushroom production facility including canned mushroom processing operation with an anticipated production of 9,000 metric tons of canned mushrooms annually. There are scores of smaller units of 200-500 TPA capacity (about 35-40 units) with climate controlled facility growing button mushrooms in almost all regions of the country in India, growing mainly button mushrooms for local market. These smaller units are located in Tamil Nadu, Haryana, Punjab, Uttar Pradesh, Maharashtra, Gujrat, Himachal Pradesh, West Bengal, Sikkim, Assam and other places in the country, besides the seasonal growing activity in western plains of India in winter months.

Indonesia: The Indonesian mushroom-canning industry has accounted for a major share of US canned mushroom imports since 2003-04, with nearly all such imports in retail-size cans, and the US market continues to be the principal export market for Indonesian products. ETIRA, an integrated mushroom growing, harvesting, and processing operation, grows 12775 tons of fresh mushrooms annually for canning and export. A number of other firms, including Aneka Janur, Jamur Bersaudara Dara PD, Jamur TC 99 Toko, Indo Evergreen Agro Business Corp. PT, and PT Karya Kompos Bagas, are selling canned mushrooms produced in their own or other firm's canneries.

Mushroom production, consumption and trade in European Union countries-2012: The EU is a global producer of mushrooms and has been for a number of years. In EU countries, the largest canned production in 2007 were from The Netherlands, Spain, Poland, France, and Italy, with The Netherlands accounting for nearly one-fourth of total EU production in 2007. Most mushrooms produced in the EU are traded within member countries and the EU is not a global exporter to non-EU countries. Countries shipping the greatest share of canned mushrooms within the EU in 2007 were the Netherlands, Spain, Poland, France, and Germany. Since its entry into the EU, Poland has become the EU's largest-volume producer of fresh mushrooms, growing more than 238000 tons of mushrooms annually. The mushroom industry in Poland is composed of more than 2000 mushroom farms, ranging in size from many small family-run farms to some large operations. The mushrooms produced are of high quality and the production facilities are technologically advanced. The costs of labour (mainly Romanian workers), energy, and supplies are less than those in the United States, and the capital investment by Polish growers, especially in their composting operations, is high. The combination of high-quality product and low production costs has enabled mushroom growers in Poland to ship fresh mushrooms even to some non-EU member countries.

Germany's per capita annual mushroom consumption of 3.2 kg is one of the highest, while as the quantity of mushrooms produced in the country is not very large (JA Lelle, Jan, 2014). In 2012, German production was nearly 62,000 tons, well below the production figures of the leading European nations such as Poland (238,000 tons), the Netherlands (250,000 tons), France (108,000 tons) and Spain (98,000 tons). Based on a survey by the German Mushroom and Edible Fungus Growers Association, to which nearly all German mushroom growers belong, 54,000 tons of the total production was sold on fresh markets and 8,000 tons in processed form. These production levels remained stable compared to the previous

year, although some producers have expanded their capacity while others have ceased mushroom production altogether. Since the previous year yielded very poor straw, which (along with horse manure) is the basis of the mushroom cultivation substrate, net revenues for the past two years were slightly lower than usual. The key edible fungus cultivated in Germany is still the common white button mushroom *Agaricus bisporus*. Although other cultivated fungi such as the king oyster mushroom *Pleurotus eryngii*, the commonly grown oyster mushroom *Pleurotus ostreatus* and shiitake mushroom *Lentinula edodes* are slowly catching up, production of these mushrooms in Germany for the year 2012 totalled only about 2000 tons.

Changing Consumption scenario in Germany: Mushroom consumption is increasing slowly but steadily. In 2011, sale of fresh mushrooms in Germany increased by nearly 2% following an 8% rise in the previous year. In 2010, a generally tight supply of vegetables in the country had a distinctly stimulating effect on the market for the button mushroom, by far the most important fungal species. When the food trade was searching for cheap promotional items in 2010, they often resorted to mushrooms. This additional impulse faded away in 2011 because fresh vegetables were conveniently available throughout the year. Nevertheless, sales rose by 2%, in line with the growth rate for fresh vegetables overall. Consumer prices for mushrooms have now risen again slightly after a drop in 2010. Consumer spending grew by almost 5%, which is clearly a better result compared to that for fresh vegetables (-4%). A point worth noting is that demand for chestnut mushroom shiitake is increasing slowly but steadily in Germany. According to Mr Jochen Winkhoff, General Manager of the German Mushroom and Edible Fungus Growers Association, the high point of the market for chestnut mushrooms is generally reached in the autumn. Since chestnut mushrooms have a higher dry matter content, their taste is particularly intense. A survey of distributors in the trade press suggests that the increasing consumption of chestnut mushrooms is not the white button mushroom. Therefore, it is possible to state that there is a slow increase in the overall consumption of mushrooms in Germany. This trend is also supported by the relevant statistics. The trend towards buying chestnut mushrooms continued in 2011 and 2012. Market share by volume purchased increased from 19% to almost 22%. In terms of expenditure, it is now as much as 29%. The market share of organic mushrooms has increased too, but their proportion of overall mushroom consumption is still low. The share of organic products is now just under 3% of the volume of fresh mushrooms purchased. It is over 6% in terms of spending, although most of the organic mushrooms sold are of expensive chestnut variety. The majority of mushrooms consumed are imported. A good 50% of the fresh mushrooms consumed in Germany are imports. As already stated, domestic production for the fresh market is about 54,000 tons. Of this, however, approximately 6,000 tons are exported. An additional 8,000 tons of German mushrooms are processed. Imports of fresh mushrooms were about 62,000 tons in both 2011 and 2012. The Netherlands and Poland are the main suppliers of fresh mushrooms, each accounting for approximately 30,000 tons. Hungary follows in third place, but with only 500 tons. Hardly any other edible mushrooms are imported into Germany in any quantity. One exception is fresh *Cantharellus cibarious* mushroom collected from wild, large quantities of which are brought into Germany from eastern Europe (Poland, Belarus and the Baltic States).

Canada: As in the United States, the most commonly grown mushroom throughout Canada is the common white mushroom. Mushroom production is located principally in two provinces, with Ontario and British Columbia accounting for 57 and 37 per cent, respectively. Of the total in 2008.76 tons In 2008, Canada was a net exporter of fresh mushrooms but a net importer of canned mushrooms.

Mexico: Although extensive data on the mushroom growing and processing industry throughout Mexico are not available, Mexico is growing in importance as a supplier of fresh and canned mushrooms to the U.S. market. Commercial mushroom production of common white, portabella, and cremini mushrooms is cantered in the San Miguel de Allende area of Guanajuato state. A US -owned facility in San Miguel de Allende that grows and processes mushrooms is described as the single largest growing operation in Mexico. The company covers about 90 acres and accounts for the growing and processing of about 20 million pounds of mushrooms annually. The facility is vertically integrated, consisting of a spawn centre, large composting areas, growing rooms, cold storage for warehousing fresh mushrooms, and a cannery. The production of fresh mushrooms for shipment principally to the United States has risen since 2003/04, encouraged by the strength of the U.S. dollar vis-à-vis the Mexican peso and supported by US investment from Monterey Mushrooms, Inc.

REGIONAL MUSHROOM *MELA* AND *KISAN GOSHTHI*: ENABLING FARMERS TO ACCESS TECHNOLOGY AND ADVISORY SERVICES

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ABSTRACT

Melas (Field days) and *kisan goshthis* (Farmer-expert interactions) have been key activities of agriculture research institutes to spread awareness about the technologies among clients and to address their problems.The Directorate of Mushroom Research initiated the concept of regional mushroom *mela* and *kisan goshthis* during 2010. Unlike the national mushroom mela, the regional mushroom melas were organised among the cluster of mushroom growers in Haryana, Punjab and Jammu & Kashmir with three key objectives of spreading the advanced technology among the rural farmers, addressing the problems encountered by mushroom growers in cultivation by expert advises and promoting mushroom consumption among masses. Five such *melas* were organised during 2010-11 with prior planning, preparation and publicity. About 200-350 mushroom growers other farmers, entrepreneurs, several officials, scientists and technical staff from different government organisations, financial institutions, krishi vigyan kendra took part in the mela. It was observed that, less than 10 per cent of women participated in the mela except one place (Khanpura, Punjab) where nearly 18 per cent women attended. With respect to the age group, the young people aged between 15-35 years outnumbered the other age groups in all the five melas. The event was organised to synchronise with the seasonal mushroom growing activities in the area. The majority of the participants at all the venues expressed happiness and approval about the timing of the event, benefits for participants, participation in future events, facilities and arrangements during the mela and recommending to others for participation in future events.

Keywords: mushroom, regional *mela*, *kisan goshthi*, consumption fair, advisory services

INTRODUCTION

Extension programs seek two general objectives. The first is to provide technical education services to farmers through demonstrations, lectures, contact farmers and other media. The second is to function in an interactive fashion with the suppliers of new technology, by providing demand feedback to technology suppliers and technical information to farmers to enable them to better evaluate potentially useful new technology and ultimately to adopt (and adapt) new technology in their production systems [1]. Agricultural research institutes and State Agricultural Universities in India organize *melas* (Field days) and *kisan goshthis* (Farmer-expert interactions) as means of extension programmes to spread awareness about the technology generated at institute among their clients and to address their problems.

The ICAR-Directorate of Mushroom Research, Solan (HP) organizes a National Mushorom Mela during September every year at its campus in Solan. This mela aims mainly to showcase the improved technologies in the production, protection and post harvest processing of different cultivated mushrooms. A *kisan gosthi* is organized during the mela with an objective to provide opportunity for all the stakeholders to interact with the scientists and experts of the Directorate and other organizations. The annual mushroom mela is attended by large number of mushroom growers, entrepreneurs, spawn and compost suppliers, mushroom businessmen and scientists. An impact assessment of this *mela* for more than ten years revealed that, the mushroom growers and entrepreneurs felt the need of organising such *melas*, *kisan gosthis* and promoting mushroom consumption at regional level especially among the cluster of mushroom growers [2].

Sensing this felt need of the stake holders, the Directorate of Mushroom Research, initiated the concept of regional mushroom *mela* and *kisan goshthis* during 2010. Unlike the national mushroom mela, the regional mushroom melas were organised among the cluster of mushroom growers in Haryana, Punjab and Jammu & Kashmir with key objectives of spreading the advanced technology among the rural farmers, addressing the problems encountered by mushroom growers in cultivation by expert advises and promoting mushroom consumption among masses. The present paper aims to evaluate

five such regional mushroom melas organised during 2010-11.

METHODOLOGY

The regional mushroom *mela/ kisan goshthi*/ mushroom consumption fair were organised at a central place accessible to large number of mushroom growers in the region. The preparations for organising the mela were done with the support of progressive mushroom grower as the host of the event, officials of department of horticulture/ agriculture, financial institutes, development agencies, etc. Wide publicity was given in the region to make the growers aware about the mela being organised. The latest technologies developed in the mushroom cultivation were presented in the form of demonstrations, displaying of posters, distribution of samples, etc. In order to bring awareness about the mushrooms among common people, several posters were displayed to show the health benefits of mushroom, variety of value added products from different types of mushrooms, cultivation technologies of different mushrooms, spawn production technology, etc. A registration kit containing all the complementary literature of ICAR-DMR, Solan, a writing pad and a pen was given to all the people attending the mela. All the publications of ICAR-DMR including the mushroom recipes were kept for sale in the stall. Mushroom fortified biscuits, mushroom pickle and other protein enriched processed products of mushrooms were kept for sale for the visitors. Mushroom recipe was served in the lunch as a means to show the delicacy of the vegetable. Either a State Minister, Secretary of the government department or key officials of ICAR or other University was invited to all the events as guest of honour.

In the present study, the evaluation of all the regional *melas* was carried out with respect to participation by mushroom growers/ entrepreneurs and their distribution, chief source of information about the mela, the feedback by respondents attending the *mela* about the season or timing of the event, usefulness of the event, and suggestions for future organisation of the event. Descriptive statistics is employed for analysis and to draw suitable conclusions.

RESULTS AND DISCUSSION

The results of the study are analysed and discussed under various headings as listed in the methodology. The details of the events along with the timing, host of the event, place and date are presented in Table 1.

Table 1. Details of Mushroom *mela*, *kisan goshthi* and mushroom consumption fairs during 2010-11

Sl. No	Month and Year	Details of the event	Host of the event	Place and state
1	January, 2010	Regional mushroom *mela* and *kisan goshthi*	K S Chauhan, progressive farmer, mushroom grower	Aterna village, Sonepat District, (Haryana)
2	January, 2010	Regional mushroom *mela* and *kisan goshthi*	Vijender Dhankad, progressive farmer, mushroom grower	Gannaur village, Sonepat District, (Haryana)
3	February, 2011	Regional mushroom *mela* and mushroom consumption fair	Harsunjith Singh Thiara, progressive mushroom grower	Thiara mushroom farm, Khanpura village, Dt Hoshiarpur (Punjab)
4	February, 2011	Regional mushroom *mela* and mushroom consumption fair	HAIC (Cooperating centre of DMR, Solan)	Haryana Agro Industrial Corporation, Murthal (Haryana)
5	October, 2011	Regional mushroom *mela* and *kisan goshthi*	Department of Agriculture, Govt. of J&K	Krishi Bhavan, Talab tillo, Jammu (Jammu & Kashmir)

Field days are usually opportunities to hold demonstrations on a slightly larger scale, and are usually run in a more informal and less highly structured manner. The purpose is often to introduce a new idea and a new crop, and to stimulate the interest of as many farmers as possible. Experimental stations or other government centres may be used for field days, but it is more usual and profitable for them to be held on the land of a local farmer. There is a greater chance of making an impact if the field day is held on a farmer's land, and if the farmer plays a part in running it and explaining the purpose [3]. Hence, the regional mushroom melas were mostly organised at progressive growers field to have good impact over others and to

demonstrate improved practices. At Murthal and Jammu, the premises of Haryana Agro Industrial Corporation (HAIC) and Department of Agriculture, Government of Jammu were made use to organise these melas as they were most accessible to large number of mushroom growers in the adjacent locality.

Participation of mushroom growers/ entrepreneurs

The participants attending the mushroom *mela* or *kisan goshthi* were either mushroom growers or entrepreneurs involved in supplying inputs for mushroom cultivation like spawn, wheat straw, gypsum, inputs for construction of mushroom houses/ huts, machinery suppliers etc. The data on the distribution of participants attending the *mela* is presented in Table 2 and as average age and gender in Fig.1. The number of officials and scientists attending the events were not included in the data.

Table 2. Distribution of participants attending the mushroom *mela*/ *kisan goshthi* based on gender and age group

Sl. No	Place and state	By gender		By age group in years			Total participant farmers
		Male	Female	15-35	36-50	51 or above	
1	Aterna village, Sonepat district, (Haryana)	224 (91.80)	20 (8.20)	118 (48.36)	83 (34.02)	43 (17.62)	244
2	Gannaur village, Sonepat district, (Haryana)	206 (92.79)	16 (7.21)	101 (45.50)	68 (30.63)	53 (23.87)	222
3	Thiara mushroom farm, Khanpura village, Dt Hoshiarpur (Punjab)	150 (82.42)	32 (17.58)	74 (40.66)	66 (36.26)	42 (23.08)	182
4	Haryana Agro Industrial Corporation, Murthal (Haryana)	325 (91.04)	35 (9.80)	171(47.90)	126 (35.29)	60 (16.81)	357
5	Krishi Bhavan, Talab tillo, Jammu (Jammu & Kashmir)	230 (91.63)	21 (8.37)	112 (44.62)	96 (38.25)	43 (17.13)	251

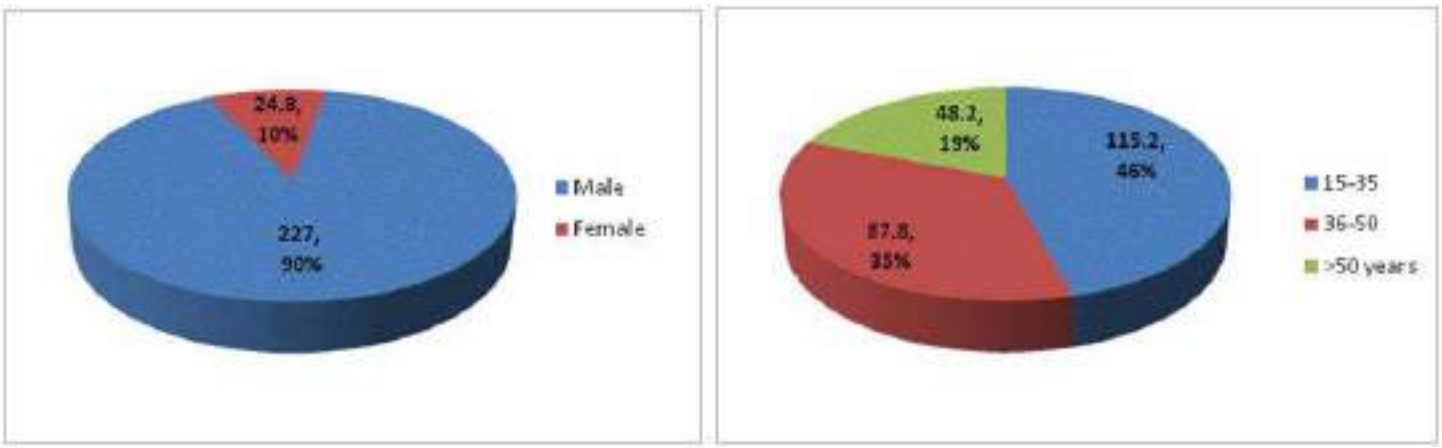

Figure 1. Distribution of participants attending mushroom *mela* (average 5 melas) according to gender and age group

From the data, it is observed that less than 10 per cent of women participated in all the *melas* except at one place (Khanpura, Punjab) where nearly 18 per cent women attended. With respect to the age group, the young people aged between 15-35 years outnumbered the other age groups in all the five melas. Participants between 36-50 years were also in considerable proportion (30-40 per cent) followed by the people above 50 years. Though, the participation of young aged people is a welcome trend, the lesser participation of women needs to be addressed as mushroom cultivation is

deemed as women friendly enterprise, offering potential employment and livelihood opportunities for women folk. The social and cultural constraints could be possible reason for lesser participation of women in such events. Similar results were observed by Khan *et al.* [4] and Koyenikan and Edioghon [5] under similar circumstances

Source of information

The organisation of the events were preceded by wide publicity through letters, e-mails, farm to farm contact, news paper advertisement, banners at various places and publicity through officials of department of agriculture/ horticulture and other institutions vested with such work. Though, the farmers were apprised about the event by multiple sources, the single chief source of information as mentioned by the respondent is mentioned in Table 3.

Table 3. Chief source of information about the *mela* for respondent farmers

Sl. No	Place and state	Chief source of information about mela					Total responding farmers
		DMR staff	Dept official	News paper	Neigh-bours	Others	
1	Aterna village, Sonepat District, (Haryana)	62	18	9	29	22	140
2	Gannaur village, Sonepat district, (Haryana)	76	12	2	12	9	111
3	Thiara mushroom farm, Khanpura village, Dt Hoshiarpur (Punjab)	80	46	2	10	14	152
4	Haryana Agro Industrial Corporation, Murthal (Haryana)	28	74	14	17	7	140
5	Krishi Bhavan, Talab tillo, Jammu (Jammu & Kashmir)	23	127	5	22	4	181
	Total (Per cent)*	269 (H ≈ 37%)	277 (H 38%)	32 (4H %)	90 (12H %)	56 (8H %)	(724)

* Per cent figure given in parenthesis is rounded off to nearest round figure.

The majority of the farmers indicated that, either DMR staff (37 per cent) or officials of state department of agriculture and horticulture (38 per cent) were the chief source of information about the mela. Sizeable proportion of people said that, they got the information from their neighbours (12 per cent) and few from the newspapers (4 per cent) and other sources (8 per cent).

Impact of regional mushroom *mela*

Besides, the number of mushroom growers participated; the impact of the regional mushroom mela and the kisan goshthi is assessed based on the timing of the event, feedback on facilities and arrangements, perceived benefits among the respondents and their willingness to suggest others to participate in such events in the future. The details of the feedback are presented in Table 4.

Season or timing of the events

The organisation of such melas and goshthis were synchronised with seasonal growing of white button mushroom (*Agaricus bisporus*) in North Indian states of Haryana, Punjab, Himachal Pradesh and adjacent areas of national capital New Delhi. Farmers in this area take up seasonal mushroom production starting from October to March extending at times up to April depending upon prevailing weather conditions. It was observed that, generally composting and shed construction begins in October and cropping starts from November. Coinciding with second month of first cropping, they prepare another lot of compost and fill it after disposing compost of first crop. However, in Himachal Pradesh peak winter month- December is

Table 4. Feedback of respondents' perception about the mela

Sl. No	Farmers' perception about the event	Locations									
		Aterna		Gannaur		Khanpura		HAIC		Jammu	
		Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
1	Appropriate nos. of timings of the event	58 (41.4)	82 (58.6)	64 (48.9)	67 (51.1)	89 (62.2)	54 (37.8)	42 (30.7)	95 (69.3)	125 (82.2)	27 (17.8)
2	Benefitted with the regional melas/goshthis	115 (82.1)	25 (17.9)	103 (78.6)	28 (21.4)	123 (92.5)	10 (7.5)	98 (71.5)	39 (28.5)	142 (93.4)	10 (6.6)
3	Willingness to participate in the next regional mela	124 (88.6)	16 (11.4)	107 (81.7)	24 (18.3)	119 (89.5)	14 (10.5)	106 (77.4)	31 (22.6)	136 (89.5)	16 (10.5)
4	The facilities and arrangements in the mela were good	131 (93.6)	9 (6.4)	91 (69.5)	40 (30.5)	120 (90.2)	13 (9.8)	119 (86.9)	18 (13.1)	131 (86.2)	21 (13.8)
5	Willing to recommend to others to participate in such events in future	130 (92.9)	10 (7.1)	107 (81.7)	24 (18.3)	122 (91.7)	11 (8.3)	105 (76.6)	32 (23.4)	135 (88.8)	17 (11.2)

(values in parenthesis are per cent values)

avoided for cropping as the temperature dips very low (<10 ºC). In Jammu, single crop is taken by majority of the farmers during November–January.

The majority of the participants from three places disagreed to the statement that, the timing of the event was appropriate. The majority of the respondents at Khanpura and Jammu stated that, the timing was appropriate (Table 4). Since, the season was half way at the time of the organisation of mushroom mela in the first four events, the majority felt that, such an event at the beginning of the season will help the growers. The mela and kisan goshthi was planned ahead of the season elicited better response among the farmers with respect to the timing of the event.

Impact of the mela

The majority of the farmers agreed that they were benefitted with such *melas* and *goshthis* and would recommend to others to participate in such events in the future (Table 4). The majority of the respondents also expressed willingness to participate in the next regional mushroom *melas* and *kisan goshthis*. The respondents expressed satisfaction over the facilities and arrangements during the mela.

Suggestions for future events

In the open ended response, several suggestions were elicited from the farmers. Few of the suggestions with higher frequency and relevant to the event are discussed below. The majority of the respondents suggested to plan and organise such event either before the start of the winter season or immediately after the onset of winter season. The suggestions at the earlier events, helped to plan the event at Jammu during October month of 2011. Some suggested to give publicity through television and other news media. The live demonstrations of different varieties near the venue were requested for seeing the results for comparison. Few suggestions were received on logistics and other arrangements. It emerged during the organisation of these events that, a local growers achievements must be recognised in the form of appreciation or an award.

CONCLUSION

Such regional melas, focussing the location specific issues and problems can be highly beneficial to the farming community. Besides helping farmers to access the advances in mushroom cultivation, people get to aware about the importance of mushrooms in the diet and thus augmenting mushroom consumption in our country. The concurrent organisation of *kisan gosthi* becomes a welcome interface and facilitate direct and face to face interaction of farmers with scientific community.

Further innovations and amendments to suit to; each component of mushroom enterprises (spawn, compost, machinery, post harvest processing), stakeholders, region, constraints prevailing, etc. would bring higher impact among the mushroom farming community in the rural areas.

ACKNOWLEDGEMENTS

The authors acknowledge the contribution and support of HAIC, Murthal, Department of Horticulture, Government of Haryana and Punjab, Department of Agriculture, Government of Haryana, Punjab and Jammu and Kashmir, Progressive mushroom growers of the region, officials from KVKs and other organisations in organising the regional mushroom mela and mushroom consumption fair in these three States of India.

REFERENCES

[1] Evenson RE. (2000). Economic Impacts of Agricultural Research and Extension. In: Handbook of Agricultural Economics, Gardner B and Rausser, G. (Eds). Elsevier Science, Amsterdam, Ch 11, pp. 574- 616

[2] Mahantesh Shirur *et al*. (2011). Impact Assessment of National Mushroom Mela. *Mushroom Res.* (20)2: 95-102.

[3] Oakley P and Garforth C. (1985), Guide to extension training. FAO, Rome. http://www.fao.org/docrep/t0060e/t0060e07.htm

[4] Khan M *et al*. (2012). Participation of women in agriculture activities in district Peshawar. *Sarhad J. Agric*. 28(1): 121-127.

[5] Koyenikan MJ and Edioghon CO. Gender participation in agricultural extension activities of ondo state agricultural development programme (adp): implications for sustainable developmenthttp://www.uniben.edu/sites/default/files/abstracts/gender_participation_in_agricultural_extension_activities_of_ondo_state_agricultural_development_programme.pdf

STATUS AND SCOPE OF MUSHROOM CULTIVATION IN HARYANA-CONSTRAINTS AND FUTURE OUTLOOK

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ABSTRACT

Agaricus bisporus (white button mushroom), being a temperate mushroom was initially grown only in the hilly regions of India, particularly Himachal Pradesh and Kashmir, under seasonal conditions. Seasonal cultivation of this mushroom was introduced in North-Indian plains including Haryana in 80s. Since, then the state has achieved a tremendous increase in mushroom production with current production of more than 10,000 tonnes per annum. Though the production technologies for *Agaricus bisporus, A. bitorquis* (button mushroom), *Pleurotus* spp. (oyster mushroom), *Calocybe indica* (milky mushroom) and *Volvoriella volvacea* (paddy straw mushroom) have been developed, yet all these mushrooms could not achieve the commercial status except white button mushroom (*A. bisporus*). This mushroom is cultivated in India under both controlled and natural conditions. Even though, centralized facilities have been developed in some parts of the state to provide spawn and pasteurized compost, the quality of spawn, low and variable productivity and lack of industries involved in post-harvest processing/ value addition are the problems faced by many growers.

Keywords: seasonal cultivation, button mushroom, oyster mushroom, milky mushroom, paddy straw mushroom

Indian agriculture is known for its multi-functionalities of providing employment, livelihood, food, nutritional and ecological securities. Mushroom production leads to the bioconversion of agro-residues into nutritious food. India produces about 600 million tonnes of agricultural byproducts, which can profitably be utilized for the cultivation of mushrooms [1]. Currently, we are using 0.04% of these residues for producing around 1.2 lakh tons of mushrooms of which 85% is button mushroom. India contributes about 3% of the total world button mushroom production [2]. In the wake of increasing population, increase in awareness about health benefits of mushroom and changing food habits, the demand for various mushrooms is likely to increase sharply. Mushroom cultivation can contribute in achieving nutritional and social security along with promotion of value addition, canning and export industries also. Some of the mushrooms like oyster, paddy straw and milky mushroom have simple cultivation technology. The cultivation methods for these as suited to Haryana conditions have also been standardized. Mushroom being rich in protein is considered very important particularly for people of Haryana as majority of its population is vegetarian. The cultivation of mushroom has been adopted in the recent years by the farmers of Haryana because the economic returns are much higher compared to other agriculture crops and has great scope because of the proximity to Delhi.

The results presented are based on different studies of seasonal mushroom farms undertaken in Haryana. The 34.9% of the total investment is on fixed inputs while 65.1% is on variable inputs. In the fixed investment, major share is spent on construction of mushroom sheds. In the variable costs, maximum proportion is spent on labour (28.09%) followed by that on straw (14.96%) and bran (8.96%). A perusal of studies revealed that net returns per kg of mushroom produced was ₹ 9.50 on an average selling price of ₹ 30.00 per kg. Since benefit cost ratio over variable cost, total production cost and total cost were greater than the mushroom production is a highly profitable proposition. Mushroom produced in the state is sold in Delhi market through commission agents and is also sold directly either to the processing firms or the traders. But these are not transparent and the farmers remain at the receiving end. The marketing cost of mushroom is quite high and costs such as washing, weighing, packing etc have necessarily to be made by the farmers. The major marketing costs are commission charges (41.05%) and transportation (36.52%). These charges can be rationalized through collective efforts of farmers and government. Mushroom growers also pointed out the unauthorized charging of commission fee for selling their mushroom from designated 'farmers sheds' where they are not supposed to pay any commission charges. Direct marketing of mushroom by farmers to processing firms and traders also involves manipulative and unethical practices by these like reduction in predetermined contract price on flimsy excuses of quality and delay / even default in payments. When prices are low, they do not purchase from the contracted farmers. Keeping in view, the farmers vulnerability, they have to put joint or group efforts and these may or may not be formal but should be the practical ones. The quality at

competitive price is the key to success and farmers have to make an assertive shift from 'production mode' to 'production with quality mode' by applying latest production and marketing techniques. Vertical integration of agriculture and food markets from farm to firm is the best way to achieve efficiency and serve the interest of each stakeholder in the chain i.e. the farmer, the processor, the retailer and the consumer. There is no organized assistance available for marketing of mushrooms in India. Every export-oriented unit has its own individual arrangement for marketing, and the mushrooms are preserved in brine and canned in large containers of 3-5 litres (or bigger) capacity for export. A long-term strategy has to be developed to help the industry.

As India itself is a big market, vigorous extension activities are required to make the people aware of mushrooms, which can help in developing domestic market. This is the key of success of Chinese mushroom industry as they consume more than 80% of mushroom produced by them. Mushroom revolution is going to happen as a result of the improved productivity as well as vast increase in the number of environment controlled and seasonal growing units. The share of the button mushroom is bound to decline with more and more specialty mushrooms becoming available for seasonal cultivation. With the increased production, there is every likelihood that an organized mushroom marketing channel is established. A significant quantity of mushrooms is likely to be utilized for production and consumption of the value added products, as compared to present trend of fresh mushroom utilization. The venture will also be designed and directed towards complete recycling of the agro-wastes for production of food (mushroom), feed (cattle feed), fuel (biogas) and fertilizer (organic manure).

Majority of the farmers are cultivating mushrooms only during particular seasons. Farmers in the plains of North India cultivate white button mushrooms during winter only and stop the mushroom cultivation during summer and dismantle their temporary growing houses. It is also paradoxical to note that India is largely a tropical country and we mainly cultivate temperate mushroom. The tropical and sub tropical mushrooms like oyster (*Pleurotus* spp.), paddy straw (*Volvariella* sp.), milky (*Calocybe* sp.), reishi (*Ganoderma* sp.), wood ear (*Auricularia* spp.) etc. are not cultivated on a larger scale. Hence, the continuous cultivation of different mushrooms depending on the season is certain to increase the economic returns of the mushrooms growers. Round the year cultivation assumes much significance especially for rural livelihood security.

Button mushroom compost is prepared using variety of base materials like wheat straw, paddy straw, sugarcane bagasse, chicken manure, various cakes and brans, as per their availability in different regions of the country. In major parts of Haryana wheat straw is widely used whereas in some parts, paddy straw is used which is available in abundance. Poultry manure, an important component meeting the nitrogen needs, is available throughout the state and is used mostly in short method of composting. Growers following long method of composting generally use chemical fertilizers in place of poultry manure for balancing the nitrogen requirement in the compost. Poultry manure is a carrier of nematodes and competitor moulds and hence, is not preferred for unpasteurized compost. Haryana farmers have successfully adopted low cost technologies developed by Haryana Agriculture University (involving thatched structures, bed cultivation and prolonged cropping using compost prepared by long method) for seasonal cultivation and contribute over 10,000 tons of mushrooms annually.

The cultivation technique of white button mushroom requires an indoor temperature between 15 to 25 ^{o}C i.e. 22-25 ^{o}C (vegetative growth) and 14-18 ^{o}C (fruiting). In North India it can be grown conveniently during October to February under natural conditions. The compost is prepared by mixing various raw materials (wheat/paddy/brassica straw) in specific proportions either by long or short method of composting. Since preparation of compost by short method requires specialized unit which is not feasible for small and marginal farmers; hence, only long method, which takes 28 days is being used. The wheat straw can be replaced by paddy or brassica straw depending on the formulae to reduce the cost of cultivation. For spawning, spawn is available in polypropylene bags. For casing, generally, a mixture of FYM and garden soil/ field soil is used. But a mixture of burnt rice husk and garden soil/ field soil (1:1) has been found to be cost-effective. As burnt rice husk needs no sterilization, only garden/field soil is sterilized. Temperature around 25 ^{o}C till 1 week after casing is most desirable and subsequently temperature should be 14-18 ^{o}C and relative humidity of 80-90% (maintained by spraying water) in the mushroom house during entire fruiting period. Mushroom is picked in button stage; and for marketing generally packaging is done in polythene bags containing 200 g mushrooms and few holes are made in the packets to avoid condensation of the moisture in the bags.

Both the production systems viz., seasonal and controlled environment growing are in practiced. Environment controlled growing of button mushrooms may not be fully successful in our country if implemented as such without bringing in necessary modifications needed for Indian conditions. The following aspects that need attention are: development of environment

friendly composting process and shortening composting period by total indoor/single phase and use of microbial consortium etc; eco-friendly methods of pasteurization and standardisation of growing houses for optimum yields especially for low cost structures; improved casing materials, having easy and uniform application qualities; mechanization, as in future manual operations will become costlier.

Cultivation of oyster mushroom (locally known as Dhingri) in Haryana has tremendous potential, which is yet to be fully harnessed. Its cultivation technology is very simple as no special compost preparation and casing is needed. During the year many crops can be taken. Its production is more than the white button mushroom. It can be consumed fresh as well as can be sun dried for future use. Milky mushroom; due to its robust size, milky white colour, flavour and long shelf life has attracted attention of both consumers and prospective growers. Its nutritive value is also at par with other edible mushroom. The cultivation method is similar as that of oyster mushroom, except that it requires casing. For promotion of these, efficient low-cost region-specific growing systems and species for seasonal and round- the-year commercial cultivation should be developed and use of spent substrate for recycling as manure or cattle feed may be promoted [3].

Diversification of mushroom portfolio in the state and the country is required for round-the-year cultivation of different mushrooms to ensure environment and employment sustainability. Promotion of medicinal mushrooms *Ganoderma lucidum* and *Grifola frondosa*, which are very popular medicinal mushrooms and have great demand in the international market is also necessary. These can be cultivated under natural conditions on small as well as industrial scale.

Spawn acts as the seed, and is the most crucial input for successful cultivation of mushroom and there only two government institutes involved. However, in India the spawn industry is an un-organized venture and needs research support in the years to come so that it may attain quality standards and competitiveness comparable to multi-national companies. Development and enforcement of spawn standards in the country should be done at the earliest in the interest of mushroom growers.

Mushroom like other crops are also attacked by different diseases, insect-pests which affect the quality and yield. So, in the interest of mushroom growers researchers should pay attention to the following: Use of botanical pesticides, bio-control agents genetic resistance to manage diseases and pests. Development of quick diagnostic tools for detection of nematodes infestation, and standards for residual toxicity of chemicals used by the mushroom industry.

Increased productivity demands proper post harvest infrastructure to enhance shelf life and marketability. Mushrooms are delicate and highly perishable in nature. Short shelf life of mushroom poses unique problems in packaging, marketing and preservation of mushrooms. The retail packaging for fresh marketing is highly crude and is done in hand sealed polypropylene bags [4]. Similarly, canning in tin cans for button mushrooms and sun drying for other mushrooms are the most common methods of preservation employed. The aspects which have to be given greater attention are; low cost drying technology for the domestic and state-of-the-art technology for international market, modified atmosphere packaging and controlled atmosphere packaging, use of recyclable and biodegradable packing material, reduction in blanching losses during canning, development of low cost freeze-drying and IQF technologies; and ready-to-cook recipes, value-addition and product diversification to cover pharmaceutical, cosmetic and fast food industries.

The spent mushroom substrate (SMS) left after final crop harvest is a matter of concern as it creates various environmental problems including ground water contamination and nuisance. As mushroom production is increasing, so is the SMS generation, which calls for alternative management of this waste. Fortunately, SMS has many positive attributes still left for its potential uses. The material has been found to be a good nutrient source for field and horticultural crops because of its nutrient-status.

REFERENCES

[1] Singh M *et al.* (2011). Mushroom cultivation, marketing and consumption. Directorate of Mushroom Research (ICAR) Chambaghat, Solan (HP) India. pp. 3-14.

[2] Chang ST. (2006). The world mushroom industry-trends and techonogical development. *Intl. J. Med. Mushrooms*. 8: 297-314.

[3] Ahlawat OP *et al.* (2010). Bioremediation of fungicides by spent mushroom substrate and its microflora. *Indian J Microbiol.* 50(4): 390-395.

[4] Banga G *et al.* (2013). Marketing practices of mushroom growers in Punjab. *Mushroom Res*. 22(2): 137-143.

EVALUATION OF MUSHROOM TYPES SUITED FOR KUTTANAD THROUGH PARTICIPATORY TECHNOLOGY DEVELOPMENT

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ABSTRACT

The present study was undertaken with a view to evaluate mushroom types best suited for cultivation in Kuttanad during rainy and summer seasons based on Participatory Technology Development (PTD), nutrient analysis and consumer survey. Results confirmed that pink oyster, *Pleurotus eous* (875.5 g/bed) was significantly superior in mean yield over *Pleurotus florida* (786 g/bed) and *Pleurotus sajor- caju* (671.7g/bed). During the rainy season (July- August 2012), highest biological efficiency (87.5%) was exhibited by *Pleurotus eous* compared to *Pleurotus florida* (78.6%). Mean yield of pink oyster (790 g) was on par with milky mushroom *Calocybe indica* (800g). Nutrient analysis of different types of mushroom harvested from different types of substrate revealed that pink oyster was nutritionally better as it contained lower carbohydrate, higher protein and highest dietary fibre compared to *Pleurotus florida*, *Hypsizygus* sp. and *Pleurotus sajor-caju*. Mineral content was also optimum when compared to other mushrooms under study with a good amount of potassium (2800 ppm). In the comparative analysis of nutrients of different mushroom types grown on steam sterilized paddy straw , concentration of proximates like carbohydrate (15%) and fibre (3.4%) was maximum in pink oyster when compared to *Pleurotus florida* and *Pleurotus sajor-caju*. With regard to mineral content also, *Pleurotus eous* showed maximum concentration of magnesium (174 ppm) and zinc (16.9 ppm). In consumer survey, pink oyster ranked second in consumption (56%) and was mainly due to its less availability when compared to *Pleurotus florida.* Based on the PTD trials, nutrient analysis and consumer survey, pink oyster- *Pleurotus eous* can be recommended for cultivation in Kuttanad region of Kerala for both rainy and summer seasons. *Pleurotus florida* also can be recommended for rainy season and *Pleurotus eous* and *Calocybe indica* for summer season of Kuttanad.

Keywords: Kuttanad, nutrient analysis, proximate, minerals.

INTRODUCTION

Kuttanad is a special agro ecological zone representing the water logged lands spread over 69 panchayaths of Alappuzha, Kottayam and Pathanamthitta districts of Kerala state. Large area of this land is below, at, or just above mean sea level. Climate is tropical humid monsoon type with a mean annual temperature of 27.6 ^{o}C and rainfall 2746 mm. Humidity in general is very high. Wet lands of Kuttanad, the rice bowl of Kerala stretch over an area of 35,500 ha where rice is cultivated. Total production of rice vary from 1.6 lakh tons to 1.75 lakh tons and straw from 2.25 lakh tons to 2.5 lakh tons. Usually this large quantity of straw is left in the field for incorporation in the soil. A viable alternative for Kuttanad farmers for recycling of agro waste into protein rich food is mushroom cultivation. Hence, the present study was undertaken with a view to evaluate mushroom types best suited for cultivation in Kuttanad during rainy and summer seasons based on Participatory Technology Development (PTD), nutrient analysis and consumer survey, since no previous work has been conducted so far in this regard.

MATERIALS AND METHODS

Identification of Mushroom Types Suited For Kuttanadu

Mushroom varieties used for PTD Trials were shown in Plate 1

Plate 1. Mushroom varieties used
Identified by Dr. D. Geetha , Professor, College of Agriulture, Vellayani

Rainy Season: Ten farmers from Alappuzha and Pathanamthitta districts who were engaged in mushroom cultivation for over two years were selected based on the recommendation of the Agricultural officer of the concerned Krishi Bhavan. Varieties used are given in Plate 1. The experiment was laid out in Complete Randomised Design with three treatments and six replications under natural conditions. 10 beds were used for each variety. Chemically sterilized paddy straw was used. Weight of each mushroom bed was fixed as 1 kg of dry substrate. They were prepared as per the standard procedure of multilayered spawn running technique. Mushroom beds were kept in a mushroom shed of length 6m and breadth 4m. Polythene bags were removed after completion of spawn running stage. Observations on yield, number of days for spawn running, days for first harvest, interval between first and second harvest, cropping period, mean maximum temperature and relative humidity of the shed were recorded. Data was tabulated, statistically analysed and inferences were made.

Summer Season: The experiment was laid out in Completely Randomised Design in 10 locations with five treatments and six replications under natural conditions. Trial was also conducted at research station. Observations recorded were same as above. Data was tabulated, analysed statistically and inferences were made.

Nutrient Analysis

Nutritional analysis of mushrooms grown on chemically sterilized paddy straw and also from different substrates like paddy straw, rubber wood saw dust, banana pseudostem waste were conducted at the Quality Control Lab of Central Institute of Fisheries technology, Kochi on payment basis. AOAC methods were followed . Proximates like carbohydrate, fat, protein, fibre and mineral like sodium, potassium, calcium, magnesium, iron, molybdenum, selenium and zinc were analyzed.

Consumer Survey

A consumer survey was conducted with a sample size of 100 among the mushroom farmers of Kottayam and Alappuzha districts of Kuttanad for assessing the preference in terms of consumption. Beneficiaries were selected from the consumers of mushroom farmers who were members of Mushroom Club, RRS, Moncompu.

RESULTS AND DISCUSSION

PTD trials: The Rainy Season (June-July, 2012)

Data on yield and biological efficiency of mushrooms under study i.e. *P. florida, P. eous* and *P. sajor-caju* are given in Table 1. From table 1, it was observed that pink oyster *P. eous* gave the highest mean yield of 875.50g/ bed and was significantly superior over *P. florida* and *P. sajor-caju*. The same result was also obtained with biological efficiency *P. eous* ranked first (80.5%).

Table 1. Yield and biological efficiency of mushrooms

SI. No	Mushroom	Yield (gm)	Biological efficiency (%)
1	*Pleurotus florida*	786	78.6
2	*Pleurotus eous*	875.50	87.5
3	*Pleurotus sajor-caju*	671.33	67.1
	CD value	79.01	7.9

Mushroom shed characters: Mean Maximum Temperature of the shed inside 24±2 °C,
Mean Relative Humidity: 90%

With regard to the period for completion of spawn running, *P. eous* took the least time i.e. 10 days after bed preparation followed by *P. florida* with 10 days as evidenced from Table 2. Pink oyster took the least time for attaining first harvest also ie 12 days after bed preparation. Time interval between first and second harvest was the least for *P. eous* (6 days) followed by *P. florida* i.e. 7 days. From the results of the PTD trial, pink oyster can be recommended for rainy season cultivation in Kuttanad.

Table 2. Other observations of PTD trial

Sl. No.	Varieties	No. of days for spawn running	Days for 1st harvest	Interval between 1st and 2nd harvest (days)	Interval between 2nd and 3rd harvest (days)	Cropping period days
1	*Pleurotus florida*	16	19	8	6	47
2	*Pleurotus eous*	10	12	6	7	*46*
3.	*Pleurotus sajor-caju*	20	25	10	8	43

***** Spawn used: First generation

For Summer Season

Data from farmer's field were not satisfactory due to many factors that impaired successful cultivation like high temperature, low quality of irrigation water (due to high acidity and salinity) and severe attack of rats. For summer season statistical

Table 3. Yield data of mushroom

Sl. No	Name of Mushroom	Mean Yield (gms)	Biological Efficiency (%)
1	*Pleurotus florida*	570	57
2	*P. eous*	730	73
3	*Calocybe indica*	800	80
4	*Hypsizygus sp.*	632	63.2
5	*P. sajor-caju*	610	61
	CD value	50.21	6.8

Mushroom shed characters: Mean maximum temperature inside: 34±2 °C
Mean relative humidity: 68%

Table 4. Other observations of PTD trial

Sl.	Varieties	No. of days for spawn running	Days for 1st harvest	Interval between 1st and 2nd harvest (days)	Interval between 2nd and 3rd harvest (days)	Cropping period days
1	*Pleurotus florida*	22	25	12	12	55
2	*Pleurotus eous*	15	17	6	9	39
3.	*Calocybe indica*	27	33	10	15	58
4	*Hypsizygus sp.*	21	25	11	13	49
5	*Pleurotus sajor-caju*	22	26	13	8	47

analysis of data from Rice Research Station showed that yield of Pink oyster, *P. eous* (790g/bed) was on par with milky mushroom - *Calocybe indica* (800g/bed).

Time taken to complete spawn running stage was enhanced during summer and lowest period was observed in pink oyster i.e. 15 days. It was maximum for milky mushroom (*Calocybe indica*) i.e. 27 days. The same trend was observed with number of days for first harvest. Interval between first and second harvest was the least for pink oyster.

Nutrient Analysis

(a) Mushrooms from chemically sterilized paddy straw

Results of Table 5 showed that nutrient status of mushrooms harvested from the same substrate i.e. paddy straw varied with mushroom types. With respect to proximate, milky mushroom had the highest concentration of carbohydrate (3.32g/100g). Lowest content of fat i.e. 0.09 per cent was noticed in *Hypsiygus* sp. pH of mushrooms ranged between 5.2 and 5.8.

Table 5. Relative concentration of nutritional factors of mushroom types cultivated in Kuttanad

Sl. No	Parameter(g)	Type of Mushroom				
		P. florida	*P. eous*	*Calocybe indica*	*Hypsizygus* sp.	*P. sajor-caju*
1.	Carbohydrate	3.17	2.59	3.32	2.61	2.59
2.	Protein	3.03	2.72	2.10	2.76	2.96
3.	Fat	0.09	0.21	0.26	0.04	0.54
4.	Fibre	1.5	2.9	1.86	1.79	0.52
	pH	5.52	5.5	5.75	5.60	5.23

**Substrate : Chemically sterilized paddy straw*

Relative concentration of minerals are presented in Table 6. *P. sajor-caju* contained the highest concentration of all minerals under study i.e. sodium, potassium, calcium, magnesium, iron, molybdenum, selenium and zinc. Selenium, the antioxidant mineral was present only in *P. sajor-caju*. All mushroom varieties were found to be a good source of potassium (1840-3300 pm). The K content was higher in comparison with Sodium, which is an advantage from the nutritional point of view. The crude fat content was even less than one per cent as similar to report of Khanna and Garcha [1]. Mushrooms grown in the present study were observed to be almost fat free especially when grown on paddy straw and therefore can be consumed safely in fresh state by patients suffering from diabetes and atheroscelerosis.

Table 6. Relative concentration of minerals in mushroom types cultivated in Kuttanad

SI.No	Parameter (ppm)	Type of mushroom (chemically sterilized mushroom bed)				
		P. florida	***P. eous***	***Calocybe indica***	***Hypsizygus* sp.**	***P. sajor-caju***
1	Na^+	100	40	100	50	100
2	K^+	1840	2800	3200	2500	3300
3	Calcium	9.03	1.67	4.30	1.31	*118*
4	Magnesium	141.46	65.62	60.14	59.43	191.1
5	Iron	10.19	*10.91*	4.73	12.95	75.26
6	Molybdenum	BDL	BDL	BDL	BDL	BDL
7	Selenium	BDL	BDL	BDL	BDL	0.871
8	Zinc	5.85	9.2	0.91	5.05	29.27

* BDL- Below detective level

Mushroom from beds of different substrates

From Table 7, it was confirmed that in general proximates were higher in quantity in *P. florida* and *P. eous* harvested from rubber wood sawdust. pH of all varieties ranged between 5 and 6. Banana waste showed the lowest content of carbohydrate and fat.

Table 7. Relative concentration of nutritional factors in fruit bodies of *P. florida* and *P. eous* upon cultivation on variable substrates

SI. No	Parameter	Type of mushroom						
		P. florida			***P. eous***		***Hypsizygus* sp.**	
		Paddy straw	**Saw dust**	**Banana waste**	**Paddy straw**	**Sawdust**	**Paddy straw**	**Saw dust**
1.	Carbohydrate	3.17	7.59	1.83	2.59	2.59	2.61	1.87
2.	Protein	3.03	5.46	4.65	2.72	2.8	2.76	2.47
3.	Fat	0.09	0.46	0.19	0.21	3.3	0.04	0.13
4.	Fibre	1.5	1.69	0.54	3.11	0.99	1.79	0.72
	*pH	5.52	5.43	5.32	5.5	5.18	5.6	5.28

For minerals, all mushroom varieties from rubber sawdust gave the highest content. Selenium, the prominent antioxidant mineral was present in all the mushroom types harvested from saw dust beds only. So rubber wood sawdust can be considered as a better substrate for getting mushrooms of higher nutrient value. Crude fibre contents varied between mushroom between substrates. It was noted that highest fibre content was seen in *P.eous* when grown on paddy straw. Mushrooms from sawdust that recorded highest content and hence pink oyster grown in rubber wood saw dust can be recommended to meet dietary fibre need of patients suffering from stomach and internal disorder. Beelman *et al*. [3]

described that nutritive composition of the mushroom varied with species, strain, type of substrates on which it was grown, the maturity of the fruiting body, the methods of analysis and the environmental conditions on which it was grown.

Table 8. Relative concentration of minerals in mushroom types cultivated in Kuttanad

SI. No	Parameter	Type of mushroom						
		P. florida			*P. eous*		*Hypsizygus* sp.	
		Paddy straw	Saw dust	Banana waste	Paddy straw	Sawdust	Paddy straw	Saw dust
1.	Sodium	100	**250**	100	40	**70**	50	**60**
2.	Potassium	1800	**3050**	5800	2800	800	59.43	**2200**
3.	Calcium	9.03	**67.07**	*0.01	1.08	0.005	12.95	**233.8**
4.	Magnesium	141.46	**196.45**	259.3	65.62	**203.2**	BDL	**272**
5.	Iron	10.19	**41.89**	37.24	10.23	**146.3**	**BDL**	**70.39**
6.	Molybdenum	BDL	BDL	2.02	BDL	BDL	5.05	BDL
7.	Selenium	BDL	**7.07**	BDL	BDL	**0.28**	**0.005**	**9.84**
8.	Zinc	5.85	**15.39**	10.44	11.95	**59.95**	0.25	**31.95**
	pH			5.33	5.5			

Studies by Verma *et al*. [2] reflected mushroom contains significant amounts of magnesium, calcium, phosphorous and iron. Proximate compositional variations were apparent among all mushroom types under study grown on variable substrates and may be affected by certain intrinsic physiological and biochemical variabilities induced by the usage of variable substrates for cultivation. In general, mushrooms are established good source of proteins [1].

Consumer Survey: Survey was conducted among consumers from Kuttanad (Alappuzha and Kottayam districts) with a view to identify the most preferred mushroom varieties and other consumption details. Results were interpreted based on percentage data. Friends formed the major source of motivation for consuming mushroom (Table 9).

Table 9. Motivation for consuming mushroom

SI. No	Source of motivation	Frequency (n=100)	Rank
1	Friends	48	1
2	Media	31	2
3	Classes	21	3

All the respondents were aware of the nutritional and medicinal properties of mushroom. *P. florida* ranked first in mushroom consumption based on availability. Pink mushroom was consumed by 56 per cent of the respondents while milky mushroom was used by 20 per cent. Trainings formed the major source of inspiration (37 per cent) for getting information about medicinal value of mushroom (Table 10). 90 per cent of the consumers bought mushrooms directly from farmers (Table 11).

Table 10. Source of Information about Medicinal value of mushroom

SI. No	Source of motivation	Frequency (n=100)	Rank
1	Training	39	1
2	Other sources	35	2
3	Media	19	3
4	Books	16	

Table 11. Source of purchase of mushroom

SI. No	Source of motivation	Frequency (n=100)	Rank
1	Direct purchase	90	1
2	Home delivery	8	2
3	Vegetable shop	2	3
4	Super market	0	4

Among the consumers 92 per cent purchased 200g mushroom at a time. Sale price of mushroom was Rs. 200/kg for 91 per cent of the respondents. From Table 12 it was found that 69 per cent of the consumers were continuously using mushrooms for the past one year.

Table 12. Period of utilisation of mushroom

SI. No	Source of motivation	Frequency (n=100)	Rank
1	1 year	69	1
2	6 months	23	2
3	2 years	8	3

With regard to the dishes prepared all the consumers used mushroom in the form of "Thoran". (Table 12) 93 per cent of the consumers were not aware of the use of mushroom as "snacks". With regard to the recipes 100 per cent of the consumers used mushroom in the form of 'Thoran'. For 100 samples mushroom was consumed by all family members.

Table 13. Type of mushroom dishes prepared

SI. No	Source of motivation	Frequency (n=100)	Rank
1	Thoran	100 %	1
2	Theeyal	61 %	2
3	Soup	30 %	3
4	Fried	7 %	4
5	Other curry	5 %	5

Considering the health benefits after mushroom consumption, reduction in body weight was observed by 52 per cent of the respondents while in 28 per cent of the samples increased digestion was reported. Reduction in cholesterol level was experienced by 79 per cent while lowering of blood pressure was noticed in one per cent of the samples (Table 14).

Table 14. Benefits of consumption

SI. No	Source of motivation	Frequency (n=100)	Rank
1	Weight reduced	52 %	1
2	Improved digestion	28 %	2
3	Indigestion problems	20 %	3
4	No specific effect	20%	4
5	Weight increased	0	5
6	Headache	0	5

CONCLUSION

The present study identified pink mushroom i.e. *Pleurotus eous* as the most suited mushroom variety for cultivation during rainy season in Kuttanadu region of Kerala state with an average yield of 875.5g/bed. With regard to the period for completion of spawn running, it took the least time i.e. 10 days after bed preparation followed by *P. florida* with 10 days. Nutrient status of mushrooms harvested from the same substrate i.e. paddy straw varied with mushroom types. With respect to proximate, milky mushroom had the highest concentration of carbohydrate. Highest amount of protein was noticed in *P. florida*. Lowest content of fat i.e. 0.09% was noticed in *Hypsiygus* sp. It was confirmed that in general, proximates were higher in quantity in *P. florida and P. eous* harvested from rubber wood sawdust. pH of all varieties ranged between 5 and 6. Banana waste showed the lowest content of carbohydrate and fat. Selenium, the prominent antioxidant mineral was present in all the mushroom types harvested from saw dust beds only. So rubber wood sawdust can be considered as a better substrate for getting mushrooms of higher nutrient value. Results of consumer survey showed that friends formed the major source of motivation for consuming mushroom. *P. florida* ranked first in mushroom consumption based on availability. Trainings formed the major source of inspiration for getting information about medicinal value of mushroom. Sale price of mushroom was ₹ 200/kg for 91 per cent of the respondents. 90 per cent of the consumers bought mushroom directly from the farmers. With regard to the recipes 100 per cent of the consumers used mushroom in the form of 'Thoran'. For 100 samples mushroom was consumed by all family members. The results of the present study will pave the way for increasing domestic cultivation of mushroom and enhancing the consumption as a protein food source in Kuttanad region.

ACKNOWLEDGEMENTS

Government of Kerala for providing financial assistance.

REFERENCES

[1] Garcha HS and Khanna P. (1981). Nutritional values of mushroom. *P. florida. Mush. Sci.* 11: 561-71.

[2] Verma A *et al*. (2002). Relative production and biochemical compositions of fruit body of *Pleurotus florida* upon cultivation in variable substrates. *Ind. J. Mush*. 20 (1 & 2): 36-40.

[3] Beelman RB *et al*. (2003). Bioactive components in button mushroom *Agaricus bisporus* (J.Lge) Impact of nutritional, Medicinal and biological importance (Review). *Int. J. Med Mush.* 5: 321-327.

ISSUES AND OPPORTUNITIES IN SPREAD OF MUSHROOM ENTERPRISE IN ODISHA

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ABSTRACT

In Odisha, mushroom production is widely spread in all 30 districts producing 8000 MT of straw mushroom per annum. In the state, paddy straw mushroom is more popular both with respect to consumer preference and production volume. Along with production of straw mushroom during hot and wet months, oyster is produced during winter months. Button mushroom is produced under controlled condition at a very limited scale. The initial spread of the enterprise was fast, mostly among the small, marginal, landless, women farmers and unemployed youths. The reason for fast spread of the enterprise could be attributed to certain advantages like better taste, short production period, easy and simple cultivation method, higher profitability and potentiality of the enterprise to provide gainful employment. In spite of many accelerating bio physical and socio economic factors to support spread of this enterprise, the growth and stability has remained limited during recent years. In order to ascertain the growth limiting factors and opportunities, a study was undertaken in the state of Odisha covering 300 mushroom growers spread in three districts namely Bhadrak, Dhenkanal and Puri. The major issues identified during the study were comparatively poor resource base of the farmers to take it up at commercial scale, lack of specific project or programme to promote the enterprise, lack of ownership of line departments in spread of the technology, unmatched support of banking and insurance institutions, reducing yield potential of spawn, non availability of quality spawn in desired quantity, high perishability of the produce, lack of cold chain, unstable market price, presence of traders as middlemen in the supply chain in both management of input and produce, yield fluctuations due to climate changes, reducing yield due to continuous cropping, substandard marketing facility and absence of fixed price for mushroom as opined by majority of the respondents. Majority of the respondents (44.77%) perceived limited access to finance as the prime issue closely followed by limited availability of production technology, inadequate schematic support for production and post harvest and Inadequate market support. Apart from socio-personal and marketing issues, lack of proper cataloging of mushroom growers, spawn producers and established input agencies were identified to be the major issues in technology dissemination attempts. In the context of opportunities, abundant availability of substrate found to be the most important reason in continuing mushroom production with mean score of 8.08, followed by growing demand for mushroom , increasing number of agencies and promoters to promote, emerging market channels and diversifying food habit from non-vegetarian to vegetarian were rated better than average and have been perceived to constitute the basket which in future will boost the enterprise More specifically, the demand for the produce by vegetarians and diabetics being the major source of vegetarian protein, potentiality of the enterprise in creating large scale employment in rural and peri-urban areas, ease of production process, over and above the growing acceptability and inclusion of mushroom in the food habit are certain opportunities for the enterprise in the days to come. Government strategies and future programmes will be the catalytic factors in enhancing the enterprise in the state.

Keywords: mushroom, technology, constraints, issues

INTRODUCTION

Mushroom farming is popular in coastal states like Odisha, AndhraPradesh, Tamil Nadu and West Bengal because of congenial agro climatic factors and natural demand for mushroom being a part of the food habit of people. In Odisha, mushroom cultivation is an important agro-based enterprise for the farming community. The agro-climatic conditions of Odisha are conducive for production of paddy straw during March-October, oyster mushroom from November-February, milky mushroom from March-October and button mushroom during winter seasons. Among these varieties, paddy straw mushroom is widely accepted among the farming community because of its excellent taste and flavour, simple and easy production technology, abundance of raw material, shorter crop duration (15 days), higher profitability and potentiality of

the enterprise to provide gainful employment to small, marginal, landless farmers, farm women and unemployed youth. This enterprise is less dependant on land and has the ability to grow on a variety of residual agricultural waste and is a potential food source to fight malnutrition as well as in enhancing farm household income. The total annual mushroom production of Odisha is 12,333.20 MT (CTMRT-2013) out of which 98% is consumed as fresh and a very little portion is processed. Apart from research and promotion of activities by CTMRT, Bhubaneswar, efforts have been made by all the Krishi Vigyan Kendras, private spawn producers, line departments, ATMAs, NGOs and some farmers to promote the enterprise in a larger scale.

Despite of the favourable agro ecological conditions, support by several institutions and agencies, mushroom farming is not spreading at a pace matching the market demand. There are certain inherent problems or bottlenecks, which limit the growth of the enterprise in the state. The present study was undertaken in the state of Odisha to identify the issues and opportunities in spread of mushroom enterprise.

MATERIALS AND METHODS

The study was undertaken in three districts namely Bhadrak, Dhenkanal and Puri of Odisha state of India. A stratified multistage sampling procedure was followed to select the samples for the study. Personal interview using interview schedule and focused group discussion techniques were used for collection of information.

RESULTS AND DISCUSSION

Socio-personal characteristics

The study was undertaken by involving the mushroom growers in the study area. Even though all the respondents were involved in the enterprise, they vary with respect to socio economic parameters as indicated in table below.

Age distribution of the respondents presented in Table 1 indicate that, majority of the respondents (53.33%) belong to the age group upto 35 years considered to be young age whereas 40% belong to middle age group (36 to 50 years) and rest 6.67% belong to old age group (more than 50). Majority of the respondents (85.67%) were male and 15.67% were female. Even though mostly the female farmers take care of household activities and contribute to a larger extent in managing the day to day activities of the farm, male as the head of the family were more prominent in decision making activities and considered to be better in adopting mushroom enterprise than female. The education level of the respondents as observed from table above, majority of respondents (49.33%) were up to 10th class, followed by up to graduation (22.00%), up to 7th class (20.33%), can read and write (6.33%), above graduation (1.33%) and illiterate (0.67%). The data indicate that majority of respondents (43.67%) were in the enterprise for more than 6 years, while 37.67% in between 3-5 years and 18.67% for two years only. The figures in table reveal that, 50% of the respondents were in the medium income group generating an average annual income ranging from ₹ 40100/- to ₹ 101527/- exclusively from mushroom enterprise, where as 18.33% of them were in the high income group getting

Table 1. Socio economic profile

	Parameter	Percentage
(A)	**Age Group**	
	Up to 35 years	53.33
	36-50 years	40
	Above 50 years	6.67
(B)	**Sex**	
	Male	85.67
	Female	15.67
(C)	**Level of Education**	
	Illiterate	0.67
	Can read and write	6.33
	Up to 7th	20.33
	Up to 10th	49.33
	Up to graduation	22.00
	Above graduation	1.33
(D)	**Age of Enterprise**	
	Up to 2years	18.67
	3 to 5 years	37.67
	6 years and above	43.67
(E)	**Income from the enterprise ***	
	High (>101527.06)	18.33
	Medium(40099.6- 101527.06)	50.00
	Low (<40099.6)	31.67

*Mean- ₹ 70813.33, SD - 30713.73

average annual income of more than ₹ 101527/- and 31.67% were in the low income group generating average annual income of less than ₹ 40100/- from mushroom enterprise.

Issues in spread of the technology

Spread of the mushroom enterprise has remained limited because of certain issues associated with the spread. The major issues identified during the study were comparatively poor resource base of the farmers to take it up at commercial scale, lack of specific project or programme to promote the enterprise, lack of ownership of line departments in spread of the technology, unmatched support of banking and insurance institutions, reducing yield potential of spawn, non availability of quality spawn in desired quantity, high perishability of the produce, lack of cold chain, unstable market price, presence of traders as middlemen in the supply chain in both management of input and produce, yield fluctuations due to climate changes, reducing yield due to continuous cropping, substandard marketing facility and absence of fixed price for mushroom as opined by majority of the respondents. These issues have been broadly categorized in consultation with selected sample and experts in the field. The responses of the respondents have been analyzed and presented in Table 2.

Table 2. Issues in spread of the enterprise (N=300)

Sl. No.	Issues	Frequency	%	+/- than average
1.	Limited applicability of available mushroom production technology	60	20.00	-
2.	Limited availability of production technology	134	44.67	+
3.	Poor availability of inputs	86	28.67	-
4.	Limited access to finance	134	44.77	+
5.	Inadequate market support	114	38.00	+
6.	Inadequate schematic support for production & post harvest	133	44.33	+

The data presented in Table 2 indicate that out of the six identified issues, majority of the respondents (44.77%) perceived limited access to finance as the prime issue closely followed by limited availability of production technology, inadequate schematic support for production and post harvest and Inadequate market support. However, issues like limited applicability of available mushroom production technology and poor availability of inputs were rated as less important issue.

Opportunities in spread of the enterprise

All these issues which were identified during the field study and depicted in the above table give a definite framework to think about the approaches and solutions for getting rid upon those problems. During the course of investigation responses were also obtained on opportunities for spread of the enterprise and are presented in Table 3.

Table 3. Opportunities in spread of the enterprise (N=300)

Sl.No	Opportunities	Mean score	+/- than average
1	Increasing number of agencies and promoters to promote	7.03	+
2	Availability of substrate	8.08	+
3	Growing market demand	7.06	+
4	Diversifying food habit from non-vegetarian to vegetarian	6.52	+
5	Upcoming schematic supports	4.13	_
6	Emerging market channels	6.97	+

MOS = 10, Overall mean score = 6.47

In the above table the mean score analysis of the parameters against which reactions were studied indicate that, abundant availability of substrate found, to be the most important reason in continuing mushroom production with mean score of 8.08, followed by growing demand for mushroom with mean score 7.06, increasing number of agencies and promoters to promote (7.03), emerging market channels (6.97) and diversifying food habit from non-veg. to veg. The respondents gave least importance to up coming schematic support as an opportunity.

Further analysis of the information obtained indicate that opportunities like availability of substrate, growing demand for mushroom, increasing number of agencies and promoters to promote, emerging market channels and diversifying food habit from non-vegetarian to vegetarian (all+) were rated better than average and have been perceived to constitute the basket which in future will boost the enterprise.

CONCLUSION

From the initial years of introduction when mushroom was accepted as a backyard activity by the resource poor farm families, it has now being recognized as a potential commercial activity in rural areas of the state of Odisha. However, the potentiality shown in productivity, profitability and market demand has not been exploited fully. On one hand the enterprise has spread like anything, on the other hand farmers do express reservation to proceed further. Even if with so many supporting factors for accelerating the spread of the enterprise, some of the constraints faced by the farmers limit expansion to the full potential level. The issues like limited access to finance, availability of production technology, inadequate schematic support for production and post harvest, and inadequate market support need to be addressed and lot more support will be required from public as well as private sector to address and overcome these issues. At the same time the study has revealed important opportunities like abundant availability of substrate, growing demand for mushroom, increasing number of agencies and promoters to promote the enterprise, emerging market channels and diversifying food habit from non-vegetarian to vegetarian, which can be strategically exploited to achieve higher spread and stability of the enterprise in the state.

REFERENCES

[1] Anonymous (2011). Vision- 2030, Directorate Mushroom Research, July 2011.

[2] Chang ST and Miles PG. (1991). Recent trends in world production of cultivated mushroom. *Mushroom Journal* 50: 15-17.

[3] CTMRT (2010). Information on Status of Mushroom in Orissa, All India Coordinated Research Project (AICRP) on Mushroom

[4] Das RN (2009). Livelihood security in rice based farming system, National seminar on Managing Rural Livelihood in India: Challenges and Opportunities, Orissa University of Agriculture and Technology, Bhubaneswar. pp. 110-123.

[5] Economic Survey of India, 2011-12, Government of India

[6] Hugar LB *et al.* (1992). Constraints in diffusion of new farm technology, An economic evaluation. Agriculture Situation in India, pp. 15-19.

[7] Kusabs N *et al.* (2003). Opportunities to Measure Consumer Preference and Retail Shelf Performance Using. Individual Product Id, *Acta Horticulturae* No.: 604.

[8] Pradhan L and Nayak MP. (2009). Mushroom cultivation: livelihood support for farm families, National seminar on Managing Rural Livelihood in India : Challenges and Opportunities, Orissa University of Agriculture and Technology, Bhubaneswar. pp. 220.

[9] Singh Nasib *et al.* (2008). Constraints in mushroom production technology in Haryana, Agricultural *Science Digest.* 28: 2.

[10] Tamilselvi G and Kumar JV. (2009). Problems of rural women entrepreneur in agriculture based enterprises, Agriculture Update 2009, Vol.4 No.1/2 pp. 28-31.

[11] Truong Thi *et al.* (2002). Factors affecting farmers' adoption of technologies in farming system: A case study in OMon district, Can Tho province, Mekong Dsssssssssselta, Omonrice 10: 94-100.

MUSHROOM ACCEPTABILITY AND CONSUMPTION INTENTION FOR THE MAIN MUSHROOMS PRODUCED IN BRAZIL

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ABSTRACT

Mushrooms are known for their functional and nutritional importance. However, there are few works about their acceptance as food by Brazilian consumers, which are important as directives for mushroom producers. The objective of this study was to evaluate *Agaricus bisporus*, *Pleurotus ostreatus*, *Agaricus blazei* and *Lentinula edodes* for mushroom acceptability and consumption intention. A dish with rice and *A. bisporus*, *P. ostreatus*, *A. blazei* or *L. edodes* mushrooms was prepared. The dishes (samples of 20 g) were given to 192 randomly-chosen untrained panelists. The acceptability was determined on a 5-point hedonic scale, and the habit of consumption and purchase intention were evaluated. The mushroom global score acceptability was 3.61 for *A. bisporus*, 3.48 for *A. blazei*, 3.24 for *P. ostreatus* and 2.89 for *L. edodes*. For *L. edodes* the color was the main rejected characteristic. There were no differences to the mushroom acceptability according to the panelists socio-economic characteristics. Although most of the panelists did not have the habit of buying mushrooms, the majority (90.6%) was willing to purchase mushrooms and 38.5% were willing to pay as much as US$ 80 per dried kilogram of mushrooms. Due to the high commercial prices of mushrooms in Brazil - at least US$ 100 per dried kilogram they are still considered exotic and are likely to be bought as functional food, because of health benefits than sensorial characteristics.

Keywords: sensory analysis, basidiomycete, food consumption, functional food

INTRODUCTION

Mushrooms are an important product in global trade with an estimated production of 3.4 million metric tons in 2007. China is the biggest producer with a market of US$ 82.9 million in 2008 and 2009 [1]. In Brazil, the mushroom marketing is still a challenge due to low production technology and the need for consumer market development. There are few data about Brazilian mushroom consumption, but it was 8 g *per capita* in 1984 and 80% of the cultivated mushroom was *Agaricus bisporus* [2]. The main produced and commercialized mushrooms in Brazil are *A. bisporus* (Lange) Imbach, *P. ostreatus* (Jacq. ex Fr.) Kumm., *A. blazei* Murrill *ss.* Heinem (*A. brasiliensis* Wasser *et al*.; *A. subrufescens* Peck) and *Lentinula edodes* (Berk.) Pegler. *A. bisporus* mushrooms are the most cultivated and consumed worldwide [1] and in Brazil [2]. *P. ostreatus*, *A. blazei* and *L. edodes* mushrooms, despite having an exotic and peculiar taste, are much less consumed. Nutritionally, mushrooms have low caloric value, and high protein and fiber content. Moreover, they are a source of immune modulator compounds [3] with antioxidant [4], anti-inflammatory [5] and antitumor [6,7] activities. The action mechanisms of those bioactive substances are still not well understood. Nevertheless, it was suggested that the main active principle is beta-D-glucan, which is known as potent activator of the immunological system [3].

Despite the functional importance of these basidiomycetes there are few studies on mushroom acceptability by Brazilian consumers. Escouto *et al.* [8] evaluated *A. blazei* mushroom acceptability by Brazilian consumers but without comparing it with other mushrooms. Thus, due to mushroom functional importance, economic potential, and lack of information on mushroom acceptability, this study aimed to compare the acceptability of *A. bisporus*, *P. ostreatus*, *A. blazei* and *L. edodes*. This research provides information about consumers' acceptability of mushrooms and allows pointing out the commercial potential of the most cultivated mushrooms in Brazil.

MATERIALS AND METHODS

P. ostreatus, *A. bisporus*, *A. blazei* and *L. edodes* were produced according to Aguilar-Rivera *et al*. [9], Mamiro and Royse [10], Colauto *et al.* [11] and Royse and Sanchez [12], respectively. They were harvested with closed basidiocarp, before rupture of the inner veil, except *P. ostreatus* that was harvested with closed basidiocarp edge and *L. edodes* that was harvested with 50-70% opened basidiocarps, after rupture of the inner veil, and pileus edge slightly convex. After drying at 60 °C in an oven with air circulation, a 35 g sample of each mushroom was rehydrated in 700 ml of boiling water for 20 min. Mushrooms were sliced (1 cm large), *sautéed* with 12 ml of soybean oil for 3 min. Afterwards 200 g of rice (*Oryza sativa*, cultivar Atalanta, Embrapa), 500 ml of leftover water from mushroom-rehydration and 4 g of salt was added. The pan was maintained semi covered at low heat for 15 min until completely cooked.

The research was approved by the Ethic Committee involving Humans (registration number 11500). Consumer acceptability was done in individual booths illuminated with white lighting in the morning at 10-11 h and in the afternoon at 15-17 h. The samples (20 g) were assigned with three-digit random numbers and served under identical conditions. The randomly chosen participants (n = 192) were male and female adults who voluntarily agreed for the study. They signed a consent agreement to provide socioeconomic data and frequency of purchase and consumption of mushrooms at home and restaurants. The panelists evaluated the dishes with a 5-point hedonic scale from 1 ("dislike extremely") to 5 ("like extremely") [13]. Each consumer evaluated the sample for color, aroma, consistency and flavor. After sensorial evaluation, a sample of dried mushrooms (50 g) was submitted to each participant and they were asked about how much they were willing to pay for it. The results were submitted to analysis of variance (Analysis of One-way Variance – ANOVA) followed by Tukey's test ($p<0.05$).

RESULTS AND DISCUSSION

A. bisporus, *P. ostreatus* and *A. blazei* received the highest ($p < 0.05$) appreciation rates for color (Table 1). *L. edodes* had the highest ($p<0.05$) rejection for color with 49.5% for the sum of "I disliked it" and "I disliked it very much" (Table 1). *P. ostreatus*, *A. blazei* and *L. edodes* presented the lowest "indifferent" percentage for color (Table 1). These mushrooms had distinct colors such as light caramel, caramel and dark brown (close to black), respectively, differently. *A. bisporus* had a very light caramel color. *A. bisporus* and *P. ostreatus* had light colors, whereas *A. blazei* had medium color. Light and medium colors are considered common for food, known and accepted by consumers. However *L. edodes* has a dark brown color – which is less common in food – reducing acceptance. Dark-colored foods could be rejected because they are associated with roasting and spoilage. Thus, the high rejection rate of *L. edodes* could be associated with its dark color.

Many authors described the role of light in mushroom cultivation [14-16]. For *A. bisporus* light is not essential for fruiting and may inhibit mycelial growth [17,18]. On the other hand, light is necessary for *L. edodes* for its vegetative and fruiting stages [19]. Excessive light exposure can reduce the number of fruit bodies and produce dark mushrooms, whereas a lack of light reduces pileus diameter, stipe length and promotes a light beige color in basidicoarps. According to Tokimoto and Komatsu [18] fruiting bodies apparently develop abnormalities and sporulation reduction when *L. edodes* are grown under filtered light using colored cellophane papers. Thus, light is an important factor in determining the color of *L. edodes* fruiting bodies and could be used by mushroom growers in order to produce a less dark mushroom as an alternative for consumers.

For aroma the highest ($p<0.05$) values for acceptability were for *A. bisporus* and *A. blazei*. *L. edodes* presented the highest ($p<0.05$) rejection rate for aroma with 52.1% for the sum of "I disliked it" and "I disliked it very much" (Table 1). The highest acceptability of *A. bisporus* aroma might be associated to its familiarity by Brazilians [2] and worldwide cuisin [1]. Also *A. bisporus* aroma is dependent of at least eight compounds of carbon-epoxy, including benzyl isothiocyanate, and 1-3 octanol, responsible for 59.0% of the mushroom aroma [20].

For *A. blazei* the high acceptability for aroma (Table 1) is related with seven volatile compounds, mainly by the benzaldehyde that is responsible for 43.2% of the flavoring [21]. This is the main compound related to almond aroma for this mushroom

[22]. Other important compounds are benzyl alcohol and methyl benzoate that probably contribute to the sweet floral note of this mushroom [22]. *A. blazei* has 41.67 mg kg^{-1} of 1-3-octanol, an aliphatic volatile constituent of other mushroom aromas [22]. Therefore, the aroma complexity of *A. blazei* compounds characterizes it as an exotic mushroom with distinct traits easily noticed by panelists. Differently from other mushrooms, *A. blazei* stood out in the dish for its distinct aroma and flavor. According to Escouto et al. [8], *A. blazei* had 68% acceptability and tasters "like it" or "dislike it" but very few chose "indifferent".

Table 1. Answers (n = 192) to hedonic scale for *Agaricus bisporus*, *Pleurotus ostreatus*, *Agaricus blazei* and *Lentinula edodes* mushroom sensorial evaluation

Fungus	"I liked it" "I liked it very much" (%)	"Indifferent" (%)	"I disliked it" "I disliked it very much" (%)	Mean scores for acceptability	F	*p*-Value
Answers for color						
A. bisporus	55.67*	29.90	14.43	3.61[a]	6.3	< 0.001
P. ostreatus	58.76	14.43	26.81	3.47[a]		
A. blazei	56.70	14.43	28.87	3.41[a]		
L. edodes	39.94	10.57	49.49	2.85[b]		
Answers for aroma						
A. bisporus	48.95	32.29	18.76	3.45[a]	5.1	< 0.01
P. ostreatus	39.58	17.71	42.71	2.95[b]		
A. blazei	47.91	10.42	41.67	3.09[a]		
L. edodes	33.33	14.58	52.08	2.71[c]		
Answers for consistency						
A. bisporus	71.14	16.49	12.37	3.88[a]	5.3	< 0.01
P. ostreatus	56.70	14.43	28.87	3.41[b]		
A. blazei	59.79	16.49	23.71	3.54[b]		
L. edodes	48.45	14.43	37.11	3.16[c]		
Answers for flavor						
A. bisporus	54.63	23.71	21.65	3.50[a]	5.7	<0.001
P. ostreatus	46.39	17.53	36.09	3.15[b]		
A. blazei	41.24	10.31	48.45	2.90[c]		
L. edodes	37.12	15.46	47.42	2.85[c]		

*Different letters in the same column for the same sensorial descriptor indicate statistical differences by Tukey's test ($p<0.05$).

Similar to *A. bisporus*, the main compound responsible for *L. edodes* aroma is 1-3 octanol whose concentration is reduced when the basidiocarp is opened [23]. Opened basidiocarps have 44% of total volatiles represented by 1-3 octanol whereas closed basidiocarps have 63%. In Brazil *L. edodes*, differently from *A. bisporus* and *A. blazei*, is mainly consumed with opened basidiocarps. In general, it is an advantage for growers that get more biomass for their product but it has low quality aroma. Moreover, it is possible that *L. edodes* mushroom color had negatively affected the aroma-evaluation of this mushroom for first sensorial perception by panelists. Also, in China, the biggest mushroom producer and consumer in the world [24], *L. edodes* is the second most cultivated mushroom [1]. It suggests that cultural characteristics might be a variable to be analyzed in future experiments.

For consistency, *A. bisporus* was the most ($p<0.05$) accepted followed by *P. ostreatus* and *A. blazei*. *L. edodes* had the highest ($p<0.05$) rejection with 37.1% for the sum of "I disliked it" and "I disliked it very much". *A. bisporus* and *A. blazei* have an average of 28% and 20.6% of total dietary fiber. On the other hand, *L. edodes* has 53.3-57.5% of total dietary fiber and 14.4% of them is chitin, an insoluble long-chain polymer of a N-acetylglucosamine with great resistance to biting [25]. Thus, the biting resistance is higher for *L. edodes* (0.81 to 1.23 kg mm^{-2}) whereas it is just 0.33 kg mm^{-2} for *P. ostreatus* [26]. Mushroom rehydration is a process that does not allow a full restoration of the mushroom and it is different to each species. Apparently, it is more difficult for *L. edodes* mushrooms.

Regarding flavor, the answers were divided between "I liked it" and "I disliked it" with polarized perception for mushroom flavor. *A. bisporus* had the highest ($p<0.05$) acceptability followed by *P. ostreatus*, and after *A. blazei* and *L. edodes*. Edible mushrooom flavor primarily consists of nucleotids, soluble sugars, polyols and mainly free aminoacids such as glutamate. The glutamate and nucleotide concentration, responsible for umami taste, varies from 6.04 to 13.50 mg g^{-1} in *A. bisporus* [24, 27], 1.28 to 4.4 mg g^{-1} in *A. blazei* [22, 24], 0.84 to 2.14 mg g^{-1} in *P. ostreatus* [24] and 1.30 to 1.71 mg g^{1} in *L. edodes* [24, 28]. The glutamate and nucleotide concentration varies enormously with strain, cultivation condition and with maturity stage of the mushroom. Mushrooms harvested at various maturity stages had different nonvolatile component profile depending on the mushroom species. Investigations on the umami ingredients at different maturity stages of *A. bisporus* indicated that the two umami amino acids - Asp and Glu - showed an increase at stage two (closed basidiocarp). After stage two Asp content gradually decreased, while the Glu content increased with maturity stage [24]. Therefore, the highest glutamate concentration in *A. bisporus* can be an important factor to its acceptance; moreover, 18 free amino acids participate in the flavor formation for *A. bisporus* [29], 13 for *P. ostreatus* in which two were sweet, four were bitter and eight were flavorless [28], and 12 for *A. blazei* [30] and *L. edodes* [28]. On the other hand, the greatest acceptance of *A. bisporus* flavor (Table 1), described as a smooth taste, can also be related to the panelists' familiarity since *A. bisporus* is popular in pizzas and other dishes in Brazil.

The global score (average for color, aroma, consistency and flavor answers) were 3.61± 0.19 for *A. bisporus*, 3.48 ± 0.33 for *A. blazei*, 3.24 ± 0.24 for *P. ostreatus* and 2.89 ± 0.19 for *L. edodes*. It indicates the general order of acceptability for the most cultivated mushrooms in Brazil. There were 192 panelists, most of them female, with education background equal or superior to high school, over 30 years old and with income under US$ 690 per month (Table 2). The socio-economic factors did not affect the mushroom acceptability level i.e. regardless of economic status, gender, age or education background, mushroom acceptance was based on personal criteria.

Most of the volunteers do not have a habit of buying and consuming mushrooms since 60.4% do not "buy mushrooms at the supermarket", and 65.6% do not "order dishes with mushrooms at restaurants". Despite the reduced consumption habit, 13.5% of the panelists "know somebody who consumed mushrooms to treat diseases". The most cited answers were for the treatment of stomach diseases, cancer, diabetes, weight loss and as immune stimulator.

Table 2. Analysis of variance and significance level for the hedonic scale for the acceptance of a dish with rice and mushroom in function of gender, education background and age of 192 panelists

Panelists	**Number of individuals**	***p*-Value**
Male	84	0.5828
Female	108	
Primary education	24	0.0856
High school education or higher	168	
Income lower than US$ 690	122	0.3079
Income higher than US$ 691	70	
30 years old or higher	72	0.2901
29 years old or lower	120	

Although most of the panelists do not have the habit of buying mushrooms, the majority (90.6%) would like to buy this food. *A. bisporus* mushroom presented the greatest ($p<0.05$) purchase intent of 42.7% followed by *P. ostreatus* with 16.7%, *A. blazei* 15.6% and *L. edodes* 15.6%. Among the answers, most of them intend to purchase industrialized (canned) *A. bisporus* but not fresh one because "it is difficult to find them at local supermarkets".

When the panelists were asked how much they were willing to pay for 50 g of dried mushrooms, 26.0% were willing to pay US$ 2.00; 38.5% US$ 4.00 and, surprisingly, 27.1% more than US$ 4.00. In Brazil, 50 g of dried mushrooms are sold at *Pão de açúcar*-delivery market (http://www.paodeacucar.com.br/) for US$ 9.59 for *A. bisporus*, US$ 6.62 for *P. ostreatus* and from US$ 2.22 to 13.00 for *L. edodes*, and, at *DEC Enterprises Comercial Ltda*. (http://www.agaricusblazeimurrill.com.br/) for US$ 11.13 for *A. blazei*. The reduced purchase intent by panelists associated with the higher commercial value at markets indicates that mushrooms still have limited consumption and are likely bought for consumption due to healthy benefits than sensorial characteristics.

CONCLUSION

The most accepted mushroom is *A. bisporus* followed by *P. ostreatus*, *A. blazei* and *L. edodes*. There are no differences for mushroom acceptability according to the panelists' socioeconomic characteristics. Most of the volunteers do not have a habit of buying and consuming mushrooms regularly, even though 90.6% are willing to purchase mushrooms and 38.5% are willing to pay as much as US$ 80 per dried kilogram of mushrooms. Mushrooms in Brazil are still considered exotic and are likely to be purchased as functional food, for their healthy benefits, rather than their sensorial characteristics.

ACKNOWLEDGMENTS

The authors thank Paranaense University for the financial support and fellowship.

REFERENCES

[1] Li Y. (2012). Present development situation and tendency of edible mushroom industry in China. In: *Int. Soc. Mush. Sci*. Zhang J. *et al.* Eds. 18, 3-9.

[2] Bueno FS. (2010). The production and consumption of edible and medicinal mushrooms in Brazil. In: Haciaun Desarrollo Sostenible del Sistema de Producción-Consumo de los Hongos Comestibles y Medicinales en Latinoamérica: Avances y Perspectivas en el Siglo *XXI*.pp 359-379, ISBN970-9752-01-4.

[3] Chen J and Seviour R. (2007). Medicinal importance of fungal β-(1-3), (1-6)-glucans. *Mycol. Res.* 111: 635-653.

[4] Mourão F *et al.* (2011). Antioxidant activity of *Agaricus brasiliensis* basidiocarps on different maturation phases. *Braz. J. Microbiol.* 42: 197-202.

[5] Mourão F *et al.* (2011). Anti-inflammatory activity of *Agaricus brasiliensis* in different basidiocarp maturation phases. *Food Agric.Immunol.* 22:325-333.

[6] Jumes FMD *et al.* (2010). Effects of *Agaricus brasiliensis* mushroom in Walker-256 tumor-bearing rats. *Can. J. Physiol. Pharmacol.* 88:21-27.

[7] Mourão F *et al.* (2009). Antineoplasic activity of *Agaricus brasiliensis* basidiocarps on different maturation phases. *Braz. J. Microbiol.* 40: 901-905.

[8] Escouto LFS *et al.* (2005). Acceptability of the sensory characteristics of the Brazilian mushroom *Agaricus brasiliensis*. *Braz. J. Food Technol.* 8: 321-325.

[9] Aguilar-Rivera N *et al.* (2012). Production of *Pleurotus ostreatus* (oyster mushroom) grown on sugar cane biomass (trash, bagasse and pith). In: *Mushrooms: types, properties and nutrition.* pp. 77-103, ISBN 9781614701309 161470130X.

[10] Mamiro DP and Royse DJ. (2008). The influence of spawn type and strain on yield, size and mushroom solids content of *Agaricus bisporus* produced on non-composted and spent mushroom compost. *Bioresour. Technol.* 99: 3205-3212.

[11] Colauto NB *et al.* (2010). Alternative to peat for *Agaricus brasiliensis* yield. *Bioresour. Technol.* 101: 712-716.

[12] Royse DJ and Sanchez JE. (2007). Ground wheat straw as a substitute for portions of oak wood chips used in shiitake (*Lentinulaedodes*) substrate formulae. *Bioresour. Technol.* 98: 2137–2141.

[13] Anzaldùa-Morales A. (1994). *La Evaluación Sensorial de los Alimentos en la Teoría y la Prática.* Zaragoza: Acribia, ISBN 8420007676 9788420007670.

[14] Eger G *et al.* (1976).The action of light and other factors on the sporophore initiation in *Pleurotus ostreatus. Mush. Sci.* 9: 575-583.

[15] Leatham GF and Stahmann MA. (1987). Effect of light and aeration on fruiting of *Lentinula edodes*. *Trans. Br. Mycol. Soc.* 88: 9-20.

[16] Miyazaki Y *et al.* (2011). Light-stimulative effects on the cultivation of edible mushrooms by using blue led. In: *Mush. Biol. Mush. Prod.*vol. 2, Savoie JM. *et al.* Eds. 7, 58-67.

[17] Miles PG and Chang ST. (1997). Mushroom Biology: Concise Basics and Current Developments. Singapore: World Scientific, ISBN 981-02-2877-5.

[18] Tokimoto K and Komatsu M. (1978). Biological nature of *Lentinula edodes*. In: *The Biology and Cultivation of Edible Mushrooms.* ISSN 0121680509 9780121680503.

[19] Przybylowicz P and Donoghue J. (1988). Shiitake Growers Handbook: the Art and Science of Mushroom Cultivation. Kendall: Hunt Publishing, ISSN 1739-1377.

[20] Macleod AJ and Panchasara SD. (1983). Volatile aroma components, particularly glucosinolate products, of cooked edible mushrooms(*Agaricus bisporus*) and cooked dried mushrooms. *Phytochemistry.* 22: 705-709.

[21] CaiZY. (2003). Analysis on the volatile flavor compounds in *Agaricus blazei* by GC-MS. *Zhong Yao Cai* 26: 789-791.

[22] Stijve T *et al.* (2004). Characterization of flavor and taste compounds in *Agaricus blazei* Murrill *sensu* Heinem., the cultivated almond mushroom. *Aust. Mycol.* 22: 116-122.

[23] Cho DB *et al.* (2003). Analysis of the volatile flavor compounds produced during the growth stages of the shiitake mushrooms (*Lentinus edodes*). *J. Food Sci. Nutr.* 8: 306-314.

[24] Zhang Y *et al.* (2013). Recent developments on umami ingredients of edible mushrooms - a review. *Trends Food Sci. Technol*. 33: 78-92.

[25] Dikeman CL *et al.* (2005). Effects of stage of maturity and cooking on the chemical composition of select mushroom varieties. *J. Agric. Food Chem.* 53:1130-1138.

[26] Çaðlarirmak N. (2007). The nutrients of exotic mushrooms (*Lentinula edodes* and *Pleurotus* species) and as estimated approach to the volatile compounds. *Food Chem.* 105: 1188-1194.

[27] Tsai ST*et al.* (2007). Nonvolatile taste components of *Agaricus bisporus* harvested at different stages of maturity. *Food Chem.*103: 1457-1464.

[28] Yang HJ *et al.* (2001). Non-volatile taste components of several commercial mushrooms. *Food Chem.*72:465-471.

[29] Hallock RM. (2007).The taste of mushrooms. *McIlvainea* 17: 33-41.

[30] Chang HL *et al.* (2001). Non-volatile taste components of *Agaricus blazei*, *Antrodia camphorate* and *Cordyceps militaris* mycelia. *Food Chem.* 74: 203-207.

SEASONAL GROWING OF BUTTON MUSHROOM IN NORTH INDIA

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ABSTRACT

Establishment of a mushroom project is an expensive affair but another cheap way of growing mushrooms is seasonal growing in temporary sheds during the winter season. Hundreds of growers in North India grow mushrooms in bamboo-paddy straw huts. The paper deals with the problems, innovative solutions like change of place of cultivation at regular intervals to avoid disease build-up and the scope of seasonal cultivation.

Keywords: seasonal cultivation, button mushroom, hut cultivation

Setting up a mushroom farm is an expensive and complex affair. It involves building of insulated growing rooms along with metal racks. For control of temperature, RH and fresh air, air handling units are installed for individual rooms connected with a central cooling unit comprising of compressors, condensers, etc. A composting unit comprised of Phase I tunnels, Phase II tunnels and a platform is also a part of the mushroom farm.

In North India, we get a fairly extended winter and it is possible to take one extended crop or even two crops, from end of November to end of March without sophisticated climate control. Hundreds of farmers produce button mushroom in North India with this method, with Haryana on the top of the list. The compost is made by long method and growing is done in bamboo-paddy straw huts. Right formulations and right procedures not only ensure good production, but also eliminate the need of adding nematicides and insecticides to the compost. Unskilled labour is available but not as easily as three to four years ago and getting too expensive unlike process of mushrooms. Seasonal growing is highly labor intensive.

Hundreds of farmers around Delhi, including me have been growing mushrooms seasonally for the last ten years, making compost by long method and growing in temporary huts made of bamboos and paddy straw. In the 80s, a small village emerged on the map of India, as the village of mushroom as most of the families of village Badana, Sonepat in Haryana were engaged in this profession, not any more. In fact some of the mushroom growers are shifting from seasonal growing to modern mushroom farms with climate control.

In Haryana, farmers shift the location of mushroom units at regular intervals. Advantage of starting up a new mushroom farm at a new place is always there. As the farm and the site get older, spores of green mould (*Trichoderma*, *Aspergillus*, *Penicillium* species), yellow mould (*Myceliophthora lutea*), plaster mould (*Scopulariopsis fimicola*) etc. accumulate in the air, soil even on the bamboos. They are ready to attack mercilessly on a unpasteurized compost not made properly or "selective" in other words. The monster of "wet bubble" is always looming and mushroom flies do appear as soon as warm weather begins in the month of March. If bubble attack occurs at an early stage, it can spoil the game as the economics of seasonal growing is based on prolonged period of cropping. Good hygiene standards are not possible to maintain in temporary huts erected among paddy fields. Therefore shifting the growing site every season helps to a certain extent.

The farmers talk of poor yields, citing various reasons. Many farmers are still making compost with very old formulation using inorganic nitrogen fertilizers like potash, calcium ammonium nitrate, super phosphate, etc. instead of chicken manure. Not only this formula is expensive, but the available nitrogen is not utilized by microbes working in late composting, therefore not much useful to the mushroom mycelium. With right formulation and modifications, when compost temperature touches 75 to 80°C in the core of compost pile plus high levels of ammonia, nematodes and insect eggs get killed and there is no need to add nematicide and insecticide except to take care of cold layer of compost.

Technical advice is not available to the farmers. A farmer is expected to learn the vast field of mushroom growing by attending small courses organized by research organisations. Since it is a seasonal activity, farmer gets only two to three months for composting and three months for mushroom growing, where one has to rush against the time. "Skill" is hard to

acquire - whether it is the moisture in compost or picking speed or watering regime during cropping. So, instead of giving subsidies to the farmers, they should be strengthened by providing them with technical knowledge.

Labor availability is becoming a major problem. Again being a seasonal activity, skilled labor is not available who prefer to work in climate control mushroom farms for the whole year. The reality of changing time is labour shortage and cost. The focus of agricultural labor is confined to rice transplantation, grain harvest or sugarcane harvest as these activities are more rewarding in terms of money.

Our marketing system is not conducive to farmers where the middle man's margins are high. The perishable nature of mushroom makes the things even more difficult. Like other agriculture crops, there is no provision of insurance of mushroom crops against damage of huts due to excessive rains, high winds and fire. Despite the odds, there are a handful of growers who are doing well. With experience they have learnt the art of making compost, kept their costing under control and found better market outlets in other towns besides Delhi. Like in any other business, there are some good years and some bad years. Bamboos are one time investment as they last for five to seven years. Each year when September comes, these bamboos lure the farmer and he again plans one more time even if preceding year was not so profitable!

There is a need for strong cooperative of seasonal mushroom growers, which could help the farmers get a better price for the produce by direct sale in fresh markets all over the country and a tie up with processing units. By highlighting the nutritional and medicinal value of mushrooms we may be able to increase the per capita consumption. In a rapidly developing country like ours, there is a tremendous scope with a huge market for home as well as export, in waiting. Considering this, seasonal mushroom growing will continue to have some potential for some more time to come.

TECHNOLOGICAL AND MARKETING FISSURES OF BUTTON MUSHROOM AT TRADITIONAL AND SCIENTIFIC KNOW-HOW IN MID HILLS OF UTTARAKHAND STATE, INDIA

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ABSTRACT

Mushroom growers from Pauri, Khirsu, Kaproli and Thailisain of mid hills of Uttarakhand State of India, were randomly selected for white button mushroom cultivation trials to find out tech-fissure, market-fissure and benefit:cost ratio. It was found that all these growers recorded longer period of spawn and less yield, than that recorded at Bharsar centre. Out of total yield obtained during 7 cropping weeks, 44.83-58.79% yield was obtained within first 2 cropping weeks from all trials, as compared to 60.13% yield obtained from Bharsar for the same period of time. There were negative/inferior tech-fissures with all mushroom growers when calculated on period of spawn run, casing and first harvest interface, yield, number and average weight of fresh mushroom fruit. Mushroom growers were sold their fresh mushroom in local market at local prevailing rates against fixed invested cost of ₹ 992.18. Growers from Pauri sold mushroom relatively at much higher rates of ₹120/kg and earned highest net income of ₹1355.02 q^{-1} compost with 8.15% positive market-fissure and 2.36:1 benefit:cost ratio over that of Bharsar centre, in which ₹ 1252.82 was earned as a net income with 2.26:1 benefit:cost ratio. Both, tech and market-fissure were recorded most negative from the Village Kaproli with very poor net income and benefit:cost ratio in comparison to the rest of the trials located in urban and semi-urban towns like, Pauri, Khirsu, Thailisain and Bharsar.

Keywords: technological-fissure, market-fissure, benefit:cost ratio (BCR)

INTRODUCTION

White button mushroom is most popular mushroom in India contributing about 85% of the total 1,000,00 ton production of mushrooms in the country. It involves both seasonal and high-tech growing system. Uttarakhand is one of the States of India, ranked second by producing 8,000 ton mushroom in same year after Punjab [1]. Mushroom industry of this State is dominating with marginal, small mushroom growing units and their mushroom productivity varies from 14-18 kg q^{-1} compost/ 8 weeks of harvesting period, if owner purchased compost from composting units [2]. Although, mushroom growing principles and methods are same for all marginal and small mushroom growers. Despite of this growers of mid hills have great difference in their mushroom production and sale that of. Therefore, present investigation was led down to see the technological and marketing gap in form of tech-fissure and market-fissure, respectively among 4 seasonal mushroom growers in Pauri District of Uttarakhand State, India. Technological fissure was based on the comparative study of spawn run, casing- first harvest interface, cropping pattern, yield and numbers of white button mushroom. However, marketing fissure was established on net income earned by selected growers along with benefit:cost ratio.

MATERIALS AND METHODS

Selection of Mushroom Growers

Four mushroom growers designated as MG1, MG2, MG3 and MG4 each from Pauri, Khirsu, Kaproli and Thailisain of mid hills of Uttarakhand State of India, were randomly selected for white button mushroom cultivation. The places selected in this study had high proportion of demand and supply of button mushrooms in both winter and summer months of the year. Selected mushroom growers were thought to be well acquainted with growing techniques of white button mushroom, as they adopted it as an entrepreneur at small level years back. Additionally mushroom unit located at Vir Chandra Singh Garhwali, College of Horticulture Bharsar centre was chosen as a standard check to make comparison with results received

from other mushroom growers. All selected places were described under social status, latitude, longitude, altitude, average temp and mountain facing direction (Table 1). Social status and geographical information (longitude, altitude and mountain facing) was gathered from district head quarter, Pauri. However, average temperature of the experiment was recorded from thermometers supplied to the mushroom growers.

Table 1. Social and geographical information of selected places

Name of place	Social Status	Latitude	Longitude	Altitude (msl)	Temp (°C)	Mountain facing direction
Pauri	Urban	29.80 °N	78.74 °E	1814	17-21	North-East
Khirsoo	Semi-Urban	30.17 °N	78.86 °E	1700	18-20	East
Kaproli	Village	30.03 °N	79.02 °E	1800	22-22.5	East-South
Thailisain	Semi-Urban	30.02 °N	79.04 °E	1700	18-21	South
Bharsar (Check)	Village (College)	30.05 °N	78.99 °E	1900	16-20	North-East

Preparation and Supply of Raw Materials

Full matured compost, sterilized casing, spawn and polybags were supplied to the selected farmers to lay down the experiment by using their inherent techniques and skills of mushroom cultivation. Each mushroom grower was facilitated with 100 kg compost, 20 kg sterilized casing, 650 gm spawn, 10 polybags of 10 kg capacity, 1kg capacity balance, knife, KMS, data sheets etc. They were strictly taught about making 10 replications and 7 weeks cropping period. Compost was prepared using long method of composting [3] and casing was prepared using standard procedure, therein humus rich forest litter and 2.5 year old spent mushroom substrate were used in equal proportion [4].

Tech-fissure, Market-fissure and Benefit:Cost Ratio

Tech-fissure was established by calculating per cent difference within identical parameters namely days of spawn run, casing and first harvest interface in days, yield q^{-1} compost, number of fresh mushrooms q^{-1} compost and average weight of fresh mushroom fruit in g among the selected locations. Tech-fissure was said to be either per cent superior or inferior in the excellence of adaptation and functioning of mushroom growing technology by mushroom growers over to that of Bharsar centre and calculated using formula:{(value of selected parameter of Bharsar-value of selected parameter of MG)/ value of selected parameter of Bharsar} x 100. However, Bhatia described it as a gap occurred between the level of recommendation and extent of their adoption [5]. Market fissure was based on the per cent negative/positive difference in net income earned by the mushroom growers over to that of Bharsar Centre. It was calculated with the formula {(Net income of Bharsar-Net income of MG)/ Net income of Bharsar} x 100. Benefit:Cost Ratio of the present investigation was based on the ratio of gross income earned by the MGs/total cost involved in mushroom production. The total cost was fixed of ₹992.18 to all mushroom growers as all inputs were supplied to them from Bharsar Centre.

RESULTS AND DISCUSSION

Quantitative Parameters

Data obtained from 5 different places were summarized and arranged in the Table 2 in form of period of spawn run, casing-first harvest interphase, yield, number and average weight of fresh mushroom fruit. The duration of spawn run (18-20 days) and casing and first harvest interface (16-24 days) was found more in all the mushroom growing farms over Bharsar Centre in which a minimum period of 15 days was recorded in both the cases. The trial conducted at Vir Chandra Singh Garhwali College of Horticulture, Bharsar was best and gave 22.45 kg fresh mushrooms q^{-1}compost followed by 19.56 kg fresh mushrooms q^{-1} compost from MG1. The yield received from MG2 and MG4 were statistically at par with 14.23 and

15.29 kg q^{-1}compost. Though, MG3 from Kaproli village was stood most inferior with12.57 kg yield of fresh mushroom. The mushroom numbers obtained from Bharsar and MG1 were statistically identical and counted maximum 2826.78 and 2748.21q^{-1}compost. However, MG3 from Kaproli was again inferior in production of mushroom numbers, in which only 1942.85 mushrooms were sum up. Almost similar average fruit body weight was calculated from all mushroom growers that ranged from 6.5-7.19 gm in contrary to 8.02g average fruit body weight recorded from Bharsar centre. Previous results have shown 14 and 15-25 days for spawn run and casing and first harvesting interphase, respectively and yield and numbers of mushroom were found less but with more average weight of fruit body in comparison to our findings [6].

Table 2. Quantitative observations of selected parameters

Name of Place	Spawn run (days)	Interface of casing and Ist harvesting	Yield Kg q^{-1} compost	Fruit number sq^{-1} compost	Weight/fruit body (gm)
Pauri (MG1)	19	22	19.56	2748.21	7.19
Khirsu(MG2)	18	16	15.29	2364.28	6.51
Kaproli (MG3)	20	24	12.57	1942.85	6.55
Thailisain (MG4)	18	24	14.23	2203.57	6.5
Bharsar (Check)	15	15	22.45	2826.78	8.02
CD at 5%			1.05	294.17	0.72

Cropping and Production Pattern

Seven weeks cropping pattern on yield and numbers of *Agaricus* fruits was also studied and mentioned in the Fig. 1 and 2, respectively. The seven weeks cropping pattern on yield basis for Bharsar and MG1 was almost similar with their highest peaks got at the end of I week and then it started decline gradually to its shortest peaks at VII week. The rest trials were shown their highest peaks of the yield at the end of II week then declined steadily and attained similar shortest peaks as found with Bharsar centre and MG1 (Fig. 1). Number of mushroom fruits of all trials were exponentially increased by the II week and then decline gradually to their minimum number ranged from 80.36-198.86/q compost to the last week of the crop (Fig. 2). Dhar *et al*., demonstrated that out of 10 treatments of casing mixtures, 8 treatments showed highest yield at the end of I week, followed by II week and 5 treatments showed highest numbers of mushroom fruits/quintal compost followed by I week [6]. Thus, the cropping pattern of yield and numbers of 4 different places were seemed to be almost similar to that of Bharsar centre but it was very interesting to note that the major share of yield and number of mushrooms in all trials was achieved within 2 previous weeks of cropping period. Highest 60.13% and 56.79% yield and numbers of mushroom, respectively was recorded from Bharsar centre for first two initial weeks out of total yield and number of mushroom obtained in an entire cropping period of seven week. Rest four trials also produced 44.83-58.79% yield and 36.54-57.62% number of mushrooms within a time frame of 2 initial weeks. Ahlawat also recorded about 60% yield within

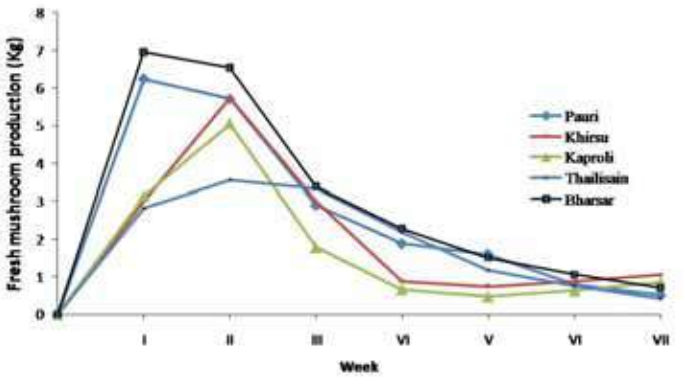

Figure 1. Cropping Pattern of Fresh Mushroom Production (Kg) q^{-1} Compost

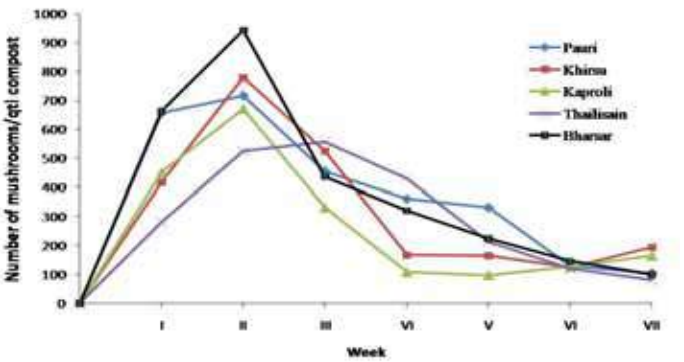

Figure 2. Cropping Pattern of Number of Button Mushroom q^{-1} Compost

2 initial flushes of button mushroom [7]. Therefore, major share of yield and numbers was achieved within first two initial weeks of 6 weeks period crop.

Tech-fissure

In the present investigation it was found that, all 4 locations of the trial showed inferior/poor adoptability in mushroom growing techniques in comparison to Bharsar centre on quantitative parameters. Table 3 revealed that Kaproli was found most inferior by scoring highest 33.33 and 60% tech-fissure in spawn run and casing-first harvest interphase, respectively against to that of very negligible inferior tech-fissure of 6.66% recorded from Khirsu. Yield and number based tech-fissure of Kaproli was also recorded poorer by 44% and 31.16%, respectively in comparison to least inferior tech-fissure for yield (12.87%) and mushroom numbers (2.77%) observed from Pauri. However, on average fruit body weight basis, at least 0.81 per cent inferior tech-fissure was measured in all locations except Pauri (0.89%). In another study minimum 14% tech-gap in spawning and spawn run to maximum 40% tech-gap in compost preparation followed by casing mixture (29%) was recorded out of 6 different practices of mushroom production tested. Psychological traits such as age, family education, socio-economic status, extension contacts, mass media exposure, change proneness, risk orientation and fatalism-scienticism were thought to be major reasons for tech-gap [8].

Table 3. Technological fissure

Name of Place	Negative/Inferior Technological-fissure (%)				
	Duration of spawn run	Casing and Ist harvesting interphase	Yield	Numbers	Fruit body weight
Pauri (MG1)	26.66	46.66	12.87	2.77	0.89
Khirsu (MG2)	20	6.66	31.89	16.36	0.81
Kaproli (MG3)	33.33	60	44.00	31.16	0.81
Thailisain (MG4)	20	60	36.61	22.04	0.81

Market-fissure

Marketing fissure was based on the per cent negative/positive difference in net income earned by the mushroom growers over to that of Bharsar Centre. Positive marketing fissure was achieved only with the MG1 of Pauri in which mushroom grower was achieved 8.15% more income. Remaining 3 MGs showed negative marketing fissures (Fig. 3). Out of them MG3 of Kaproli Village was scored 88.89% negative market-fissure followed by 54.25 and 57.15% negative market-fissure was calculated with MG2 and MG4, respectively. Maximum 2.36:1 BCR was calculated from MG1 followed by Bharsar Centre as it had 2.26:1. Kaproli was known to poorer BCR ratio of 1.14:1. The results of BCR of the present investigation were encouragious than to the BCR of 1.55:1 [9]. Rural and urban status of places and quantity produce were appeared as main causes of market-fissure. The places like Pauri have urban status was found almost equal to the Bharsar centre followed by Khirsu of semi urban status in view to market fissure and benefit:cost ratio. Both tech and market-fissure were recorded most negative from Village Kaproli with very poor net income and benefit: cost ratio. Crop management skill, harvesting time, social relations of grower were seemed as major hurdles resulted poor performance in Village Kaproli.

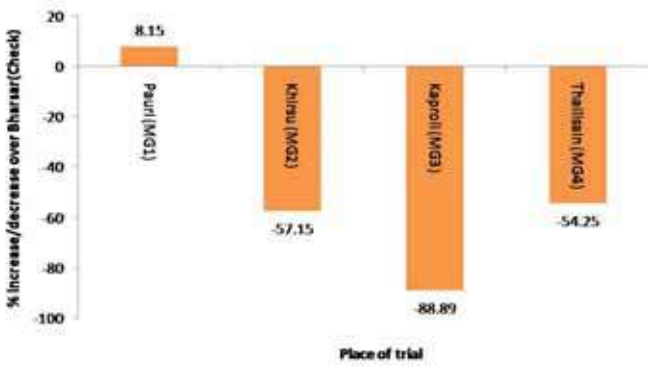

Figure 3. Market fissure

It was quite evident from the present findings that marginal and small mushroom growers of Uttarakhand State of India had rather enough difference in the adaptation and application of mushroom growing technology and marketing strategies in form of tech and marketing fissures, respectively when calculated against scientific know how. Mushroom growers followed the variations in all 5 parameters tested, but parameters like casing-I harvest interphase and yield were seemed to be more efficient to establish quite firm technological fissure among the mushroom growers due to higher variation occurred in them in contrast to the finding of minimum 14% tech-gap was recorded with spawning and spawn run [8]. However, rural and urban status of places and quantity produce were appeared as main causes of market-fissure. Places like Pauri of urban status was found almost equal to the Bharsar centre followed by Khirsu town of semi urban status in view to market fissure and benefit:cost ratio. However, the tech and market-fissure was recorded most negative in the trial located in a village Kaproli with very poor net income and benefit cost ratio.

REFERENCES

[1] Singh M. (2011). Mushrooms Production: An Agribusiness Activity. Mushrooms Cultivation, Marketing and Consumption. pp 1-10.

[2] Singh RP *et al*. (2007). Status and prospects of mushroom cultivation in Uttaranchal, India. In: Mushroom Biology and Biotechnology, pp. 313-319.

[3] Shandilya TR. (1995). Compost and composting for mushrooms. In: Mushroom Production Technology, pp. 27-34.

[4] Mishra SK. (2008). Effects of locally available decomposed materials on casing run and yield of *Agaricus bisporus* (Lange) Imbach. *Indian Phytopathol.* 61(3): 384-385.

[5] Bhatia JN *et al*. (2011).Technological gap in adoption of improved mushroom cultivation practices in Haryana. *Agri. Updt.* 6(3): 206-209.

[6] Dhar BL *et al*. (2006). Casing layer as related to mushroom yield and quality in *Agaricus bisporus* in India. *Mushroom Res.* 15(2): 111-117.

[7] Ahlawat OP. (2002). Fluctuations in pH and temperature in the compost and compost-casing interface their effect on yield of *Agaricus bisporus. Mushroom Res.* 11(1): 11-14.

[8] Singh S *et al*. (2001). Technological gap in adaptation of mushroom cultivation in Haryana. In: Modernizing Indian Agriculture in 21st Century Challenges, Opportunities and Strategies, pp 233-239. ISBN 81-7022-905-7.

[9] Barmon BK *et al*. (2012). Economics of mushroom (*Agaricus bisporus*) production in a selected Upazila of Bangladesh. *The Agri.* 10(2): 77-89.

NOTES