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Laboratory of Conservation and Utilization for Bio-resources
Key Laboratory for Microbial Resources of the Ministry of Education,
Yunnan University, Kunming 650091.
School of Life Science,
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China

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Dr. Nicole Wolter
National Institute for Communicable Diseases and University of the Witwatersrand,
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South Africa

Dr. Marco Antonio Nogueira
Universidade Estadual de Londrina
CCB/Depto. De microbiologia
Laboratório de Microbiologia Ambiental
Caixa Postal 6001
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Department of Biochemistry and Microbiology,
Faculty of Pharmaceutical Sciences,
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Bangkok 10330
Thailand

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Post Graduate Department of Botany,
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India

Dr. Shihua Wang
College of Life Sciences,
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China

Dr. Victor Manuel Fernandes Galhano
CITAB-Centre for Research and Technology of Agro-Environment and Biological Sciences, Integrative Biology and Quality Research Group,
University of Trás-os-Montes and Alto Douro,
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Dr. Suppasil Maneerat
Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90112
Thailand

Dr. Francisco Javier Las heras Vazquez
Almeria University
Spain

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Chang Gung memorial Hospital, Chang Gung University
Taiwan

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DDU Gorakhpur University, Gorakhpur-273009 (U.P.)
India

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Central University of Technology, Free State
South Africa

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Brazil

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Balikesir University
Turkey

Dr. Marija Stankovic
Institute of Molecular Genetics and Genetic Engineering
Republic of Serbia

Dr. Scott Weese
University of Guelph
Dept of Pathobiology, Ontario Veterinary College,
University of Guelph,
Guelph, Ontario, N1G2W1,
Canada

Dr. Sabiha Essack
School of Health Sciences
South African Committee of Health Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban 4000
South Africa

Dr. Hare Krishna
Central Institute for Arid Horticulture,
Beechwal, Bikaner-334 006, Rajasthan,
India

Dr. Anna Mensuali
Dept. of Life Science,
Scuola Superiore
Sant'Anna

Dr. Ghada Sameh Hafez Hassan
Pharmaceutical Chemistry Department,
Faculty of Pharmacy, Mansoura University,
Egypt

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Biochemistry and Molecular Biology
Universidade Federal de Goiás
Brasil

Dr. Abdel-Hady El-Gilany
Public Health & Community Medicine
Faculty of Medicine,
Mansoura University
Egypt

Dr. Hongxiong Guo
STD and HIV/AIDS Control and Prevention,
Jiangsu provincial CDC,
China

Dr. Konstantina Tsaousi
Life and Health Sciences,
School of Biomedical Sciences,
University of Ulster

Dr. Bhavnaben Gowan Gordhan
DST/NRF Centre of Excellence for Biomedical TB Research
University of the Witwatersrand and National Health Laboratory Service
P.O. Box 1038, Johannesburg 2000,
South Africa

Dr. Ernest Kuchar
Pediatric Infectious Diseases,
Wroclaw Medical University,
Wroclaw Teaching Hospital,
Poland

Dr. Hongxiong Guo
STD and HIV/AIDS Control and Prevention,
Jiangsu provincial CDC,
China

Dr. Mar Rodriguez Jovita
Food Hygiene and Safety, Faculty of Veterinary Science.
University of Extremadura,
Spain

Dr. Jes Gitz Holler
Hospital Pharmacy,
Aalesund. Central Norway Pharmaceutical Trust
Professor Brochs gt. 6. 7030 Trondheim,
Norway

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College of Life Sciences,
Wuhan University
Wuhan 430072, P.R.China

Dr. Anchalee Tungtrongchitr
Siriraj Dust Mite Center for Services and Research
Department of Parasitology,
Faculty of Medicine Siriraj Hospital,
Mahidol University
2 Prannok Road, Bangkok Noi,
Bangkok, 10700, Thailand

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Anti-*Leishmania* spp. antibodies in stray dogs housed in a private shelter in the Northwest Region of Parana State, Brazil

Edson Gerônimo[1], Daniela Dib Gonçalves[1]*, Aristeu Vieira da Silva[2], Fernanda Evers[4], Beatriz de Souza Lima Nino[4], Adriana Lebram Von Söhsten[2], Italmar Teodorico Navarro[4], Roberta Lemos Freire[4], Rodrigo Assunção Moura[1] and Fabiana Maria Ruiz Lopes-Mori[3]

[1]Department of Preventive Veterinary Medicine and Public Health, Universidade Paranaense (UNIPAR), Umuarama, Paraná, Brazil.
[2]Department of Biological Sciences, Universidade Estadual de Feira de Santana (UEFS), Feira de Santana, Bahia, Brazil.
[3]Department of Preventive Veterinary Medicine and Public Health, Centro Universitário Filadélfia (UNIFIL), Londrina, Paraná, Brazil.
[4]Department of Preventive Veterinary Medicine, Universidade Estadual de Londrina (UEL), Londrina, Paraná, Brazil.

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The aim of this study was to detect anti-*Leishmania* spp. antibodies in stray dogs housed in a private shelter in the city of Umuarama, Paraná, Brazil. This study included serum samples from all stray and asymptomatic dogs housed in a private shelter in the period between March and May 2011. In order to detect anti-*Leishmania* spp. antibodies, indirect immunofluorescence assay was performed using blades containing promastigote forms of *Leishmania amazonensis*. From the 173 serum samples analyzed, 38.73% were considered reactant, which is 71.64% for titer 40, 25.37% for titer 80 and 2.98% for titer 160. The reagent serology for *Leishmania* spp. in stray dogs in the city of Umuarama (PR) indicates that there might be circulation of the etiologic agent, and consequently, exposure of these animals to the parasite; however, new serological, parasitological and molecular studies with this animal species must be performed in order to better clarify the participation of dogs in the American Tegumentary Leishmaniasis (ATL) transmission chain in the mentioned city.

Key words: Dog, diagnosis, *Leishmania amazonensis, Leishmania* spp., zoonosis.

INTRODUCTION

Leishmaniasis is an anthropozoonosis caused by protozoa belonging to the Kinetoplastida order, Trypanossomatidea family of the genus *Leishmania* (Baneth et al., 2008). It is transmitted by dipteran from the Psychodidae family and constitutes a global public health problem (Hotez et al., 2007; Locatelli et al., 2014).

In Brazil, it is caused by different species, such as *Leishmania (Viannia) braziliensis*, *Leishmania (L.) amazonensis*, *Leishmania (V.) guyanensis, Leishmania (V.) shawi, Leishmania (V.) lainsoni*, *Leishmania (chagasi)* among others, and it is considered an emerging disease found not only in woods and forests, but also in urban peripheral areas, usually associated with social-economic and sanitary problems (Souza, 2007; Souza et

al., 2010).

The American Tegumentary Leishmaniasis (ATL) has extensive and diverse clinical epidemiology because its vectors are present in different locations and environments. This characteristic affects the probability of the ATL to occur in low or high rates. Furthermore, the *Leishmania* species inoculated by the vectors can cause different clinical signs in the affected human or animal. These signs may vary from skin lesions to the visceral form of infection (Brazil, 2006, 2007; Saridomichelakis, 2009).

In the ATL epidemiological chain, the role of dogs is still scarcely established. However, it has been observed that in endemic areas with the human disease, dogs also demonstrate infection by this protozoan (Brazil, 2007; Pittner et al., 2009; Zulpo et al., 2012).

Researches have described that in dogs, ATL can be characterized as a scar lesion in the ear pinna, nose, scrotum or even in any place where the vector bites; however, ulcerated lesions, non-ulcerated nodular lesions and polyarthritis manifestations have already been reported (Pirmez et al., 1988; Heusser-Júnior et al., 2010; Mitropoulos et al., 2010; Hoffmann et al., 2012).

In Brazil, studies of ATL seroprevalence in dogs using domestic or stray dogs has already been performed in the states of São Paulo (SP), Rio Grande do Sul (RS), Bahia (BA), Rio de Janeiro (RJ), Santa Catarina (SC) and Paraná (PR) showing varied prevalence (Savani et al., 1999; Jesus et al., 2006; Souza, 2007; Pereira et al., 2008; Cerino et al., 2010; Greca et al., 2010; Heusser-Júnior et al., 2010; Reis et al., 2011; Zulpo et al., 2012).

Considering the absence of regional data for ATL in stray dogs and notifications from the local health service of autochthonous human cases, the aim of this paper was to detect anti-*Leishmania* spp. antibodies in stray dogs housed in a private shelter in the city of Umuarama, Paraná, Brazil.

MATERIALS AND METHODS

Study and sample location

The shelter is located in the peri-urban area in the city of Umuarama, located in the northwestern region in the state of Paraná (PR). In a distance of 50 m from the shelter, there is the Sanitary Landfill for the Municipality of Umuarama (PR) where there is the presence of many residues (dry leaves, branches and decomposing material), as well as trash.

The shelter studied is private, kept by employees and visitors, which collect stray dogs only in the city of Umuarama (PR) and sent to the shelter awaiting future adoption. At the shelter, the dogs are lodged in collective kennels (up to ten animals) regardless of breed, gender, age or size, and the animals that are debilitated or presenting signs of any infectious disease were kept in individual kennels, isolated from the other animals.

This study includes all stray asymptomatic or symptomatic dogs, regardless of breed, gender or age in the private shelter in the period between March and May 2011. For each animal, a clinical form was filled out, containing information of the presence of clinical signs. It was not possible to obtain information on origin, exact age and sanitary status of the respective dogs, since these animals were found wandering in the streets in the city of Umuarama (PR) at the time they were taken to the shelter.

This study was approved by the Ethics Committee for Animal Experiments (CEPEEA) of the University of Paraná (UNIPAR) under process number 20678/2011.

Collection of material

The collection of 10 mL blood from each dog was performed by veterinarians by means of puncturing the external, cephalic or brachial jugular veins. The blood samples were identified and sent to the Preventive Veterinary Medicine and Public Health Laboratory at Universidade Paranaense (UNIPAR), where they were centrifuged to obtain the serum and each sample was stored in sterile vial, duly identified and stored at -20°C until the moment of use. Serological exams were performed at the Zoonosis and Public Health Laboratory in the Department of Preventive Veterinary at State University of Londrina (UEL).

Serological test

The presence of anti-*Leishmania* spp. antibodies was detected using the indirect immunofluorescence assay (IFA) (Marzochi et al., 1980; Oliveira et al., 2008). For the analyses, blades containing promastigote forms of *Leishmania amazonensis* produced by the Biological Sciences Center at UEL, dog anti-IgG conjugate (Sigma Chemical®) labeled with fluorescein isothiocyanate (SIGMA CHEMICAL Co. St. Louis, USA) standardized in a 1:40 dilution as previously described (Reis et al., 2011) and positive control from naturally infected animals for the *L. amazonensis*, previously analyzed, were used.

Sera of this study were tested in serial dilutions 1:40; 1:80 and 1:160. The samples showing fluorescent tachyzoites at a titer ≥ 40 were considered reactive.

Statistic analysis

The variables were analyzed in the EpiInfo 3.5.1 program, using the Fisher's exact test to verify the statistic significance and the odds ratio as the association measure between infection by *Leishmania* and the variables gender and presence of clinical signs, with 95% confidence interval. A significance level of 5% was used (Dean et al., 1996).

RESULTS

From the total of 173 serum samples analyzed, 29 belonged to male and 144 to female dogs, of unknown breeds, with age ranging from six months to 13 years.

It was observed that 38.73% (67/173) of the samples

*Corresponding author. E-mail: danieladib@unipar.br.

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Table 1. Prevalence of antibody titers in relation to gender detected in indirect immunofluorescence assay (IFA) for leishmaniasis in 67 reactive serum samples of dogs housed in a private shelter at the city of Umuarama, Paraná, 2011.

Antibody titer (dilution)	Prevalence		Total (%)
	Female	Male	
1/40	37	11	48 (71.65)
1/80	13	04	17 (25.37)
1/160	01	01	02 (2.98)
Total	51 (76.12%)	16 (23.88%)	67 (100)

Table 2. Result from indirect immunofluorescence (IFA) for the anti-*Leishmania* spp. antibody survey associated with gender and presence of clinical signs in 67 serum samples of reagent dogs in a private shelter, in the city of Umuarama, Paraná, 2011.

Variable	IFA results				
	Positive	Negative	Total	p	OR (95% IC)
Gender					
Male	16	13	29	0.074	2.24 (0.93-5.543)
Female	51	93	144		
Clinical signs					
Present	02	01	03	0.5603	3.23 (0.22-91.92)
Absent	65	105	170		

$p \leq 0.05$ or 95% IC in OR that does not include value 1, indicate association between the variables and the test result. Values of p calculated by Fisher's Exact Test for an $\alpha = 0.05$.

were reagent in IFA indicating the presence of antibody against this parasite, being 71.64% (48/67) for titer of 40, 25.37% (17/67) for titer of 80 and 2.98% (2/67) for titer of 160 (Table 1).

Among all the animals studied, only three 1.73% (3/173) had mucocutaneous clinical signs (ulcers and periocular alopecia) at the time of blood collection. Regarding the variables gender and presence of clinical signs, none was presented as statistically significant when associated with the serological reactions by IFA (Table 2).

DISCUSSION

Due to the strict contact with men and the possibility of asymptomatic clinical presentation, dogs play an important role in the maintenance, dissemination and transmission of different zoonotic parasitary agents, a situation that causes concern for public health, mainly when related to stray dogs, since these animals wander freely in the streets and cover long distances searching for food, water and shelter (Pittner et al., 2009; Mergener et al., 2013; Miller, 2013; Quadros, 2013; Dantas-Torres and Otranto, 2014; Gizzi et al., 2014).

Researchers have shown the frequency of anti-*Leishmania* spp. antibodies in stray dogs in different regions in the country, with the objective of helping in the understanding of this disease epidemiology in each location studied (Savani et al., 1999; Jesus et al., 2006; Souza, 2007; Pereira et al., 2008; Heusser-Júnior et al., 2010); however, in the city of Umuarama (PR), this is the first time anti-*Leishmania* spp. antibodies are researched in dogs characterized as stray dogs. This work was done after the confirmation of ATL cases in humans in the urban and peri-urban perimeter in the mentioned city in the year 2012, as published by the 12th Health Regional Office in the municipality (Brazil, 2012).

Prevalence of anti-*Leishmania* spp. antibodies detected in this paper was 38.73%, which was higher than those found by Savani et al. (1999) in São Paulo (SP) and Pereira et al. (2008) in Rio de Janeiro (RJ), which detected, respectively 0.0 and 2.16% seropositivity also in stray dogs; however, these results are similar to those found by Lonardoni et al. (2006b) that detected 37.00% of positivity in stray dogs in the northern region in the state of Paraná (PR). The prevalence of anti-*Leishmania* spp. antibodies detected in this study is an alert for

assessing the risk conditions that these animals may represent to humans. Moreover, this data may be used as sentinels for the occurrence of this parasitary agent in the local ecosystem, since dogs have been considered ATL indicators in men (dos Santos et al., 2005). In the state of Ceará, Cunha et al. (2006) associated ATL cases in human beings with the presence of housedogs infected by *Leishmania* spp.; therefore, these results support the hypothesis of domiciliary transmission of American tegumetary leishmaniasis to humans, and show the importance of knowing the canine species in the epidemiology and transmission chain for this zoonotic disease.

The ATL antibody research has been an important tool for diagnosis, as well as for seroepidemiological surveys in animals and IFA, and a standard technique for detecting ATL in humans, it is also the reference technique for canine ATL in Brazil, thus it being chosen for this work. This technique has already been used by other researchers studying canine ATL in areas with the presence of endemic disease in humans (Marzochi and Barbosa-Santos, 1988; Pereira et al., 2008).

Despite the prevalence of 38.72% positivity in IFA, the possibility of false-positive results cannot be discarded, since IFA show cross-reactions with *Trypanosoma cruzi*, the casual agent for Chagas disease, and *Leishmania chagasi*, the casual agent of visceral leishmaniasis. However, even with the possibility of cross-reactions, this result suggest a strong indication of the etiological agent circulation in the city of Umuarama (PR), and diagnosis confirmation is necessary, not only through serological methods, but also through parasitological and molecular methods, since in the present date, there is no serological method with 100% of specificity and sensitivity for this parasitary disease (Leontides et al., 2002; Hoffmann et al., 2012).

In the city of Umuarama (PR), there are a few ecological and environmental factors that must be considered, since these may influence the appearance of anti-*Leishmania* spp. antibodies in stray dogs. These factors are:

1. The city of Umuarama has woods with preserved residues from original forest in different regions in the city. These have a broad variety of vectors that consequently establish strict contact with humans, providing a favorable environment for the maintenance and dissemination of this etiological agent.
2. Next to the shelter studied, there is a large area being deforested for urban settlement, which has caused expressive environmental imbalance, and it might be contributing to the prevalence detected in this work, since with environmental imbalance, phlebotomines seek men and pets to feed themselves on (Gomes et al., 1990; Marzochi et al., 1994);
3. The private shelter where the dogs are kept is located at approximately 50 m from Umuarama's landfill, which has the presence of residues (leaves, wood, rubble) and trash, allowing the development of phlebotomines in organic matter and humid and shadow environments (Reis et al., 2011).

Despite the factors mentioned above, the authors cannot state when or where these dogs have been infected, since they were found wandering in the streets of the city at the time of their capture.

Regarding the variables, gender and presence of clinical signs associated with IFA, none was significant. Regarding animal gender, there were no significant differences (p= 0.074), which corroborates with the results (p=0.0559) of Reis et al. (2011) also in the state of Paraná (PR). Regarding the presence of clinical signs (ulcers and periocular alopecia), there were no significant differences (p=0.5603); however, it is important to highlight that at the time of the blood collection, two (0.86%) animals presented mucocutaneous signs, and one (0.57%) biocular alopecia (dos Santos et al., 2005), that is, clinical signs suggestive of ATL (Tab. 2). Lonardoni et al. (2006a, b) stated that even if the dogs do not develop lesions suggesting the disease, the presence of subclinical form of canine ATL cannot be discarded, which highlights the importance of seroepidemiological surveys in asymptomatic animals.

Conclusion

The reactive serology for *Leishmania* spp. in stray dogs in the city of Umuarama (PR) may indicate the circulation of the ATL etiologic agent, and consequently, the exposure of these animals to the parasite; however, new serological, parasitological and molecular studies with these animals species must be performed in order to clarify the participation of dogs in the ATL transmission chain in the location studied. Moreover, studies with different phlebotomine species would be important for the real knowledge of the parasite cycle in the respective city.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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In vitro evaluation of some fungicides and bioagents against common bean anthracnose (*Colletotrichum lindemuthianum* Sacc. & Magnus*)* Briosi & Cavara

Sileshi Fitsum, Mohammed Amin*, Thangavel Selvaraj and Adugna Alemayehu

Department of Plant Sciences, College of Agriculture and Veterinary Sciences, Ambo University, Ambo, P. O. Box 19, Ethiopia.

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Bean anthracnose caused by *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara is one of the most devastating seed-borne diseases of common bean (*Phaseolus vulgaris* L.) in Ethiopia. The aim of the present investigation was to evaluate the antifungal activities of bioagents and fungicides which can be used to control bean anthracnose. Three fungicides viz., Mancozeb, Folpan and Mancolaxyl, and three bioagents viz., *Trichoderma harzianum* Rifai, *Trichoderma viride* Pers. Fr. and *Pseudomonas fluorescens* Migula, were screened *in vitro* for their antifungal activities against common bean anthracnose, *C. lindemuthianum* using the dual culture and microtitre double-dilution techniques. Antagonistic effects of the three bioagents tested by the dual culture method showed highly significant (P<0.01) percentage of inhibition of the mycelia germination of *C. lindemuthianum.* The highest percentage of inhibition of the mycelia germination (80.39%) was obtained from *T. viride,* followed by 75.49% from *T. harzianum* and 40.2% from *P. fluorescens*. Similarly, highly significant (P<0.01) differences were observed in the radial growth of mycelia of *C. lindemuthianum.* The highest growth of mycelia (3.4 cm) was measured from the control (*C. lindemuthianum*), whereas the least (0.67 cm) was obtained from the dual culture containing *T. viride.* The *in vitro* assays revealed that all the antagonistic bioagents produced siderophores which were capable of inhibiting mycelia growth of the pathogen. The mancozeb fungicide was found to be fatal to *C. lindemuthianum* at four different concentrations poisoned on potato dextrose agar medium.

Key words: Bio agents, *Colletotrichum lindemuthianum*, dual culture, fungicides, *in vitro, Phaseolus vulgaris.*

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an important food grain legume crop, cultivated in almost every part of the world (Ibarra-Perez et al., 1997). For many households in the tropics, the crop is a good source of cash and food nutrients (Popelka et al., 2004). Globally, common bean is cultivated in nearly 28 million hectares of land, producing about 20 million tons annually (FAOSTAT, 2008). The highest production and area

*Corresponding author. E-mail: yonias_1986@yahoo.com.

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under common beans in Africa is in the east and central regions, where beans are mainly grown by resource poor farmers (Wortmann et al., 1998). In Ethiopia, common bean is mainly cultivated in the eastern, southern, south-western and rift valley regions of the country (Habtu et al., 1996). Annual area coverage is estimated to be around 200,000 ha (CSA, 2010). The crop is increasingly becoming important to the national economy and to the farmers as food and cash income. Despite its economic significance and wide area of production, the national annual average yield of common bean in Ethiopia is low, ranging from 0.615-1.487 tons/ha between the years 2004 and 2010 (CSA, 2010). Such a low figure is far below the corresponding yield recorded at research sites (2.5-3 tons/ha) using improved varieties (EEPA, 2004). The low national yield could be attributed to various constraints as low adoption of improved agricultural technologies, drought, diseases and pests, lack of improved seed varieties, poor cultural practices, shortage of land and environmental degradation (Legesse et al., 2006, Kutangi et al., 2010). Due to extreme differences in agro-climatic conditions and cropping practices in Ethiopia, these production constraints could vary from one region to the other.

Common bean production is influenced by both biotic and abiotic stresses; biotic factors are responsible for major losses. Six major diseases (anthracnose, rust, angular leaf spot, common bacterial blight, Bean Golden Mosaic Virus and Bean Common Mosaic Virus) are known to hamper common bean production. However, the most important among these is anthracnose which is found in almost every bean growing region of the world (Kelly et al., 1994). It is considered as one of the most destructive disease (Pastor-Corrales and Tu, 1989). Anthracnose of the common bean is caused by *Colletotrichum lindemuthianum,* a hemibiotrophic fungus. The disease generally occurs by contaminated seeds or infected plant debris (Dillard and Cobb, 1993). This disease may lead to major or total crop loss, particularly in a case where a susceptible variety is grown (Fernandez et al., 2000; Sharma et al., 2005).

Chemical and biological methods are very useful alternatives among the different strategies for plant growth promotion and disease suppression.

Bioagents (living antagonistic organisms) however, can be safer, more biodegradable and less expensive to develop as compared to synthetic fungicides (Amin et al., 2014). The use of *Pseudomonas fluorescens* and *Trichoderma* species are becoming increasingly common as an effective, economic and environment friendly approach and also effectively controlling many seed and soil borne pathogens including *Colletotrichum.* Padder et al. (2010) reported that seed dressing or soil application of *Trichoderma viride*, *Trichoderma harzianum* and *Gliocladium virens* caused significant inhibition of mycelial growth of *C. lindemuthianum*, effectively controlling the seed borne infection and increasing the seed germination; extracellular metabolites like siderophores, antibiotics, lytic enzymes and volatile compounds produced by rhizobacteria (*Pseudomonas fluorescens* and *Bacillus capacia*) effectively reduced lesions and damages caused by *C. lindemuthianum* on bean plants.

Recent innovations showed that biological control of crop diseases is getting increased attention as an environ-mentally sound approach. But in Ethiopia, the method has received comparatively little attention. Apparently, the management of bean anthracnose through biocontrol agents, particularly*, P. fluorescens, T. viride* and *T. harzianum* has not been studied so far in Ethiopia. Therefore, the present work was carried out with the objective of the evaluation of some fungicides and bioagents for the management of common bean anthracnose disease under *in vitro* conditions.

MATERIALS AND METHODS

Project location

In vitro evaluation of the antagonistic activities of three bio agents: *T. harzianum*, *T. viride* and *P. fluorescens* and the efficacy of three fungicides; Mancozeb (Unizeb 80% WP), Folpan 80% WDG and Mancolaxyl 72 WP (Mancozeb + Metalaxyl) against common bean anthracnose disease was carried out in Plant Science Research Laboratory, Ambo University, Ethiopia. All the three bioagents and fungicides were obtained from the Department of Plant Science of the university. Ambo is located 120 km west of Addis Ababa at 8°98' South latitude and 37°83' North longitude. It has a total geographical area of 83,598.69 km^2, with elevation ranging from 1380-3300 m above sea level.

Sample collection and isolation of common bean anthracnose pathogen

Anthracnose infected common bean variety Mexican-142 pods were collected from the University farm and washed in running tap water. About 1 g of the diseased sample was surface sterilized in a solution of 1% hypochlorite and macerated three times in sterile distilled water and the filtrate was further diluted using sterile distilled water. From the appropriate dilutions, 0.1 ml of an aliquot was spread-plated in duplicates on pre-dried surfaces of Potato Dextrose Agar (PDA) medium and incubated at 26°C for 10 days. The colonies of *C. lindemuthianum* was picked up from the culture plates and further sub-culturing and purified through repeated spread-plated on the PDA medium (Das et al*.,* 2003).

Antifungal assay (dual culture method)

The dual culture method was used to evaluate the antagonistic effects of the bioagents. A 5 mm diameter agar disc of each of the three bio-agents (*T. harzianum, T. viride* and *P. fluorescens*) were taken from five day old cultures and placed, separately, at the periphery of Petri plates (90 mm, diameter) containing solid PDA. A similar size of agar disc of *C. lindemuthianum* was placed at the periphery of each Petri plate with the bioagents, but on the opposing end. *C. lindemuthianum* agar disc was placed in a similar manner on a fresh PDA plate, as control. All pairings were carried out on three replicates and incubated at 25°C. Antagonistic activity was assessed after five days of incubation by measuring the radius

Figure 1. Bean crops with anthracnose infected pods.

Figure 2. *C. lindemuthianum* growth on agar plates.

$$\text{PIMG (\%)} = \frac{R1-R2}{R1} \times 100$$

of the mycelia growth (R2) of *C. lindemuthianum* in the direction of the antagonistic colony and the radius of *C. lindemuthianum* in the control plate (R1). The two readings, R1 and R2 were transformed into percent inhibition of mycelium growth (PIMG) according to Skidmore and Dickinson (1976). The number of days taken for the antagonists to overgrow the whole colony of *C. lindemuthianum* was also recorded.

Siderophore production assay

The procedure of Schupp et al. (1988) was used for detection of siderophore production by the three bioagents. Whatman No.1 filter paper soaked in an indicator solution containing 1% ammonium ferric sulfate $[FeNH_4(SO_4)_2.12H_2O]$ in sulfuric acid was placed on 4-day old single colonies of each of the three bioagents grown on Des-4 medium containing; 2% Dextrin, 2% Manitol, 1.2% L-asparagine, 0.025% L-lysine, 0.01% L-methionine, 0.01% L-theronine, 0.5% $CaCO_3$, 0.025% $MgSO_4$, 0.05% K_2HPO_4, 0.005% $ZnSO_4$ and 2% agar. Brown halo formation around the colonies of each bio agent was recorded and used as detection for siderophore production.

Efficacy of fungicidal assay

The efficacies of each of the three fungicides, Mancozeb, Folpan and Mancolaxyl against *C. lindemuthianum* were evaluated using the Food Poisoned Technique, as described by Nene and Thapliyal (1979). Potato dextrose agar (PDA) liquid medium was mixed with different concentrations (100, 250, 500 and 1000 ppm) of each of the three fungicides, poured into sterilized Petri plates and allowed to solidify. A disc of 7 mm diameter of *C. lindemuthianum* grown on a solid PDA medium was cut with the help of a sterilized cork borer and placed aseptically in the center of each of the Petri plates containing the test fungicides and incubated at room temperature for 7 days. Culture discs grown under the same conditions on PDA without the test fungicides were used as controls. The radius of the mycelia growth in each fungicidal concentration was measured.

Statistical analysis

Analysis of variance (ANOVA) was performed for the antifungal antagonistic assay using Statistical Analysis System (SAS) version 9.1.3 software (SAS Institute, 2002). Least significance difference (LSD) was used to separate treatment means ($P<0.05$).

RESULTS AND DISCUSSION

Isolation of the pathogen

The target anthracnose pathogen was isolated from freshly collected common bean pod samples (Figure 1) at the University farm. All the isolates produced characteristic central blackish surrounding whitish on PDA medium (Figure 2) and this observation was confirmed with the observation of Ainsworth et al. (1973).

Antifungal assay

Antagonistic effects of the three bio agents tested showed highly significant ($P<0.01$) percentage of inhibition of the mycelia growth (PIMG) of *C. lindemuthianum.* The highest PIMG (80.39%) was obtained from *T. viride,* followed by 75.49% from *T. harzianum* and 40.2% from *P. fluorescens*. Similarly, highly significant ($P<0.01$) differences were observed in the mycelia growth of *C. lindemuthianum.* The highest growth of mycelia (3.4 cm) was measured from the control, whereas the least (0.67 cm) was from the dual culture containing *T. viride* (Table 1; Figure 3). This is similar to the works of Padder et al. (2010) who, in India, recorded mycelia growth inhibition of 69.21 and 64.2% with *T. viride* and *T. harzianum*, respectively, against a local strain of *C. lindemuthianum*.

T. viride was overgrown on the whole colony in dual culture plates and started sporulation in 10 days and *T. harzianum* in 12 days after inoculation of the dual culture. Contrarily, the bacterial antagonistic, *P. fluorescens* did

Table 1. Effect of dual cultures of bioagents on mycelial growth, PIMG and the number of days taken for the antagonists to overgrow the whole colony of *C. lindemuthianum* on PDA.

Bioagents used	Mycelial growth (cm)	PIMG (%)	Overgrowth of colony (days)
T. viride	0.67[c]	80.39[a]	10
T. harzianum	0.83[c]	75.49[a]	12
P. fluorescens	2.03[b]	40.2[b]	-
Control	3.4[a]	-	-
C.V	13.5	11.12	-
LSD (0.05)	0.47	16.48	-

PIMG= Percentage inhibition of mycelial growth, PDA = potato dextrose agar.

Figure 3. Mycelial inhibition in dual cultures; A = *T. viride* vs. *C. lindemuthianum*, B = *T. harzianum* vs. *C. lindemuthianum* and C = *P. fluorescens* vs. *C. lindemuthianum*.

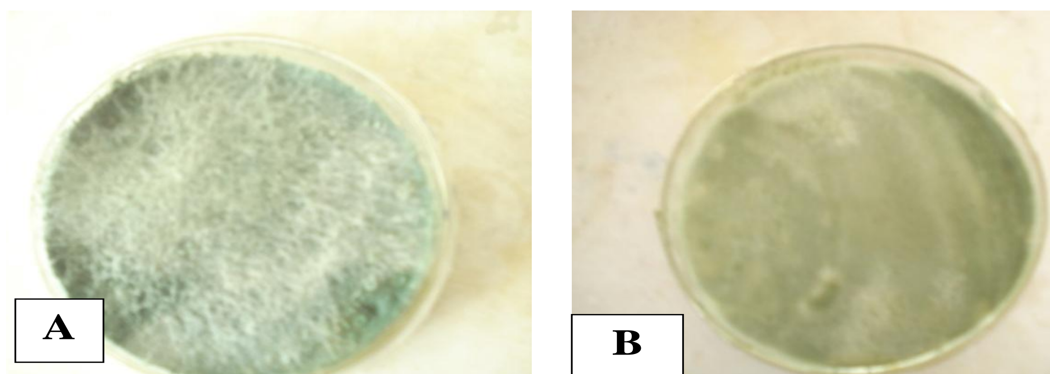

Figure 4. Overgrowth of colony of *C. lindemuthianum* and sporulation; A = *T.* viride, 10 DAI and B = *T. harzianum*12 DAI, Where: DAI = days after inoculation.

not show any signs of colony overgrowth (Table 1; Figure 4).

Siderophore production

The *in vitro* detection of siderophore production by the three bioagents viz., *T. viride*, *T. harzianum* and *P. fluorescens* revealed that all produced the extracellulariron chelating metabolites. Cultures of the three bioagents formed brown halo around their colonies immediately after addition of the indicator, soaked filter papers. Cultures of *T. viride*, formed dark brown halo and *P. fluorescens* formed light brown halo, whereas cultures of *T. harzianum* formed an intermediate brown halo around their colonies (Figure 5).

O'Sullivan and O'Gara (1992) reported that *P. fluorescens* inhibit the plant pathogens through the production of antibiotics, chitinolytic enzymes and

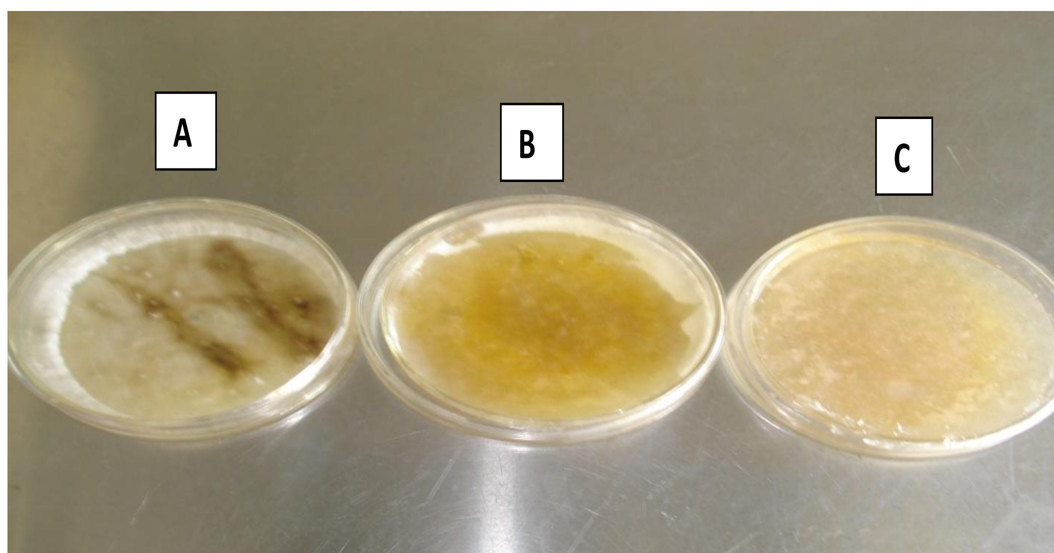

Figure 5. Brown halo formation around colonies of bioagents; A = *T. viride*, B = *T. harzianum* and C = *P. fluorescens*.

Table 2. Effect of different concentrations of test fungicides on the radial growth of mycelia of *C. lindemuthianum* using the food poison technique.

Test fungicide	Mycelial growth of *C. lindemuthianum* in different fungicidal concentrations (cm)			
	100 ppm	250 ppm	500 ppm	1000 ppm
Mancolaxyl	3.0	2.63	0.67	0
Folpan	3.2	2.97	2.4	0
Mancozeb	2.4	2.23	0	0
Control	3.5	3.5	3.5	3.5

ppm= parts per million.

siderophores; extracellular metabolites like siderophores, antibiotics, lytic enzymes and volatile compounds produced by rhizobacteria (*P. fluorescens* and *Bacillus cepacia*) effectively reduced lesions and damages caused by *C. lindemuthianum* on bean plants. Siderophore production by different *Trichoderma* strains is documented (Heidrun et al., 1991). Padder et al. (2010) suggested a hyphal interaction mechanism in which hyper parasitism along with the production of antibiotics and secondary metabolites like siderophores to have contributed to the inhibition of mycelial growth, and control of seed borne infection.

Efficacy of fungicides

Four different concentrations of the three synthetic fungicides evaluated via the food poison technique showed inhibition of mycelial growth to a varying extent. No growth of *C. lindemuthianum* was observed in any of the three fungicides at a concentration of 1000 ppm. Mancozeb showed the least mycelia growth with 2.4 cm at 100 ppm and 2.2 cm at 250 ppm concentrations, whereas the growth of mycelia was not allowed at both 500 and 1000 ppm. Mancolaxyl showed comparatively better inhibition at 100, 250 and 500 ppm fungicidal concentrations than folpan (Table 2).

Conclusions

Anthracnose has been reported as a serious threat to bean production in the major common bean growing regions of Ethiopia, especially in areas like Ambo. Frequent rainfall and moderate temperature that prevail during the main cropping season predispose the crop to attack by various pathogens including *C. lindemuthianum*. *In vitro* evaluation of fungicides and bioagents provide useful preliminary information regarding the efficacy against a particular pathogen within a short period of time

and therefore serve as a guide for further field testing in the future. The production of siderophores and mycelial inhibition by either overgrowing or exhibiting inhibition zones that was detected *in vitro* by all the bioagents are considered to be one of the mechanisms that controlled the development of anthracnose in the future experimenttal field study. Among the tested fungicides, Mancozeb was the most effective, showing a strong inhibition even at the lowest dosage. The bioagents evaluated in this study were found to be economically important options that need to be further investigated.

Conflict of interest

The authors declare that they have no conflict of interest.

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Biological activities of secondary metabolites from *Emericella nidulans* EGCU 312

Neveen M. Khalil[1]*, Emad A. Shalaby[2], Dalia M. I. A. Ali[1], Enas M. Ali[1] and Ahmed M. Aboul-Enein[2]

[1]Botany and Microbiology Department, Faculty of Science, Cairo University, Giza 12613, Egypt.
[2]Biochemistry Department, Faculty of Agriculture, Cairo University, Giza 12613, Egypt.

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The fungus, *Emericella nidulans* was isolated from soil. The ITS region of 5.8S rRNA of the isolated fungus was amplified and sequenced. *E. nidulans* EGCU312 was given an accession number: KC511056 in the NCBI GenBank. Twenty one (21) fractions were obtained from the ethyl acetate extract of fungal filtrate. Fraction no. 12 showed the highest antioxidant activity with 81.54% at 200 µg/ml. High anticancer activities (against EACC cell line) ranging between 64.3 and 87.7% at 200 µg/ml, were exhibited by fractions no. 1, 2, 4, 9, 12 and 20. The mode of action of anticancer activity was studied by measuring activities of lactate dehydrogenase (LDH) and caspase-3. Fraction no. 12 gave the highest effect (2249.2 U/l) in LDH released as compared to control cells (1127.7 U/l) and caused a 1.56-fold increase in caspase-3 activity. Interestingly, fraction no. 12 caused 100% inhibition of *Staphylococcus aureus* and *Escherichia coli* at 50 µg/ml, and *Aspergillus fumigatus* at 100 µg/ml. The minimum bactericidal concentrations (MBC) of this fraction were 4 and 10 µg/ml for *S. aureus* and *E. coli*, respectively, while the minimum inhibitory concentration (MIC) was 45 µg/ml against *A. fumigatus*. GC-MS profile of fraction no. 12 showed 21 compounds, six of which, that is, 2-methylbenzylamine, N-heptyl-N-octyl; naphthalene, 2,3,6-trimethyl-; octadecanoic acid, ethyl ester; 1,2-benzenedicarboxylic acid, butyl octyl ester; tributylacetylcitrate; 1,2- and benzenedicarboxylic acid, diisooctyl ester, were of known biological activities.

Key words: *Emericella nidulans* EGCU 312, antioxidant, anticancer, antimicrobial.

INTRODUCTION

Secondary metabolites are natural products distinguished from primary metabolites, which are small compounds of intermediary metabolism needed for growth, development and reproduction of a living organism. On the other hand, secondary metabolites play non-essential roles (Vining, 1992). They are often used in defense against predation and habitat encroachment, or even used in communication. Therefore, these natural compounds endow the organisms that produce them, survival advantage over non-producing species.

*Corresponding author E-mail: neveen@sci.cu.edu.eg. Tel: +201003643976.

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Secondary metabolites are largely found in bacteria, fungi, plants, dinoflagellates, mollusk sponges and insects. The fungal kingdom, encompassing many species, is a rich source of natural products with important medicinal properties. So far, 1,500 compounds in fungi have already been isolated, and more than half of these natural products have antibacterial, antitumor or antifungal activity (Pelaez, 2005; Wang et al., 2013; Shen et al., 2014). Many well-known drugs have been isolated from a variety of fungal species, such as penicillin, an antibiotic, lovastatin, cholesterol lowering drug, and cyclosporine, an immunosuppressant (Hoffmeister and Keller, 2007). Due to the structural diversity of these fungal secondary metabolites, discovery of novel fungal natural products may lead to a variety of new medicines. There are four major classes of fungal secondary metabolites, categorized by their biosynthesis pathway: non ribosomal peptides (NRP), polyketides (PK), terpenes and indole alkaloids (Keller et al., 2005).

Soil has the largest population of microbes. Cultured soil microbes have been an incredibly productive source of drugs, for example the cancer chemotherapeutics doxorubicin hydrochloride, bleomycin, daunorubicin and mitomycin. Aspergilli represent a group of filamentous fungi that plays a key role in industrial biotechnology. *Emericella nidulans* (teleomorph of *Aspergillus nidulans*) serves as a working horse in industrial production of enzymes and chemicals. Although, studies related to the biopotential activities of antimicrobial, antioxidant, and anticancer metabolites from this fungus based on drug discovery are limited.

Antimicrobial agents have been widespread and largely in effective therapeutic use since their discovery in the 20th century. However, the emergence of multi-drug resistant pathogens now presents an increasing global challenge to both human and veterinary medicine. It is now widely acknowledged that there is a need to develop novel antimicrobial agents to minimize the threat of further antimicrobial resistance (Hearst et al., 2009).

Free radicals are implicated in the pathogenesis of various human diseases such as arteriosclerosis, cancer, diabetes mellitus, liver injury, inflammation, skin damages, coronary heart diseases, and arthritis (Moon et al., 2006). Antioxidants serve as the defensive factor against free radicals in the body. Synthetic antioxidants such as butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are usually used as food additives by the food industry to prevent lipid peroxidation. However, their application has been limited because of possible toxic and carcinogenic components formed during their degradation. In view of these health concerns, finding safer, more effective and economic natural antioxidants is highly desirable (Mathew and Abraham, 2006). A number of microorganisms are commonly known to produce antioxidants, these include *Penicillium roquefortii*, *Aspergillus candidus*, *Mortierella* sp., *Emericella falconensis*, *Acremonium* sp., *Colletotrichum gloeosporioides* (Rios et al., 2006), *Mycelia sterilia* (Mathew and Abraham, 2006), *Antrodia camphorata*(Song and Yen, 2002), *Chaetomium* sp., *Cladosporium*s p., *Torula* sp*., Phoma* sp. etc. (Huang et al., 2007). A lot of fungi still needs to be explored as the production, downstream processing of actual bioactive phytochemicals from plants is quite tougher as compared to microbes.

During the last decades, more and more work have been done by researchers in the search for drugs against cancer, seeing that the disease is becoming a major cause of death among the population of developed countries (Szekeres and Novotny, 2002). The various forms of cancer require multiple approaches for their treatment, which opens a wide field of research that has to be explored. Natural products have therefore been recognized as one promising source for antitumor compounds. A substantial amount of research into cytotoxic natural products has been carried out in the last 50 years, and significant advances in cancer treatment have been achieved (da Rocha et al., 2001). Filamentous fungi can be considered as a useful source for production of antitumor secondary metabolites which therefore can be used as a novel therapeutic strategy for treatment of cancer.

Keeping the above in mind, the present study was planned to screen and expand the spectrum of *E. nidulans* having antioxidant, anticancer and antimicrobial compounds.

MATERIALS AND METHODS

Isolation and identification of fungal isolate

Soil samples, obtained from an agricultural soil from Cairo University, Giza, Egypt, were used as inocula for soil dilution plate method (Johnson et al., 1960). They were plated on modified solid Jackson̍s medium containing: glycerol 1.5%, sucrose 1.5%, peptone 0.6%, yeast extract 0.15%, NaCl1.5%, KH_2PO_4 0.06%, $MgSO_4.7H_2O$ 0.5%, $CuSO_4.5H_2O$ 0.0001%, $FeSO_4.7H_2O$ 0.0003%, and agar, 1.5%. Streptomycin (30 µg/ml) was added to the above medium after sterilization by autoclaving at 121°C and 1.5 bars for 15 min. Plates were incubated at 28°C for 4 days. Fungal colonies were purified by sub-culturing on modified solid Jackson̍s medium.

Identification of one of the developed fungal isolates was carried out by morphological and microscopic examinations (such as color, texture of mycelia, spore formation pattern, etc.). This was followed by nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing. Genomic DNA was isolated using Qiagen kit. Internal transcribed spacer (ITS) region of 5.8SrRNA was amplified using the primer ITS5 with sequence 5'- GGA AGT AAA AGT CGT AAC AAG G. Sequencing of PCR amplified product was performed at Macrogen (South Korea). The resulting sequence was entered into the BLAST algorithm of National Centre of Biological Information (NCBI) database to obtain closely related phylogenetic sequences and a phylogenetic tree was constructed. The obtained sequence was then submitted to the GenBank of NCBI database.

Fungal production of secondary metabolites

A two-step culture was performed for secondary metabolites

Table 1. Fractions collected from ethyl acetate crude extract of *E. nidulans* EGCU 312 using column chromatography.

Fraction no.	Hexane (%)	Chloroform (%)	Ethyl acetate (%)
1	100	0	
2	90	10	
3	80	20	
4	70	30	
5	60	40	
6	50	50	
7	40	60	
8	30	70	
9	20	80	
10	10	90	
11	0	100	0
12		90	10
13		80	20
14		70	30
15		60	40
16		50	50
17		40	60
18		30	70
19		20	80
20		10	90
21		0	100

production from *E. nidulans* EGCU 312. First, cultivation was carried out in 250-Erlenmeyer flasks each containing 100 ml modified Jackson's medium used as seed medium. Inocula were prepared by harvesting spores from 7-day-old PDA slants of *E. nidulans* EGCU 312 in sterilized solution containing 0.9% (w/v) NaCl and 1% (v/v) Tween-80. The concentrations of spore suspensions were determined in a hemocytometer and adjusted to 2 x 10^6 spores/ml. Each flask was inoculated with 1 ml spore suspension. The flasks were incubated for 24 h at 30°C in a shaking incubator (180 rpm). The produced culture was used as 10 % inocula for second step of cultivation. Fermentation was performed in 500-Erlenmeyer flasks, each containing 250 ml modified Jackson's medium. Flasks were incubated for seven days at 30°C in a shaking incubator (180 rpm). The fermented whole broth was filtered through cheesecloth to separate into supernatant and mycelia. The former was used in the following step.

Preparation of fungal extracts

Fifteen liters of the fungal culture filtrate were subjected to extraction with ethyl acetate, three times and solvent layer was separated. Collected solvent extract was evaporated under vacuum using rotary evaporator (40°C) to dryness and then weighed.

Separation of active gradients (secondary metabolites) from fungal extract

Ten grams of the *E. nidulans* EGCU 312crude ethyl acetate extract were fractionated over a Vacuum Liquid Chromatographic Column (VLC, 15 x10 cm, i.d packed with VLC silica gel H (100 g). Gradient elution was carried out with hexane, chloroform and their mixture with an increased polarity pattern with ethyl acetate (100% hexane to 100% chloroform and finally 100% ethyl acetate)). Fractions (200 ml of each) were collected (as shown in Table 1). The biological activities of each fraction were processed as antioxidant, anticancer and antimicrobial.

Antioxidant activity

DPPH method

The 2,2 diphenyl-1-picrylhydrazyl (DPPH) test was carried out for the 21 fractions as described by Burits and Bucar (2000). 1 ml of fungal extract/fractions (100 and 200 µg/ml) was mixed with 1 ml DPPH reagent (0.002% (w/v) /methanol solution). After an incubation in the dark at room temperature for 30 min, the absorbance was measured at 517 nm (using Jenway 6130 spectrophotometer). Butylated hydroxyl toluene (100 and 200 µg/ml) was used as positive control. This test was carried out in triplicate and the antioxidant activity was calculated as follows:

Activity (%) = A_c-A_t / A_c x 100

Where, A_t is the absorbance of samples and A_c the absorbance of methanolic DPPH solution.

Anticancer activity (viability test)

Induction of tumor cell line

Female Swiss albino mice (kept under environmental and nutritional conditions for two weeks) were injected intraperitoneal (i.p) by Ehrlich

ascites carcinoma cells (EACC), for preparation of tumor cell line. EACC resistant to endoxan were used. The parent line was first supplied, courtesy of Dr. G. Klein, Amsterdam, Holland. The tumor line was maintained in the National Cancer Institute, Egypt in Female Swiss Albino mice by weekly transplantation of 2.5 x 10^6 cells which were centrifuged at 1000 xg for 5 min at 4°C. The pellet was washed with saline (0.9% NaCl), then the needed number of cells was prepared by suspending the cells in the appropriate volume of saline.

The viability percentage of tumor cells was measured by the modified cytotoxic trypan blue exclusion technique (Bennet and Catovsky, 1976). The culture medium used was prepared using RPMI medium, 10% fetal bovine serum and 10% l-glutamine. Trypan blue (0.4%) was prepared then kept in brown closed glass bottle. The viability percentage (V %) of tumor cells was measured after incubation with the tested fungal extracts as well as DMSO as control. 2 ml of cells (4x10^6 cells) were transferred into a set of tubes, then different fungal extract/fraction (100 and 200 µg/ml) were added into the tubes as well as DMSO. The tubes were incubated at 37°C for 2 h. Then, in a test tube containing 80 µl saline and 10 µl trypan blue, 10 µl of cell suspension were added and mixed then the number of living cells was calculated using a hemocytometer.

The mechanisms of tested fungal extracts as anticancer were subjected to the promising fractions only, that is, the fractions which showed high cytotoxicity to EACC (Fractions 1, 2, 4, 9, 12 and 20). Each of these six fractions was evaluated as membrane destructors for cancer cells, that is, release of lactic acid dehydrogenase (LDH) out of cells. In addition, the action of fungal extracts (6 promising fractions) as apoptosis compounds against cancer cells was tested using caspase activity assay.

LDH activity

In EACC samples, cells were counted microscopically and the lactic dehydrogenase (LDH; EC 1.1.1.27) activity was determined in the ascites solution by method of Kaplan and Pesce (1996).

Caspase activity assay

After centrifugation of the treated cells as previously described, caspase-3 enzymatic activity was determined in treated and untreated cancer cells using Caspase Apoptosis Assay Kit (Cat. #-786-200/50) (Geno Technology Inc. St. Louis MO, USA). Prior to use, caspase kit reagents were first prepared, followed by lysis of the treated cells according to a modification of the manufacturer's protocol. In this study, cells were lysed with a sonicator (Misonix, Farmingdale, NY, USA), and caspase-3 enzymatic activity in the lysates was determined as described. Briefly, microtiter wells were set up in duplicates for controls, blank, and test cells (lysates). Then 50 pL of 2 x Caspase assay buffer were transferred into each well followed by addition of 50 ~tL of the cell lysate to the wells, and addition of 5 pL of the caspase substrate, Ac-DEV-AFC. A few minutes were allowed for reaction, and the plate was read (at a zero initial time) on ELIZA micro-plate reader (NX 1001 multi-font) at 405 nm. The plate was then incubated at 37°C for 2.5 h and the absorbance read again at 405 nm wavelength. The level of caspase-3 enzymatic activity in the cell lysate was directly proportional to the color reaction. Therefore, to quantify the enzyme in the lysates, the fold increase in caspase-3 protease activity was determined by comparing the absorbance from the treated samples with the non-treated controls. To further confirm, compare and establish non-specific protease activity, control experiments were repeated and run with or without caspase-3 specific inhibitor, ZVAD- FMK. Briefly, reaction wells of the MTP were prepared to contain the following: a) 5 pL lysate + 50 }.tL of 2 x assay buffer + 1 btL of Z-VAD-FMK + 5 gL Ac-DEVAFC conjugate; b) 50 pL of 2 x assay buffer + 5 btL Ac- DEV-AFC +1 pL distilled water; and c) 5 btL cell lysate + 50 btL of 2 x assay buffer + 1pL distilled water. The plate was incubated at 37°C for 2.5 h and the absorbance read at 405 nm as described above. The inter-treatment data were compared to ascertain and confirm the effect of ZVAD- FMK on caspase-3 enzymatic activity.

Antimicrobial activity

Each of 21 fractions previously mentioned was tested as antibacterial and antifungal agents as follows:

Antibacterial assays

The method of Jiang et al. (2005) was adopted with some modifications. One loopful of fresh bacteria (*Staphylococcus aureus* ATCC6538 or *Escherichia coli* ATCC8739) was suspended in an appropriate amount of sterilized saline solution, forming a bacterial cell suspension. The viable cell number in the suspension was controlled *via* the turbidity comparison method. This suspension was diluted to a prescribed cell concentration with sterilized distilled saline solution, thus preparing a bacterial cell suspension that was directly used for the antibacterial tests for the fractionated extract. In a 96-well microtiterplate, 150 µl of bacterial cell suspension were added per well.

The fractions (dissolved in 1% DMSO) were tested at a final concentration of 50 µg/ml. The negative control system contained 1% DMSO instead of the fraction tested. Streptomycin (at a final concentration of 50 µg/ml) was tested as positive control. The microtiter plate was incubated for 2 h at 30°C. 100 µl of the suspension was pipetted and quickly mixed with sterilized saline solution. The viable cell number in each of the tested fraction/-bacterial suspension systems at the contact time was determined by conventional spread-plate method. Replicates were made and colonies were counted after 24 h of incubation at 37°C on nutrient agar medium (0.5% peptone, 0.3% beef extract, 0.5% NaCl, 1.5% agar). The percentage of growth inhibition was counted as follows:

$$\text{Inhibition (\%)} = \frac{\text{Negative control} - \text{Treatment}}{\text{Negative control}} \times 100$$

The minimum bactericidal concentration (MBC) of fraction no. 12 or streptomycin against *S. aureus* or *E. coli* was determined. The above mentioned antibacterial assay was followed. The MBC values were determined as the lowest concentration that inhibited colony formation.

Antifungal assays

Spore suspensions of *Aspergillus fumigatus* were obtained from their respective 7-day-old PDA slants in sterilized solution containing 0.9% (w/v) NaCl and 1% (v/v) Tween-80. The concentrations of spore suspensions were determined in a hemocytometer and adjusted to 2 x 10^6 spores/ml. The spore germination assay was conducted. 50 µl of spore suspension were transferred to each well of amicrotiter plate containing 100 µl liquid Czapek-Dox medium with fraction to yield a final concentration of 100 µg/ml.

DMSO (1%) and amphotericin B replaced tested fractions were used as negative and positive controls, respectively. The plate was incubated at 30°C for 16 h. All tests were conducted in replicates. Spores were considered to be germinated when the germ tube extended to at least twice the length of the spore itself (Griffin,

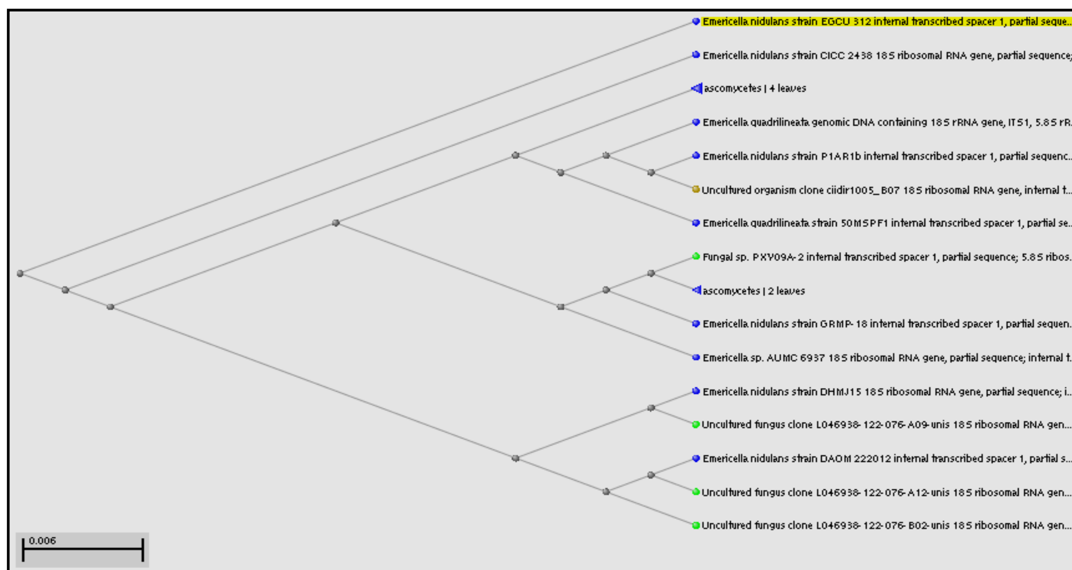

Figure 1. Phylogenetic tree showing genetic relationship between the isolate *E. nidulans* EGCU 312 (in highlight) and other closely related reference microorganisms.

1994). Germinated spores were counted using a hemocytometer. About 100 spores per replicate were observed to detect spore germination. The percentage of inhibition of spore germination was counted as mentioned before. This antifungal assay was then used to find the minimum inhibitory concentration (MIC) of fraction no. 12 oramphotericin B against *A. fumigatus*. The minimum concentration that inhibited spore germination was considered as the MIC value.

Inocula of *Candida parapsilosis* were prepared from cultures on malt agar slants (3% malt, 1.5% agar) incubated at 37°C for 16-18 h. The yeast cells were washed in sterile saline, centrifuged and resuspended in saline. The number of blastospores/ml of suspension was determined by hemocytometer. A suitable volume of suspension was seeded into 250 ml Erlenmeyer flasks containing 100 ml molten malt agar medium and poured in sterilized Petri plates. A sterilized cork borer (6 mm diameter) was used to punch wells in solidified medium and filled with extracts of 40 µl of 100 µg/ml final concentration of fractions. DMSO (1%) was used as negative control. The efficacy of extracts against *C. parapsilosis* was compared with the antibiotic amphotericin B (positive control). The plates were incubated at 37°C for 24 h. Replicates were assayed for each sample. The anticandidal activity was interpreted from the size of diameter of inhibition zone measured to the nearest millimetre (mm) as observed from the clear zones surrounding the wells.

GC-MS analysis

From the six promising fractions, fraction no. 12 which was obtained by successive extraction (90% chloroform and 10% ethyl acetate) of ethyl acetate crude fungal extract of *E. nidulans* EGCU 312 gave the highest anticancer and antioxidant activities at the two concentration test. In addition, the same fraction showed very strong antimicrobial activity. Therefore, the identification of the active principles of this fraction was taken into consideration using GC-MS analysis. GC/MS analysis was performed on a Thermoquest-Finnigan trace GC-MS equipped with a DB-5 (5% (w/v) phenyl) methylpolysiloxane column (60 m \ 0.25 mm i.d., film thickness 0.25 µm). The injection temperature was 220°C and the oven temperature was raised from 40°C (3 min hold) to 250°C at a rate of 5°C/min, then held at 250°C for 2 min; transfer line temperature was 250°C. Exactly 1 µl of sample was injected and helium was used as the carrier gas at a flow rate of 1.0 ml/min. The mass spectrometer was scanned over the 40 to 500 m/z range with an ionizing voltage of 70 eV and identification was based on standard mass library of National Institute of Standards and Technology (NIST Version 2.0) to detect the possible extract components.

RESULTS AND DISCUSSION

Isolation and identification of fungal isolate

In the present study, five different fungal isolates were obtained. Purification and identification was performed for fungal isolate no. 1. It was identified as *E. nidulans* through morphological and microscopic examinations. Identification was further confirmed by molecular technique. ITS region is the most widely sequenced DNA region in fungi. It is most useful for molecular systematics at the species level, and even within species (Meenupriya and Thangaraj, 2011).

In this study, DNA was isolated from isolate no. 1 and the ITS region of 5.8SrRNA was amplified using the specific primer ITS5. The sequence was determined using automated sequencers. Amplifi-cation and sequen-cing of fungal rRNA gene resulted in 381 bp long nucleotide sequence. NCBI Blast search sequence similarity was conducted against the existing non-redundant nucleotide sequence database. This showed maximum identity (88%) with *E. nidulans* species. The phylogenetic tree was constructed (Figure 1) to show sequence alignment with available sequences from NCBI

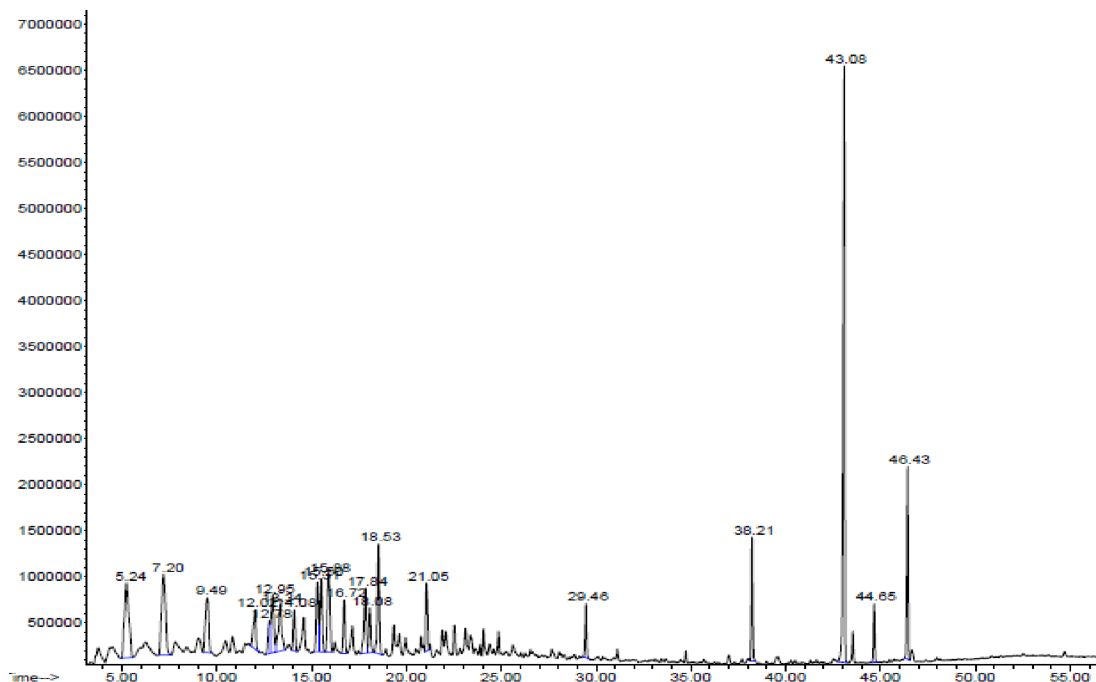

Figure 2. GC/MS chromatogram of fraction no. 12 of ethyl acetate extract from *E. nidulans* EGCU 312.

data bank (first 15 hits in Blast results). The 381 bp long nucleotide sequence from this work was deposited in NCBI GenBank and was given a strain identifier, *E. nidulans* EGCU 312, with accession number: KC511056.

The ethyl acetate extract of *E. nidulans* EGCU 312 was fractionated on silica gel column. Twenty-one fractions (Fr.1-21) were obtained (Table 1). All fractions were tested for their antioxidant, anticancer and antimicrobial activities.

GC-MS analysis

GC-MS profile of fraction no.12 is shown in Figure 2 and its retention time (RT) and percentage of peak of individual compounds are presented in Table 2. Fraction no. 12 showed eight major compounds, that is, tributylacetylcitrate (22.95%), hexadecane (7.87%), pentadecane (7.81%), 1, 2-benzene dicarboxylic acid, diisoctyl ester (6.43%), 2-methyl benzyl amine, N-heptyl-N- octyl (5.23%), benzene, (1-methyl undecyl) (4.81%), heptadecane (4.56%) and 1, 2-benzene dicarboxylic acid, buty (4.05%). These compounds could be responsible for different biological activities.

Tributylacetylcitrate was detected in *Casimiroa edulis* leaf extract and showed insecticidal activity against *Spodoptera littoralis* larvae (Barakat, 2011). Also, 1,2-benzene dicarboxylic was reported to possess anti-inflammatory (Li et al., 2004) and antibacterial activity (Modupe et al., 2010). Moreover, Senthilkumar et al. (2011) found that 1,2-dicarboxylic acid anddiisooctyl ester along with other compounds would have suppressed the growth of *F. oxysporum.*

Antioxidant activity

The DPPH scavenging assay was performed to test the percentage of antioxidant activity of the twenty-one separated fractions of *E. nidulans* EGCU 312 (Table 3). The separated fraction no. 12 showed the highest antioxidant activity with 60.47 and 81.54% at 100 and 200 µg/ml and followed by fractions no. 1, 2, 4 and 20 (with activity ranged 77.0, 64.30, 76.80 and 79.41% at 200 ug/ml, respectively) when compared with butylated hydroxyl anisole as standard antioxidant (88.75% at 200 ug/ml).

Twenty-one (21) compounds were identified in the promising fraction (fraction no. 12) of *E. nidulans* EGCU by GC-MS analysis. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented in Table 2. The spectrum of the unknown components of fraction no. 12 was compared with the spectra of known components stored in the NIST library. Six compounds with their biological activities were found in *E. nidulans* EGCU (Figure 2 and Table 2). Moreover, two from these compounds (benzenedicarboxylic acid, butyl octyl ester and 1,2-benzenedicarboxylic acid, diisooctyl ester, with concentration 4.05 and 6.43%, respectively) had high antioxidant activity as reported by Senthilkumar et al. (2011) and Shanab et al. (2010, 2011).

Table 2. List of major components and their biological activities of promising fraction (12) obtained from *E. nidulans* EGCU 312 through GC-MS study.

RT	Compound name	Relative concentration	Reported activities
5.0-12.02	Alkane derivatives	22.83	No activity
12.78	Benzene, (1-pentylhexyl)-	1.72	No activity
12.95	Benzene, (1-butylheptyl)	3.77	
13.34	Benzene, (1-propyloctyl)-	3.77	
14.08	Benzene, (1-ethylnonyl)-	2.01	
15.31	Benzene, (1-pentylheptyl)-	3.79	
15.50	Benzene, (1-butyloctyl)-	3.69	
15.88	2-Methylbenzylamine, N-heptyl-N-octyl	5.23	Antimicrobial
16.72	Benzene, (1-ethyldecyl)-	2.36	No activity
17.83	Benzene, (1-pentylheptyl)-	3.54	
18.08	Benzene, (1-butylnonyl)-	2.12	
18.53	Benzene, (1-methylundecyl)-	4.81	
21.05	Naphthalene, 2,3,6-trimethyl-	3.12	Antimicrobial, Anticancer
29.47	Octadecanoic acid, ethyl ester	1.88	Antioxidant, Anticancer
38.22	1,2-Benzenedicarboxylic acid, butyl octyl ester	4.05	Antimicrobial, antifouling
43.09	Tributyl acetylcitrate	22.95	Antimicrobial activity
44.65	2,5-Cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-ethylidene-	1.93	No activity
46.43	1,2-Benzenedicarboxylic acid, diisooctyl ester	6.43	Antibacterial, antioxidant, antitumor, cancer preventive, immunostimulant, chemo preventive, lipoxygenase-inhibitor and pesticide

Anticancer activity

Viability test assay was used to assess the anticancer activity of the 21 derived fractions against EACC cell line. Fractions no. 1, 2, 4, 9, 12 and 20 showed acceptable potency against EACC cell line with high anticancer activity ranging 77.0, 64.3, 84.1, 87.7, 77.5 and 76.7% at 200 μg/ml, respectively (Table 3).

From the identified chemical constituents of fraction no. 12 as a promising fraction in most biological activities, three compounds were known to have anticancer activity namely, naphthalene, 2, 3 6-trimethyl (3.12%); Octadecanoic acid, ethyl ester (1.88%) and 1,2-Benzenedicarboxylic acid, diisooctyl ester (6.43%). These results were in agreement with the results obtained by Senthilkumar et al. (2011) and Shanab et al. (2010, 2011).

LDH activity

The cytotoxic effect of fungal fractions was tested *via* the lactate dehydrogenase (LDH) release assay, based on the extent of LDH leakage into the medium. The augmented release of LDH into the media is reflective of cell membrane damage. Therefore, we conducted this experiment in order to estimate the release of LDH after treatment with various concentrations of fungal extract/fraction. As expected, fungal promising fractions (1, 2, 4, 9, 12 and 20) caused cytotoxicity in a dose dependent manner. Fraction no. 12 gave the highest effect by a 2249.2 U/L increase in LDH-release when compared with control cells (1127.7 U/L) as shown in Table 4.

Caspase-3 activity

Caspase-3 is an effector caspase that plays a central role in the mitochondrial-mediated cell death pathway and is responsible for the breakdown of several cellular components involved in DNA and its repair and regulation. The caspase-3 activityin cell line was measured after 24 h of incubation with the six promising fractions (Table 4). The obtained data revealed that, the highest activity of caspase enzyme was obtained by fractions no. 2 and 12 (1.92 and 1.56 relative to control 1.0, respectively).

The results revealed that these compounds enhance cancer cell damage and death. Therefore, from the results of LDH and caspase-3 we can suggest that the active compounds separated from the isolated fungus have anticancer activity by damaging the cancer cells by programmed cell death (apoptosis).

Table 3. Antioxidant and anticancer activity (%) of different fractions from *E. nidulans* EGCU 312.

Fractions no.	Conc. (µg/ml)	Antioxidant activity (%)	Anticancer activity %
1	100	50.56 ± 2.30	39.5 ± 1.85
	200	77.0 ± 3.45	88.5 ± 6.45
2	100	45.97 ± 2.0	50.3 ± 2.4
	200	64.30 ± 4.65	81.5 ± 0.89
3	100	15.64 ± 0.5	41.6 ± 2.15
	200	24.61 ± 1.50	75.9 ± 1.78
4	100	51.62 ± 1.89	60 ± 5.12
	200	76.80 ± 3.65	84.1 ± 3.65
5	100	52.64 ± 5.42	27.65 ± 0.15
	200	54.67 ± 2.10	55 ± 3.25
6	100	12.60 ± 0.54	20.3 ± 2.0
	200	34.61 ± 2.63	41.65 ± 4.89
7	100	27.0 ± 1.45	31.65 ± 1.78
	200	29.01 ± 2.0	48.6 ± 3.6
8	100	24.65 ± 1.62	25.8 ± 2.5
	200	44.0 ± 1.78	52.5 ± 3.9
9	100	35.80 ± 2.15	64.2 ± 2.5
	200	55.74 ± 2.68	87.7 ± 3.9
10	100	34.62 ± 1.54	32.2 ± 1.87
	200	59.60 ± 2.69	58.6 ± 2.8
11	100	23.4 ± 1.54	27.6 ± 0.8
	200	45.72 ± 2.45	57.9 ± 2.8
12	100	60.47 ± 2.63	48.9 ± 1.74
	200	81.54 ± 4.58	77.56 ± 2.6
13	100	24.60 ± 1.20	13.6 ± 0.8
	200	39.87 ± 1.47	66.2 ± 2.0
14	100	12.7 ± 1.02	16.98 ± 1.7
	200	28.64 ± 2.8	62.5 ± 3.6
15	100	27.64 ± 1.25	14.52 ± 2.4
	200	45.67 ± 2.47	48.6 ± 3.7
16	100	19.0 ± 0.65	28.3 ± 2.0
	200	27.89 ± 1.45	57.6 ± 1.45
17	100	45.65 ± 2.98	36.5 ± 3.6
	200	55.87 ± 3.65	55.9 ± 2.5
18	100	34.61 ± 1.45	22.4 ± 1.65
	200	59.87 ± 3.67	58.7 ± 3.8
19	100	22.16 ± 1.48	10.32 ± 1.6
	200	37.89 ± 2.78	55.65 ± 4.9
20	100	53.97 ± 1.52	21.65 ± 1.2
	200	79.41 ± 6.98	76.7 ± 3.7
21	100	33.02 ± 4.3	33.5 ± 2.0
	200	56.65 ± 2.45	62.2 ± 3.8

Data are mean ± standard error.

Antimicrobial activity

Antimicrobial activities of the 21 fractions are shown in Table 5. The Gram positive bacterium *S. aureus* ATCC6538, the Gram negative bacterium *E. coli* ATCC8739, the human pathogenic fungus *A. fumigatus*

Table 4. Lactate dehydrogenase and caspase-3 enzymes activities as U/L in treated (with promising fractions) and untreated cells.

Fraction no.	LDH (U/L)	Caspase-3 (U/L)
Control (untreated cells)	1127.7 ± 20.6	0.27 ± 0.05
1	1161.7 ± 12.5	0.38 ± 0.02
2	1103.9 ± 10.6	0.52 ± 0.07
4	1096.7 ± 32.5	0.25 ± 0.0
9	816.9 ± 12.8	0.26 ± 0.0
12	2249.2 ± 23.0	0.42 ± 0.03
20	889.3 ± 21.4	0.28 ± 0.01

Data are mean ± standard error

Table 5. Bioassay monitoring of antimicrobial metabolite production by *E. nidulans* EGCU 312.

Fraction no.	Test organism			
	S. aureus **(% inhibition)**	***E. coli*** **(% inhibition)**	***A. fumigatus*** **(% inhibition)**	***C. parapsilosis*** **(inhibition zone, mm) (well diameter, 8 mm)**
-ve Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0
+ve Control	100 ± 0	100 ± 0	100 ± 0	27 ± 1.02
1	100 ± 0	6 ± 1.30	0 ± 0	21 ± 1.34
2	10 ± 1.14	0 ± 0	26 ± 1.77	19 ± 1.23
3	93 ± 2.32	0 ± 0	31 ± 2.17	16 ± 1.58
4	0 ± 0	0 ± 0	90 ± 3.01	18 ± 1.31
5	8 ± 1.22	8 ± 1.91	87 ± 2.14	0 ± 0
6	95 ± 2.18	60 ± 1.99	14 ± 2.33	22 ± 1.68
7	100 ± 0	0 ± 0	52 ± 1.91	17 ± 1.45
8	75 ± 1.43	0 ± 0	0 ± 0	16 ± 1.73
9	0 ± 0	0 ± 0	100 ± 0	12 ± 1.05
10	60 ± 1.76	47 ± 2.43	0 ± 0	16 ± 1.53
11	100 ±0	52 ± 2.08	100 ± 0	0 ± 0
12	100 ±0	100 ± 0	100 ± 0	0 ± 0
13	100 ± 0	0 ± 0	99 ± 0.58	0 ± 0
14	60 ± 1.68	0 ± 0	30 ± 1.94	0 ± 0
15	80 ± 2.02	15 ± 1.82	0 ± 0	0 ± 0
16	15 ± 1.39	0 ± 0	0 ± 0	17 ± 1.27
17	51 ± 2.17	22 ± 1.62	21 ± 1.53	0 ± 0
18	99 ± 1.00	58 ± 1.57	0 ± 0	0 ± 0
19	100 ± 0	0 ± 0	0 ± 0	0 ± 0
20	98 ± 1.15	65 ± 2.13	0 ± 0	0 ± 0
21	90 ± 2.11	63 ± 2.21	0 ± 0	0 ± 0

Data are mean ± standard error; -ve Control: 1% DMSO. +ve Control: Streptomycin in the case of *S. aureus* and *E. coli*; amphotericin B in the case of *A. fumigatus* and *C. parapsilosis*.

and the human pathogenic yeast fungus *Candida parapsilosis* were used as test organisms. The inhibitory effect of these fractions was compared with standard antibiotics. The isolated fractions showed different degrees of growth suppression of the tested microorganisms. A hypothesized mechanism of antimicrobial activity could be change in cell permeability due to interaction between the fungal secondary metabolites in the tested fractions and the electronegative charges on the cell surfaces. The interaction leads to leakage of intracellular electrolytes and proteinaceous constituents. Another mechanism is interaction of the extracted secondary metabolites with the microbial DNA, leading to inhibition of mRNA and protein synthesis. From the results in Table 5, it appeared that the Gram positive bacterium *S. aureus* was more sensitive to the tested fractions when compared with the Gram negative bacterium *E. coli*. This could be attributed to their markedly different cell wall structure (Feng et al., 2000). The peptidoglycan in the cell walls of the Gram positive *S. aureus* is much thicker than that in *E. coli*, while the lipopolysaccharide (LPS) layer is much thicker in *E. coli.* The LPS layer is thought to provide protection to the cell wall against antibiotics. Concentrations of the tested frac-

Table 6. Antimicrobial activity (MBC or MIC) of fraction no. 12 against pathogenic microorganisms.

Antimicrobial activity	MBC (µg/ml)		MIC (µg/ml)
	S. aureus	*E. coli*	*A. fumigatus*
+ve Control	9	20	70
Fraction no. 12	4	10	45

+ve Control: Streptomycin in the case of *S. aureus* and *E. coli*; amphotericin B in the case of *A. fumigatus*.

tions were doubled in the case of fungi. Generally, fungi were more resistant to the tested fractions than bacteria. The structure of fungi and bacteria differ in very significant ways (such as the diploid nature of most fungi and the longer generation time of fungi as compared to bacteria), and the available antibacterial and antifungal agents target structures and functions most relevant to the organisms to be inhibited (Ghannoum and Rice, 1999). Another difference between bacteria and fungi lies in the cell wall structure, where the fungal cell wall is primarily composed of chitin, glucan, mannans and glycoproteins (Bowman and Free, 2006).

Obviously, fraction no. 12 was the most potent inhibitor. This fraction (50 µg/ml) caused complete inhibition of cell viability of *S. aureus* and *E. coli* (100 %). Interestingly, at 100 µg/ml, it showed 100% inhibition of spore germination of *A. fumigatus*. However, it showed no effect on the growth of *C. parapsilosis*.

The antimicrobial test was performed with different concentrations of fraction no. 12 (from 1 to 50 µg/ml in the case of *S. aureus* and *E. coli*, and from 1 to 100 µg/ml in the case of *A. fumigatus*). The results of MBC and MIC values of fraction no. 12 were very promising (Table 6). The MBC values by this fraction were 4 and 10 µg/ml, as compared to 9 and 20 µg/ml by streptomycin for *S. aureus* and *E. coli*, respectively. While the MIC values were 45 and 70 µg/ml by fraction no. 12 and amphotericin B, respectively, against *A. fumigatus*. It can be speculated that one or more of the active ingredients found in fraction no. 12 might mediate the observed inhibitory activities.

The data in Table 2 reveals that, the current fraction was rich in antimicrobial agents e.g: 2-methyl-benzylamine, N-heptyl-N-octyl (5.23%), naphthalene, 2,3,6-trimethyl- (3.12%), 1,2-benzenedicarboxylic acid, butyl octyl ester (4.05%), tributyl acetyl citrate (22.95 %) and 1,2-benzenedicarboxylic acid, diisooctyl ester (6.43%). The cytotoxicity of these compounds appeared also, by the anticancer effects as previously mentioned.

From the foregoing, it can be concluded that valuable secondary metabolites could be extracted from the soil fungus *E. nidulans* EGCU 312. The applicable importance of such metabolites lies in the fact that they showed distinguishable antioxidant, anticancer and antimicrobial activities.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Amplified ribosomal DNA restriction analysis and repetitive element polymorphism-polymerase chain reaction (rep-PCR) DNA fingerprinting of members of *Frankia* genus

Faten Ghodhbane-Gtari* and Abdellatif Boudabous

Laboratoire Microorganismes et Biomolécules Actives, Faculté des Sciences de Tunis, Université de Tunis El Manar, Campus Universitaire, 2092, Tunis, Tunisia.

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Slow growing actinobacteria of the genus *Frankia* are best known for their nitrogen-fixing mutualism with dicotyledonous host plants called actinorhizal plants. Twenty nine (29) strains obtained from diverse host plants and geographic area, have been studied based on amplified ribosomal DNA restriction analysis (ARDRA) and repetitive element polymorphism-polymerase chain reaction (rep-PCR) DNA fingerprinting using BOX A1R primer. The collection has been classified into 28 ARDRA haplotypes clustered into three genogroups. The first genogroup 1 contains *Frankia* strains infecting *Elaeagnus*, genogroup 2 includes strains infecting *Casuarina*, while *Frankia* strains infective on *Alnus*, *Comptonia* and *Myrica* were grouped in genogroup 3. The results of BOX-PCR fingerprinting, supported the observation that BOX-PCR seems to be able to discriminate *Frankia* at strain level but are not useful for assigning strain to their respective genogroups or host infection groups.

Key words: *Frankia*, ARDRA, BOX-PCR, host infection groups.

INTRODUCTION

Frankia genus contains diazotrophic actinobacteria that are able to establish root nodules with diverse dicotyledonous host plants known as actinorhizal plants that have attracted interest with regard to the input of fixed nitrogen on marginal soil where indigenous legumes are absent (Gtari and Dawson, 2011). *Frankia* strains grow slowly with doubling times of 15 to 48 h or more leading to difficulty in arranging strains into phenotypically related groups (Benson and Silvester, 1993). However, the host-specific responses of the *Frankia* strains remain mostly the only useful criteria to group them into four major host infectivity groups (HSGs) (Backer, 1987). HSG 1 is composed of strains that infect *Alnus*, *Comptonia* and *Myrica*; HSG 2 strains infect members of

*Corresponding author. E-mail: feten.ghodhbane@fst.rnu.tn. Tel/Fax: +21670860553.

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the *Allocasuarina*, *Casuarina* and *Myrica*; HSG3 strains infect members of the Elaeagnaceae, Rhamnaceae, *Gymnostoma* and *Myrica* and HSG4 contained strains that nodulate members of the Elaeagnaceae but not *Myrica*. Phylogenetic analysis based on entire 16S rRNA gene sequences permitted to assign *Frankia* strains to four clusters (Normand et al., 1996). Cluster 1 includes *Frankia* strains which form nodules on members of Betulaceae, Myricaceae and Casuarinaceae. In cluster 2 are grouped *Frankia* strains that only infect members of the Coriariaceae, Datiscaceae, Rosaceae and Ceanothus of the Rhamnaceae. Cluster 3 strains form effective nodules on members of the Myricaceae, Rhamnaceae, Elaeagnaceae and Gymnostoma of the Casuarinaceae. Despite the fact that *Frankia* strains from cluster 1 and 3 are being routinely isolated and cultivated, those from cluster 2 have not been isolated in pure culture despite many attempts and remain, therefore, considered as an obligate symbionts. Atypical *Frankia* strains that are unable to infect or fix nitrogen are included in cluster 4. These clustering have been confirmed by other molecular studies such as intertranscribed spacers (ITS) 16S-23S rRNA (Ghodhbane-Gtari et al., 2010), *gyr*B (Nouioui et al., 2011) and *gln*II (Gtari et al., 2004; Nouioui et al., 2011) gene sequence analysis. Due to the slow growth of *Frankia* strains and the limited funding for maintaining several collections especially those containing unidentified and uncharacterized strains, there is need for reducing the costs of shortages without risk of losing biodiversity (Lumini and Bosco, 1999). As a result of this, the present study tests the efficiency of some genetic fingerprinting and low cost based techniques for worldwide and routine characterization of *Frankia* isolates.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Twenty nine (29) *Frankia* strains were used in the present study (Table 1). Cultures are routinely subcultured at 28°C in DPM medium (Baker and O'Keefe, 1984) modified to contain as carbon source in addition to Na-propionate, Na-pyruvate, Na-succinate, Na-acetate and glucose to accommodate strain specific requirements.

DNA extraction, PCR amplification and amplified ribosomal DNA restriction analysis

DNA extraction was made from one month old liquid culture of the *Frankia* strains after forcing several bacterial colonies (from a culture volume of 1-5 ml) through a 0.7 × 30 mm sterile needle to homogenize the mycelium. After centrifugation, the resulting cell pellet was washed twice with sterile distilled water, incubated for 30 min in DNA extracting buffer (100 mmol l^{-1} Tris-HCl, pH 8; 20 mmol l^{-1} EDTA, pH 8.2; 1.4 M NaCl, and 2% w/v cetyl trimethyl ammonium bromide (CTAB), chloroform extracted and ethanol precipitated. The DNA pellet was dissolved in 50 µl TE (10 mmol l^{-1} Tris-HCl, pH 8; 20 mmol l^{-1} EDTA, pH 8.2). PCR reaction of the 16S rRNA gene was carried out by using FGPS56-352 and FGPS1509'-153 following conditions described by Normand et al. (1996). PCR amplification were performed in 100 µl final reaction volume containing 10 ng genomic DNA, 1X Taq polymerase buffer, 1.5 mmol l^{-1} $MgCl_2$, 0.1 µM each dNTP, 0.2 µM each primers and 2 U Taq DNA polymerase. The thermal program consisted of three min at 95°C followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45s. PCR products were digested with restriction enzymes; *Alu*I, *Hae*III and *Rsa*I, overnight at the optimal conditions recommended by the manufacturer. Repetitive element polymorphism-polymerase chain reaction (rep-PCR) was performed in 25 µl final volume using 50 ng genomic DNA, 1X Taq polymerase buffer, 2.5 mmol l^{-1} $MgCl_2$, 0.5 µM each dNTP, 0.5 µM BOX-A1R primer (Versalovic et al., 1994), 0.04 U µl^{-1} Taq DNA polymerase and 5% (v/v) DMSO, and subjected to a thermal program: 95°C for 5 min, 35 cycles consisting of 1 min at 94°C, 1 min at 45°C and 2 min at 72°C. Amplified ribosomal DNA restriction analysis (ARDRA) and BOX-PCR products were electrophoresed in 2.5% agarose in TBE buffer (Sambrook et al., 1989), ethidium bromide stained and photographed under ultraviolet light.

Fingerprints were analyzed using GelCompar II v. 6.5 (Applied Maths NV) and dendrograms were constructed using unweighted pair group method with arithmetic mean (UPGMA) algorithm based on Dice similarity coefficient for ARDRA and Pearson's correlation coefficient for BOX-PCR similarity matrix.

RESULTS AND DISCUSSION

Beside difficulties in isolating *Frankia*, the nitrogen-fixing actinobacteria and symbionts of actinorhizal plants, identifying and conserving diversity of cultured strains remain problematic in view of scarcity of financial supports in several still devoted laboratories in the field. Moreover, the non useful phenotyping methods especially the time consuming of plant infecting experiments suggest for developing a low cost and world widely used method for routine characterization of *Frankia* isolates. In the present study, 29 *Frankia* reference strains and isolates were characterized by ARDRA and repetitive element polymorphism-PCR (BOX-PCR) methods. Individual *Alu*I, *Hae*III and *Rsa*I restriction patterns of the 16S rRNA gene amplicon and UPGMA dendrogram were shown in Figure 1. The first genogroup 1 contains *Frankia* strains NRRL-B16219, NRRL-B16306, NRRL-B16316, NRRL-B16412, EAN1pec, BMG5.3, BMG5.11, BMG5.13 and BCU110501 infecting *Elaeagnus*, genogroup 2 includes strains; KB5 and CcI3 infecting *Casuarina*, while *Frankia* strains infective on *Alnus* (ArI3, AvcI1, AvsI1, ACN14a and ACN1), *Comptonia* (CpI1) and *Myrica* (NRRL-B16386) are grouped in genogroup 3. Moreover, this grouping based on ARDRA is driven by host plant infectivity rather than host plant origin of isolation. Some *Casuarina* (NRRL-B16306 and NRRL-B16412) and *Ceanothus* (NRRL-B16316) strains that are non infective on the latter host plant but infective on *Elaeagnus* (Baker, 1987) grouped accordingly to genogroup 1. Likewise strains isolated from different host plants that cross-infect the same plant sets are included in the same genogroup such as those isolated from *Alnus*, *Comptonia* and *Myrica* that cluster with *Alnus* infective strains. Such as CpI1 and NRRL-B16386 isolated

Table 1. *Frankia* reference strains and isolates used in this study.

Host infectivity	Registration	Acronym	Host plant of origin	Provenance	Reference
Alnus	ULQ010201401	ACN14a	*Alnus crispa*	Tadoussaq (Canada)	Normand and Lalonde, 1982
	ULQ0102001007	ACNl	*Alnus crispa*	Orléans (France)	
	HFP013003	ArI3	*Alnus rubra*	Oregon (USA)	Berry and Torrey, 1979
	DDB01020110	AvcI1	*Alnus viridis*	Ontario (Canada)	Baker et al., 1980
	LLR 160401	NRRL B-16386	*Myrica californica*	California (USA)	Lechevalier, umpublished
	DDB 01361310	NRRL B-16406 =AvsI4	*Alnus viridis*		Baker, 1987
	HFP070101	CpI1	*Comptonia peregrina*	Massachusetts (USA)	Callaham et al., 1978
	BMG5.40		*A. glutinosa*	Tunisia	This study
	BMG5.41		*A. glutinosa*	Tunisia	This study
	BMG5.42		*A. glutinosa*	Tunisia	This study
Elaeagnus	ULQ130100144	EAN1pec	*Elaeagnus angustifolia*	Ohio, U.S.A.	Lalonde et al., 1981
	DDB 000320	NRRL B-16219 = Cj	*Ceanothus jepsonii* (rhizosphere)	Wyoming, USA	Baker, 1987
	LLR 02022	NRRL B-16306 = R43	*Casuarina*		Baker, 1987
		NRRL B-16316= Cal1	*Ceanothus americanus*	Vermont, USA	Lechervalier umpublished
	LLR 020601	NRRL B-16412	*Casuarina equisetifolia*	Great Stirrup Cay, Bahamas	Lechervalier umpublished
	BCU110501	Dt501	*Discaria trinervis*	Argentina	Chaia, 1998
	BMG5.12	BMG5.12	*E. angustifolia*	Tunisia	Gtari et al., 2004
	BMG5.11	BMG5.11	*E. angustifolia*	Tunisia	Gtari et al., 2004
	BMG5.3	BMG5.3	*E. angustifolia*	Tunisia	Gtari et al., 2004
	BMG5.7	BMG5.7	*E. angustifolia*	Tunisia	This study
	BMG5.8	BMG5.8	*E. angustifolia*	Tunisia	This study
	BMG5.15	BMG5.15	*E. angustifolia*	Tunisia	This study
	BMG5.16	BMG5.16	*E. angustifolia*	Tunisia	This study
Casuarina	HFP020203	CcI3	*C. cunninghamiana*	Florida (USA)	Zhang et al., 1984
		KB5	*C. equesitifolia*	Kings bore (Australie)	Rosbrook et al., 1989
	BMG5.22	BMG5.22	*Casuarina glauca*	Tunisia	This study
	BMG5.20	BMG5.20	*Casuarina glauca*	Tunisia	This study
	BMG5.21	BMG5.21	*Casuarina glauca*	Tunisia	This study
	BMG5.23	BMG5.23	*Casuarina glauca*	Tunisia	This study
	BMG5.24	BMG5.24	*Casuarina glauca*	Tunisia	This study

from *Comptonia perigrena* and *Myrica californica* associated with *Alnus* strains or BCU110501 isolated from *Discaria trinervis* is grouped with *Elaeagnus* strains. The newly isolates from *Elaeagnus angustifolia* (BMG5.7, BMG5.8, BMG5.15 and BMG5.16), *Casuarina glauca* (BMG5.20, BMG5.22, BMG5.23 and BMG5.24) and *Alnus glutinosa* (BMG5.40, BMG5.41 and BMG5.42) grouped with their respective host plant groups. Repetitive element polymorphism-PCR using BOX- A1R primer applied for the 29 *Frankia* strains and isolates is shown in Figure 2. Twenty nine (29) unique profiles were obtained with one to three common bands. The remarkable differences observed by rep-PCR fingerprinting even among strains sharing the same ARDRA haplotypes

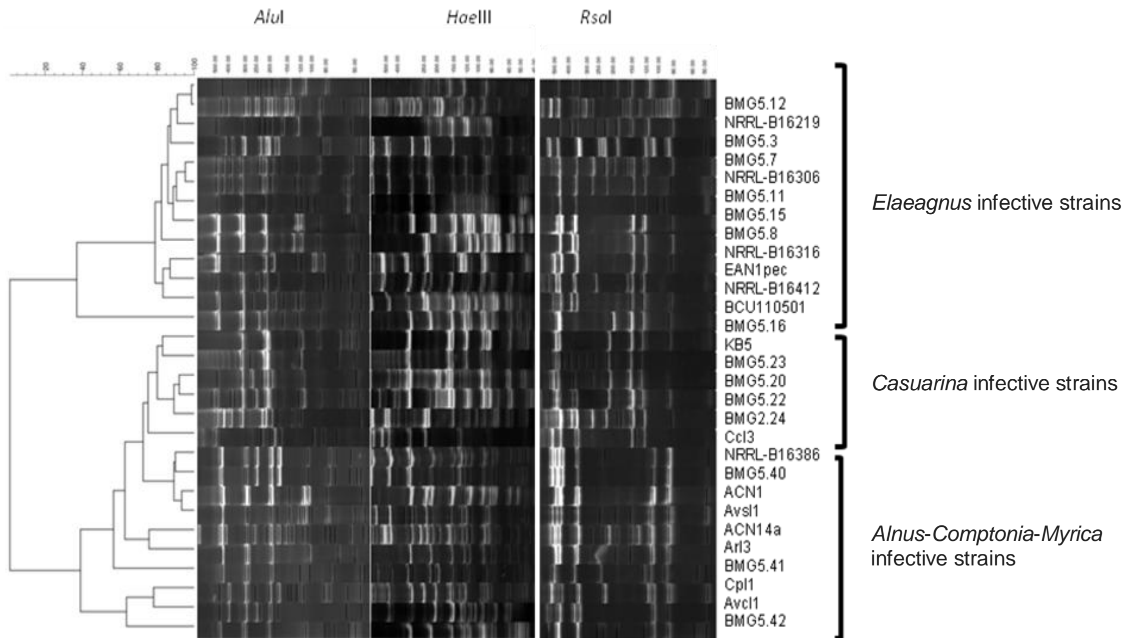

Figure 1. UPGMA cluster analysis using Dice coefficient of ARDRA digitized banding patterns generated by restriction digestions with *Alu*I, *Hae*III and *Rsa*I enzymes of the 16S rRNA gene amplicon from *Frankia* reference strains and isolates.

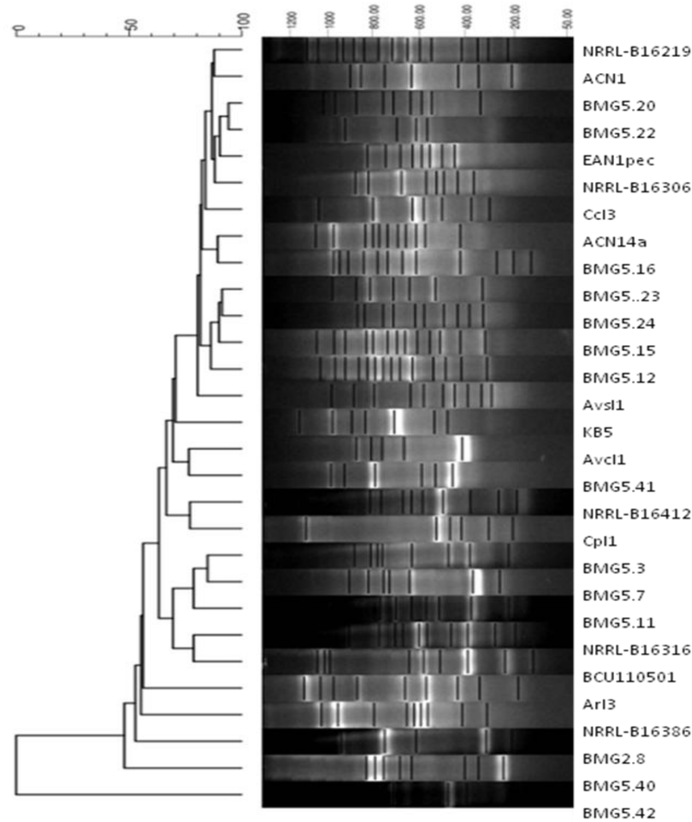

Figure 2. Cluster analysis of BOX-PCR fingerprints of *Frankia* reference strains and isolates using UPGMA algorithm based on Pearson’s correlation coefficient.

(example *Casuarina* strains) indicate noteworthy genome variability among *Frankia* strains. While grouping is possible at high cutoff, the generated genogroups are not correlated to host infection groups that are determined in this study based on amplified ribosomal DNA restriction analysis. The high differences observed by BOX-PCR even on closely related *Frankia* strains was previously reported by Gtari et al. (2004), Murry et al. (1987) and Jeong and Myrold (1999) and seems to be a general feature among *Frankia* genus that reflect soil effect rather than host effect on *Frankia* genome variability. This suggests that repetitive element polymorphism-PCR using BOX-A1R primer is a reliable technique for strains discrimination among *Frankia* genus. Amplified ribosomal DNA restriction analysis has not been often used to characterize *Frankia* strains. Excepting Gtari et al. (2007) who performed an *in silico* ARDRA of entire diversity of *Frankia* 16S rDNA sequences retrieved from GenBank covering all host specificity groups and Huguet et al. (2004) who were interested only on Myricaceae isolated and uncultured strains directly in root nodules. Our ARDRA study may be the first report on assessment of *Frankia* diversity on a large collection of reference strains and isolates. The study demonstrated the feasibility and utility of ARDRA as fast and low cost based techniques for worldwide and routine characterization of *Frankia* isolates.

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Full Length Research Paper

Evaluation of wild *Cicer* species accessions for resistance to three pathotypes of *Ascochyta rabiei* (Pass.) Labr. in Algeria

Ibrahim Elkhalil Benzohra[1]*, Boubekeur Seddik Bendahmane[1], Mokhtar Youcef Benkada[1] and Mohamed Labdi[2]

[1]Laboratory of Plant Protection, Department of Agronomy, University Abdelhamid Ibn Badis of Mostaganem, Mostaganem, Algeria.
[2]INRAA/URO, Algerian National Institute of Agricultural Research / Western Research Unity, Sidi Bel Abbes, Algeria.

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Twenty-five (25) genotypes of five wild *Cicer* species (*Cicer judaicum, Cicer bijugum, Cicer cuneatum, Cicer echinospermum* and *Cicer reticulatum*) were screened for resistance to ascochyta blight disease caused by *Ascochyta rabiei*, by artificially inoculating the germplasm under glasshouse. Highly significant effect (P<0.01) was observed on their reaction to three pathotypes of *A. rabiei* (Mos02 'pathotype III: highly aggressive', At02 'pathotype II: moderate aggressive', and Sba02 'pathotype I: least aggressive'), there is a difference in genotypes reaction to *A. rabiei* isolates but very important resistance was observed (>50% of accessions collection). All five *C. judaicum* accessions are resistant to *A. rabiei* isolates, two resistant accessions in the wild species *C. echinospermum* (ILWC0 and ILWC246) and three accessions in *C. reticulatum* (ILWC81, ILWC104 and ILWC247), *C. cuneatum* (ILWC37, ILWC40 and ILWC232) and *C. bijugum* (ILWC195, ILWC285 and ILWC286).

Key words: *Ascochyta rabiei*, *Cicer arietinum*, *Cicer* sp., aggressiveness, resistance.

INTRODUCTION

Chickpea is an important food legume crop in the Central, West Asia and North Africaregion (CWANA), accounting for 29% of the total food legume production (Singh, 1990; Zohary and Hopf, 2000; Kerem et al., 2007). It serves as a source of inexpensive high quality production in the diets of many people and provides a rich crop residue for animal feed (Singh et al., 1992).

In the Mediterranean region, chickpea is traditionally sown in spring and, as a consequence of the low rainfull during the growth period in dry summers, these results in poor biomass development (Kanouni et al., 2011). Work on cold tolerance in chickpea has been intiated since, the advantages of fall-sown crop over traditional spring sown crop were realized (Singh et al., 1997). Winter sowing expands the vegetative growth period and improves the seed yield up to 2 tonnes/ha (Singh et al., 1995; Singh

*Corresponding author. E-mail: ibrahimelkhalil@live.fr. Tel: +213 791 54 66 99.

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Table 1. Wild *Cicer* species accessions origined from ICARDA.

Wild *Cicer* species	Accession
C. judaicum	ILWC4, ILWC43, ILWC148, ILWC168, ILWC256
C. bijugum	ILWC0, ILWC195, ILWC241, ILWC285, ILWC286
C. cuneatum	ILWC37, ILWC40, ILWC185, ILWC187, ILWC232
C. echinospermum	ILWC0, ILWC180, ILWC181, ILWC235, ILWC246.
C. reticulatum	ILWC81, ILWC104, ILWC237, ILWC247,ILWC290

ILWC: International Legume Wild *Cicer*.

Table 2. *Ascochyta rabiei* isolates with their origin, date of isolation and pathotype groups.

Isolates	Origin	Dates of isolation	pathotypes
Sba01	Sidi Bel abbes	March 2008	I (least aggressive)
At02	Ain Temouchent	November 2008	II (moderately aggressive)
Mos02	Mostaganem	June 2009	III (Highly aggressive)

and Reddy, 1996), but is rarely adopted by the farmers because the cool and wet weather, typical for Mediterranean winters, favors the development of fungal diseases. The ascochyta blight caused by *Ascochyta rabiei* (Pass.) Labr. (Teleomorph, *Didymella rabiei* Kov. v. Arx.), is the major disease that affects the chickpea fields in Algeria and other Meditrranean countries (Singh and Reddy, 1990). Data of many years of prospections, showed the presence and extension of ascochyta blight with fals of output which can go upto 100% (Bouznad et al., 1996). Mabsoute et al. (1996) annouced that in algeria like other Maghreb countries, the ascochyta blight remains the major constraint of chickpea.

Fungicides such as chlorothalonil are sometimes used to control the disease, but their use is often uneconomical under epiphytotic conditions, because a minimum of four to six sprays can be required (Reddy and Singh, 1983). The use of resistant cultivars appears to be the best management option for this disease (Porta-Puglia et al., 1996). The use of resistant chickpea cultivars is the most effective and economical management strategy for ascochyta blight since the application of fungicide is not economical (Gan et al., 2006). Therefore, breeding of resistant chickpea cultivars against ascochyta blight is efficace to control this disease in chickpea fields. However, limited resistance in existing chickpea germplasm has prompted the search for new sources of resistance to ascochyta blight (Reddy and Singh, 1984). Wild relatives of crops often possess genes that confer resistance to biotic stresses (Malhotra et al., 2000). Sources of resistance to ascochyta blight have been found in a limited number of annual wild *Cicer* species, as reported for *Cicer pinnatifidum* Jaub. & Sp. and *Cicer judaicum* Boiss. (Singh et al., 1981), for *Cicer bijugum* K. H. Rech. (Haware et al., 1992), for *Cicer bijugum*, *Cicer echinospermum* P. H. Davis and *Cicer reticulatum* Ladiz. (Stamigna et al., 1998), and for *Cicer judaicum* and *Cicer pinnatifidum* (Singh and Reddy, 1993). The aim of this study was to evaluate resistance of wild *Cicer* species accessions to three pathotypes of *A. rabiei* from north west region of Algeria.

MATERIALS AND METHODS

Wild *Cicer* species accessions

Accessions of wild *Cicer* species including *C. judaicum, C. bijugum*, *C. echinospermum*, *C. reticulatum* and *C. cuneatum* (Table 1) were obtained from International Center for Agricultural Research in the Dry Areas (ICARDA). A total of 25 wild *Cicer* accessions was screened for resistance to three pathotypes of *A. rabiei* in glasshouse trials.

Fungal isolates

The isolates of *Ascochyta rabiei* used in this study were obtained by isolation from samples of stems, sheets and chickpea pods presenting of the symptoms of ascochyta blight (Table 2).

Obtaining seedlings and inoculum preparation

The seeds of chickpea and its wild relatives used are sterilized with Sodium hypochlorite (at 0.2%) for 10 min and washed 3 times with sterile distilled water. They were then sown in pots of 10 cm height and 6 cm in diameter, containing a sterile peat-moss, at rate of 2 seeds per pot and 4 repetitions for each particular treatment.

Three isolates of *A. rabiei* were used in this study (Table 2), each one of them represents one pathotype. The cultures of isolates were flooded with sterile distilled water and spores were scraped with sterile glass spatula. The concentrated spores' suspensions were filtered through filter paper to remove mycelia fragments. Spores suspensions were adjusted to 5×10^5 conidia ml^{-1} using a hemacytometer (Iqbal et al., 2003). All isolates used in this study originate from single conidia.

Figure 1. Rating scale of ascochyta blight disease's severity.

Inoculation of seedlings

Two weeks old seedlings of each line were inoculated with the isolates of *A. rabiei* using 4 pots of 2 plants per isolate. In each experiment, as control, inoculated set of plants were sprayed with sterile distilled water by pressure sprayer in growth chamber (Pieters and Tahiri, 1986). After spraying, plants were inoculated by spore suspension. In order to maintain humidity, seedlings were sprayed with sterile distilled water 2 times a day with a humidifier (Setti et al., 2009).

Rating scale

1: No lesion is visible on the whole of the plants.
3: Visible lesions on less than 10% of the plants, the stems are not reached.
5: Lesions on 25% of the plants, with damage on approximately 10% of the stems.
7: Lesions on all the plants, approximately 50% of the stems are reached, which results in the death of certain plants because of serious damage.
9: Lesions diffused on all the plants, the stems are reached in proportions higher than 50% with the death of the majority of the plants.

The chickpea lines rated 1.0 to 4.9 were considered resistant and those rated 5.0 to 9.0 were considered susceptible (Türkkan and Dolar, 2009) (Figure 1).

Statistical analysis

The variances (σ^2), averages and standard deviation (SD) of various repetitions were calculated and analyzed by the software of statistics (STAT BOX 6.0.4. GRIMMERSOFT) and the device used are the global bifactorial randomization (two studied factors, F1 is aggressiveness and F2 is chickpea germplasm and wild *Cicer* species accessions reactions) by the test of Newman and Keuls ($P_{0.05}$ and $P_{0.01}$). Isolates were classified in three groups by their aggressiveness on three chickpea lines, and chickpea lines were classified according to their reaction to ascochyta blight disease. Mean disease scores for control accessions were subjected to analysis of variance (ANOVA) in order to detect differences between separate trials. For each separate trial, differences between mean disease scores of ILC1929, the susceptible control, and mean disease scores of individual accessions were calculated using *t*-tests.

Table 3. Aggressiveness of three pathotypes of *A. rabiei* on chickpea germplasm and wild *Cicer* accessions.

Organism	Aggressiveness (Mean ± SD)			F value	C.V.
	Sba 02	At 02	Mos 02		
Wild *Cicer* accessions	4.01^{c} ± 0.5	4.69^{b} ± 0.66	5.01^{a} ± 1.01	95.11**	20.7%

**Highly significant effect at *P*<0.01, SD: standard deviation, C.V.: Coefficient of variation, a, b and c: homogenate groups.

Table 4. Reaction of 25 wild *Cicer* accessions to pathotype I (Sba02) of *A. rabiei*.

Species	Genotypes	Mean ± SD
Cicer arietinum	ILC1929	6.5^{a} ± 2.51
	ILWC4	3.5^{c} ± 1
	ILWC43	3^{c}
C. judaicum	ILWC148	4^{bc} ± 1.15
	ILWC168	3^{c}
	ILWC256	4^{bc}
	ILWC0	4.5^{abc} ± 1
	ILWC195	3^{c}
C. bijugum	ILWC241	4.5^{abc} ± 1
	ILWC285	3.5^{c} ± 1
	ILWC286	3^{c}
	ILWC37	3.5^{c} ± 1
	ILWC40	3.5^{c} ± 1
C. cuneatum	ILWC185	4^{bc} ± 1.15
	ILWC187	5^{abc}
	ILWC232	3.5^{c} ± 1
	ILWC0	4^{bc} ± 1.15
	ILWC180	5^{abc}
C. echinospermum	ILWC181	6^{ab} ± 1.15
	ILWC235	3.5^{c} ± 1
	ILWC246	3.5^{c} ± 1
	ILWC81	3^{c}
	ILWC104	3^{c}
C. reticulatum	ILWC237	5^{abc}
	ILWC247	3.5^{c} ± 1
	ILWC290	5^{abc}
F value		4.11**
C.V.		23.63%

**Highly significant effect (*P*<0.01, Test of Newmann-Keuls at 1%) ; SD: sandard deviation ; C.V.: Coefficient of variation.

Table 5. Reaction of 25 wild *Cicer* accessions to pathotype II (At02) of *A. rabiei*.

Species	Genotypes	Mean ± SD
Cicer arietinum	ILC1929	8.5^{a} ± 1
	ILWC4	4.5b ± 1
	ILWC43	4^{b} ± 1.15
C. judaicum	ILWC148	5^{b}
	ILWC168	4.5^{b} ± 1
	ILWC256	4.5^{b} ± 1
	ILWC0	5^{b}
	ILWC195	4.5^{b} ± 1
C. bijugum	ILWC241	5^{b}
	ILWC285	4.5^{b} ± 1
	ILWC286	4^{b} ± 1.15
	ILWC37	4^{b} ± 1.15
	ILWC40	4.5^{b} ± 1
C. cuneatum	ILWC185	4^{b} ± 1.15
	ILWC187	5.5^{b} ± 1
	ILWC232	4^{b} ± 1.15
	ILWC0	4.5^{b} ± 1
	ILWC180	6^{b} ± 1.15
C. echinospermum	ILWC181	6.5^{b} ± 1
	ILWC235	5.5^{b} ± 1
	ILWC246	4.5^{b} ± 1
	ILWC81	5^{b}
	ILWC104	4^{b} ± 1.15
C. reticulatum	ILWC237	5.5^{b} ± 1
	ILWC247	4^{b} ± 1.15
	ILWC290	5.5^{b} ± 1.91
F value		3.82**
C.V.		20.99%

** Highly significant effect (*P*<0.01, Test of Newmann-Keuls at 1%); SD : sandard deviation ; C.V.: Coefficient of variation.

RESULTS

Higly significant effect (*P*<0.01) was observed on chickpea germplasm and wild *Cicer* accessions reaction to *A. rabiei* isolates (Tables 3, 4, 5 and 6). The mean diseases scores and their standard deviations (SD) for all chickpea germplasm and wild relatives accessions tested in four separate trials are mentionned in the Tables 3, 4,

Table 6. Reaction of 25 wild *Cicer* accessions to pathotype III (Mos02) of *A. rabiei*.

Species	Genotypes	Mean ± SD
Cicer arietinum	ILC1929	9^{a}
	ILWC4	$4.5^{cd} \pm 1$
	ILWC43	$4^{cd} \pm 1.15$
C. judaicum	ILWC148	$4.5^{cd} \pm 1$
	ILWC168	$4^{cd} \pm 1.15$
	ILWC256	$5.5^{cd} \pm 1$
	ILWC0	5^{cd}
	ILWC195	$3.5^{d} \pm 1$
C. bijugum	ILWC241	$5.5^{cd} \pm 1$
	ILWC285	$4.5^{cd} \pm 1.91$
	ILWC286	$4^{cd} \pm 1.15$
	ILWC37	$4^{cd} \pm 1.15$
	ILWC40	$4^{cd} \pm 1.15$
C. cuneatum	ILWC185	5^{cd}
	ILWC187	6^{bcd}
	ILWC232	$4.5^{cd} \pm 1$
	ILWC0	$5.5^{cd} \pm 1$
	ILWC180	$6^{bcd} \pm 1.15$
C. echinospermum	ILWC181	$8^{ab} \pm 1.15$
	ILWC235	$6^{bcd} \pm 1.15$
	ILWC246	$4^{cd} \pm 1.15$
	ILWC81	$3.5^{d} \pm 1$
	ILWC104	$3.5^{d} \pm 1$
C. reticulatum	ILWC237	$6.5^{bc} \pm 1$
	ILWC247	$4^{cd} \pm 1.15$
	ILWC290	$6^{bcd} \pm 1.15$
F value		6.55**
C.V.		21 28%

** Highly significant effect (*P*<0.01, Test of Newmann-Keuls at 1%) ; SD: sandard deviation ; C.V.: Coefficient of variation.

5 and 6. The mean disease score that were significantly different (P<0.01) from the susceptible line ILC1929, are also cited in the Tables 4, 5 and 6.

The wild *Cicer* species accessions showed important and interesting source of resistance to *A. rabiei* isolates (>50% of accessions collection (Table 7); but unfortunately, only two species (*Cicer reticulatum* and *C. echinospermum*) are fertile and can be used as a source of resistance (Collard et al., 2003). The evaluation of the resistance in wild *Cicer* species reaction showed broad but important, which will be used in the future in the program of creation of new hybrids of chickpea cultivars resistant to ascochyta blight disease. There was very important resistance was observed_ in the accessions of wild species *Cicer judaicum*, *C. cuneatum* and *C. reticulatum*, compared to others. For the 25 accessions of five species (Figure 2), 13 accessions were resistant to Sba02, 15 to At02 and 14 accessions to Mos02.

DISCUSSION

The primary objective of this research was the screening of wild *Cicer* species accessions for resistance to *A. rabiei*. Many reports on the screening of Wild *Cicer* species for resistance to ascochyta blight have appeared in the literature and a long list would be required to mention all wild *Cicer* accessions that have been reported to be resistant.

The screening of chickpea germplasm was reported from many countries including India (Reddy and Singh, 1984; Singh et al., 1984; Singh and Reddy, 1990; Haware et al. 1995), Syria, Lebanon (Reddy and Kabbabeh, 1985; Udupa et al., 1998; ICARDA, 2003), the Palouse region of USA (Jan and Wiese, 1991; Chen et al., 2004), Italy (Porta-Puglia et al., 1996), Pakistan (Jamil et al., 2002; Iqbal et al., 2003; Iqbal et al., 2004; Malik et al., 2005; Ilyas et al., 2007; Ghazanfar et al., 2010), Spain (Navas-Cortes et al., 1998), Australia (Khan et al., 1999; Nasir et al., 2000), Tunisia (Hamza et al., 2000), Canada (Chongo et al., 2004; Vail and Banniza, 2008), Turkey (Dolar et al., 1994; Türkkan and Dolar, 2009) and Algeria (Zikara-Zine and Bouznad, 2007).

Udupa and Weigand (1997) suggested that is possible to determine the resistance and sensitivity of chickpea germplasm according to their reaction to the three pathotypes of *A. rabiei*, consisting of pathotype I to determine the susceptible chickpea lines, pathotype II for tolerant and pathotype III for resistant chickpea lines.

In Pakistan, the sensitivity of chickpea germplasm ILC 263 was reported by Iqbal et al. (2004), and ILC 1929 by Reddy and Kabbabeh (1985). The chickpea cultivars ILC 3279 and ICC 3996 which were recorded as resistant to ascochyta blight for many years of world chickpea production (Singh et al., 1984; Labdi, 1995; Nasir et al., 2000), became susceptible in these last years (ICARDA, 2003). Thus, our results confirm this sensitivity reaction. Despite the importance of use the resistant cultivars to control this disease, it’s difficult to obtain a stable resistance (Iqbal et al., 2003). The causes of this rapid breakdown varietal resistance are due to pathogenic variability of pathogen agent and the presence of the teleomorph *Didymella rabiei* (Kov. v. Arx.) under fields conditions (Navas-Cortes et al., 1990; Trapero-Casas and Kaiser, 1992). Ascospores of *D. rabiei* (Perfect stage of *A. rabiei*) are a major source of primary inoculums which play an important role in the pathogenicity and

Table 7. A wild *Cicer* species accessions showing their Sensibility or resistance to three pathotypes of *A. rabiei*.

Species	Genotype	Reaction[a]		
		Pathotype I	Pathotype II	Pathotype III
Cicer arietinum	ILC1929	S	S	S
C. judaicum	ILWC4	R	R	R
	ILWC43	R	R	R
	ILWC148	R	S	R
	ILWC168	R	R	R
	ILWC256	S	R	S
C. bijugum	ILWC0	S	S	S
	ILWC195	R	R	R
	ILWC241	S	S	S
	ILWC285	R	R	R
	ILWC286	R	R	R
C. cuneatum	ILWC37	R	R	R
	ILWC40	R	R	R
	ILWC185	S	R	S
	ILWC187	S	S	S
	ILWC232	R	R	R
C. echinospermum	ILWC0	S	R	S
	ILWC180	S	S	S
	ILWC181	S	S	S
	ILWC235	S	S	S
	ILWC246	R	R	R
C. reticulatum	ILWC81	R	S	R
	ILWC104	R	R	R
	ILWC237	S	S	S
	ILWC247	R	R	R
	ILWC290	S	S	S

[a]Wild *Cicer* accessions reaction was rated 1.0 to 4.9 for resistant (R) seedlings and those rated 5.0 to 9.0 for susceptible (S) (Türkkan and Dolar 2009).

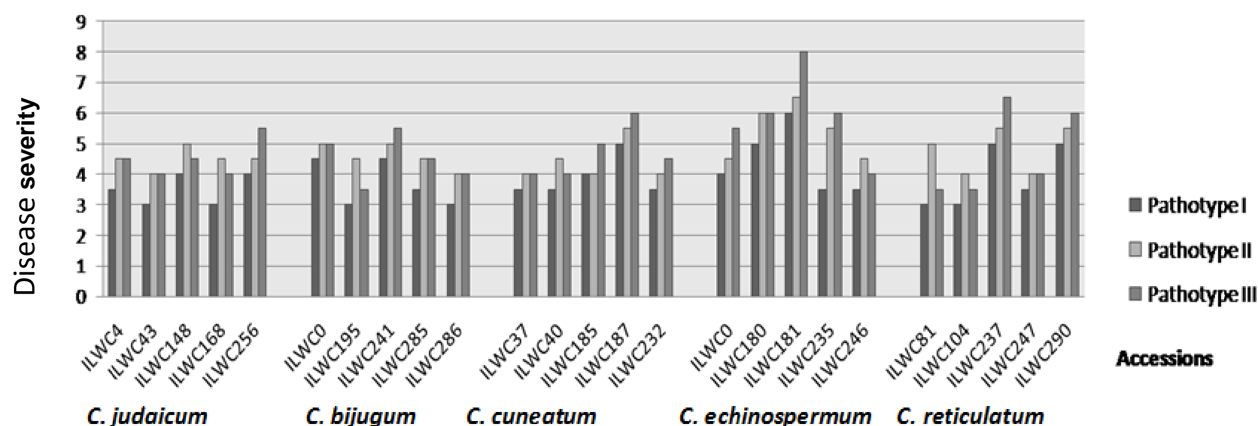

Figure 2. Aggressiveness of three pathotypes of *A. rabiei* against 25 wild *Cicer* species accessions.

epidemiology of *A. rabiei* (Nasir et al., 2000).

The tolerant chickpea germplasm ILC 482 and ILC 483, which are become susceptible to pathotypes II and III of *A. rabiei*. Similarly, the sensitivity behavior of these two chickpea germplasm was reported by other authors like Singh and Reddy (1990).

Many authors around the world have reported the importance of wild *Cicer* species in resistance to different stresses that affect the culture of chickpea (Nene and Haware, 1980; Haware et al., 1992; Singh and Reddy, 1993; Singh and Weigand, 1994 ; Singh et al., 1994; Singh et al., 1998; Shah et al., 2005; Pande et al., 2006; Aryanmanesh 2007; Trapero-Casas and Kaiser, 2009; Saeed et al., 2010).

In the pathological aspect, there is a wide spectrum of variability among isolates of *A. rabiei* (Navas-Cortes et al., 1998; Chongo et al., 2004 ; Banniza and Vail, 2008; Türkkan and Dolar, 2009). We must therefore use the screening test 2 or 3 aggressiveness classes of the pathogen to facilitate the interpretation of results (Udupa et al., 1998). In our test, we used three isolates representing the three pathotypes of *A. rabiei* according to their degree of aggressiveness (Table 2).

Similarly, in Australia, Collard et al. (2001) used one isolate for screening test accessions and reported the existence of significant resistance among these wild species to this isolate. The *C. Judaicum* accessions have a greater resistance than other species. These results have also been reported by Singh et al. (1991) in Syria, Lebanon and Turkey. We note that the majority of accessions tested in our test, have not been studied elsewhere, except seven lines showed a similarity in their reaction against *A. rabiei*. The accession ILWC 81 (*Cicer reticulatum*) seems resistant in our test, but sensitive in the results of Stamigna et al. 1998 and Collard et al. (2001).

The lack of results completely similar with other research, may be linked to the methods chosen (number of isolates, nature and concentration of the inoculum, seedlings inoculated with isolates separately or mixed etc.).

Conclusion

The screening of wild *Cicer* species accessions showed a different behavior to three pathotypes of *A. rabiei*. The evaluation of wild *Cicer* species accessions for resistance to *A. rabiei* showed the presence of significant resistance compared to known cultivars of chickpea (ILC 3279, and ILC72 ICC3996) in different countries (Labdi, 1995; Aryamanesh, 2007).

The wild species *C. Judaicum*, *C. bijugum* and *C. reticulatum* gave a very high level of resistance to ascochyta blight disease. But just *C. reticulatum* can be used in the future to transfer its resistance traits important chickpea cultivars by hybridization or other appropriate methods. Due to the fact that more resistant chickpea cultivars selected and cultivated in the world for several years later became susceptible when they were cultivated on a large scale.

Such results could be useful for choosing representative pathotypes that may be used to identify specific resistant groups for utilization in breeding program. It's necessary to apply this test on commercial chickpea cultivars for reduce crop damage caused by this disease. The knowledge generated on *A. rabiei* resistance in chickpea germplasm indicated that can be exploited for disease control by building disease resistance pyramids due to complex nature of ascochyta blight disease.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Biotechnological potential of *Candida* spp. for the bioconversion of D-xylose to xylitol

Marcus Venicius de Mello Lourenço[1]*, Francisco Dini-Andreote[2,3], Carlos Ivan Aguilar-Vildoso[3] and Luiz Carlos Basso[1]

[1]Department of Biological Science, “Luiz de Queiroz” College of Agriculture, University of São Paulo, Piracicaba, São Paulo, 13418-900, Brazil.
[2]Department of Soil Sciences, “Luiz de Queiroz” College of Agriculture, University of São Paulo, Piracicaba, São Paulo, 13418-900, Brazil.
[3]Department of Genetics, “Luiz de Queiroz” College of Agriculture, University of São Paulo, Piracicaba, São Paulo, 13418-900, Brazil.

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In the present study, 28 yeast isolates were obtained from sugarcane filter cake material collected across several ethanol industrial areas located in the State of Sao Paulo, Brazil. First, isolates were taxonomically affiliated by sequencing and analysis of the D1/D2 region of the 26S rRNA gene as *Candida tropicalis* (24 isolates) and *Candida rugosa* (four isolates). Second, five phylogenetically distant isolates were selected and quantitatively tested for their capacity to bioconvert D-xylose to xylitol (*C. tropicalis* MVP 03, *C. tropicalis* MVP 16, *C. tropicalis* MVP 40, *C. rugosa* MVP 17 and *C. rugosa* MVP 21). The fermentation processes yielded xylitol production ranging from 5.76 to 32.97 g L^{-1}, from an initial D-xylose concentration of 50 g L^{-1}, with the volumetric production (Qp) ranging from 0.06 to 0.35 g L^{-1} h^{-1}. The measurement of these parameters allowed the determination of the conversion efficiency of D-xylose to xylitol (η), which showed values ranging from 6 to 61%. Remarkable, the yeast isolate *C. tropicalis* MVP 16 presented the highest efficiency among tested lines, yielding up to 32.97 g L^{-1} of xylitol (Qp = 0.35 g L^{-1} h^{-1}, η = 61%) after 96 h of fermentation. These results describe the biotechnological potential of yeast populations naturally occurring in filter cake substrates. Further studies at the genomic level are required, in order to enhance our understanding on yeast metabolisms involved in the xylitol bio-conversion/production, a currently high-added-value product.

Key words: Fermentation efficiency, 26S rRNA gene, yeast isolates, sugarcane residues.

INTRODUTION

Sugar-derived alcohols comprise a class of polyols obtained from carbonyl (aldehyde/ketone), which is reduced to the corresponding hydroxyl group (Akinterinwa et al., 2008). These alcohols have a broad application in pharmaceutical, odontological and food industries (Chen et al., 2010; Sampaio et al., 2009; Silveira and Jonas,

*Corresponding author. E-mail: mv_pole@yahoo.com.br. Tel: 05519-34294169.

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2002), mostly due to their intrinsic characteristics (for example, sweetness) similar to those of sucrose. There are multiple potential health benefits for using in replacement of sucrose, maltodextrins and glucose syrups. This is mostly related to the concepts of glycaemia and insulinaemia, reduced energy, low calorie content, caries reduction and digestive health (Granstrom et al., 2007; Schiweck et al., 2003; Ghosh and Sudha, 2012).

Xylitol is a five-carbon pentahydroxy alcohol broadly occurring in nature, for example in fruits at low concentration rates (approximately 9 mg g^{-1}) (Schiweck et al., 2003), and in mammals as an intermediate metabolic compound (for example, in human, metabolism is being produced at rates of 5 to 15 g per day) (Pepper and Olinger, 1988). Industrially, the synthesis of xylitol is carried out by the chemical reduction of xylose from natural sources, which is well-known to be a high cost process due to the low initial sugar availability in current used substrates, combined with the high cost in further required purification steps (López et al., 2004). In this sense, there is an increasing demand to establish and standardize an efficient fermentative process to obtain xylitol, for instance by using different sources of organic material as initial substrates (Parajó et al., 1997). The advantage of such strategy arises mostly in the no requirement of pure xylose syrup at the initial fermentative step, since low-cost hemicellulosichydrolysates can be applied (Parajó et al., 1997; Roseiro et al., 1991).

Biochemically, the bioconversion of D-xylose to xylitol is performed by the action of the xylose reductase enzyme (XR, EC 1.1.1.21), which catalyses the first step in the D-xylose assimilation. This metabolic capacity has been commonly described to be present in several yeast species (for example, *Debaryomyces hansenii*, *Meyerzyma guilliermondii* and *Candida parapsilosis*) (Girio et al., 1996), particularly in those belonging to the genus *Candida* (for example, *Candida tropicalis* and *C. parapsilosis*) (Faria et al., 2002; Latif and Rajoka, 2002; Silva and Roberto, 1999; Walther et al., 2001).

In the current industry, the major amount of xylitol is produced from birch and other three species commonly found in Scandinavian countries. Alternatively, the reduction of xylose has also been applied at different substrates obtained from a range of other natural sources (for example, corn cobs, sugarcane, bark, seeds and nuts) (Makinen, 2000; Nair and Zhao, 2010).

Due to the increasing industrial demand for xylitol, the aims of this study were to isolate and test yeast isolates obtained from sugarcane filter cake material, regarding their potential use in the bioconversion of D-xylose to xylitol. We posited filter cake substrate as a source of specialized yeasts to be assessed regarding their potential use in industrial processes. In this sense, the obtained isolates were firstly identified by sequencing and analysis of the D1/D2 region of the 26S rRNA gene, and further analytically tested in fermentative essays.

MATERIAL AND METHODS

Yeast isolation

Samples were collected in triplicate at four industrial areas located at the State of São Paulo, Brazil. A total of 500 g of sample was randomly collected in the area of the deposit of filter cake material. Samples were homogenized and subsamples containing 5 g were transferred to vials containing 50 mL of sterile distilled water and mixed in a shaker for 2 min (orbital shaking at 100 g). In order to obtain yeast isolates, serial dilutions were carried out (from 10^8 to 10^1). Aseptically, 100 µL of each dilution was transferred to Petri dishes containing solid YEPX medium (yeast extract 1%, peptone1% and xylose 2%) amended with chloramphenicol and tetracycline, both at the concentration of 100 µg mL^{-1}, to avoid bacterial contamination (Iak and Hahn, 1958; Muthaiyan et al., 2011). Plates were incubated at 30°C for 24 h. After this period, isolated colonies presenting yeast macroscopic characteristics were transferred to new Petri dishes containing YEPX media, and subjected to the exhaustion technique. Pure colonies were named and stored in tubes containing liquid medium YEPX amended with 40% of glycerol at -80°C.

DNA extraction from yeast isolates

A total of twenty-eight yeast isolates were obtained and subjected to total DNA extraction. First, each isolate was grown separately in culture tubes containing 10 mL of liquid YEPD medium (yeast extract 1%, peptone 1% and glucose 2%) for 24 h at 30°C, in orbital shaker at 100 g. Aliquots of 1.5 mL of each culture were transferred to 2.0 mL microtubes and centrifuged at 10,000 g for 5 min. The supernatant was discarded and the precipitated cells were eluted and homogenized in 0.2 mL of buffer A (Triton X-100 2%, SDS 1%, 100 mMNaCl, 10 mM Tris-HCl, 1 mM EDTA and pH 8.0). To the obtained suspension it was added 200 mg of glass beads 0.1 mm (glass beads Biospec products TM®) and 0.2 mL of clorofane (phenol: chloroform: isoamyl alcohol 25: 24: 1), and the mixture was mixed by vortex for 3 min. The microtubes were then centrifuged at 12,000 g for 5 min and obtained supernatants were transferred to a clean tube. 1 mL of ethanol (100 %) was added and tubes were centrifuged under the same parameter. Precipitated DNAs were eluted in 0.4 mL of TE buffer, 10 µL of 4M ammonium acetate and 1 mL of ethanol (100%). The tubes were centrifuged and obtained DNAs were eluted in 50 µL of TE buffer. All DNA samples were stored at -20°C for further analysis.

PCR amplification and sequencing of the yeast 26S rRNA gene

For the amplification of the D1/D2 region of the yeast 26S rRNA gene, PCR reactions were performed containing 1x PCR buffer, 3.0 mM $MgCl_2$, 1.0 U of Taq DNA polymerase, 1.0 mMdNTPs, 20 pmol of each primer – NL1 (5' GCCATATCAATAAGCGGAGG 3') and NL4 (5' GGTCCGTGTTTCAAGACGG 3'), and sterilized deionised water to the final volume of 50 µL, as previously described (O'Donnell, 1993). The thermal cycler machine was a PTC-100 MJ – Research, under the following conditions: one cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles of amplification at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension cycle of 72°C for 7 min. The expected amplicon size was approximately 600 bp. After PCR amplification, obtained amplicons were purified with polyethylene glycol solution (PEG 8,000 20% and 2.5 mMNaCl), and aliquots of each amplicon were checked by electrophoresis in agarose gel 1.2% in TBE buffer, further stained in ethidium bromide solution and visualized under UV light. Amplicons presenting the right size were then subjected to sequencing with the chain termination method. The

sequencing reaction (20 µl) contained 1x sequencing buffer (Applied Biosystems), 2.0 µl of Big Dye Terminator mix and 0.1 µM of the primer NL4. Ready-to-sequence DNA templates were isopropanol cleaned and eluted in 10 µl HiDiformamide. After denaturation at 95°C for 3 min, sequencing of PCR products were performed with the MegaBACE™ 1,000 (GE Healthcare Life Sciences). Standard running procedure recommended by the manufacturer was used.

For the taxonomic affiliation of yeast isolates, obtained chromatograms were trimmed for quality using Lucy (available at the ribosomal database project: website at http://rdp.cme.msu.edu/), using a threshold of base score of >20. Furthermore, trimmed sequences were compared against the NCBI database using the algorithm BLAST nt/nt against the non-redundant database (available at http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis of yeast isolates

Phylogenetic analysis was performed with the software MEGA v.4.0 (Tamura et al., 2007). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distances between the sequences were computed using the Kimura-2 parameter (Kimura, 1980) and are in the units of the number of base substitutions per site (note scale bar). The statistical support of phylogenetic trees was obtained with bootstrap analyses (1,000 replications).

Analytical screening of yeast isolates

Based on the molecular identification and phylogenetic reconstruction of yeast isolates, it was possible to distinguish among five phylogenetically different isolates to be tested regarding their capacity and efficiency to convert D-xylose substrate to xylitol: *C. tropicalis* MVP 03, *C. tropicalis* MVP 16, *C. tropicalis*MVP 40, *C. rugosa* MVP 17 and *C. rugosa* MVP 21. The fermentative screening and efficiency measurement were performed in comparison with three other isolates well-known regarding their capability to produce xylitol. These so-called 'control' strains were obtained from the culture collection of the Department of Biological Sciences at the University of Sao Paulo (ESALQ/USP): *Kluyveromyces marxianus* IZ 1339, *C. tropicalis* IZ 1824 and *C. guilliermondii* FTI 20037.

The fermentation processes were performed in 125 mL Erlenmeyer flasks containing 50 mL of medium UPX (Urea 2.3 g L^{-1}, peptone 6.6 g L^{-1}, D-xylose 50 g L^{-1}, pH 6.0). All flasks were incubated in orbital shaker at 100 g for 96 h at 30°C. Time-series samples (0, 24, 48, 72 and 96 h after incubation) were collected in triplicate and subjected to analytical measurements of biomass accumulation, D-xylose consumption and xylitol production.

The cell concentration was determined as optical density (OD) at 600 nm using a HitaCHi U1800 model spectrophotometer. An OD of 1 unit is equivalent to 0.24 g of dry cells per litter of D-Xylose. The xylitol concentration was determined using high-performance liquid chromatography in a BIO-RAD aminex HPX-87H (300 x 7.8 mm) column at 45°C, containing 0.005 M sulfuric acid as eluent, flow rate of 0.6 mL min^{-1}, refraction index detector and 20 µL of sample volume. For HPLC measurements, 1 mL of each sample was prepared by centrifugation for 5 min at 12,000 g, followed by 100-times dilution in sterile water and filtering in Milipore membrane (0.22 µm). Values were statistically tested by one-way analysis of variance using Tukey's test ($P<0.05$).

Determination of fermentative parameters

The xylitol yield factor (Yp/s, measured in g g^{-1}) was determined as the ratio of xylitol and D-xylose consumed, at the end of each fermentative process. The cell yield factor (Yx/s, in g g^{-1}) was determined as the ratio of formed cells and the total consume of the substrate (D-xylose) after 96 h. The volumetric productivity of xylitol (Qp, in g L^{-1} h^{-1}) was expressed as the amount of xylitol produced per hour. The efficiency of bioconversion of D-xylose to xylitol (η, in %) was calculated as the ratio of Yp/s with the theoretical value of 0.917 g g^{-1}, as previously proposed by Barbosa et al. (1988).

RESULTS AND DISCUSSION

Taxonomic affiliation of yeast isolates

The taxonomic affiliation was performed by pairwise comparison of sequences of the D1/D2 region of the yeast 26S rRNA gene. Surprisingly, all the twenty-eight obtained yeast isolates belonged to the genus *Candida*, with *C. tropicalis* encompassing 85.71% (24 isolates) and *C. rugosa* 14.29% (4 isolates). Phylogenetic reconstruction also revealed a close association among our sequences to those well-described sequences available in the NCBI database (Figure 1).

Regarding the fermentation of D-xylose, special attention has been given to yeast strains belonging to the genus *Candida* (Faria et al., 2002; Ikeuchi et al., 1999; Latif and Rajoka, 2001; Meyral et al., 1991; Silva and Roberto, 1999; Walther et al., 2001). In this sense, we exploit the filter cake substrate as a source of genetic diversity for the occurrence of specialized yeast strains belonging to *Candida* spp. This yeast genus facilitated the investigation regarding its efficient capability to produce different bio-products by industrial fermentative processes. We also suggested that *Candida* spp. are likely to present a generalist occurrence, possibly leading to their dominance in our isolation approach and also in the filter cake material. Despite relative work have been done to improve our recovery of different yeast species, no significant results were obtained (data not shown). Thus, we reinforce our point that sugarcane filter cake is a suitable substrate for exploration of specialized yeast strains, with further potential use in the industry.

The bioconversion of D-xylose to xylitol mediated by *Candida* spp.

Several studies have already evaluated the efficiency of different yeast strains for the bioconvertion of D-xylose to xylitol (Sirisansaneeyakul et al., 1995; Winkelhausen and Kuzmanova, 1998). However, since a wide range of variables can interfere in the fermentative efficiency (for example, initial concentration of D-xylose, fermentation volume, temperature and pH), comparisons among studies are likely to be strongly biased. To circumvent such limitation, we used three 'control' yeast strains, well-known regarding their efficiency to convert D-xylose to xylitol. These strains were used to supply a more reliable comparative basis, in order to infer the efficiency of our five selected isolated during the analytical measurements.

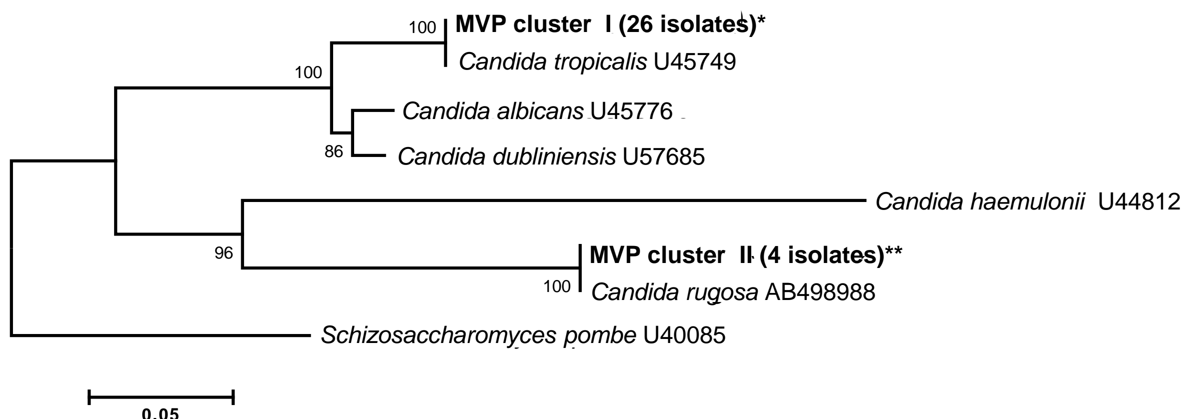

Figure 1. Phylogenetic tree based on sequence analysis of the D1/D2 region of the yeast 26S rRNA gene. The tree encompasses sequences from members of the genus *Candida*, and the clustering was obtained by the application of the Neighbor-joining method and Kimura-2 parameter, with a bootstrap analysis of 1,000 replications. *Cluster I encompass isolates (MVP 03, 06, 07, 09, 10, 12, 13, 14, 15, 16, 18, 19, 22, 23, 25, 26, 27, 28, 29, 30, 32, 33, 34, 37, 39 and 40); **Cluster II encompass isolates (MVP 17, 21, 24 and 35).

All tested isolates and control strains significantly increased biomass during the fermentation process, with a discrete peak observed after 72 h of incubation for the isolate MVP 16, reaching 6.47 g L^{-1}. On the other hand, the lowest biomass yield was observed for the isolate MVP 17, reaching 3.82 g L^{-1} after 96 h of incubation (Figure 2a). Regarding D-xylose consumption, the isolates MVP 03, MVP 16, MVP 40 and the strain FTI 20037 completely consumed the initial substrate input (50 g L^{-1}) after 72 h of fermentation. The isolates MVP 17 and MVP 21 showed the lowest efficiency for D-xylose consumption (Figure 2b). The opposite trend was observed for xylitol production, where the isolate MVP 16 reached the highest production (value of 33.67 g L^{-1} after 72 h of fermentation) (Figure 2c). Surprisingly, the efficiency of this strain exceeded the rate of production obtained by the 'control' strains, which showed values ranging from 5.12 (strain IZ 1824) to 24.12 g L^{-1} (strain FTI 20037) after the same period of time (Figure 2c). In addition, it is worthy to mention that the slight decrease in xylitol concentrations after 96 h of fermentation, occurred due to xylitol consumption by yeast cells after a long period of incubation.

Analytical measurements of fermentative parameters

We calculated the fermentative parameters after 96 h of incubation. We assumed that after this period of time, tested strains had sufficient time to consume most of (or entirely) the initial D-xylose substrate. Remarkably, the 'control' strain *Candida guilliermondii* FTI 20037, a well-studiedy east strain used as a standard control for xylitol production (Carvalho et al., 2004; Rodrigues et al., 2003; Sarrouh et al., 2009; Soleimani and Tabil, 2013; Cortez and Roberto, 2014), together with strains MVP 03, MVP 16 and MVP 40, consumed entirely the D-xylose substrate after 72 h of fermentation. Higher values of conversion efficiency (η) were also obtained for these three last isolates (MPV 03, MPV 16 and MPV 40), reaching 56, 62 and 55%, respectively. These values were even higher than those obtained for the 'control' strains, for instance the strain FTI 20037, which presented the highest η among tested 'control' strains, reached η = 47% (Table 1).

We observed the volumetric productivity (Qp) to range from 0.01 g L^{-1} h^{-1} for the isolate MVP 17 to 0.34 g L^{-1} h^{-1} for MVP 16. The xylitol yield factor (Yp/s) presented the highest value for the isolate MVP 16 (Yp/s = 0.67 g g^{-1}) and lowest for MVP 17 (Yp/s = 0.06 g g^{-1}). Conversely, the cell yield factor (Yx/s) ranged from 0.08 g g^{-1} for the isolate MVP 03 to 0.28 g g^{-1} for MVP 21 (Table 1).

As previously mentioned, comparison among different studies may be strongly biased, due to differences in fermentative parameters and set-up, and initial amount of D-xylose substrate. As an example of such bias, Silva and Roberto (1999) showed that an increase in the initial substrate input of D-xylose from 60 to 120 g L^{-1} can severely decrease the production of xylitol under the course of the fermentative process. In this sense, not only highly-efficient yeasts strains are required, but also the establishment of an effective and standardized procedure for xylitol production.

We argue that such procedure must take into account the selection of highly-specialized yeasts strains, able to perform such bioconversion at a determined threshold trade-off between initial substrate concentration and conversion efficiency. Also, as previously mentioned by Silva and Roberto (1999), osmophilic effect is an important factor to be considered through the course of the interaction between the organism and the substrate availability, since it can repress some enzymes involved

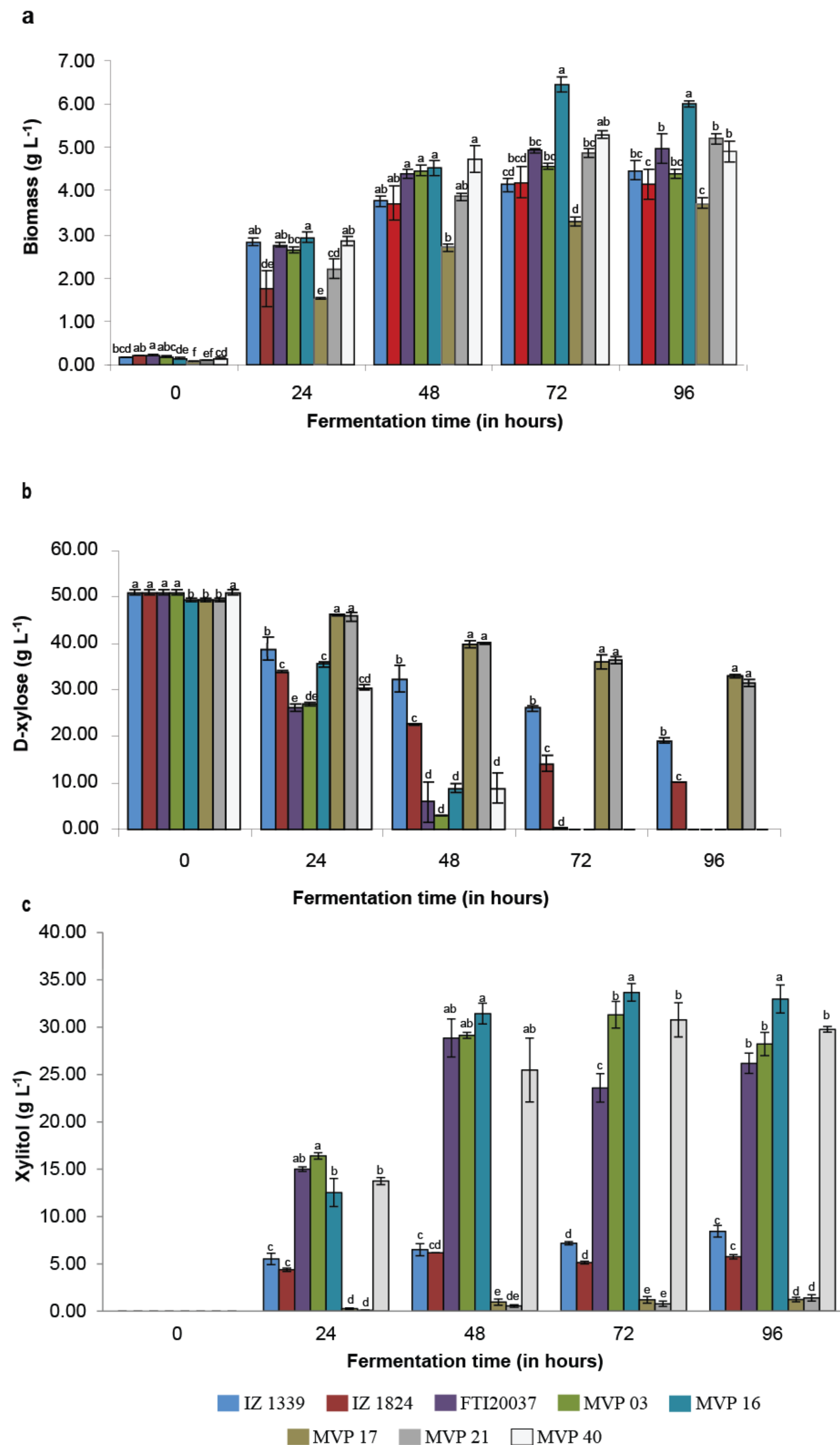

Figure 2. Values of (a) biomass accumulation (dry weight) (g L^{-1}); (b) D-xylose consumption (g L^{-1}); and (c) xylitol production (g L^{-1}) obtained from time-series sampling during the fermentative process in medium UPX at 100 g and 30°C. Values indicate the average from three replicates and bars display the standard deviation. Bars presenting different letters are statistically different ($P<0.05$).

Table 1. Analytical measurements of fermentative parameters calculated for the yeast isolates and 'control' strains after 96 h of fermentation.

Yeast isolates/strains	Yp/s[a]	Qp[b]	Yx/s[c]	η^d (%)
K. marxianus IZ 1339	0.26 ± 0.029	0.09 ± 0.008	0.13 ± 0.009	24
C. tropicalis IZ 1824	0.14 ± 0.005	0.06 ± 0.003	0.10 ± 0.007	13
C. guilliermondii FTI 20037	0.51 ± 0.023	0.27 ± 0.014	0.09 ± 0.007	47
C. tropicalis MVP 03	0.61 ± 0.028	0.33 v 0.018	0.08 ± 0.003	56
C. tropicalis MVP 40	0.60 ± 0.040	0.32 ± 0.023	0.09 ± 0.005	55
C. tropicalis MVP 16	0.67 ± 0.040	0.34 ± 0.020	0.11 ± 0.002	62
C. rugosa MVP 17	0.06 ± 0.005	0.01 ± 0.001	0.22 ± 0.001	6
C. rugosa MVP 21	0.09 ± 0.008	0.02 ± 0.001	0.28 ± 0.011	8

[a]Yp/s (g g^{-1}) g xylitol produced/g xylose consumed; [b]Qp (g L^{-1} h^{-1}) volumetric productivity of xylitol; [c]Yx/s (g g^{-1}) g cell mass produced/g xylose consumed; [d]efficiency calculated assuming the theoretical maximum of 0.917 g g^{-1} (Barbosa et al., 1988).

in D-xylose metabolism, as well as other substances commonly found, hemicellulosic hydrolysates substrate.

Conclusions

There is a long-standing effort in the literature aiming to develop a standardized fermentative process to efficiently produce xylitol for industrial application (Cadete et al., 2012; Sampaio et al., 2009; Winkelhausen and Kuzmanova, 1998). In this sense, many studies have been investigating a wide range of possibilities including enzymatic and fermentative processes. While chemical reduction of xylose has been proving to be a costly method (López et al., 2004), researches focusing on the isolation and screening of efficient microbial strains (in this case, yeast isolates) to perform such bioconversion are urgently required. This can lead to further establishment of an efficient and low-cost method for xylitol acquisition.

In this sense, a range of yeast species were already described as presenting the ability to bioconvert D-xylose to xylitol, such as *Scheffersomyces (Pichia) stipitis, Candida shehatae, Candida lignosa, Candida insectosa, Candida tenuis, Pachysolen tannophilus* (Agbogbo and Coward-Kelly, 2008; Wohlbach et al., 2011)*, Spathaspora passalidarum* (Nguyen et al., 2006) and *Spathaspora arborariae* (Cadete et al., 2012; Da Cunha-Pereira et al., 2011). We described here, the isolation of twenty-eight *Candida* spp. yeast strains obtained from sugarcane filter cake material, and further characterize five of them in terms of analytical fermentative essays.

The increasing market demand facilitated a more sophisticated and efficient industrial production of xylitol. Our results show that sugarcane filter cake can be explored as a source of yeast isolation to be further used in this industrial application. We describe a highly efficient yeast isolate (*C. tropicalis* MVP 16), which under our established fermentative parameters, is able to yield up to 32.36 g L^{-1} of xylitol (with Qp value of 0.35 g L^{-1} h^{-1}, Yp/s of 0.65 g g^{-1} and Yx/s of 0.11 g g^{-1}), reaching a conversion efficiency of 62%. However, it is worth mentioning that variation in the initial D-xylose concentration and fermentative parameters were not tested. These, together with further studies at the genomic level are still required for a better understanding of this yeast strain metabolism.

Conflict of interest

The authors declare that they have no conflict of interest

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Full Length Research Paper

Probiotic attributes of a yeast-like fungus, *Geotrichum klebahnii*

Poonam Syal and Ashima Vohra*

Department of Microbiology, Institute of Home Economics, University of Delhi, HauzKhas Enclave, New Delhi, India.

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***Geotrichum klebahnii*, a filamentous yeast-like fungus, was isolated from a cheese sample. Several *in vitro* tests were carried out for its probiotic characterization. This isolate showed high survival rate of 100 ± 1.8% at low pH (pH 2) and 100 ± 0.9% at high oxbile concentration (1%) and also grew well at 37°C. It showed an auto-aggregation ability of 100.00 ± 1.5% after 20 h of incubation at 37°C, as well as 36.43 ± 0.70% and 52.13 ± 1.50% cell surface hydrophobicity with xylene and n-hexadecane, respectively. It had inhibitory activity against food-borne pathogens such as *Salmonella* sp., *Vibrio* sp. and *Staphylococcus aureus*. *G. klebahnii* produced the enzymes phytase and inulinase. It was a producer of vitamin B_{12} and exoploysaccharides. It assimilated up to 29.42 ± 2.1% cholesterol after 48 h of incubation at 37°C. The organism did not produce gelatinase and DNase assay, indicating its safety as a probiotic microorganism. This is the first report of the probiotic potential of yeast like fungus, *G. klebahnii*.**

Key words: Probiotics, yeast, antimicrobial, phytase, inulinase.

INTRODUCTION

Yeasts contribute to the fermentation of a broad range of other commodities, where various species may work together with bacteria and/or filamentous fungi. Fermented milk products that are manufactured using starter cultures containing yeasts include acidophilus-yeast milk, kefir, Koumiss, Leban and cheese (Lang and Lang, 1975). *Saccharomyces* spp. For example *Saccharomyces burnetii, Saccharomyces kluveri, Saccharomyces byanus, Saccharomyces rosinii, Saccharomyces cerevisiae* and *Saccharomyces boulardii* may be isolated from a variety of dairy products including milk, yogurt, cream, dahi, cheese and kefir. *Saccharomyces* spp. cannot ferment lactose so they develop in milk as a secondary flora, after bacterial growth. Lactic acid produced by lactic acid bacteria creates a high acid environment, creates a selective environment for yeast growth (Fleet, 1990). *Saccharomyces cerevisiae*, which according to The European Food Safety Authority (EFSA) has a Qualified Presumption of Safety (QPS) status, is the most common yeast used in food fermentation where it has shown various technological properties.

Probiotics have been defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Fuller, 1989). *S. boulardii* is the only yeast with clinical effects and the only yeast preparation with proven probiotic efficiency in double-blind studies (Sazawal et al., 2006).

Geotrichum sp., filamentous yeast-like fungus, is ubiquitous and is found in a wide range of habitats such as plant tissues, silage and soil (Jacques and

*Corresponding author. E-mail: vohra_ashi@yahoo.co.in. Tel: +919811642765.

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Caseregola, 2008). This genus belongs to the class Hemiascomycetaceae, order Saccharomycetales and family Dipodascaceae. It is also a component of the natural flora of human mouth, skin and gastrointestinal tract. Species belonging to this genus have been commonly isolated from dairy products such as cheese, fermented milk and also from juices. One of its species, *Geotrichum candidum* has been reported by the International Dairy Federation and European Food and Feed Culture Association as a microorganism with a documented history of use in dairy products (Mogensen et al., 2002). Sarkar et al. (1994) reported the population of *Geotrichum candidum* in 40-50% of market samples of Kinema (a soyabean fermented food) to be 0.8-4 x 10^4cfu/g. Nago et al. (1998) isolated 54 strains of yeasts from Beninese ogi, out of which *Geotrichum* spp. accounted for 26% of the yeast isolates. *Geotrichum penicillatum*and *Geotrichum candidum* have been reported to be isolated from Boza, a beverage consumed in Bulgaria, Albania (Vasudha and Mishra, 2013; Gotcheva et al., 2000).

Geotrichum klebahnii ATCC 42397 (previously named *Trichosporon penicillatum*SNO-3) is a yeast-like fungus originally isolated from mandarin peel (Rojas et al., 2008). This study is the first report of probiotic properties of *G. klebahnii,* isolated from cheese sample.

MATERIALS AND METHODS

All the chemicals used were of AR grade and procured from Qualigens and Himedia (Mumbai, India).

Isolation procedure and Identification

A 25 g sample of the cheese sample was homogenized in 225 ml of sterile phosphate buffered saline (PBS; g/L: NaCl 8.0, KCl 0.2, disodium phosphate 1.44 and potassium phosphate 0.24), pH 7. 10-fold serial dilutions of the sample were prepared in sterile PBS, pH 7. The dilutions were spread plated on MYPG medium (g/L: malt extract 3.0, yeast extract 3.0, peptone 5.0, glucose 10.0 and agar 20.0, pH 5.6) and the plates were incubated at 30°C for 24-48 h and observed for microorganisms with yeast-like morphology under microscope.

The culture was identified at IMTECH, Chandigarh, India, using BioLog identification kits.

Acid tolerance test

The culture was inoculated (1%) into PBS maintained at low pH (2, 2.5 and 3) using 1 N HCl. Samples were taken after 5 h of incubation at 37°C and spread plated on MYPG. The survival rate was calculated as the percentage of colonies grown on MYPG medium after exposure to low pH as compared to the initial cell concentration.

Bile salt tolerance test

PBS tubes containing 0.3, 0.5 and 1% oxbile were inoculated with the yeast culture and incubated at 37°C for 5 h. The survival rate of each strain was expressed as the percentage of viable cells in the presence of bile salt as compared to that without bile salt.

Comparison of growth at 30 and 37°C

Growth at both temperatures was compared by determining the number of viable cells by the plate count method after incubation for 48 h.

Auto-aggregation assay

G. klebahnii was grown for 48 h at 37°C in MYPG broth. The cells were harvested by centrifugation at 7000 rpm for 10 min, washed twice and resuspended in PBS. Cell suspensions (4 mL) were mixed by vortexing for 10 s and auto-aggregation was determined after 3 and 20 h of incubation at 37°C. An aliquot (0.1 mL) of the upper suspension of PBS after incubation was transferred to another tube with 3.9 mL of PBS and the absorbance (A) was measured at 600 nm (Del et al., 2000). The auto-aggregation percentage is expressed as: $1-(A_t/A_0) \times 100$, where A_t represents the absorbance at time t = 3, or 20 h and A_0 the absorbance at t = 0.

Cell surface hydrophobicity

One milliliter of the hydrocarbon, that is, n-hexadecane and xylene were added to tubes containing cell suspensions (3 ml). The cells were vortexed for 120 s. The suspension was then kept undisturbed at 37°C for 5 min to allow phase separate, and the hydrocarbon layer was allowed to rise completely. After 5 min, the aqueous phase was removed carefully and the absorbance (OD) was measured using a spectrophotometer at 600 nm (Rosenberg et al., 1980). The decrease in the absorbance was taken as a measure of the cell surface hydrophobicity (hydrophobicity (%)), calculated using the equation given below:

$$\text{Hydrophobicity (\%)} = (OD_{initial} - OD_{final}) / OD_{initial} \times 100$$

Where, $OD_{initial}$ and OD_{final} are the absorbances (at 600 nm) before and after extraction with the hydrocarbons.

Inhibitory action against enteric pathogens

Double layer method, was used to evaluate the antagonistic activity of *G. klebahnii* against enteric pathogens, that is, *Escherichia coli, Salmonella* sp., *Staphylococcus aureus, Vibrio cholerae* and *Pseudomonas* sp. obtained from the Departmental Culture Collection Centre. An overnight culture of *G. klebahnii* was prepared in MYPG broth at 30°C and inoculated onto plates by swabbing a 1 inch by 1.5-inch area in the center of each plate. The plates were incubated at 30°C for 48 h. The growth in each plate was then overlaid with 10 mL of molten nutrient agar (0.7% agar) previously inoculated with 1 mL of the prepared test pathogen cultures. The agar was allowed to solidify and the plates were incubated aerobically at 37°C for 24 h and examined for growth inhibition (Maia et al., 2001).

Enzyme based screening

Phytase

Phytase assay was performed according to the method described by Vohra and Satyanarayana (2001) using phytate minimal medium (g/L: glucose 15.0, sodium phytate 5.0, $(NH4)_2SO_4$ 5.0, $MgSO_4.7H_2O$ 5.0, KCl 5.0, $FeSO_4.7H_2O$ 0.01, $MnSO_4.4H_2O$ 0.01 and agar 20.0).

The plate was incubated at 30°C for 48 h. After incubation, the plate was flooded with 2% cobalt chloride and then kept at room temperature for 5 min. After removing cobalt chloride solution, a freshly prepared solution containing equal volume of 6.25% ammonium molybdate and 0.42% ammonium vanadate was flooded onto the plates. They were incubated at room temperature for 5 min after which, the solution was removed. The plates were observed for zone of hydrolysis.

Inulinase

Inulin (1%) and phenol red (0.01%) was incorporated into medium with the following components, g/L: NH_4NO_3, 2.3; $(NH_4)_2HPO_4$, 3.7; KH_2PO_4, 1.0; $MgSO_4$, 0.5; yeast extract, 1.5. The pH of the medium was adjusted to 7.3-7.4 by using 1 N NaOH. Plate was point inoculated with a fresh culture of *G. klebahnii* and incubated at 37°C for 48 h. It was then observed for change in color from red to yellow around the colony of *G. klebahnii*.

Vitamin B_{12} assay

To determine vitamin B_{12} production, *G. klebahnii* was streaked on vitamin B_{12} assay medium, containing all the vitamins except vitamin B_{12}, followed by incubation at 37°C. The growth of the organism on the assay plate was taken as positive for vitamin B_{12} production.

Exopolysaccharide production

Exopolysaccharide production assay was performed according to the methods described by Mora et al. (2002). Freshly activated culture of *G. klebahnii* was streaked on the surface of plate containing ruthenium red milk agar (g/L: skim milk powder 100.0, sucrose 10.0 and ruthenium red 0.08 and agar 20.0). After incubation at 37°C for 24 h, the plate was observed for white (exopolysaccharide producing) or red (non-exopolysaccharide producing) colony.

Cholesterol reduction assay

The ability of *G. klebahnii* to assimilate cholesterol was determined according to the method described by Searcy and Bergquist (1960). The percentage assimilation was calculated using the formula:

Assimilation (%) = [(Concentration of cholesterol in control - Concentration of cholesterol in sample) / Concentration of cholesterol in control] x 100

Safety assessment

Gelatinase production

Gelatinase production was determined by point inoculating culture of *G. k*lebahnii on plate containing tryptone-neopeptone-dextrose (TND) agar (g/L: tryptone 17.0, neopeptone 3.0, dextrose 2.5, NaCl 5.0, K_2HPO_4 2.5 and agar 20.0), supplemented with 0.4% gelatin. The plate was incubated at 37°C for 48 h and was then flooded with saturated ammonium sulfate solution. Development of clear zones around the spots against the opaque background indicated a positive reaction (Gupta and Malik, 2007).

DNase production

DNase agar medium (HiMedia) was used to check production of DNase enzyme and was streaked with *G. klebahnii* culture, followed by plate incubation at 30°C for 48 h. After incubation, a clear pinkish zone around the colonies against dark blue background was considered positive for DNase production (Gupta and Malik, 2007).

Statistical analysis

All the experiments were performed in triplicate. The data were assessed using analysis of variance (ANOVA) with a level of significance at $p < 0.05$. The results are presented as means ± standard division. All statistical analysis was performed using 'Design Expert 6.0' software (Stat-Ease, Inc., Minneapolis, MN, USA).

RESULTS AND DISCUSSION

Morphological characteristics

Colonies on MYPG medium were white-cream, slightly raised, circular, fimbriate margins, with velvety appearance. Under microscope, septate hyphae and cylindrical individual cells were observed.

Acid and bile salt tolerance of *G. klebahnii*

Probiotics after ingestion are subjected to and need to survive the unfavourable physiological conditions of the gastrointestinal tract such as the extremely acidic environment and the detergent effect of bile secretions. The pH of the human stomach is around 1.5-3.5; whereas the physiological concentration of bile salts in the small intestine is between 0.2 and 2.0% (Gunn, 2000; Sahadeva et al., 2011). Sufficient survival of the probiotic microorganisms through the transit to GI tract is crucial to confer any health benefits on the host. In this investigation, *G. klebahnii* showed high acid tolerance with survival rate of 100 ± 1.5%, 100 ± 2.3% and 100.37 ± 1.8% at pH 3, 2.5 and 2, respectively, after 5 h of incubation at 37°C. It was also highly tolerant to bile salt with a survival rate of 100 ± 0.5%, 100 ± 1.1% and 100 ± 0.9%, at 0.3, 0.5 and 1.0% oxbile, respectively, after 5 h of incubation at 37°C.

Comparison of growth at 30 and 37°C

For a probiotic organism, it is also necessary to grow at human body temperature of 37°C and *G. klebahnii* showed comparable growth at both temperatures (37 and 30°C). While most *S. cerevisiae* strains grow and metabolize at a temperature of 30°C, *S. boulardii* is a thermotolerant yeast that grows optimally at 37°C (Czerucka et al., 2007).

Autoaggregation and cell surface hydrophobicity of *G. klebahnii*

Adhering potency of probiotics to the intestinal tract of

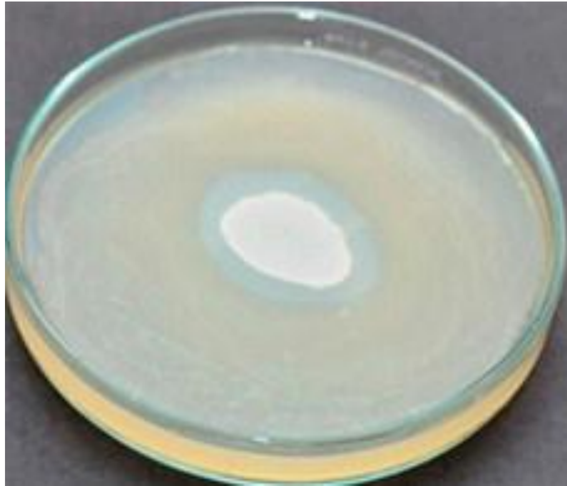

Figure 1. Plates showing zones of inhibition against *Staphylococcus aureus.*

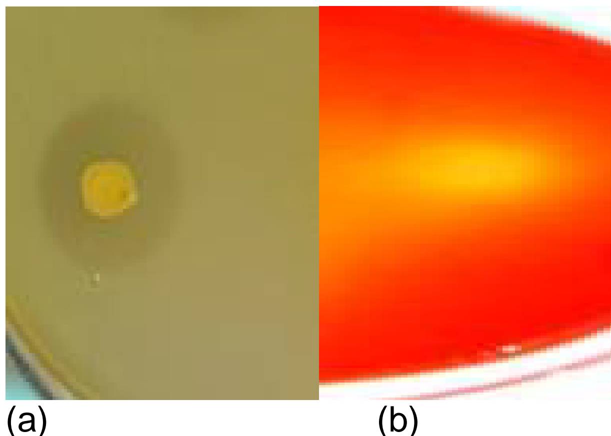

Figure 2. Plates showing enzyme production. (a) Phytase and (b) Inulinase.

host is believed to be crucial in order to ensure their maintenance in the intestinal tract for a longer period of time. Adhesion is initially based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesions (usually proteins) and complementary receptors. Aggregation ability is one of the factors related to cell adherence properties. It has been reported that auto aggregation ability above 80% is considered to be strong auto-aggregation (Radulovic et al., 2010). In this investigation, *G. klebahnii* showed 94.31 ± 3.5% autoaggregation ability, after 3 h of incubation at 37°C which further increased to 100.00 ± 1.5% after 20 h of incubation. Cell surface hydrophobicity is another factor responsible for adhesion to host tissues. In this study, in vitrocell surface hydrophobicity was observed to be 36.43 ± 0.7% and 52.13 ± 1.5%, with xylene and n-hexadecane, respectively. This method has been reported to be qualitatively valid to estimate the ability of a strain to adhere to epithelial cells (Pizzolitto et al., 2012; Keily and Olson, 2000). Isolates having good autoaggregation ability in conjunction with the good hydrophobicity values can strongly be related to the adhesion ability of these microorganisms. Though these traits are independent of each other; they are still related to adhesion property of a particular microbe (Rahman et al., 2008).

Antimicrobial activity

Probiotics might prevent infection by competing with pathogens for binding sites on epithelial cells (O'Sullivan et al., 1992). Probiotics might also inhibit the growth of pathogenic bacteria by producing bacteriocins such as nisin (Del Miraglia and De Luca, 2004). Antibacterial properties are one of the most important selection criteria for probiotics. In this study, *G. klebahnii* had antimicrobial activity against *Salmonella* sp., *Vibrio* sp. and *Staphylococcus aureus*. Antimicrobial activity was observed as a clear zone of inhibition (16-36 mm diameter) around the colony of *G.klebahnii* (Figure 1). *S. boulardii* have been shown to protect against various enteric pathogens and members of the family Enterobacteriaceae in animal studies (Czerucka and Rampal, 2002) such as *E. coli*, *Shigella*, *Salmonella typhimurium*, *Pseudomonas aeruginosa, S. aureus* and *Entamoeba histolytica.*

Enzyme based screening

Probiotics improve host digestive processes by producing extracellular enzymes. Phytases are nonspecific phosphatase enzymes, which catalyze the dephosphorylation of phytate and release free inorganic phosphate (Pi) and inositol phosphate esters (Vucenik and Shamsuddin, 2006). Phytic acid or phytate, the primary storage form of phosphorus in mature seeds of plants, has antinutritional properties since it has a strong chelating capacity and lowers the bioavailability of divalent ions such as iron, zinc, calcium and magnesium by forming insoluble complexes and also can negatively influence the functional and nutritional properties of proteins such as digesting enzymes (Reddy and Pierson, 1994). Inulinases catalyse the hydrolysis of inulin, producing inulo-oligosaccharides, fructose and glucose as main products. Inulo-oligosaccharides are prebiotics and their positive effect on human health has been widely acknowledged (Vohra and Satyanarayana, 2003). In this study, *G. Klebahnii* was observed to produce phytase (Figure 2a) and inulinase (Figure 2b).Several fungi such as *F. verticillioides*, *Aspergillus* sp., *Penicillium* sp. and *Emericella* sp., have been r eported to be phytase

Table 1. Probiotic properties of *G. klebahnii.*

Acid tolerance	100% tolerant at pH 2-3
Bile salt tolerance	100% tolerant at 0.3-1.0% oxbile concentration
Ability to grow at 37ºC	Yes
Autoaggregation ability	100% after 20 h of incubation
Cell surface hydrophobicity	36.43 ± 0.7% with xylene and 52.13 ± 1.5% with n-hexadecane
Antimicrobial activity	Inhibited the growth of *Salmonella* sp., *Vibrio* sp. and *Staphylococcus aureus*
Enzymes produced	Phytase and inulinase
Vitamin B_{12} assay	Positive
Exopolysaccharide production	Positive
Ability to assimilate cholesterol	29.42 ± 2.1%
Gelatinase and DNAse production	Negative

producers (Marlida et al., 2010). Mughal et al. (2009) reported 45.65 IU/mL inulinase production from *G. candidum*.

Vitamin B_{12} assay

Another benefit to human health is the ability to provide growth factors such as vitamin B_{12} (Abramov et al., 2003), which play a key role in the normal functioning of the brain and nervous system and for formation of the blood. Most bacteria and yeasts produce vitamin B_{12}. There are few fungi with the ability to synthesize vitamin B_{12}, *Agaricusbisporus* being one of them. In this investigation, *G. klebahnii* was observed to produce vitamin B_{12}, which has not been reported till now. Whole yeast cells of *S. cerevisiae* have nutritive value since they are an excellent source of amino acids, good source of mineral and vitamin B complex (Yamada and Sgarbieri, 2005)

Exopolysaccharide production

Exopolysaccharides (EPSs) are exocellular polymers are thought to play a role in protection against desiccation, toxic compounds, bacteriophages, osmotic stress and to permit adhesion to solid surfaces and biofilm formation (Vuyst and Degeest, 1999). Another physiological benefit is that EPS is retained longer in the gastrointestinal tract, so that colonization by the probiotic microorganisms can be enhanced. EPS has immunostimulatory and anti-tumoral activities, and phosphate groups in EPS play an important role in the activation of macrophages and lymphocytes (Uemura et al., 2003). In this study, *G. klebahnii* was observed to be positive as its colony appeared white against red background. EPS production by fungi including *Alternaria* sp., *Aspergillus* sp., *Botrytis* sp., *Cladosporium* sp., *Mucor* sp., *Ganoderm alucidum, Agaricus blazi,* Cordyceps sp., *Lentinus edodes*, *G. frondosa* and many others have been reported (Mahapatra and Banerjee, 2013).

Cholesterol reduction

High levels of serum cholesterol have been associated with the risk of coronary heart disease and also in inducing colon cancer. It has been recorded that even 1% reduction in cholesterol can reduce the risk of cardiovascular diseases by 2-3% (Manson et al., 1992). In this study, *G. klebahnii* was found to assimilate up to 29.42 ± 2.1% cholesterol thus can be considered as a promising probiotic agent. One study in hypercholesterolemic mice showed that administration of low levels of the probiotic bacteria *Lactobacillus reuteri* for 7 days decreased total cholesterol and triglyceride levels by 38 and 40%, respectively, and increased the high-density lipid : low density lipid ratio by 20% (Taranto et al., 1998). The cholesterol-lowering potential of *L. acidophilus* has been widely studied (Lin et al., 1989).

Gelatinase activity and DNase production test

According to FAO/WHO (2002) every probiotic strain needs to be assessed for safety to be used as a food or feed supplement. Gelatinases (MMP-2 and MMP-9) are MMPs capable of degrading almost all ECM and basement membrane components and might provide suitable substrate for further activity of human gelatinases or other bacterial proteinases (Zhao et al., 2011). Extracellular DNase provides a growth advantage to the pathogen by enlarging the pool of available nucleotides by DNA hydrolysis helping in the dissemination and spread of the pathogen by liquifying pus and also aids the evasion of the innate immune response by degrading neutrophil extracellular traps (NETs) (Hasegawa et al., 2010). A microorganism should not produce these enzymes so as to be used as a probiotic in food and feed. In this investigation, *G. klebahnii* was found to be negative for gelatinase and DNase activity even after the longest incubation period, validating their relative safety as probiotic candidates. A summary of the probiotic properties of *G. klebahnii* is presented in Table 1.

In conclusion, *G. klebahnii* isolated from cheese has been observed to have excellent qualities of a probiotic microorganism, such as its ability to survive conditions similar to the human gastrointestinal tract (acidic pH, high bile salt concentration, growth at 37°C, high auto-aggregation ability and cell surface hydrophobicity). With its great antimicrobial activity, it can be helpful in curing infections and reducing antibiotic use. It produces phytase enzyme that degrades phytate (an antinutrient), improves nutritional status of food and aids digestion. It also produces inulinase, an enzyme that produces inulo-oligosaccharides which have anti-cancerous effect. It also has the ability to produce vitamin B_{12}, exopolysaccharides (immunostimulants) and to assimilate cholesterol reducing chances of hypercholesterolemia and heart diseases. Being a non-gelatinase and non-DNase producer, it proves to be safe as a probiotic and possesses the ability to confer health benefits on the host.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Prevalence of antibiotic resistant *Salmonella* isolates, *Entermoeba histolytica* and *Giardia lamblia* in Harar, Eastern Ethiopia

Dinkineh Abebe Adimasu*, Ameha Kebede and Sissay Menkir

Ethiopian Health and Nutrition Research Institute, Bacteriology and Mycology Research Case Team, Haramaya University P.O.Box: 12 42, Harar, Ethiopia.

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The objective of this study was to investigate the prevalence and to track associated risk facters of antibiotic resistant *Salmonella*, *Entamoeba histolytica* and *Giardia lamblia* in Harar, Easthen Ethiopia. A total of 384 stool samples were collected from Harar Hiwot-Fana Hospital and analyzed in Harari Regional Laboratory. The results of the study show that 96 (25%), 80 (20.6%) and 56 (14.6%) of the samples were positive for *Salmonella*, *E. histolytica* and *G. lamblia*, respectively. The antimicrobial sensitivity test showed that all (100%) of the *Salmonella* isolates were sensitive to ciprofloxacin while 85% were sensitive to nalidixic acid. Of the 56 (14.6%) *Salmonella* isolates, 100, 100, 85 and 71.2% were resistant to ampicillin, tetracycline, trimethoprim-sulfamethoxazole and chloramphenicol, respectively. This study indicates that *Salmonella*, *E. histolytica* and *G. lamblia* were prevalent in Harar and this enterophathogens should be considered routinely in the diagnosis of patients with diarrhoea. Moreover, physicians should also prescribe appropriate drugs either after sensitivity testing or in areas where there are no facilities for culturing; they have to refer updated information on local sensitivity patterns.

Key words: Prevalence, *Salmonella*, *E. histolytica*, *G. lamblia*, antibiotic resistance, diarrhoea, Entropathogens.

INTRODUCTION

Infectious gastrointestinal illnesses cause significant morbidity, mortality and socioeconomic burden worldwide (Guerrant et al., 2002). *Salmonella*, *G. lamblia* and *E. histolytica* are the most common aetiological agents of human diarrhoeal diseases worldwide, and account for a significant proportion of morbidity and mortality in developing countries (Heyworth, 1992). It is estimated that up to two hundred million people are chronically infected with *Giardia lamblia* globally, and 500,000 new cases are reported annually (WHO, 1998). The prevalence of the disease varies from 2 to 5% in developed and from 20 to 30% in developing countries (Flanagan, 1992).

Amebiasis is also one of the world's most prevalent and

*Corresponding author. E-mail:abebedinkineh@gmail.com. Tel: 251920460564, +25111 2 13 34 99. Fax: +00251 1 2754744 / 757722.

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fatal infectious diseases. Next to malaria and schistosomiasis, amebiasis ranks third on the list of parasiticcauses of death worldwide (Walsh, 1986). Around 500 million peoples are infected worldwide while 75,000 die of the disease annually (Walsh, 1986). Salmonellosis causes more disease burden than any other food borne pathogen worldwide. An estimated 93.8 million cases of gastroenteritis caused by *Salmonella* species occur globally each year (Majowicz et al., 2010). The other major epidemiological development in Salmonellosis is the emergence of multiple-antibiotic resistant *Salmonella* in the developing countries (Okeke et al., 2005).

In Ethiopia, various studies invariantly concluded that diarrhoeal disease is the cause of morbidity and mortality among infants, childrens, adults and elder WHO (2005). About 39 million episodes of diarrhoea per year were estimated to occur in Ethiopia out of which 230,000 would be children below five years of age and these would result to death (WHO, 2004). Harar is one of ancient and historical towns in Eastern Harerge, Ethiopia. In Eastern Hararge, particularly in Harar town and the surroundings, risk factors which lead to gastrointestinal infections are common.

According to Health Management Information System (2010), annual records of Harar Hiwot Fana Hospital revealed that the incidenceof diarrhoeal disease was very high. There is, however, information gaps on major aetiological agents of diarrhoeal diseases in the study area.

Therefore, the objective of this study was to determine the prevalence of three entropathogens (*Salmonella, E. histolytica* and *G. lamblia*) and evaluate the drug resistance patterns of *Salmonella* isolates from diarrhoeal patients in Hiwot Fana Hospital.

MATERIALS AND METHODS

Description of the study area

The study was conducted in the Harar Hiwot Fana Hospital in Harar town, Eastern Ethiopia which is located 525 km to the east from Addis Ababa. It has an altitude of 1850 m-above sea level with 596 mm mean annual rainfall and 24.02°C average annual temperature. Hiwot Fana Hospital was selected purposefully for this study due to its high patient influx.

Study design

A hospital based cross sectional study was conducted to determine the prevalence of three entropathogens. Data was collected using apre-tested structured questionnaire and laboratory based diagnosis. A pre-tested structured questionnaire was used to collect data regarding socio-demographic factors and risk factors of the study subjects. Standard bacteriological and parasitological techniques were used at the Harer Reginal Laboratory (HRL), to detect the presence of *Salmonella* species, *E. histolytica* and *G. lamblia.*

Study population

All patients who came to the out patients departments (OPD) of Hiot Fana Hospital (HFH) with complaints of diarrhoea were enrolled. In this study, patients less than 15 years of age were considered as children and their stool samples were collected with the help of their parents / care takers.

Sample size determination

Since there was no previous investigation conducted on the same title in the study area, a P value of 0.5 was taken to ensure the sample size was large enough to satisfy the precision and confidence constraints. By taking this into consideration, the sample size for the unknown population was calculated based on the 95% confidence limits and 5% sampling error using a formula described by Hassan (1991).

$$n = \frac{(Z\ \alpha/2)^2\ P(1-P)}{d^2}$$

Where: n = number of sample size, P = prevalence of *Salmonella*and selected intestinal protozoan parasites, d = marginal error between the sample and the population, $Z_{\alpha/2}$ = Critical value at 95% certainty (1.96), considering 5% non responsive rate. Therefore, the calculated sample size for this study was 384.

Sampling method

A serial sampling method was used where all patients coming to the OPD with diarrhoeal cases were recruited as they came until the required sample size was reached.

Specimen collection, handling and transport

Before collection of stool samples, patients above 15 years of age and children's parents or care-takers were given orientation on how to take samples.The laboratory technician also provided them with materials to place the stool specimens. In addition, about 2 g of fresh stool sample were collected by the principal investigator and assistant data collectors together from each study subject on the same day of enrolment. Each sample was labeled with the code on the corresponding patients' questionnaire. Finally, the collected stool specimens were delivered to the Harari Regional Laboratory, which is very close to HFH, without using transport media for analysis on the same day.

Culture and isolation procedures of *Salmonella*

About 1 g of stool sample was added in a tube containing sterile saline solution to prepare a faecal suspension. A loopfuls of feacal suspension was transferred to 9 ml selenite broth and incubated aerobically at 37°C for 18 h. A loopful of faecal suspension from selenite broth was sub-cultured on xylose lysine deoxycholate citrate (XLD) agar and incubated at 37°C in aerobic incubator for 18 h.

Typical colonies with black centres and a lightly transparent zone with a reddish color were considered as presumptive *Salmonella* species. Typical colonies with the above morphology were further confirmed by urease test, triple sugar iron agar, kligler iron agar test and lysine iron agar motility test, indole test and citrate utilization test. *Salmonella* species are urease negative, citrate positive, LIA positive, motile, indole negative and yields a red slope (alkaline)

Table 1. Distribution of the study population (n = 384) by age group and sex that was examined for the presence of *Salmonella* species, *E. histolytica* and *G. lamblia*.

Age group (years)	Male	Female	Total
0.25 - 4	96 (46.2%)	80 (45.5%)	176 (45.8%)
5-14	64 (30.8%)	56 (31.8%)	120 (31.3%)
≥15	48 (23.1%)	40 (22.7%)	88 (22.9%)
Total	208 (54.2%)	176 (45.8%)	384 (100%)

and yellow (acid) butt with/out gas or H_2S production (Cheeseborough, 2006).

Antibiotic susceptibility tests for *Salmonella*isolates

The standard Kirby-Bauer disk diffusion test (Bauer et al., 1966) was used to determine the antimicrobial sensitivity profiles of the *Salmonella* isolates. Broad spectrum antimicrobial agents were chosen since they are frequently prescriped by general practitioners to humans suffering from salmonellosis, after visiting the Hiot Fana Hospital. These antibiotics include ampicillin, tetracycline, trimethoprim-sulfamethoxole, chloramphericol, nalidixic acid and ciprofloxacin.

Nutrient broth inoculated with *Salmonella* isolates were used to prepare bacterial suspensions that was compared to a turbidity of a 0.5 McFarland standard. Mueller-Hinton agar plates were spread-plated with these bacterial suspensions. Different antimicrobial agent containing disks were placed in intimate contact with the cultures on the inoculated plates. The plates were incubated at 35°C for 18 h. The diameters (in millimeters) of the clear zones of growth inhibition around the antimicrobial disks were measured. Sensitive strains of E*scherichia coli* (Amrical type Culture Collection 25922) were used as a negative control in this experiment. The break points used to categorize isolates as resistant, intermediate resistant and sensitive to each antimicrobial agent were based on recommendations proposed by the Clinical and Laboratory Standard Institute (NCCLS, 2000).

Detection of *E. histolytica* and *G. lamblia*

Direct wet mount method

The direct wet mount with 0.85% saline solution was prepared in the laboratory and observed for motile trophozoites of *E. histolytica* and *G. lamblia* under light a microscope at 10X and 40X magnifications. Lugol's iodine staining was also used to observe cysts of *E. histolytica* and *G. lamblia*.

Concentration method

A portion of stool samples was processed using the formalin-ether concentration method (Bello, 2002). The stool sample was sieved with cotton gauze and transferred to 15 ml centrifuge tube. Then 8 ml of 10% formalin and 3 ml of diethyl ether were added and centrifuged for 2 min at 2000 rpm. The supernatant was decanted and the residues were transferred to microscope slides and observed under light microscope at 100X and 400X magnifications for the presence of cysts and trophozoites of *E. histolytica* and *G. lamblia* (Bello, 2002).

Data analysis

Quantitative data that were generated from questionnaire survey about associated risk factors, socio-demographic factors and clinical features and laboratory data of the three entric pathogens were entered into a computer using statistical package for social science (SPSS) (ver. 12.0) data analyzing software.The Chi-square test was done to associate *Salmonella*, *E. histolytica* and *G. lamblia* positivity with risk factors and observed clinical features. A p-value of < 0.05 was considered to indicate statistically significant differences. In the mean time descriptive statistic cross-tabulation of SPSS 12 version was used to analyze the distribution of study subjects by age and sex, and the frequency of positive results under each age and sex categories. Zone of inhibition differences between antibiotics for *Salmonella* isolates was calculated in comparison with the inhibition zone produced by the positive control strain *E. coli* (ATTC25922) and this was used to interpret the antimicrobial resistance of *Salmonella*.

Ethical clearance

First, the proposal was reviewed and approved by the ethical review committee of the College of Health Science, Haramaya University. Institutional consent was obtained through communication with Harari Reginal Healthy Bureau before conducting the study. The participation of patients, however, was planned to be purely a voluntary activity and they were clearly informed that about the purpose of the research issues of confidentiality and anonymity were also maintained.

RESULTS AND DISCUSSION

Distribution of the study population

During a six month study period, stool samples of three hundred eighty four (n=384) diarrhoeal patients who attended the out patient department (OPD) of Hiwot Fana Hospital (HFH) were examined for the presence *Salmonella* species, *E. histolytica* and *G. lamblia*. The distribution of the study population by sex and age is shown in Table 1. Data obtained from the present study is of epidemiological value to the study area and therefore the criteria to determine age groupswas based on Harari Regional Health Bureau data record system on diarrhoeal diseases prevalance, where 0.25 to 4, 4 to 14 and ≥15 age groups are reported as under five children, young children and adults, respectively. Data obtained in the study reveals that the diarrhoeal diseases in the HFH were more common among individauls in between three months to four years of age. This is in line with data obtained from most studies worldwide which indicate that children suffer from diarrhoeal diseases more than adults

Table 2. Prevalenceof *Salmonella* species, *E. histolytica* and *G. Lamblia* among the study population (n=384) by age and sex.

Age group and sex	Number examined		*Salmonella* species		*E. histolytica*		*G. lamblia*	
	Frequency	%	No. Positive	% Positive	No. Positive	% Positive	Positivity	% Positive
0.25-4 years								
Male	96	46.2	17	17.7	24	25	27	28.1
Females	80	45.5	15	18.7	26	32.5	29	36.25
Total	176	45.8	32	18.2	50	28.4	56	31.8
5-14 years								
Males	64	30.8	9	15.6	15	23.4	8	12.5
Females	56	31.8	7	10.7	15	26.7	8	14.2
Total	120	31.3	16	13.3	30	25	16	13.3
≥15years								
Males	48	23.1	4	8.3	9	18.7	5	10.4
Females	40	22.7	4	10	7	17.5	3	7.5
Total	88	22.9	8	9.1	16	18.8	8	9
Total for all age groups	384	100	56	14.6	96	25	80	20.8

due to a lower immune status (Workmanet al., 2006; WHO, 2011).

Prevalence of *Salmonella* species, *E. histolytica* and *G. lamblia*

The number and percentage of stool samples that were positive for the different enteropathogens based on culture and microscopic examination are shown in Table 2. In general, a total of 232 (60.41%) enteropathogens were detected and/or isolated. Among these the proportion of *E. histolytica* 96 (25%) was higher than that of *G. lamblia* 80 (20.6%) and *Salmonella* species 56 (14.6%). As shown in Table 2, intestinal protozoan parasites (*E. histolytica and G. lamblia)* were more frequently isolated than enteric *Salmonella* species

In the present study, the prevalence of *E. histolytica* reported at 25% was higher than those (8.8, to 18.5%) reported in previous studies (Getenet, 2008; Bayeh et al., 2010) conducted in North West Ethiopia, respectively. These differences imply the endemicity of *E. histolytica* in the study area because of so many associated risk factors of the pathogen. However, the data obtained in the current study is lower than prevalence reports of 38 and 34.2% obtained in a rural area in Eastern Ethiopia (Dawit, 2006) and the Vhembe district, South Africa (Samie et al., 2009) respectively. It is therefore suggested that differences in the settings and designs used in these studies may account for the patterns observed. In the current study, hospital based descriptive crossectional survey was employed compared to the previous reports that involved community based logitudinal approaches (Dawit, 2006; Samie et al., 2009).

Similarly, high level of giardiasis was observed in this study. Out of the 384 diarrhoeal patients, 80 (20.6%) were positive for *G. lamblia.* The finding of this study is higher as compared to 9.3% reported from Addis Ababa preschool children (Seyoum et al., 1981), 5.8% reported from Jimma University Hospital and Some Selected Health Centers in Addis Ababa (Getenet, 2008), 7.0% reported from Bahir Dar Town, North West Ethiopia (Bayeh et al., 2010) and 12.8% reported from Vhembe District, South Africa (Samie et al., 2009). The high prevalence in this study might be attributed to the endemicity of Amebiasis in the study area because of many risk factors which predispose the dwellers in Harar and the sourrounding. However, the finding of this study is in agreement with 21.43% reported from orphanage centers Addis Ababa, Ethiopia (Sintayehu, 2010). One would expect the rate of giardiasis to be higher in developing countries when compared to developed countries but unfortunately most individuals are usually asymptomatic (Gilman et al., 1988). Patients with asymptomatic *Giardia* infections may go unidentified and serve as carriers who potentially transmit the pathogens to healthy individuals (U.S. EPA, 1989).

The proportion of *Salmonella* species (14.6 %, 56/384) detected from diarrhoeal individuals in the out-patient unit in this study was comparable with a previous study that was conducted at Jimma University Specialized Hospital (Abebe, 2002). On the contrary, these results were higher than prevalence rates of 2.9% reported in Djibouti (Mikhail et al., 1990), 9.2% in Manila, Philippines (Adkins et al., 1987), 3.3% in Lagos, Nigeria (Ogunsanya ,1994), and 4.5 to 10.9% in Addis Ababa, Ethiopia (Afeworki, 1985). This increased prevalence of *Salmonella*in HFH may indicate that poor sanitary practices are highly common among individuals in the study area.

As indicated in Table 2, the enthropathogenic organisms investigated were isolated in all age groups. Moreover, patients below 15 years of age were positive for

these enthropathogenic organisms that are associated with diarrhea. About 18.2 and 13.3% positivity of the *Salmonella* isolates were seen in young children between the ages of 0.25 - 4 years and adult children of 5-14 years old, respectively. However, 9.1% isolates were seen in patients who were ≥15 years old. The prevalence of *E. histolytica* was also shown to vary between different age groups. Hence, 28.4 and 25% positivity was obtained from age groups 0.25 to 4 years and 5 to 14 years, respectively. However, 18.8% positivity was obtained from age groups ≥15years.The 31.8 and 13.3% positivity of *G. lamblia* from young children and adult children, respectively is higher than the 9.1% positivity from ≥15 year of age catagories (Table 4).

The significant variations in the prevalence of *Salmonella* species, *E. histolytica* and *G. lamblia* based on age groups are similar to other previous studies (Workman et al., 2006; Sorokin et al., 2007). In the previous studies and the current one, the general trend is that symptomatic enteric infections frequently affect children younger than 15 years of age and incidences decline with age, particularly in developing countries (Workman et al., 2006; Sorokin et al., 2007).

The distribution of *Salmonella* species, *E. histolytica* and *G. lamblia* infection among male and female patients revealed statistically comparable frequency. In the mean time, the prevalence of all three entropathogens was shown to have comparable result under each age categories (Table 2). This implies that both sexes were equally at risks for acquiring and suffering from *Salmonella, E. histolytica* and *G. Lamblia*in fections in the study area.

Risk factors associated with *Salmonella*, *E. histolytica* and *G. lamblia* infections in the study area

The quantitative data that was generated from questionnaire survey and laboratory results showed that infections caused by the three enteric pathogens is significantly associated with the absence of toilet facilities, the consumption of raw milk, consumption of raw vegetables and fruits, the common usage of mass catering foods outlets and the consumption of street vended foods products. Moreover, the possession of domestic animals and the cohabitation with animals were significantly associated with cases of *Salmonella* and *G. lamblia* infections among patients investigated. Statistically significant associations were also found between infection with *Salmonella* and consumption of raw meat (Tables 3A and B)

In the present study, there was a significant correlation beteween the absence of laterine and infections with all three pathogens. According to Curtis and Cairncross (2003), there was high association between the risk of contracting salmonellosis, amoebiasis and giardiasis with poor living and housing conditions.

G. lamblia and *Salmonella* infection were significantly associated with the presence and co-habitation of domestic animals. This finding agrees with the fact that *G. lamblia* and *Salmonella* are important human and animal pathogens worldwide and animals are the reservoir for these enteric pathogens (Hoelzer et al., 2011). Unlike *G. Lamblia* and *Salmonella*, *E. histolytica* did not show association with the presence and cohabitation of domestic animals. The possible explanation for this finding is that the potential sources and reservoir for this enthropathogen is human being but not domestic animals.

In the present study, there was a significant association between the consumption raw milk and the presence of *Salmonella*, *E. histolytica* and *G. lamblia* infections in the patients. Therefore, patients who consumed raw milk were at risk of presenting with salmonellosis, giardiasis and amebiasis. This is similar to a previous finding (Jayarao et al., 2006) who reported that in Pennsylvania, the occurence of infections caused by enteric pathogens in humans was as a result of the consumption of raw milk. However, in some studies, the consumption of raw meat and meat products has been identified as the principal cause of increased *Salmonella* gastroenteritis worldwide (WHO, 1988; Oliveira et al., 2002; Haeghebaert et al., 2001; Fey et al., 2000). The finding of this study is in line with previous findings. Both *E. histolytica* and *G. lamblia* revealed statistically insignificant association with consumption of raw meat.

In this study, there was a significant correlation between salmonellosis, gardiasis and amoebiasis with the consumption of raw vegetables and fruits from unhygienic sources (Table 5). Different works also showed that *Salmonella*, *E. histolytica* and *G. lamblia* are frequently isolated from raw fruits and vegetables (Robertson and Gjerde, 2001).

Reports of food borne diseases outbreaks in various countries have resulted from unhygienic food handling and preparation practices within food establishments (CDC, 2010). Bayeh et al. (2010) reported that 41.1% out of of 384 food handlers working in different food establishments of Bahir Dar Town, North West Ethiopia had intestinal parasites and 6 (1.6%) were found positive for *S. typhi*. In this study, infection of *Salmonella*, *E. histolytica* and *G. lamblia* were revealed to have high statistical significance with consumption of food from catering establishments. This may be the implication of poor sanitary condition of mass catering food establishments in the study area.

In this study the prevalence of *Salmonella, E. histolytica* and *G. lamblia* was highly associated with the consumption of street vended foods which is in line with Feglo et al. (2004) report from Ghana, Accra. The possible explanation for this finding is that street foods in Harar Town, Eastern Ethiopia are sold under unhygienic conditions, with limited access to safe water, sanitary services, or garbage disposal facilities.

Table 3A. The association between risk factors and prevalence of *Salmonella*, *E. histolytica* and *G. lamblia.*

Risk factor	*Salmonella* spp.			*E. histolytica*			*G. lamblia*		
	Number of isolates	P-value	X^2 value	No. positive	P-value	X^2 value	No. positivity	P-value	X^2 value
Residence									
Urban	24 (15.0%)	0.845	0.38	38 (22.8%)	0.735	0.326	32 (20.0%)	0.734	24.326
Rural	32 (14.3%)			58 (26.9%)			48 (21.4%)		
Educational level									
Literate	24 (14.3%)			43 (23.8%)			32 (19.0%)		
Illiterate	32 (14.8)	0.884	0.21	53 (25.9%)	0.635	0.226	48 (22.2%)	0.447	0.577
Latrine									
Present	16 (8.7%)	0.02***	9.831	32 (17.4%)	0.001***	10.908	24 (13.0%)	0.001***	12.98
Absent	40 (20.0%)			64 (32.0%)			56 (28.0%)		
Water									
Protected	24 (14.3%)	0.884	0.21	46 (24.0%)	0.637	0.223	32 (17.4%)	0.111	2.538
Unprotected	32 (14.8)			50 (26.1%)			48 (24.0%)		
Domestic animals									
Present	40 (20.0%)	0.02***	9.831	48 (24.0%)	0.223	0.637	72 (36.0%)	0.000***	58.213
Absent	16 (8.7%)			48 (26.1%)			8 (4.3%)		
Domestic animals house									
Separate	8 (6.3%)	0.01***	10.704	45 (19.0%)	0.447	0.577	8 (6.3%)	0.00***	24.758
Cohabit	48 (18.8%)			51 (22.2%)			72 (28.1%)		
Raw milk									
Used	40 (17.9%)	0.031***	4.626	72 (32.1%)	0.000***	14.629	56 (25.0%)	0.017***	5.659
Unused	16 ((10.0%)			24 (15.0%)			24 (15.0%)		

Table 3B. The association between risk factors andprevalence of *Salmonella*, *E.histolytica* and *G. lamblia*

Risk factor	*Salmonella*			*E. histolytica*			*G. lamblia*		
	Number of isolate	P-value	X^2 value	Positivity	P-value	X^2 value	Positivity	P-value	X^2 value
Raw meat									
Used	48 (24.0%)	0.000***	29.712	58 (26.0%)	0.637	0.223	48 (24.0%)	0.111	2.538
Unused	8 (4.3%)			38 (24.1%)			32 (17.4%)		

Table 3B. Cont.

Raw vegetables and fruits									
Used	48 (20.7%)	0.000***	17.544	70 (31.0%)	0.001***	11.383	64 (27.6%)	0.000***	16.205
Unused	8 (5.3%)			26 (15.0%)			16 (10.5%)		
Use of mass catering establishments for foods									
Used	40 (24%)	0.012***	8.807	72 (34.6%)	0.000***	22.447	56 (26.9%)	0.000***	18.078
Unused	16 (14.3%)			24 (14.3%)			24 (10.1%)		
Consumption of street vended foods									
Used	40 (18.5%)	0.041***	6.393	68 (32.3%)	0.000***	18.963	52 (26.9%)	0.002***	12.409
Unused	16 (11.1%)			28 (12.1%)			28 (18.2%)		

***Significant at $p<0.05$.

Table 4. Clinical symptoms and their association with positivity of *Salmonella, E. histolytica* and *G. lamblia.*

Clinical symptom	*Salmonella*			*E. histolytica*			*G. lamblia*		
	Number of isolates	P-value	X^2 value	Number of positive results	P-value	X^2 value	Number of positive results	P-value	X^2 value
Fever									
Present	32 (9.7%)	0.001***	13.530	40 (29.4%)	0.139	2.186	32 (23.5%)	0.928	0.335
Absent	24 (23.5%)			56 (22.6%)			48 (19.4%)		
Vomiting									
Present	40 (23.3%)	0.02***	12.671	46 (13.3%)	0.640	0.219	24 (20.0%)	0.876	0.73
Absent	16 (11.2%)			50 (30.3%)			56 (21.2%)		
Abdominal pain									
Present	48 (16.7%)	0.000***	4.014	80 (27.8%)	0.029***	4.741	72 (25.0%)	0.000***	12.126
Absent	8 (8.3%)			16 (16.7%)			8 (8.3%)		
Duration of dairrhoea									
1-5	40 (25.0%)	0.000***	23.892	32 (28.6%)	0.000***	28.343	24 (18.3%)	0.001***	13.974
6-10	8 (7.1%)			56 (35.0%)			40 (26.0%)		
11-15	8 (7.1%)			8 (7.1%)			16 (14.3%)		
Consistence of stool									
Watery	16 (15.4%)			20 (15.3%)			32 (33.3%)		
Bloody	24 (25.0%)	0.004***	13.558	46 (48.3%)	0.000***	87.967	8 (8.3%)	0.000***	18.882
Mucoid	8 (8.3%)			8 (7.7%)			24 (23.1%)		
Mixed	8 (9.1%)			22 (20.3%)			16 (18.2%)		
Total	56 (14.6%)			96 (25.0%)			80 (20.6%)		

***Significant at $p<0.05$.

Table 5. The proportion of resistant, sensitive and susceptible *Salmonella* isolates (n=56) to six different antibiotics.

List of antibiotics tested against *salmonella* isolates	Antibiotics susceptibility profiles of *salmonella* isolates (n=56)			
	No. of resistant isolates	% of resistant isolates	No. of intermediate resistant isolates	% of intermediate resistant isolates
Ampicillin	56	100	0	0
Tetracycline	56	100	0	0
Chloramphenicol	40	71.4	6	10.7
Co-trimoxazole	48	85.7	4	7.14
Nalidixic acid	6	10.7	2	3.5
Ciprofloxacin	0	0	0	0

S = Sensitive, I = intermediate R = resistance.

As indicated in Table 5, the statistical analysis of residence, educational level and water with contracting salmonellosis, giardiasis and amebiasis revealed insignificant correlation.

Clinical features

The clinical features of *Salmonella* infections commonly present with bloody and/or watery diarrhoea, fever, head ache and abdominal cramping (Hohmann, 2001), which are similar to the findings of our study where abdominal pain and bloody and/or watery diarrhoea were the dominant symptoms of culture positive cases. The incubation period for *Salmonella* gastroenteritis is typically from 12 to 72 h (Hohmann, 2001), which is in agreement with this study, where the duration of diarrhoea (before visiting HFH) was between one to five days in the majority of patients (Table 6).

Persons who ingested *E.histolytica* cysts most of the time may not have any symptoms at all and may function only as carriers and spreaders, contaminating the areas wherever they go. The disease symptoms usually start after a period of 7 to 15 days of infection which is called the incubation period (Petri and Singh, 1999), which is in agreement with this study where the duration of diarrhea (before visiting HFH) was between 6 to 10 days in majority of patients. Bloody diarrhoea and abdominal pain are the major symptoms of amoebiasis (Petri and Singh, 1999), which is similar to our study where abdominal pain and bloody diarrhoea were the dominant symptoms of *E. histolytica* positive patients.

Person who ingested *G. lamblia*cysts may develop acute or chronic diarrhoeal illnesses in which the symptoms occur one to two weeks (average seven days) after swallowing the cysts, which is in agreement with this study where the duration of diarrhoea (before visiting HFH) was between 6 to 10 days in majority of patients. Watery stooland abdominal cramping are the most common clinical manifestation of giardiasis (Petri and Singh, 1999), which is in line with our study where abdominal pain and watery diarrhoea were the dominant symptoms of *G. lamblia* positive patients.

Test for antibiotic susceptibility of *Salmonella* isolates

Among 56 *Salmonella* isolates, 56 (100%), 56 (100%), 48 (85.7%), 40 (71.2%) and 6 (10%) were found to have developed resistance for ampicilline, tetracycline, trimethoprim-sulphamethoxazole (co-trimoxazole), chloramphenicol and nalidixic acid, respectively. None of the isolates were, however, resistant to ciprofloxacin. Intermediate susceptibility was found only in 6 (10.7%), 4 (7.14%) and 2 (3.5%) of the tested isolates against chloramphenicol, trimethoprim-sulfamethoxazole and nalidixic acid, respectively. Furthermore 56 (100%), 48 (85.7%), 10 (17.8%) and 4 (7.14%) tested isolates were susceptible to ciprofloxacin, nalidixic acid, chloramphenicol and trimethoprim-sulphamethoxazole, respectively.

In this study the prevalnce of resistant *Salmonella* isolates to tetracycline, ampicillin, trimethoprim-sulfamethoxazole and chloramphe-nicol is much higher than previous studies reported from Addis Ababa, Ethiopia (Afeworki, 1985). However, these observations are comparable to recent reports from Addis Ababa and North West Ethiopia (Mache, 1997). The percentage of isolates resistant to ampicillin, tetracycline, trimethoprim-sulfamethoxazole and chloramphenicol in the present study was also higher than those reported from Brazil, where only 88.8%, 86.4% 56.8% and 55.3 %of the isolates were found to be resistant, respectively (Ali et al., 2003). A comparable result in trimethoprim-sulfamethoxazole and Chloramphenicol resistance was reported from

Pakistan (Ali et al., 2003) where 86.8, 70.1% out of 54 *Salmonella* isolates were resistant to this drug, respectively. In contrast, all were found to be resistant to trimethoprimsulfamethoxazole in a

study at Mollorca, Spain, during the period 1987-1991 (Reina et al., 1994).

The high resistance to ampicillin (100%), tetracycline 100%), trimethoprim-sulfame thoxazole (85.7%) andchloramphenicol (71.2%) in this study might be due to misuse of these drugs because of their easy access and affordability to the public. In this study, however ciprofloxacin and nalidixic acid were found still to have high potency agnist *Salmonella* isolate in the study area, where all *Salmonella* isolates and 85% of *Salmonella* isolates were shown susceptibility to ciprofloxacin and nalidixic acid, respectively. The possible explanation for this finding is that may be ciprofloxacin and nalidixic acid are not frequently and unnecessarily prescribed or sold over the counter in the open markets and private pharmacies without prescription. Since these drugs are not easily affordable, everywhere in Hospitals and private pharmacies and in the market, people have no easy access to ciprofloxacin and nalidixic acid to purchase. This finding is in line with Ngo (2005) who conducted a study on the prevalence and risk factors associated with antibiotic resistance of bacteria from diarrhoeal patients in Bac Ninh Hospital Northern Vietnam. Ngo (2005) concluded that the cheapest and easily affordable drugs like ampicillin, tetracycline, trimethoprim-sulfamethoxazole and chloramphenicol are widely utilized in the community with or without prescription by health personnel and as a result, the selective pressure of these commonly used antibiotics on the bacteria circulating in the community could have resulted in high frequency of resistant pathogenic bacteria, of which *Salmonellais* is the one.

Conflict of interest

The authors declare that they have no conflict of interest.

Conclusion

The findings of this research indicated that *Salmonella*, *E. histolytica* and *G. lamblia* are important enteropathogens prevalent in young children (0.25-4 years of age) and adult children (4-14 years of age) followed by adults (15 and above years of age). Therefore, these entropathogens should receive significant attention in the diagnosis and control of diarrhoeal disease caused in the study area.

The associated risk factors in contracting thus pathogens were found to be lack of latrine, possession and cohabitation with domestic animals, raw milk consumption, consumption of raw meat, consumption of raw vegetables and fruits, consumption foods from mass catering establishment and consumption of Street vended foods.

This study has also shown that 100 and 85% of the total *Salmonella* isolates were sensitive to ciprofloxacin and nalidixic acid, respectively. They were found to be 100% resistant to ampicillin and tetracycline followed by trimethoprim-sulfamethoxazole (85.7%) and chloramphenicol (71.2%).

Recommendations

Decision makers should implements awareness creation to the community regarding to the associations between risk factors and contracting *Salmonell*, *E. histolytica* and *G. lamblia*. Regulatory body should pay due attention to strengthening compliance with good manufacturing practices by mass catering food establishments and to maintaining acceptable sanitary conditions in general, and food hygiene in particular. Access to standard sanitary facilities by the general public should receive consideration. Regulatory body should also intervene with the monitoring of the health status of sick food handlers working in food establishments. Further studies should be made to identify *Salmonella* at spp. and serotype level, so that comparison with serotypes isolated from animals/ food products could be possible, for identifying the sources of infection.

Ampicillin, tetracycline, chloramphenicol and trimethoprim-sulphamethoxazole should not be used as a drug of choice for the treatment of enteric *Salmonella* without making sensitivity tests prior to treatment. Since considerable amount of *E. histolytica* were detected, advanced microbiological techniques such as ELISA and PCR should be conducted to differentiate invasive (*E. histolytica*) from non-invasive one (*E. dispar*).

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Full Length Research Paper

Nodulation efficacy of *Bradyrhizobium japonicum* inoculant strain WB74 on soybean (*Glycine max* L. Merrill) is affected by several limiting factors

Ahmed Idris Hassen*, Francina L. Bopape, Isabella H. Rong and Galaletsang Seane

Agricultural Research Council, Plant Protection Research Institute (ARC-PPRI), Private bag X134, Queenswood 0121, Pretoria, South Africa.

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Lack of indigenous soil rhizobia that colonize and nodulate soybean (*Glycine max* L. Merrill) roots is a common problem of South African soils. *Bradyrhizobium japonicum* strain WB74 has long been used as an effective commercial inoculant strain for soybean since its introduction in 1998. This paper investigates the major limiting factors involved in the nodulation efficiency of *B. japonicum* WB74 that affect soybean growth and yield in South Africa. Methods including analysis of soil physicochemical properties, farmers' management practices and quality control tests of locally manufactured inoculant products were employed. Inoculant's strain verification was conducted using phylogenetic analysis of the 16S ribosomal RNA of the *Bradyrhizobium* strains in each of the inoculant product. The major findings of this study is that nodulation failure of introduced *B. japonicum* is caused by a combination of several limiting factors such as acidic nature of the soils as well as poor soil nutrition status especially that of phosphorous. Nodulation failure was also prominent in the soybean farms where there was no proper soil management practice. Results of the viability and shelf life studies also indicate that nodulation failure is caused by using sub-standard soybean inoculants products available on the market.

Key words: *Bradyrhizobium japonicum* WB74*,* inoculants, nodulation, quality control.

INTRODUCTION

Fertilizer nitrogen (N) is one of the major agricultural inputs worldwide to meet the nitrogen requirement of several plants. However, in addition to being expensive for most small scale and subsistence farmers, heavy use of N-fertilizer is both harmful to the environment and results in depletion of fossil fuels needed for the production of nitrogen fertilizers (Bohlool et al.,1992). An alternative and more sustainable process is biological nitrogen fixation (BNF) by a group of symbiotic bacteria called Rhizobia which fix atmospheric nitrogen (N_2) and make it available to plants (Hassen et al., 2012; Zahran, 1999; Bohlool et al., 1992).

Soybean [(*Glycine max* (L.) Merrill.)] is a legume of tropical to subtropical origin and is one of the most

*Corresponding author. E-mail: HassenA@arc.agric.za. Tel: +27 12 808 8192.

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important sources of food, feed and one of nature's most versatile plants (Keyser and Li, 1992). Inoculation of soybeans with rhizobia throughout the world is estimated to be in the range of 12 - 20 x 10^6 ha/year which results in the establishment of a large rhizobial population in the rhizosphere thereby enhancing improved nodulation and nitrogen fixation (Senevirante et al., 2000). South African soils are largely devoid of rhizobia strains that are able to colonize and nodulate soybean roots and therefore need inoculation of soybeans with effective rhizobia (Bloem, 1998). More than five decades of soybean cultivation in South Africa which involves the use of commercial inoculant strains of rhizobia has resulted in the establishment of populations of *Bradyrhizobium* sp. in the soybean rhizosphere (Botha et al., 2004).

The general principle is that inoculation with rhizobia for subsequent years is often not needed if a legume has a long history of cultivation in that particular area. This is because most of these soils are believed to contain the appropriate rhizobia (Sylvia et al., 2005). However, in areas where acidic soils and high temperature limit rhizobial survival between cropping seasons, re-inoculation is crucially required, which is true for most of the acidic soils in South Africa. Research conducted on biological nitrogen fixation in South Africa resulted in the screening and selection of competitive and high nitrogen fixing soybean inoculant strain *Bradyrhizobium japonicum* WB74 which is a synonym of the Australian strain CB1809. The strain was tested on 30 different soybean cultivars under different geographical locations in the field and proved to be highly competitive and effective in nodulating soybean on South African soils since its introduction in 1998 (Bloem, 1998). *B. japonicum* strain WB74 is currently stored at the South African Rhizobium Culture Collection (SARCC) and is routinely monitored for its viability, purity as well as genetic stability in nodulating soybeans.

Recently, however, there were several concerns as to the nodulation and nitrogen fixation efficiency of this strain due to reports of nodulation failure in various soybeans fields. The main objective of this research was therefore to investigate the major reasons for the reported failure of nodulation in soybeans by introduced commercial strain *B. japonicum* WB74 in South Africa.

MATERIALS AND METHODS

Survey of the major soybean growing farms and onsite observation

In January 2012, selected soybean growing farms in nine soybean farms located in three provinces in South Africa including the Free State, KwaZulu Natal and Mpumalanga were surveyed three months after planting. In these farms where the plant growth stage was sufficient enough to start nodulation, plants were carefully dug out to evaluate the effectiveness of the nodules visually. The visual classification and scoring system was done using a modification of the scheme used by Corbin et al. (1977) for chickpea. The nodule score for soybean is determined based on the number, size, colour and position of nodules across the root. Nodule colour scoring was made using a rating scale of 0-3, where 0 = no nodule, 1 = white nodules, 2 = green nodules, 3 = pink nodules; Nodule number was scored as 3 = many nodules, 2 = average, 1 = few nodules and 0 = no nodule; Nodule position was scored as 4 = on the crown, 3 = side and tap root, 2 = side root and 1 = on root tip; Size of nodules was scored as 3 = large, 2 = intermediate, 1 = small, 0 = too small to none.

Management practices and soil sample analysis

Farmers at each soybean farm were questioned about the various management practices they followed as these could affect the expected yield to be obtained after using the rhizobium inoculant. These include the practices starting from land preparation to planting, fertilizer and fungicide/herbicide application, conservation agricultural practices and handling of the rhizobium inoculants. Soil samples were collected from different sites of the plots in each soybean farm and major physical and chemical analyses were conducted at the Institute for Soil, Climate and Water (ISCW) laboratory of the ARC in Pretoria which included particle size analysis (%), chemical analysis and cation exchange capacity (CEC).

Inoculants pH, moisture content and plate count determination of rhizobia

The moisture content and the pH of the soybean inoculants obtained from the local manufacturers were determined using standard protocols every month (Somasegaran and Hoben, 1994). Likewise, randomly chosen sachet of the inoculant from each of the four manufacturers was chosen and tested each month for a period of five months to determine the colony forming unit (counts) of the *Bradyrhizobium.* strain per gram of the inoculant (cfu g^{-1}) over the shelf life period. Ten grams of the perlite inoculant was mixed in 90 ml sterile distilled water and mixed well to give a 10^{-1} dilution. For the inoculant purchased from manufacturer IV, 10 ml of the inoculant was transferred into 90 ml sterile distilled water. The mixture was then incubated at 30°C for 45 min on a rotary shaker at 100 rpm. A tenfold serial dilution (up to 10^{-6}) was made by transferring 1 ml of this mixture into 9 ml sterile distilled water. Aliquot (1 ml) from the 10^{-3} - 10^{-5} dilution series was spread plated on sterile Yeast Mannitol Agar (YMA) containing (g L^{-1}): Mannitol (10), KH_2P0_4 (0.5), $MgSO_4$ (0.2), yeast extract (0.4), NaCl (0.1), distilled water (1L). The YMA is supplemented with 10 ml L^{-1} Congo red solution. The plates were incubated at 28°C for 3-8 days and the resulting colonies were counted to determine the cfu g^{-1} or cfu ml^{-1} of the *B. japonicum* WB74 like colonies.

Inoculants strain verification by 16S rRNA sequence analysis

Randomly selected pure colonies of the rhizobia from YMA plates were subjected to DNA extraction to amplify the conserved 16S rRNA genes by the polymerase chain reaction (PCR) using forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'-TAC CTT GTT ACG ACT TCA CCC CA-3' (Lane, 1991). The amplification was performed in a 25 µl reaction volume containing 6 µl of the template DNA, 5 µl Flexi buffer, 2.5 µl MgCl, 0.5 µl dNTPs, 0.5 µl of each of the foreword and reverse primers, 0.5 µl Taq polymerase and 9 µl of nuclease free water. Amplifications were carried out in an Eppendorf Master cycler Gradient apparatus (Applied Biosystems, USA) with an initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 1 min. PCR amplified DNAs were visualized by electrophoresis on a 1% agarose gel in TBE buffer containing

Table 1. On farm evaluation of the various management practices used by farmers and observation of the nodulation pattern and scoring.

Soybean farm	Inoculants* used	Use of** Sticker	Application of various external inputs†					Nodulation scoring (0-4)				Yield ha^{-1} (tone)¥
			Mo	P	KCl	N	Surface lime	Color	Size	Number	Position	
Bothaville 1	Peat (MI)	-	+	-	-	+	+	0	0	0	0	2.00
Bothaville 2	Peat (MI)	+	+	-	+	-	-	1	2	2	2	2.80
Viljenskroon	Peat (M III)	-	+	-	-	-	-	1	2	2	3	1.30
Reitz	Peat (MI)	-	+	+	+	-	-	1	2	2	2	0.62
Piet Retief I	Peat (MI)	-	+	+	+	-	+	3	3	4	4	3.60
Piet Retief II	Peat (MI)	-	+	-	-	-	-	3	2	3	3	2.80
Middleburg	Peat (MIII)	-	+	+	+	-	+	3	2	3	3	2.60
Leandra	Peat (M II)	+	+	-	-	-	+	3	3	4	3	1.78
Bergville	Peat (MIII Liquid (MIV)	-	+	-	+	-	-	3	3	3	3	3.80

*Inoculants purchased by farmers from manufacturers (M I, II and III) are all peat formulated while those from manufacturer IV are liquid formulation. **Stickers are mixed with peat inoculants in water to help the inoculants stick to the seeds to prevent being washed away while watering. †Mo= Molybdenum, P = phosphorous, KCl = potassium chloride, N = starter nitrogen, + = applied, - = not applied; ¥ The yield data shown is provided by each farmer after harvest.

0.5 mg/ml ethidium bromide. Sequencing of the 16S PCR products was performed at Inqaba Biotech (Pretoria, South Africa) and the resulting sequences were blast searched on the NCBI data library. After edition and proper alignments of the nucleotide sequences of the rhizobia from the inoculants and reference strains obtained from the NCBI data base library, phylogenetic tree was constructed using both the Neighbor Joining (NJ) and Un-weighted Pair Group Method (UPGMA) in MEGA5 program (Tamura et al., 2007).

RESULTS

On farm observation of soybean plants and management practices

Most of the farmers planted in November except the farm in Piet Retief, Mpumalanga and another farm in Bergville that was planted in October (data not shown). The majority of the farmers used the commonly known Pannar cultivar PAN1666R, while two farmers, one in Bothaville and another one in Leandra planted more than six soybean cultivars (data not shown). Soil surface lime application before planting was made by farmers in the Free State (Bothaville1) and Mpumalanga (Piet Retief1, Middleburg and Leandra) (Table 1). However, no surface lime application was made by the other farmers even if it was necessary to do so. The farmers also vary in their usage of fertilizer and other chemicals input except in the application of molybdenum (Mo) in which most of them applied molybdenum as a seed treatment before planting (Table 1).

Farmers in all the visited farms inoculated their soybeans with Rhizobium inoculants prepared either in the form of peat or liquid formulation supplied by local manufacturers. Among the farms surveyed in this study, one farmer in Bergville, KwaZulu Natal had also used a rhizobium inoculant called 'Rhizoliq' imported from abroad by a local distributor company (data not shown). Results of on farm observation of the nodules formation and scoring of their effectiveness based on color, size position and number is presented in Table 1 and Figure 1.

Soil physical and chemical analysis

Generally, samples collected from three soybean farms in the Free State and three farms in Mpumalanga had high sand content (Table 2). Only Bergville in Kwazulu Natal and Piet Retief in Mpumalanga had higher proportion of clay (Table 2). Two farms in the Free State and one in KwaZulu Natal were characterized by a strongly acidic soil (pH = 5.25 to 5.42), while four other soybean farms (three in Mpumalanga and one in Free State) had a moderately acidic soil (pH= 5.7 - 6.0) (Table 2). The soils in the soybean farms in

Figure 1. On farm evaluation of randomly uprooted nodules (average of three) for three soybean farms six to eight weeks after planting and inoculation. The scoring scheme of Corbin et al. (1977) was used to evaluate the effectiveness of the nodules based on color (0-3), size (0-3), number (0-4) and position (0-4) of nodules. a) Piet Retief 1 nodules with a scoring of 3, 3, 3, 4; b) Reitz nodules with a score of 1, 2, 2, 2; c) Bothaville 1 with score of 0, 0, 0, 0 (without nodules).

Table 2. Soil chemical and physical properties from nine soybean farms in three provinces in South Africa.

Soybean farms	Chemical analysis				Extractable cations (cmol (+)/kg						Particle size† (%)			
	C (%)	P*	$N\text{-}NO_3$*	Al*	Mn*	Na	K	Ca	Mg	CEC	Sand	Silt	Clay	Soil pH
Bothaville 1	0.31	4.94	1.23	62.26	14.15	0.116	0.362	2.18	1.359	5.755	79	2	19	5.98
Bothaville 2	0.29	5.19	0.47	71.66	11.49	0.070	0.240	0.670	0.572	3.081	86	3	11	5.40
Viljenskroon	0.45	72.4	0.36	65.80	17.80	0.099	0.453	5.760	0.688	3.789	84	6	10	6.93
Reitz	0.51	2.95	2.96	117.21	25.48	0.068	0.243	0.756	1.568	3.80	81	5	14	5.25
Piet Retief 1	2.18	2.40	0.21	290.00	35.91	0.147	0.445	6.491	1.943	13.06	29	17	54	6.77
Piet Retief 2	2.26	2.57	0.61	236.22	11.85	0.088	0.302	2.371	0.629	6.499	76	8	16	5.70
Middleburg	1.1	15.1	1.58	117.7	13.81	0.076	0.233	1.675	0.799	4.362	76	6	18	6.01
Leandra	1.62	2.84	0.28	131.76	43.65	0.079	0.543	3.048	1.276	7.978	66	12	24	5.80
Bergville	2.27	2.34	1.23	265.72	20.51	0.131	0.198	4.214	1.678	12.55	34	19	47	5.42

*mg/kg; CEC = cation exchange capacity†, Sand = 0-0.0 mm; Silt = 0.05-0.00 mm; Clay = <0.002 mm.

Piet Retief I (Mpumalanga) and Viljenskroon (Free State) had pH which is close to neutral (pH = 6.77 - 6.93) (Table 2). Summary of the comparison of soil physical characteristics and chemical properties including soil nitrate and aluminum level, cation exchange capacity (CEC) and extractable cations for all the soybean farms is presented in Table 1.

plate count of Rhizobia

The initial count of the *B. japonicum* WB74 like colonies in the inoculants from the three local manufacturers I, II, and III was > 10^8 cfu g^{-1} and was equivalent to a log transformed value of 8.29, 8.33 and 8.06, respectively (Table 3). During the second month, the count slightly decreased by only 0.35 log units for both manufacturer I and II and by 0.36 log units for manufacturer III (Table 3).

The decrease in the counts became very significant during the fourth month in which count from manufacturer II was less by 1.8 log units, manufacturer I by 1.82 log units and manufacturer III by 2.49 log units. The initial *Bradyrhizobium* count for manufacturer IV was 1.21 x 10^8 cfu ml^{-1} in the first month and decreased to 9.2 x 10^5 cfu ml^{-1} in the fourth month (Table 3).

Strain verification by 16S rRNA sequence analysis

The amplified product of the 16S rRNA gene of each of the *Bradyrhizobium* strain retrieved from the four soybean inoculants was subjected to sequencing reaction. After appropriate edition of the sequences using both the Bio-edit and Chromas lite program, a 930 base pair of nucleotides was generated for each sequence and aligned online using MAFFT nucleotide alignment tool. Neighbour Joining (NJ) and UPGMA (data for UPGMA tree not shown) phylogenetic trees constructed from the aligned sequences revealed that the strains from the four different soybean inoculants fall into three separate

Table 3. Plate count of *Bradyrhizobium* strain WB74 like colonies per gram of each soybean inoculants and determination of pH and moisture content over a period of five months to determine the viability of the rhizobia and the inoculant's quality in long term storage.

Inoculant/ Manufacturer	Viable plate count (cfu g^{-1})*					Inoculant *pH**				Moisture content (%)*			
	I	II	III	IV	V**	I	II	III	IV	I	II	III	IV
Manufacturer I (Perlite)	1.98 x 10^9	8.8 x 10^8	4.8 x 108	3.0x 10^7	>10^6	7.72	7.50	7.51	7.61	33	33	33	30
Manufacturer II (Perlite)	2.14 x 10^9	9.7x 10^8	5.7x 10^8	3.4x 10^7	>10^6	7.72	7.83	7.64	7.48	33	32	33	30
Manufacturer III (Perlite)	1.15 x 10^9	5.1x 10^8	3.2 x 10^8	3.8 x 10^6	>10^6	8.43	8.19	8.06	8.12	35	33	34	30
Manufacturer IV (Liquid)	1.21x 10^9	9.4 x 10^8	7.8 x 10^8	9.2 x 10^6	>10^6	7.47	7.52	7.60	5.58	NA	NA	NA	NA

*The viable plate counting was determined over a period of five months (I - V) and pH and moisture content determination was conducted over a period of four months (I-IV). **contaminants were detected at the fifth month from the 10^{-6} dilution series of the NA plates, therefore the YMA plates containing the *Bradyrhizobia* were not counted due to the presence of contaminants after the fourth month.

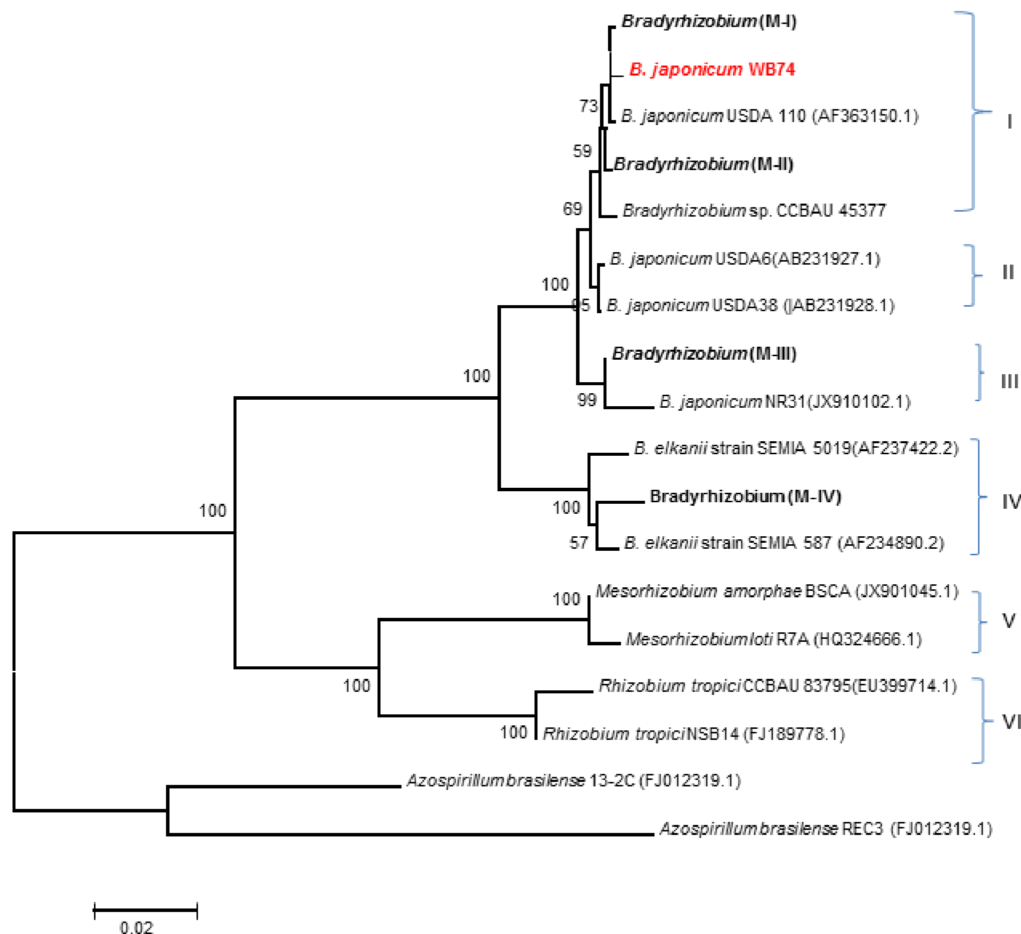

Figure 2. Neighbor-Joining tree to show the phylogenetic relationship of *Bradyrhizobium* strains used in four soybean inoculants in South Africa with *B. japonicum* strain WB74 and selected reference strains obtained from the NCBI data base. The evolutionary distances were computed using the Jukes-Cantor method and scale bars represent substitution of 2 bases per 100 nucleotide positions. The *B. japonicum* strain WB74 and the *Bradyrhizobium* strains recovered from the four inoculants were designated in bold and letters in parenthesis represent Manufacturer I - IV. The tree is rooted with *Azosprillum brasilense* strains. NB. Only strains from inoculant manufacturers I and II have grouped together with *B. japonicum* WB74 strain.

clusters (Figure 2). In both the NJ and UPGMA trees, only strains from manufacturer I and manufacturer II clustered with *B. Japonicum* WB74 (Cluster1) (Figure 2). Inoculant strain from manufacturer III clustered in a separate group with *B. japonicum* strainNR31 (JX910102.1) using the Neighbour Joining method (Figure 2) and with *B. japonicum* USDA strains in the UPGMA method (data not shown). The *Bradyrhizobium* strain from manufacturer IV clustered with *Bradyrhizobium elkani* SEMIA 5019 and *B. elkani* SEMIA 587 (Figure 2).

DISCUSSION

The major limiting factors that contributed to the failure of nodulation of soybean by introduced *B. japonicum* strain WB74 in South Africa was investigated in this study. In our survey to investigate the management practices used by the farmers, there is indication of lack of consistency among the different soybean farms. For instance, although most of the surveyed farmers have treated their seeds with molybdenum before planting, failure of legumes to nodulate under acidic soil conditions of Bothaville2, Reitz and Bergville farms was observed. In all the three farms where the soil pH is strongly acidic (5.2 - 5.4), farmers did not intend to use surface lime application before planting. Zahran (1999) reported that legumes usually fail to nodulate under acidic soil conditions leading to impaired symbiotic efficiency and reduced yield. Inoculant strains of rhizobia, no matter how competitive they are, do not express their full capacity for N_2 fixation if other limiting factors such as unfavourable soil pH, mineral toxicity,and nutrient deficiency and plant diseases impose limitations on the vigour of the host legume (Brockwell et al., 1995; Peoples et al., 1995; Thies et al., 1995).

One of the principal yield limiting nutrients in many regions in the world is phosphorous (P) which is very essential for both nodulation and nitrogen fixation (Zahran, 1999). Soils in the seven soybean farms with very low P levels ranging from 2 - 7.0mg/kg may not sufficiently support the process of BNF by rhizobia. Except for the soybean fields in Piet Retief, Middleburg and Retz, no management practices were made by the other farmers to treat the soils with added phosphorous even if the amount was too low. It should be noted that field grown soybean has a high ‘P’ requirement when it is dependent on BNF for its nitrogen supply (Keyser and Fudi, 1992). The unavailability of P may be caused by higher level of soil exchangeable Aluminium (Al). In many acidic soils, aluminium and manganese toxicities are responsible for limiting plant growth (Yang et al., 2009; Liao et al., 2006).

The low P availability in acidic soils results when free Al-oxides bind native and applied P into a form unavailable to plants (Liao et al., 2006). In the current study, the level of soil exchangeable Al in the soils of the different soybean fields was observed to be high ranging from 62.3 to 290 mg/kg. According to this study, soils from soybean fields in Mpumalanga (Piet Retief, Leandra) and KwaZulu Natal (Bergville) contained the highest amount of soil aluminium as compared to those in the Free State (Bothaville, Viljenskroon, Reitz) which showed very low nodulation. The fact that these soybean genotypes thrived well under conditions of high exchangeable Al concentration in the soil (>100mg/kg) make them highly tolerant to aluminium toxicity. It is therefore clear that ‘P’ deficiency could be one of the limiting factors for the difference in yield/ha between Piet Retief and the other soybean farms.

Soils from the soybean fields for which the exchangeable Al was high were characterized by lower level of available P. For example, the available soil P for Piet Retief soil with exchangeable Al level of 290 mg/kg was only 2.4 mg/kg, whereas Viljenskroon soil with exchangeable Al level of 65.8 mg/kg contains 72.4 mg/kg available P. A similar contrast can be made between the soils in Bergville (KZN) and Bothaville (Free State) for the relationship between exchangeable Al and available P in the soil. Under such extreme conditions, farmers’ management practices play a vital role to ameliorate the hazardous effect of aluminium toxicity in acidic soils. In general, Al and Mn toxicity becomes severe in soils with $pH < 5$, but can also occur at pH levels as high as 5.5 and limit plant growth (Rout et al., 2001).

One possible explanation for a rather high yield in Piet Retief soil with the highest level of exchangeable Al and low P level is that the soybean growing in these soils might have evolved adaptive mechanisms to grow in such low-P soils. This includes exudation of organic acids, phosphatase and other compounds that could mobilize ‘P’ from bound ‘P’ pools in the soil such as Al-P and also reduce its toxicity (Liao et al., 2006). The surface lime treatment of the soils in Piet Retief, Retz, Middleburg, Leandra and Bothaville might also have reduced the toxic effect of the high Al content in these soils. Amelioration of acidic soils rich in Al helps to increase the soil pH and decrease the concentration of the extractable Al and Mn thereby improving N_2 fixation and growth (Obiri-Nyarko, 2012; Fageria and Baligar, 2003).

The high yield obtained in Bergville with high acidic soils and low P level could be explained in terms of other features of the soil such as high CEC and soil organic matter (C%). The soil in Bergville with high organic carbon (C%= 2.27) and the high CEC probably gives it a good test level which offers a large nutrient reserve for the survival and functioning of the rhizobia and their symbiont. Moreover, the high clay content and cation exchange capacity of soils in Piet Retief and Bergville renders a high water holding capacity. This observation is supported by previous investigation which showed that the population kinetics of introduced strains of rhizobia is

a function of soil organic carbon, water holding capacity and CEC (McInns and Haq, 2007).

Bradyrhizobium strains nodulating soybeans are generally sensitive to acid soils. Thus one cause of nodulation failure in legumes is the inability of the rhizobia to persist under such conditions. In the current study, soils of Bergville, Retz and Bothaville farms with a pH range of 5.2 - 5.4 are in the strongly acidic category and are not suitable for the survival of the rhizobia without proper management to reduce the acidity. Acidity has more severe effects on rhizobia multiplication than Al stress and low-P conditions (Taylor et al., 1991). In this study for instance, Piet Retief soil with high Al level and low P condition has a near neutral pH which is conducive for the survival of the soybean *Bradyrhizobium* in the soil. The farmer's proper management of this soil especially surface lime application and treatment of the soil with added P could have contributed to the success of the inoculated rhizobia and the attainment of the high yield.

Soil NO_3^- has a negative effect on the activity of the nitrogen fixing rhizobia by inhibiting the functioning of the enzyme nitrogenase and legheamoglobin. By doing so, soil NO_3^- in general inhibits nodule formation and nitrogen fixation(*Zahran*, 1999). An interesting correlation was observed in this study (data not shown) with regard to yield and soil NO_3^- level where soybeans growing in soil with the lowest NO_3^- level in Piet Retief had the highest yield (3.6 tone ha^{-1}) and Reitz soil with the highest NO_3^- level had a yield of only 0.6 tone ha^{-1}. In a study to investigate the inhibitory effect of NO_3^- on nodulation, nodule growth was completely stopped when young soybean plants growing in hydroponic culture were supplied with 5 mM nitrate (NO_3^-) solution (Takuji et al., 2011).

In the absence of adequate number of highly effective rhizobia in many soils, the need arises to use rhizobial inoculants. It is advisable to inoculate the soil with high number of effective rhizobia to out-compete the population of ineffective native rhizobia (Deaker et al., 2004). For instance increasing the number of effective rhizobia applied to the seeds between 10-100 fold will improve nodulation and grain yield (Herridge et al., 2002). According to legume inoculants quality control procedures set for the registration of inoculant products in South Africa, any inoculant which does not comply with the standards will be rejected for marketing. The inoculants will be rejected if the number of rhizobia is $< 5 \times 10^8$ cfu g^{-1} (for peat), $< 6.5 \times 10^8$cfu g^{-1}(for perlite) and $< 2 \times 10^9$cfu ml^{-1} (for liquid inoculants); contaminants present on the 10^{-5} dilution plates; pH of carrier < 6.5 or > 7.5; rhizobial strain is doubtful (Strijdom and van-Rensburg, 1981).

The near alkaline pH of the perlite and liquid inoculants detected in this study are not favourable for prolonged survival and multiplication of the *Bradyrhizobium.* Strains of *B.japonicum* are found to be less tolerant to alkaline pH and have lower survival as the pH of the inoculant increases (Gomez et al., 1997). In addition to pH, the low level of moisture content in the three perlite inoculants from the local suppliers (30- 35%) could have subjected the rhizobia in the perlite to desiccation stress and a decrease in the number of rhizobia from the required 6.5 $\times 10^9$ to 1.15×10^8 per gram of the inoculant. Low inoculant moisture content will lead to a high mortality of the bacteria when they are inoculated to the seeds. Up on seed treatment, it will result in the average decrease of 3 log units (about 1000 rhizobia $seed^{-1}$) (Cartroux et al., 2001).

In this study, molecular characterization using sequence analysis of 16S rRNA revealed that all soybean inoculant strains from the four inoculant manufacturers fall under the *B. japonicum* cluster .However, only strains from two manufacturers were closely related to the desired *B. japonicum* WB74 strain. The inoculant strain from manufacturer III was more closely related to a different strain, that is, *B. japonicum* strain NR31 (JX910102.1) rather than with *B. japonicum* WB74, while inoculant strain from manufacturer IV clustered with *B. elkani* SEMIA 5019 (AF237422.2) and *B. elkani* SEMIA 587 (AF234890.2). Strain SEMIA 5019 is initially isolated from a high manganese soil in Reo de Janerio and has been used as an inoculum since 1979 whereas strain SEMIA 587 was isolated from Rio Grande de Sol in Brazil (Santos et al., 1999).

The recommended registered strain to be used as soybean inoculant in South Africa is *B. japonicum* strain WB74 and no other strain of rhizobia, other bacteria or fungi is allowed to be present in the inoculant without being registered (personal communication with office of the Registrar, Pretoria). Our study shows that a wide variety of soybean inoculants with strains of *Bradyrhizobium* other than the recommended WB74 strain are being manufactured and/or imported by different companies to be sold to the farmers. However, there is no research to date that indicates the adaptation of these new strains of *Bradyrhizobium* to the South African soil conditions, nor is their efficacy determined under different geographical and soil condition for at least two seasons before being registered.

In summary, it has been noted in this study that the efficacy of the inoculant strain *B. japonicum* WB74 in the nodulation of soybean on South African soils is largely dependent on the intrinsic characteristics of the inoculants, farmers' management practices and other soil variables all of which affect the nodulation process and the entire symbiotic interaction. It is therefore recommended that before introducing rhizobial inoculants into the soil, sufficient soil analysis and proper management practices be conducted to improve any limiting factors that hamper the nodulation efficiency of the rhizobia. Moreover, with the increasing production and the need to market legume inoculants in South Africa, selection of strains for effective nodulation and nitrogen fixation must be conducted under field condition. The inoculant pro-

ducts manufactured using the elite rhizobial strains should comply with the standards by undergoing at least a six month quality control test which involves, number of rhizobia, inoculant consistency (pH, moisture content), rapid strain verification and shelf life determination.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Production and purification of polyclonal antibodies against 34 kDa protein (P34) of *Mycobacterium avium* subsp. *Paratuberculosis*

Hafezeh Alizadeh[1] Rasool Madani[2*] Narges Kavid[2] Fariba Golchinfar[2] and Tara Emami[2]

[1]Department of Biology, Science & Research Branch, Islamic Azad University, Tehran-Iran.
[2]Department of Biochemistry and Proteomics, Razi Vaccine and Serum Research Institute, Karaj-Iran.

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Paratuberculosis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic enteritis in ruminants. Among molecular components of MAP, protein P34 was identified as specific and immunodominant. Here, we describe the production of polyclonal antibodies with defined specificity for P34. Polyclonal antibodies were generated from New Zealand white rabbit. Animals were immunized at a certain time period with purified P34, MAP antigens and Freund's adjuvant. Antibodies were purified from serum by ion exchange chromatography. Western blotting analysis was used for evaluation of interaction between 34 kDa protein and antibodies.

Key words: 34 kDa antigen, Paratuberculosis, Polyclonal antibodies**.**

INTRODUCTION

Mycobacterium avium subsp. paratuberculosis (MAP) is the agent of paratuberculosis or Johne's disease (JD), a granulomatous enteritis in ruminants, that causes economic losses to the dairy industry (Berger et al., 2007; Santema et al., 2011). Despite its heavy economic burden, there are still no efficacious vaccination programs against Johne's disease (Bannantine et al., 2008) characterized by intermittent diarrhea, weight loss and eventual death Mundo et al., 2008). Diagnostic methods for paratuberculosis rely on the ability of the test to detect infected animals (Mutharia et al., 1997). Ruminants affected by JD develop strong immunological reactivity against *M. avium* subsp. *paratuberculosis* antigens (Berger et al., 2006). Current immuno-diagnostics tests are based on crude antigen mixtures with poor sensitivity due to high similarity between MAP and other *mycobacterial* antigens (Kavid et al., 2012). Some MAP antigens are now being evaluated for the development of new more sensitive diagnostics tests for paratuberculosis (Willemsen et al., 2005) and several studies with these antigens have been performed to set up diagnostic tests of paratuberculosis in livestock (Gioffre et al., 2006). Therefore, it is of interest to identify and characterize specific antigens of MAP and evaluate the role of these molecules as modifiers of cellular and humoral immunity (Mutharia et al., 1997). Antigens with molecular weight of 28 to 45 kDa are the most antigenic for both cellular and humoral immunity (De Kesel et al.,

*Corresponding author. E-mail: mr_madani@yahoo.com. Tel: 0098-21-34570038. Fax: 0098-26-34552194.

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1993). De Kesel et al. (1992) and Gilot et al. (1993) identified a 34-kDa protein from the A36 complex, the major antigenic complex of *M. avium* subsp. *paratuberculosis* (Coetsier et al., 1998). P34 was specific and shown to be immunodominant (Malamo, 2011). The carboxy terminus of this protein contain species-specific epitopes (Malamo, 2006). The identification of epitopes on MAP P34 is important to determine the role of bovine antibodies in the response against MAP infection (Ostrowski et al., 2003) so P34 kDa protein has become a notified subject for more studies. The aim of this study was to characterize the humoral immune response induced by immunization with MAP antigens in New Zealand white rabbit and evaluate the immunogenicity of P34.

MATERIALS AND METHODS

The purified 34 kDa protein from *M. avium paratuberculosis* (ATCC19698) was obtained from the Department of Biochemistry and proteomics, Razi Vaccine and Serum Research Institute Karaj, Iran [7]. DEAE-Cellulose columns were purchased from Pharmacia (Sweden). Protein markers were obtained from BioRad (Hercules, USA). Other reagents and chemicals were of analytical grade from Fluka and Merck.

Animal

New Zealand white female rabbit was supplied from the Laboratory Animal Breeding Unit of Razi Vaccine and Serum Research Institute, Iran.

Protein determination

Protein concentration of purified P34 kDa was measured by the method of Lowry et al., using BSA as standard (Lowry et al., 1951).

Immunization of rabbit with whole-cell extract of MAP

A New Zealand white rabbit was immunized four times at 14 days intervals with whole-cell extract and purified p34 to obtain polyclonal antibodies. So, for per injection, 600 µl of MAP antigens (300 µl whole cell extract were mixed with 300 µl purified p34) were emulsified with equal volume of Freund’s adjuvant (Sigma). For first injection, antigens were used in Freund’s complete adjuvant and emulsion was administered subcutaneously. The second, third and fourth injection were performed on days 14, 28 and 42 in Freund’s incomplete adjuvant (Sigma). Blood sample was taken 2 weeks later from rabbit and serum was prepared (Shin et al., 2009).

Ig preparation by ammonium Sulfate

The prepared serum (3 ml) was precipitated by ammonium sulfate (35%). The suspension were centrifuged at 10000 × *g* for 30 min at 4°C, the supernatant was removed and the precipitate suspended in the PBS buffer 0.01 M (1.5 µl) and sample dialyzed against PBS buffer (pH 6.5) overnight on a magnetic stirrer at 4°C (Mutharia et al., 1997)

Purification of IgG

Ion-exchange (DEAE-cellulose) chromatography was used for purification of IgG. Ig passed through the column (1.5 × 16 cm) and washed in two steps using PBS buffer 0.01 M for first time and PBS 0.1 M buffer at pH 6.5 for the second washing step. The column was run, by upward flow, at 20 ml/h rate and fractions of 1 ml were collected. The effluent was monitored at 280 nm (Pharmacia Biotech 2000). Fractions concentrations containing IgG were determined with Lowry assay (Lowry et al., 1951).

SDS-PAGE analysis of IgG

The purity of rabbit IgG preparation was checked using Bio-Rad 10% sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) under reduced conditions as described by Laemmli (1970). 50 µl IgG (9 mg/ml) was solubilized in 50 µl of sample buffer (0.0625 M Tris-HCL, pH 6.8, 10% glycerol, 2% SDS, 0.006% bromophenol blue, 5% β-mercaptoethanol), and was boiled for 10 min and loaded on the electrophoresis gel. Electrophoresis was carried out at a constant current of 110 V for 1 h. After separation, the proteins bands were stained by Coomassie brilliant blue staining solution (0.1% Coomassie blue in 70:15:15 methanol: water: acetic acid), and destained in destaining buffer (70:15:15 methanol: water: acetic acid).

Double-Immunodiffusion Assay

The double-immunodiffusion was performed in 1.2 % agar gel prepared in the PBS 0.01 M buffer at pH 8.6 with polyclonal antisera against MAP antigens used in this work. MAP antigens were poured into central wells, and serially diluted antisera were put into peripheral ones. The Coomassie Brilliant Blue R-250 solution (0.1% Coomassie blue in 70:15:15 methanol: water: acetic acid) was used for the gel fixation and staining. The gel was destained by acetic acid solution (70:15:15 methanol: water: acetic acid) for 20 min at room temperature with shaking (Nikolayenko et al., 2005).

SDS-PAGE and Western blot analysis

50 µl of the MAP antigens (34.65 mg/ml) were mixed in 50 µl of sample buffer (0.0625 M Tris-HCL, pH 6.8, 10% glycerol, 2% SDS, 0.006% bromophenol blue, 5% β-mercaptoethanol), and was boiled for 5-10 min. Sample was separated in Bio-Rad 10% polyacrylamide gel electrophoresis at 100 V for 1.5 h by Laemmli’s method (Laemmli, 1970). For immune blotting, protein bands were transferred to nitrocellulose membrane in a semidry unit (Bio-Rad PAC 1000) at 10 V for 30 min. The membrane was blocked with 3% BSA in PBS 0.01 M buffer (blocking buffer) for 2 h at 4 °C. After washing 3 times with PBS-T (500 cc PBS 0.01 M, 250 µl Tween 20), membrane was incubated in rabbit purified anti-MAP IgG (1:20 dilution) for 1.5 h with shaking at room temperature and then incubated overnight at 4°C. After washing 4 times with PBS-T, the membrane was incubated with anti-rabbit HRP IgG conjugate (sigma, 1:1000 dilution in PBST) for 1 h at room temperature and again washed for 3 times with PBS-T. Finally, the chloronaphtol peroxidase [methanol (16 ml), chloronaphtol (18 ml), PBS (24 ml), peroxidase (6 µl)] was added to the membrane and incubated for 15 min to identify the developed band of polyclonal anti-MAP IgG (Yabe et al., 1995).

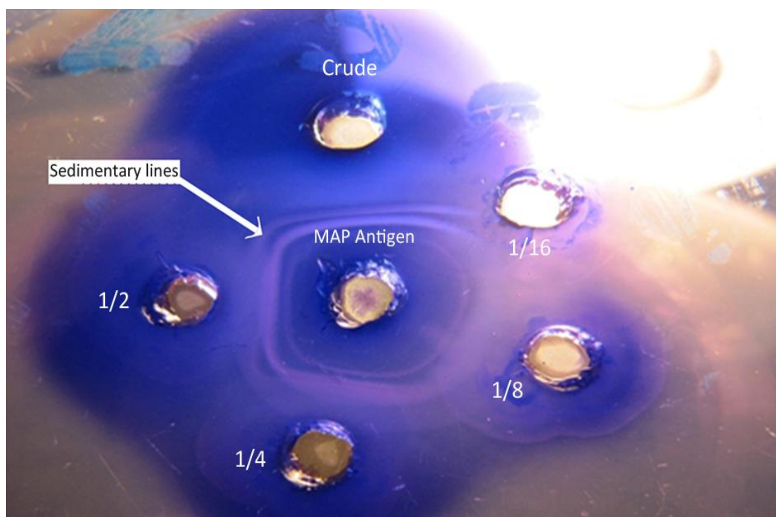

Figure. 1. Double immuno-diffusion test were applied with anti-MAP IgG in crude, 1:2, 1:4, 1:8 and 1:16 dilutions and precipitation lines between antibodies and antigens.

RESULTS

Estimation of P34 concentration

The concentration of P34 kDa was 0.096 mg/ml.

Polyclonal antibodies titer

Usually antibodies titers increase every new immunization. In this experiment the best antibodies titers were obtained from the rabbit serum collected after the fourth injection. The interaction of polyclonal anti-MAP IgG was evaluated by double-immunodiffusion test with crude, 1:2, 1:4, 1:8 and 1:16 dilutions of IgG and 3 sharp lines of precipitation were shown among MAP antigens and different titer of antibodies (till to 1:16 dilution) (Figure 1).

Purification of anti-MAP IgG

Purification of rabbit anti-MAP IgG was carried out by ion exchange chromatography on DEAE-Cellulose. In this step, as shown in the chromatogram (Figure 2) of rabbit immune-globulins, only one peak was gained. The fractions containing IgG were collected and the whole protein (IgG) concentration was obtained 9 mg/ml by Lowry assay.

SDS-PAGE analysis of purified IgG

For separating rabbit IgG, SDS-PAGE was performed under reducing condition and IgG band was evaluated in Coomassie blue stain. Figure 3 shows a single band with molecular weight about 50 kDa corresponding to rabbit IgG heavy chain (Figure 3).

Western blot analysis of MAP antigens

Western blot analyses of MAP antigens were performed with polyclonal antibodies of rabbit which showed several bands. These bands showed positives reactions between rabbit antibodies and MAP with the 34 kDa molecular weight protein identified as a sharp band (Figure 4). The specific band for P34 showed the immunodominant specificity of this protein against rabbit anti-MAP IgG.

DISCUSSION

Several antigenic proteins of MAP have been described in the pathogen and have been performed to set up diagnostic tests. Amongst whole antigens of MAP, P34 has been cloned and shown to be immunodominant antigen and have been fully characterized previously which is a good candidate to use in immunological analysis (Kavid et al., 2012; Coetsier et al., 1998). P34 has a significant role in formation of granulomata and other hypersensitivity type responses manifested in JD (Kavid et al., 2012). The carboxyl terminus of P34 containing specific epitopes with respect to MAP has been used as antigen in an ELISA for the specific diagnosis of bovine paratuberculosis (Coetsier et al., 1998). Coestier and colleagues described the preparation of polyclonal and monoclonal antibodies, directed against the 13.6 kDa carboxyl-terminal portion of the rcP34 of MAP (Malamo, 2011). Malamo (2011) used carboxyl terminous from the rcP34 of *M. paratuberculosis* to produce

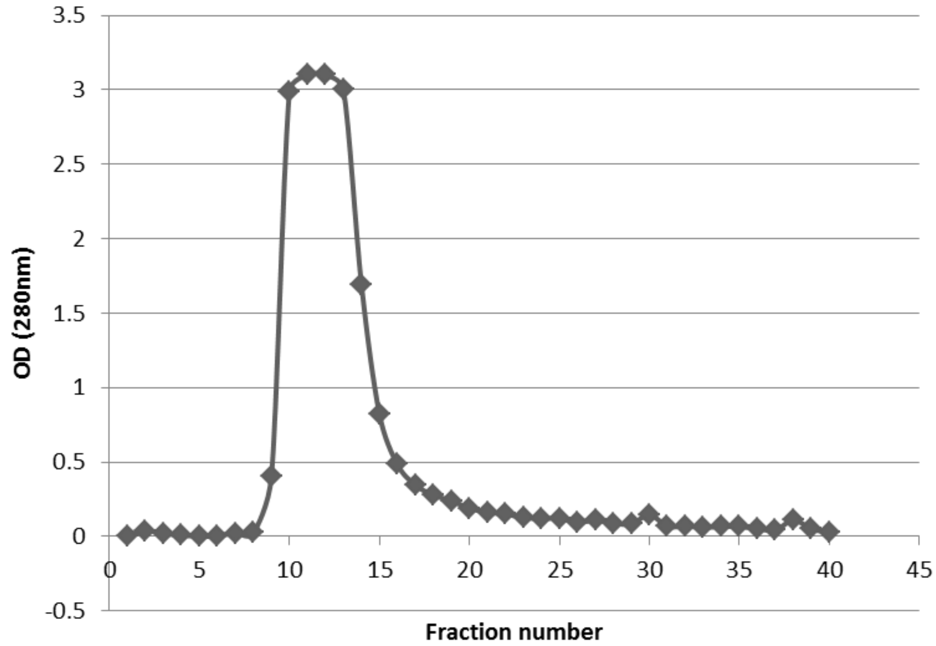

Figure. 2. Immunoglobulin solution was applied on DEAE-Cellulose column (1.5 × 16 cm). One peak was obtained from fraction 11 to fraction 17.

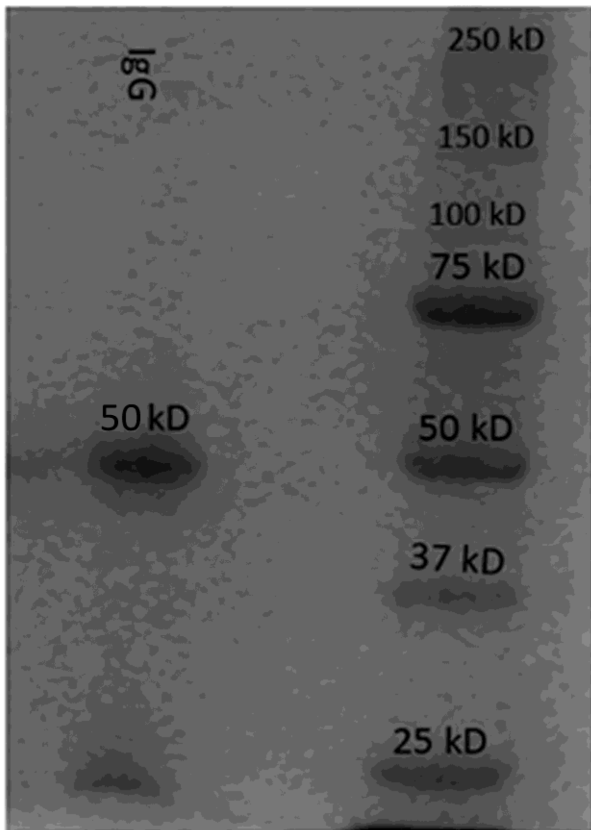

Figure. 3. SDS-PAGE: Pure IgG is shown as a single band with molecular weight about 50 kDa corresponding to rabbit IgG heavy chain.

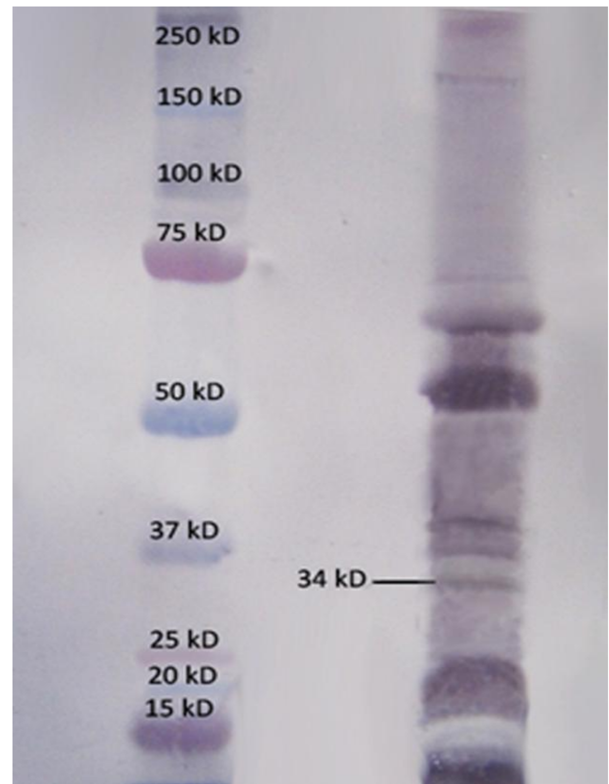

Figure 4. Western blot analysis of *Mycobacterium avium paratuberculosis* by rabbit anti-MAP IgG showed positive reactions between antigens and antibodies that P34 identified as a sharp band and this band showed the immunodominant specificity of P34.

monoclonal antibodies against MAP (Malamo, 2011). Purification of native P34 kDa protein from cell wall extracts is difficult because of aggregation and interaction of this protein with MAP cell wall proteins. P34 was previously purified by electroelution method in Biochemistry and proteomics Department of Razi Institute (Kavid et al., 2012) and in this study we used this purified protein and whole cell extracts of MAP containing P34 for produced polyclonal antibodies in rabbit. Antibodies are an important tool for investigation of protein expression and function. Polyclonal antibodies enhanced analytical sensitivity because they are directed against multiple surface antigens. Immunization of rabbit with MAP antigens was performed and blood was collected. We used ion-exchange chromatography for the purification of rabbit IgG. With Western blotting, we demonstrate the specificity of polyclonal antibodies against P34 of *M. avium paratuberculosis* and identified the immunogenicity of p34. A previous study has reported the potential of polyclonal rabbit anti-MAP antibodies to be used as a separation tool to detect MAP in milk or fecal samples (Khare et al., 2004). So we purified rabbit IgG polyclonal antibodies by ion-exchange chromatography to evaluate the reactivity of P34 by Double-immunodiffusion and western blot analysis. Double-immunodiffusion of whole MAP proteins showed three precipitate lines; these lines indicate high reactivity of antigens with rabbit anti-MAP IgG. In addition, we performed western blot analysis and result has showed several bands to MAP antigens and p34 kDa appeared as a sharp band. Moreover, the band of P34 suggests a significant role of P34 in the immunobiology of infection. Western blot confirm that P34 was immunodominant with rabbit anti-MAP IgG. In conclusion, the identification of polyclonal antibodies employing western blot analysis is of importance in studying the MAP disorder. The polyclonal anti-MAP IgG produced in this work were specific for P34 of MAP and these polyclonal antibodies could be useful as a specific antibody for future study.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Muntingia calabura botanical formulation for enhanced disease resistance in tomato plants against *Alternaria solani*

R. Rajesh*, N. Jaivel and P. Marimuthu

Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore 641 003, India.

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The present study successfully demonstrate the inhibitory activity of the medicinal plant, *Muntingia calabura* against *Alternaria solani* which causes early blight of tomato and proposes the development of a new botanical formulation (Muntingin 5EC) and its use in plant disease management after package and practice. This ecofriendly botanical formulation was developed from the purified antimicrobial metabolite (Stigmasterol) isolated from the methanol extract of *M. calabura* root. Different concentrations of Muntingin 5EC were examined on seed infection, germination and seedling vigour of tomato and it was found that two percent Muntingin 5EC increased the germination and vigour and reduced the seed infection in tomato to a significant extent. The formulation was found to possess good emulsion stability and also retain its antimicrobial activity (shelf life) for 120 days. Application of Muntingin 5EC increased the activity of enzyme such as peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and phenol content of tomato. Muntingin 5EC (2%) was found to be the optimum concentration for control of early blight of tomato under pot culture conditions. The root of *M. calabura* was found to possess good antioxidant activity. Application of this botanical formulation in plant disease management assumes special significance by being an ecofriendly and cost effective strategy, which can be used in integration with other strategies for a greater level of protection with sustained crop yields after sufficient evaluation.

Key words: *Alternaria solani,* botanical formulation, Muntingin 5EC, plant diseases control.

INTRODUCTION

In agriculture, crop loss due to plant pathogens has become a major concern nowadays. Increased usage of different chemical products to control these pathogens have resulted in problems like residual effect of chemicals in agri-based products, increased resistance for chemicals in target pathogens and environmental pollution. Plant diseases cause considerable losses in crop production and storage. The intensive and indiscriminate use of pesticides/fungicides in agriculture has caused many problems such as pollution of the

*Corresponding author. E-mail: rajtech1985@gmail.com. Tel: 91-422-6611446. Fax: 91-422-6611437.

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environment such as water, soil, animals and residual contamination of food and many others in addition to social economic problems (Stangarlin et al., 1999). Consequently, there is an increasing demand from consumers and officials to reduce the use of chemical pesticides and fungicides. In this context, biological control through the use of natural antagonistic micro-organisms has emerged as a promising alternative (Strange and Scott, 2005). Botanicals with antifungal compounds have been identified and these can be exploited for the management of diseases (Kagale et al., 2004). Botanicals have low mammalian toxicity, target specificity, biodegradability and contain many active ingredients in low concentrations, thus possess biocidal activity against several insect pests and pathogens (Kalaycioglu et al., 1997; Harish et al., 2008).

Crude extracts of some well known medicinal plants are used to control some of the plant pathogens. During the past few years, there was a growing trend all over the world to shift from synthetic to natural products including medicinal plants (Parimala devi and Marimuthu, 2011). The neglected and little known botanicals should be considered now to cure the plant diseases, which create challenging problems in agriculture and pose real economic and environmental threats.

Early blight disease of tomato by *Alternaria solani* (Jones and Grout, 2005) has become most destructive in India and yield losses due to this pathogen were up to 80% (Shanmugasundaram, 2004). The control of tomato early blight disease has been exclusively based on the application of chemical pesticides. Several effective pesticides have been recommended for use against this pathogen, but they are not considered to be long-term solutions, due to concerns of expense, exposure risks, fungicide residues and other health and environmental hazards. In an attempt to modify this condition, some alternative methods have been adopted. Recent efforts have focused on developing environmentally safe, long lasting and effective biocontrol methods for the management of plant diseases. Use of plant products and biocontrol agents has been shown to be eco-friendly and effective against many plant pathogens. A number of plant species have been reported to possess natural substances that are toxic to many fungi causing plant diseases (Lee et al., 2007). The objective of the present study was to evaluate the antimicrobial activity of root extracts from *Muntingia calabura* against *A. solani* under *in vitro* conditions and to develop a botanical formulation against plant diseases control.

MATERIALS AND METHODS

Preparation of the botanical fungicide

The partially purified methanol extract of *M. calabura* root containing the antimicrobial metabolite Stigmasterol was used to develop emulsifiable concentrates. The condensed material containing the antimicrobial metabolite, obtained after column chromatographic separation of fractions was considered as 100% concentration. The formulation was developed by using recommended quantities of surfactant (Tween 20) and co-surfactant (Ethylmethyl ketone). The 4EC formulation were prepared by adding 4 g of antimicrobial metabolite to 20 ml methanol and made up to 100 ml by adding 10 ml of Tween 20 and 70 ml of Ethylmethyl ketone.

The active fraction from methanol extract of *M. calabura* root was developed into two different emulsifiable concentrates and named as 'Muntingin 4EC' and 'Muntingin 5EC'. The formulations Muntingin 4EC and Muntingin 5EC prepared in different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%) were tested for the efficacy under *in vitro* conditions. The combined formulation was named as 'Muntingin'.

Stability, storage and antifungal studies on Muntingin 5EC

Emulsion stability

About 20 to 25 ml of emulsion concentrate of *M. calabura* 5EC was added to 70 ml of standard hard water and made up to 100 ml in a beaker. The contents of the beaker were stirred with a glass rod (4 revolutions per second) during the addition. The contents of the beaker were transferred immediately to a clean and dry measuring cylinder. The measuring cylinder with its contents was kept in a thermostat at room temperature for one hour. After 1 h, the volume of creamed matter at the top or the sediment at the bottom was measured.

Thermo stability test

The formulation was poured into a glass bottle until three fourth was filled. The bottle was closed airtight with colloid oil sealing wax to avoid any loss of volatile solvents and kept in the thermostat (50°C) for 7 days. After 7 days, the volume of the creamed matter at the top or the sediment at the bottom was measured.

Cold stability test

The formulation was poured into a glass bottle until three fourth was filled. The bottle was closed airtight with colloid oil sealing wax to avoid any loss of volatile solvents and kept it in the refrigerator (10°C) for 7 days. After 7 days, the formulation was tested for emulsion stability.

Effect of methanol extract of *M. calabura* root on mycelial dry weight of fungal pathogens

Mycelial discs (9 mm) of the pathogens (*A. solani, Fusarium oxysporum* f.sp. *lycopersici*, *Pythium* sp., and *Phytophthora* sp. were inoculated into respective broth separately containing methanol extract of *M. calabura* root (0.2, 0.4, 0.6, 0.8 and 1.0%). Conical flask without the extract was maintained as control. The treatments were replicated thrice and incubated for 21 days. The mycelium was harvested through filtration with Whatman No. 42 filter paper. The filter paper containing fungal mycelium was oven dried at 70°C for 24 h and the weight of the dried mycelium was determined (Singh and Singh, 1980).

Effect of methanol extract of *M. calabura* root on fungal spore germination

The effect of methanol extract of *M. calabura* root (0.2, 0.4, 0.6, 0.8 and 1.0%) on fungal spore germination was tested by cavity slide

method (Montagomery and Moore, 1938). Spores of the pathogen were transferred to test tubes separately by flooding with sterile water and scrapping the culture with glass rod. The transferred spore suspension was centrifuged at 2000 rpm for 10 min to remove mycelial fragments. The spore suspension was adjusted to a concentration of 10^6 per ml using a haemocytometer. One drop of the methanol extract of *M. calabura* root was added (0.2, 0.4, 0.6, 0.8 and 1.0%) to the cavity slide and allowed to evaporate. One drop of spore suspension (after thorough shaking) was added to the cavity slides and kept in a moist chamber and incubated at room temperature (28 ± 2°C). Sterile distilled water served as control. Three replications were maintained and percentage of spore germination was recorded after 24 h.

Antioxidant activity of methanol extract of *M. calabura* root

Dried powders of *M. calabura* root (10 g) were extracted in 100 ml of 50 per cent ethanol solution at 25°C for 30 min with shaking. The extract was centrifuged at 15000 rpm for 3 min and supernatant was collected. The supernatant was concentrated in a rotary evaporator and then lyophilized. The antioxidant assays carried out were ferric reducing antioxidant power assay (Benzie and Strain, 1996), DPPH scavenging activity (Blois, 1958), reducing power assay (Oyaizu, 1986) and superoxide anion radical scavenging activity (Nishikimi et al., 1972).

Antimicrobial effect against selected plant pathogens

The formulation was tested for its antimicrobial activity at different time intervals against selected plant pathogens by poison plate technique.

Assessing the biocontrol potential of Muntingin 5EC against tomato early leaf blight

Effect of Muntingin 5 EC on seed infection, seed germination and vigour of tomato seedlings were evaluated under *in vitro* condition. The treatments adopted were Muntingin 5EC at five different concentration (0.2; 0.4; 0.6; 0.8 and 1.0%), 0.2% Mancozeb (pesticide control) and standard biocontrol agent (*P. fluorescens* PF1). The tomato seeds were soaked in different concentrations of Muntingin 5EC for 2 h and twenty five seeds of each treatment were placed on moist blotters (ISTA, 1993) in Petri plate and incubated at 20 ± 2°C for 12 h of alternate natural light and 12 h of darkness. The seeds were examined for growth of seed borne pathogens on eighth day of treatment. The seed infection was expressed in percentage. The seedlings were evaluated as total number of normal seedlings and the per cent germination was evaluated. The vigour index was compared (Abdul-Baki and Anderson, 1973) and expressed as whole number.

Evaluation of Muntingin 5EC on tomato early leaf blight under pot culture condition

Effect of different concentrations of Muntingin 5EC (0.5, 1.0, 1.5 and 2.0%) together with standard practices of Mancozeb (0.2%) and biocontrol agent (*P. fluorescene* PF1) on early blight (caused by *A. solani*) was tested under pot culture condition in tomato. The extent of disease incidence is expressed as percent disease index and calculated using the disease score card for *A. solani* (Ayyangar, 1928). The peroxidase (Puttur, 1974), polyphenol oxidase (Mayer et al., 1965) and phenylalanine ammonia lyase (Zucker, 1965) were analyzed in the roots of tomato upto 10 days at 2 days interval. The phenol content was also measured at two days interval by standard procedure as described by Spies (1955).

RESULTS

The botanical formulation prepared using partially purified methanol extract of *M. calabura* containing antimicrobial metabolite stigmasterol was assessed in this study for its biocontrol potential. The formulation was found to possess good emulsion stability and also retained its antimicrobial activity (shelf life) for 120 days. The prepared formulation was tested for its efficacy in controlling the early leaf blight disease of tomato under *in vitro* and pot culture studies.

Effect of different concentrations of formulated products on fungal pathogens

The active fraction F21 from methanol extract of *M. calabura* root were developed into two different emulsifiable concentrates and named as 'Muntingin 4EC and Muntingin 5EC'. The formulations Muntingin 4EC and Muntingin 5EC were prepared in different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%). The formulation Muntingin 4EC at 2.5% and Muntingin 5EC at 2.0 and 2.5% concentration completely inhibited (100%) the growth of *A. solani, F. oxysporum* f.sp. *lycopersici, Pythium* sp., and *Phytophthora* sp. While the formulation Muntingin 4EC at 2.0% level reduced the mycelial growth up to 92.06, 90.98, 91.79 and 90.30% in *A. solani, F. oxysporum* f.sp. *lycopersici, Pythium* sp. and *Phytophthora* sp. respectively (Table 1).

Stability of EC formulations

Emulsion stability of the formulations were tested with standard hard water and the results are presented in Table 2. The standard critical limits of sedimentation of emulsion formulations were taken as 2 ml. For both formulations, *M. calabura* root, Muntingin 4EC and Muntingin 5EC, the sedimentation levels were not exceeding the critical limits of 2 ml.

The 5EC formulation of *M. calabura* root was kept at 10 and 50°C for 7 days and the sedimentation of emulsion was recorded. The visual observations confirmed the temperature stability of the EC formulations, as there was no sedimentation observed in high and low temperatures. The 2.0% solution of EC formulation had a neutral pH (7.0).

Shelf life of formulation and its effect on fungal pathogens

The formulation Muntingin 5EC was tested for its stability at room temperature (28 ± 2°C) for different periods (Table 3). The results show that the EC formulation

Table 1. Effect of different EC formulation of Muntingin (antimicrobial from *M. calabura* root) on growth inhibition of different fungal pathogens.

Formulation	Concentration (%)	*A. solani*		*F. oxysporum* f.sp. *lycopersici*		*Pythium* sp.		*Phytophthora* sp.	
		A	B	A	B	A	B	A	B
Muntingin 4EC	0.50	31.40 (± 0.12)[a]	65.38	38.32 (± 0.06)[a]	56.77	30.60 (± 0.02)[a]	66.51	32.99 (± 0.01)[a]	64.39
	1.00	22.35 (± 0.02)[b]	75.36	24.65 (± 0.02)[b]	72.19	22.00 (± 0.05)[b]	75.92	21.55 (± 0.02)[b]	76.74
	1.50	14.05 (± 0.01)[c]	84.51	16.80 (± 0.01)[c]	81.05	15.75 (± 0.02)[c]	82.76	10.98 (± 0.01)[c]	88.15
	2.00	7.20 (± 0.06)[d]	92.06	8.00 (± 0.09)[d]	90.98	7.50 (± 0.01)[d]	91.79	8.99 (± 0.02)[d]	90.30
	2.50	0.00 (± 0.00)[e]	100.00	0.00 (± 0.00)[e]	100.00	0.00 (± 0.00)[e]	100.00	0.00 (± 0.00)[e]	100.00
Muntingin 5EC	0.50	23.15 (± 0.23)[a]	74.48	23.67 (± 0.12)[a]	73.30	21.10 (± 0.09)[a]	76.90	17.86 (± 001)[a]	80.72
	1.00	12.86 (± 0.12)[b]	85.82	13.46 (± 0.05)[b]	84.82	15.30 (± 0.02)[b]	83.25	13.00 (± 0.06)[b]	85.97
	1.50	6.40 (± 0.05)[c]	92.94	7.23 (± 0.09)[c]	91.84	8.90 (± 0.01)[c]	90.26	9.70 (± 0.09)[c]	89.53
	2.00	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00
	2.50	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00	0.00 (± 0.00)[d]	100.00
Control		90.70		88.65		91.36		92.65	

A, Diameter of mycelial growth in Petri dish (mm); B, percent reduction over control. *Mean of three replications.

retained its 100% antifungal activity up to 120 days against the four pathogens tested *viz., A. solani. F. oxysporum* f.sp. *lycopersici, Pythium* sp. and *Phytophthora* sp.

Effect of methanol extract of *M. calabura* root on mycelial dry weight of fungal plant pathogens

At a concentration of 0.8%, the methanol root extract exhibiting mycelial growth reduction of 95.28, 96.39, 94.86 and 95.03% was observed in *A. solani*, *F. oxysporum* f.sp. *lycopersici*, *Pythium* sp. and *Phytophthora* sp. respectively (Table 4). Whereas in 1.0% concentration, methanol root extract of *M. calabura* and ketoconazole 100% inhibition of mycelial growth was observed.

Effect of methanol extract of *M. calabura* root on spore germination of fungal plant pathogens

At a concentration of 0.8%, the methanol root extract exhibit, inhibition of spore germination of 97.38, 96.94, 96.98 and 97.73% in *A. solani*, *F. oxysporum* f.sp. *lycopersici*, *Pythium* sp and *Phytophthora* sp., respectively (Table 5). Whereas in 1.0% concentration, methanol root extract of *M. calabura* and ketoconozole 100% inhibition of spore germination was observed.

Antioxidant activities of methanol extract of *M. calabura* roots

The methanol extract of *M. calabura* root showed good antimicrobial activity against selected plant pathogens at a concentration of 10 mg/ml. Hence, the same concentration was also used for the study of antioxidant activity (Table 6).

Effect of Muntingin 5EC on seed infection, seed germination of tomato seedlings

The seed infection by *A. solani* was reduced by 99.28% in Muntingin 5EC (2%) treated seeds. The treatments which received Mancozeb (0.2 %) and *P. fluorescens* recorded 89.28 and 90.21% reduced seed infection respectively over the control. The germination percent was increased by 16.50% in Muntingin 5EC (2%) treated seeds as compared to the control (Table 7).

The effect of various concentrations of Muntingin 5EC on growth of tomato seedlings is presented in Table 8. The maximum shoot (13.06

Table 2. Emulsion stability of different EC formulations.

Formulation	Creamy appearance/100 ml measuring cylinder		Sedimentation/100 ml measuring cylinder	
	Below 2 ml	Above 2 ml	Below 2 ml	Above 2 ml
Muntingin 4EC	+	-	+	-
Muntingin 5EC	+	-	+	-

+, Positive; -, negative.

Table 3. Shelf life of Muntingin 5EC formulation and its effect on fungal pathogens.

Days after inoculation	*A. solani*		*F. oxysporum* f.sp. *lycopersici*		*Pythium* sp.		*Phytophthora* sp.	
	A	B	A	B	A	B	A	B
30	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0
60	0.00	100.0	0.00`	100.0	0.00	100.0	0.00	100.0
90	0.01	100.0	0.01	100.0	0.01	100.0	0.01	100.0
120	0.01	100.0	0.01	100.0	0.01	100.0	0.01	100.0
150	7.54	91.28	7.23	92.42	7.75	91.95	6.36	91.70
180	12.00	85.32	10.50	85.84	11.95	85.56	10.97	85.91
Control	86.76		92.60		93.50		90.00	

ME- Methanol extract of *M. calabura*; A- Diameter of mycelial growth in petridish (mm); B- Percent reduction over control. *Mean of three replications.

Table 4. Effect of different concentrations of methanol extract of *M. calabura* root on mycelial dry weight of fungal pathogens.

Treatment	Mycelial dry weight							
	A. solani		*F. oxysporum* f.sp. *lycopersici*		*Pythium* sp.		*Phytophthora* sp.	
	A	B	A	B	A	B	A	B
ME 0.2%	34.50 (±0.72)[b]	83.81	31.82 (±0.64)[b]	82.84	40.92 (±0.59)[b]	81.55	39.80 (±1.15)[b]	84.37
ME 0.4%	28.25 (±0.43)[c]	86.74	24.62 (±0.59)[c]	81.48	32.50 (±0.75)[c]	85.13	30.20 (±0.69)[c]	88.14
ME 0.6%	13.16 (±1.16)[d]	93.82	15.23 (±1.15)[d]	95.41	17.65 (±0.61)[d]	91.92	23.58 (±1.17)[d]	90.74
ME 0.8%	10.05 (±1.18)[e]	95.28	10.15 (±1.24)[e]	96.39	11.23 (±1.16)[e]	94.86	12.65 (±0.61)[e]	95.03
ME 1.0%	0.00 (±0.00)[f]	100	0.00 (±0.00)[f]	100	0.00 (±0.00)[e]	100	0.00 (±0.00)[f]	100
Ketaconazole 0.2%	0.00 (±0.00)[f]	100	0.00 (±0.00)[f]	100	0.00 (±0.00)[e]	100	0.00 (±0.00)[f]	100
Control	213.20(±1.85)[a]		186.70 (±1.73)[a]		218.70(±1.79)[a]		254.78 (±0.59)[a]	

ME, Methanol extract; , Mycelial dry weight (mg); B, percent reduction over control. *Mean of three replications; Control, without methanol extract.

Table 5. Effect of different concentrations of methanol extract of *M. calabura* root on spore germination of fungal pathogens.

Treatment	Spore germination							
	A. solani		*F. oxysporum* f.sp. *lycopersici*		*Pythium* sp.		*Phytophthora* sp.	
	A	B	A	B	A	B	A	B
ME 0.2%	4.33 (±0.59)[b]	94.60	6.72 (±0.62)[b]	91.96	7.05 (±0.61)[b]	91.81	4.18 (±0.59)[b]	94.74
ME 0.4%	3.90 (±0.64)[bc]	95.13	4.31 (±1.16)[c]	94.85	4.40 (±0.81)[bc]	94.89	3.50 (±0.64)[b]	95.60
ME 0.6%	3.20 (±0.12) [d]	96.00	3.90 (±0.06)[c]	95.33	3.30 (±0.69)[c]	96.16	3.10 (±0.06)[c]	96.10
ME 0.8%	2.10 ± (0.06)[c]	97.38	2.56 (±0.59)[d]	96.94	2.60 (±0.69)[d]	96.98	1.80 (±0.64)[d]	97.73
ME 1.0%	0.0 ± (0.00)[e]	100.0	0.0 (±0.00)[e]	100.0	0.0 (±0.00)[e]	100.0	0.0 (±0.00)[e]	100.0
Ketaconazole 0.2%	0.00 (±0.00)[e]	100	0.00 (±0.00)[e]	100	0.00 (±0.00)[e]	100	0.00 (±0.00)[e]	100
Control	80.12 (±1.17)[a]		83.65 (±0.57)[a]		86.10 (±0.64)[a]		79.50 (±1.21)[a]	

ME, Methanol extract; A, spore germination (%); B, percent reduction over control. *Mean of three replications; Control, without methanol extract.

Table 6. Antioxidant activities of methanol extract of *M. calabura* roots.

Antioxidant activity (10 mg/ml)	Root samples
Ferric reducing antioxidant power (μM/g)	0.63 (± 0.02)
DPPH scavenging activity (%)	21 (±1.73)
Reducing power activity (%)	2.42 (± 0.02)
Superoxide anion radical scavenging activity (%)	20 (± 1.15)

Values are mean (± SE)

Table 7. Effect of Muntingin 5EC on disease infection of seed and seed germination of tomato (PKM1).

Treatment	Disease infection of Seed		Seed germination	
	Infection (%)*	Reduction over control	Germination (%)*	Increase over control
0.50% Muntingin 5EC (T_1)	10.20 (0.05)[b]	88.79	83.00	3.75
1.00% Muntingin 5EC (T_2)	7.30 (0.01)[cd]	91.97	85.80	7.25
1.50% Muntingin 5EC (T_3)	4.00 (0.06)[cd]	95.60	89.65	12.06
2.00% Muntingin 5EC (T_4)	0.65 (0.02)[d]	99.28	93.20	16.50
Mancozeb 0.2% (T_5)	9.75 (0.01)[bc]	89.28	83.50	4.37
P. fluorescens (T_6)	8.90 (0.09)[cd]	90.21	84.10	5.12
Control (T_7)	91.00 (0.02)[a]		80.00	

Values are mean (± SE) of five replicates and values followed by the same letter in each column are not significantly different from each other as determined by Duncan's multiple range test ($p \leq 0.05$).

Table 8. Effect of Muntingin 5EC on vigour of tomato seedlings.

Treatment	Shoot length (cm)*	Per cent increase over control	Root length (cm)*	Per cent Increase over control	Vigour index*	Per cent Increase over control
0.50% Muntingin 5EC (T_1)	10.08 (0.02)[b]	32.63	9.00 (0.01)[ab]	25.87	1583.64	39.45
1.00% Muntingin 5EC (T_2)	11.50 (0.12)[ab]	51.31	10.40 (0.12)[a]	45.45	1879.02	55.78
1.50% Muntingin 5EC (T_3)	12.75 (0.01)[a]	67.76	10.55 (0.02)[a]	47.55	2088.84	70.08
2.00% Muntingin 5EC (T_4)	13.06 (0.02)[a]	71.84	10.25 (0.01)[a]	43.35	2172.49	76.29
Mancozeb 0.2% (T_5)	8.18 (0.06)[c]	7.63	8.00 (0.02)[b]	1.18	1351.03	7.70
P. fluorescens (T_6)	8.30 (0.23)[c]	9.21	8.25 (0.06)[b]	1.53	1391.85	11.59
Control (T_7)	7.60 (0.01)[c]		7.15 (0.09)[b]		1180	

Values are mean (± SE) of five replicates and values followed by the same letter in each column are not significantly different from each other as determined by Duncan's multiple range test ($p \leq 0.05$).

cm) and root length (10.25 cm) were observed in the seeds which received 2.0% Muntingin 5EC. The 2.0% Muntingin 5EC treated plants recorded 71.84 and 43.35% increased shoot and root length, respectively over the control. The vigour index was also maximum with 2.0% Muntingin 5EC treatment. The treatments which received Mancozeb and *P. fluorescens* recorded 7.70 and 11.59% increased vigour index respectively over control.

Effect of Muntingin 5EC on early blight control in tomato

The tomato plants sprayed with various concentrations of Muntingin 5EC under pot culture studies were observed for the early blight disease incidence. The percent disease index of the Muntingin 5EC treated plants was significantly lesser as compared to the control. The

Table 9. Effect of Muntingin 5EC on percent disease index of early blight of tomato under pot culture condition.

Treatment	Percent disease index*	Percent reduction over control
0.50% Muntingin 5EC (T_1)	10.83 (± 0.01)^b	83.96
1.00% Muntingin 5EC (T_2)	6.56 (± 0.02)^c	90.28
1.50% Muntingin 5EC (T_3)	3.45 (± 0.26)^d	94.89
2.00% Muntingin 5EC (T_4)	0.36 (± 0.02)^e	99.46
Mancozeb 0.2% (T_5)	1.30 (± 0.03)^e	98.07
P. fluorescens (T_6)	4.18 (± 0.01)^d	93.81
Uninoculated control (T_7)	-	
Control (T_8)	67.55 (± 0.03)^a	

Values are mean (± SE) of five replicates and values followed by the same letter in each column are not significantly different from each other as determined by Duncan's multiple range test ($p \leq 0.05$).

treatment which received 2% Muntingin 5EC recorded 99.4% reduced PDI as compared to control. This was followed by the treatment 1.5% Muntingin 5EC which recorded 98.07% reduced PDI, whereas the treatment which received mancozeb (0.2%) and *P. fluorescens* recorded 94.89 and 93.81% reduced PDI, respectively (Table 9).

Effect of Muntingin 5EC spray on plant defence enzymes in roots of tomato

The changes in activities of various enzymes were monitored on 0, 2, 4, 6 and 10 days after the inoculation of *A. solani*. The PO activity reached maximum at 6 days after inoculation with 2.0% Muntingin 5EC treated plants (2.40 units/g of leaf tissue) and then declined thereafter. Though the peroxidase activities increased in inoculated control, the values were significantly lower than the plants treated with various concentrations of Muntingin 5EC, Mancozeb (0.2%) and *P. fluorescens*. In inoculated control, the activity reached maximum (1.80 units/g of leaf tissue) on second day of inoculation and then declined (Figure 1a).

Similarly, the polyphenol oxidase activity increased in plants treated with Muntingin 5EC when compared with inoculated control. Muntingin 5EC at 2.0% concentration significantly increased the activity of PPO to maximum level (2.72 units/g of leaf tissue) on the tenth day of inoculation (Figure 1b). Among the treatments, maximum phenylalanine ammonia lyase activity was observed (49.00 units/g of leaf tissue) with 2.0% Muntingin 5EC (Figure 1c). The concentration of phenol was significantly higher in plants treated with 2.0% Muntingin 5EC (188.4 µg of catechol/g of leaf tissue) than all other treatments on sixth day of challenge inoculation. In all the treatments, maximum phenol content was reached on sixth day after inoculation. However, all the treatments retained the content of phenol without much reduction even on the tenth day, but the phenol content reduced drastically in inoculated control (Figure 1d).

DISCUSSION

Plant diseases are the major biotic constraints to crop growth and cause variety of damage and significant yield loss. The disease management requires effective integration of approaches to reduce the crop loss effectively. Several strategies have been developed based on genetic, chemical, biological, cultural methods and also combined integrated diseases management framework (Arora and Kaushik, 2003).

Botanicals are materials or products of plants origin valued for their pesticidal, medicinal or therapeutic properties. Phytopesticide materials range from whole fresh plants to purely isolated bioactive phytochemicals or their formulations which are effective against pests and pathogens (Prakash and Rao, 1996). Preparation and application of botanicals for crop protection are linked to the folklores and tradition of the farmers (Anjorin, 2008). Management of disease through fungicides alone leads to soil residual problem and health hazards, besides involving higher input cost.

One of the recent approaches for plant disease management is exploitation of plant products. Inspite of the wide recognition that many plants possess antimicrobial properties, only a handful of products have been developed, because of their less persistence nature in the crop ecosystem (Kumbhar et al., 2001). According to Nagarajan (1996), the emulsifiable concentrates were the most popular formulations in India and the benefits were greater than other formulations. Reports suggest that emulsifiable concentrates are the most desirable for botanical formulations.

As part of the present study, emulsifiable concentrate (EC) formulation was developed from the partially purified antimicrobial compound obtained from methanol root extract of *M. calabura* tested for their stability under laboratory conditions. EC formulation of Muntingin was

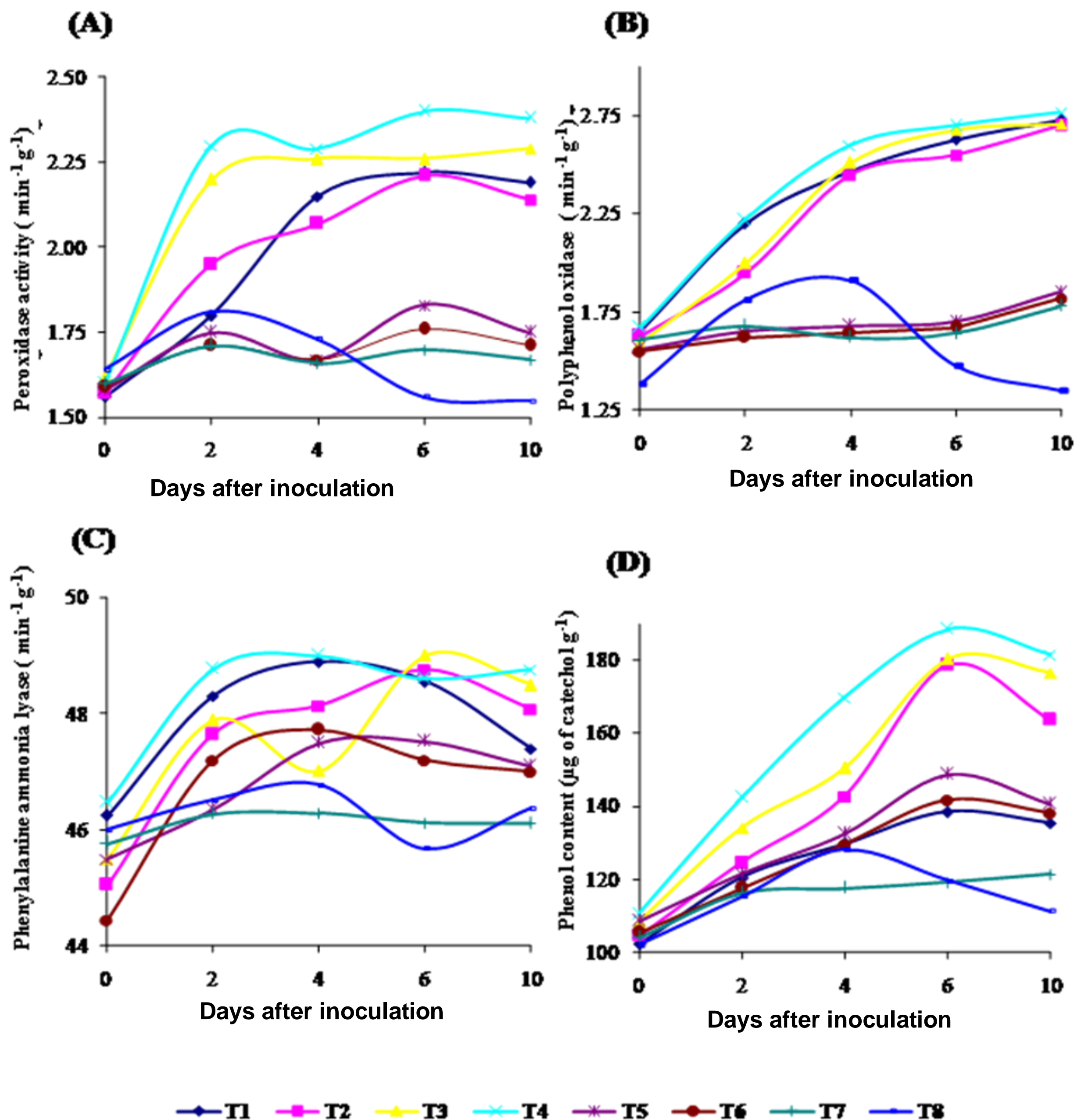

Figure 1. Effect of Muntingin 5EC on changes in plant defense metabolites of tomato under pot culture condition. A- Peroxidase; B- polyphenol oxidase; C- phenylalanne ammonia lyase; D- phenol content.

studied upto 180 days. The formulations were found to retain their maximum activity up to 120 days. The 100% reduction of fungal growth was observed upto 120 days of storage. The optimum pH of the formulation was neutral and it did not exhibit any phytotoxicity when sprayed on to the plants. The formulation was prepared as 4EC and 5EC with the name 'Muntingin'. Whereas the 5EC formulation exhibited 85% reduction of the mycelial growth against the tested fungal plant pathogen after 180 days of storage. The botanical formulation was prepared with 5EC concentration with account of 2% formulation should contain 1 mg of the active compound per milliliter of the product which is the actual MIC value for the antimicrobial compound stigmasterol.

The methanol extract of *M. calabura* root showed significant reduction in mycelial growth and spore germination of the selected fungal plant pathogens - *A. solani, F. oxysporum* f.sp *lycopersici*, *Pythium* sp. and *Phytophthora* sp. The results of the present study indicate one percent methanol root extract of *M. calabura* inhibited the spore germination of *A. solani, F. oxysporum* f.sp. *lycopersici*, *Pythium* sp. and *Phytophthora* sp. by percent.

The inhibition of spore germination by medicinal plants has been reported by many workers (Thirupathi et al., 1999). From this, it is evident that, the *M. calabura* possess antibacterial activity and capable of counteracting reactive oxygen species (ROS), which are responsible for various oxidative damages in the living system. Whereas methanol extract of *M. calabura* root exhibited 21% DPPH activity and 20% SOD activity.

Parimala devi and Marimuthu (2011) reported that the botanical formulation, Polymin 40 EC at various concentrations (0.5, 1.0, 1.5 and 2.0%) controlled *A. solani*. The p-40 at 2.0%effectively controlled the pathogens under pot culture conditions and was considered as the optimum concentration. The botanical formulation at 2% level was found to reduce the seed infection of *F. oxysporum* f.sp. *lycopersici* in tomato and also increased the vigour of tomato (76.29%) as compare to the control.

In the present investigation, the botanical formulation**,** Muntingin 5 EC at various concentrations (0.50, 1.00, 1.50 and 2.00%) was tested under pot culture conditions in controlling *A. solani* in tomato. The Muntingin 5EC at 2.0% level effectively controlled the pathogen under pot culture conditions and was considered as the optimum concentration. The increase in germination percentage and vigour of seedlings may be due to the fact that the application of Muntingin promoted the activity of seed enzymes such as amylase, catalase, etc. and also increased the metabolic activities of the seed.

The biotic and abiotic inducers play an important role in activating the defense genes in plants. Induction of defense proteins makes the plant resistant to pathogen invasion (Ramanathan et al., 2000). The results of the present study revealed that the tomato applied with Muntingin 5EC significantly induced the defense compounds (PO, PPO, PAL and phenol) as compared to control. The resistance of plants induced against the pathogens, due to the application of botanicals has been widely reported (Straub et al., 1986; Rajeswari, 2002; Kagale, 2001). Kagale et al. (2004) observed an increase in antioxidant enzymes in rice plants sprayed with *Datura metal* leaf extract. Increase in PO activity has been observed in the sesame plants treated with *Azadirachta indica* leaf extract (Guleria and Kumar, 2006). Plant phenolics are well known antifungal, antibacterial and antiviral compounds that increase the physical and mechanical strength of the host cell wall. Phenylalanine ammonia lyase is the first enzyme of phenyl propanoid metabolism in higher plants and has been suggested to play a significant role in regulating the accumulation of phenolics (Daayf et al., 1997). The formulation Muntingin 5EC containing the antimicrobial compounds led to increased biosynthesis of PO, PPO, PAL and phenols, which in turn were responsible for disease resistance in plants.

It has been concluded that the botanical formulation “Muntingin” increased the tomato germination percentage by reducing the seed infection by *A. solani*. It has also been shown, the potential inhibitory effect on the selected plant pathogen under pot culture conditions. The botanical formulation is capable of inducing the resistance in tomato plants through enhancement of defense compounds. Thus, the formulation Muntingin showed the potential for managing the early leaf blight disease in tomato. The future thrust and follow-up research efforts may aim to study the effect of Muntingin on plant disease control under field conditions and this will provide an opportunity for eco-friendly disease management on a variety of crops.

Conflict of interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Antioxidative response of the fungal plant pathogen *Guignardia citricarpa* to antimicrobial volatile organic compounds

Mauricio Batista Fialho[1], Giselle Carvalho[2], Paula Fabiane Martins[2], Ricardo Antunes Azevedo[2] and Sérgio Florentino Pascholati[1]*

[1]Departamento de Fitopatologia e Nematologia, Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Universidade de São Paulo (USP), Avenida Pádua Dias, 11, CP 09, CEP 13418-900, Piracicaba, SP, Brazil.
[2]Departamento de Genética, Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), Universidade de São Paulo (USP), Avenida Pádua Dias, 11, CP 09, CEP 13418-900, Piracicaba, SP, Brazil.

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Volatile organic compounds (VOCs) produced by the yeast *Saccharomyces cerevisiae* strain CR-1 are able to inhibit the development of the fungal phytopathogen *Guignardia citricarpa*, causal agent of the disease citrus black spot. Antimicrobial VOCs have potential applications to control plant pathogens; however, there is limited information on the action mechanisms. It is possible that VOCs trigger the accumulation of harmful levels of reactive oxygen species. Therefore, this work aimed to evaluate the effect of the artificial mixture of VOCs, constituted by alcohols and esters identified from *S. cerevisiae*, on the antioxidant system of *G. citricarpa*. The VOCs at 0.48 µl/ml of air space completely suppressed the mycelial growth and reduced the respiratory rate. Increased activity of the antioxidant enzymes superoxide dismutase and catalase in the first 24 to 48 h of exposure to the VOCs indicating intracellular accumulation $O_2^{\cdot-}$ and H_2O_2, respectively was observed; however, the antioxidant glutathione pathway was not activated. In addition, increased lipid peroxidation was detected, confirming the oxidative stress process. Therefore, it was reported for the first time the disruption of the cellular redox homeostasis in microorganisms caused by VOCs. This knowledge is important to better understand the action mechanisms of antimicrobial volatiles and to develop safer fumigants to control postharvest diseases.

Key words: Antifungal, citrus, fumigation, stress response.

INTRODUCTION

The citrus black spot caused by the ascomycete fungus *Guignardia citricarpa* (Anamorph: *Phyllosticta citricarpa*) is one of the most important diseases of citrus in producing areas of Africa, Asia, Australia and South

*Corresponding author. E-mail: sfpascho@usp.br. Tel: +55-19-34294124 ext. 210. Fax: +55-19-34344839.

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America. Several fruit symptoms are associated to the disease and infected fruits can develop them at postharvest during transport or storage. Although the disease does not cause internal decay, fruits become aesthetically damaged. This scenario limits the exporttation because citrus black spot is an A1 quarentenary disease in the European Community and the United States of America (OEPP/EPPO, 2009).

The chemical control is the main tool to control plant diseases; however, the use of carbendazim, one of the few effective fungicides to combat the citrus black spot, was banned (U.S. EPA, 2012). The impact of the chemical control on human health and environment increased the interest for alternative control methods. The use of microbial antagonists may interrupt some stage of the pathogen's life cycle by several mechanisms such as parasitism, competition for nutrients and colonization niches, and production of hydrolytic enzymes and antibiotic compounds (Sharma et al., 2009), including volatiles (Strobel, 2011).

Microbial volatile organic compounds (VOCs) are considered ideal info chemicals as their sphere of activity extends from proximal distances due to aqueous diffusion, to greater distances via aerial diffusion, affecting negatively the physiology of competitor microorganisms. Therefore, it is possible that volatile metabolites have played an important role during the microbial evolution in the context of community, population and functional dynamics (Wheatley, 2002). Potential applications of the fumigation employing safe natural VOCs include the control of pathogens in fruits, grains and seeds in closed chambers during transport and storage, and to replace the methyl bromide and others harmful fumigants to control soil-borne pathogens (Strobel, 2011). The use of VOCs is advantageous as the residual effect is limited and minimizes the product handling (Mercier and Jimenez, 2004). In addition, fungistatic VOCs can act as chemosensitizing agents by increasing the pathogen susceptibility to lower dose of fungicides; thus, reducing costs and risks of negative side effects such as acquisition of fungicide resistance and environmental impact (Kim et al., 2012).

The action mechanisms of antimicrobial VOCs are little known. There is evidence that VOCs cause DNA damage (Mitchell et al., 2010) and interfere with morphogenesis-related enzymes (Wheatley, 2002; Fialho et al., 2011). It is possible that VOCs trigger the accumulation of harmful levels of reactive oxygen species (ROS) resulting in oxidative stress. The ROS include free radicals such as superoxide anion ($O_2^{\bullet-}$), and non-radical reactive species such as hydrogen peroxide (H_2O_2). These potent oxidizing agents react specifically and rapidly with macromolecules, resulting in DNA mutations, protein oxidations and lipid peroxidation (Aguirre et al., 2005; Heller and Tudzynski, 2011).

The ROS are continuously produced during the aerobic metabolism but are maintained at basal and harmless levels by antioxidant mechanisms. Superoxide dismutase (SOD, EC 1.15.1.1) is considered the first line of defense by catalyzing the dismutation of $O_2^{\bullet-}$ to H_2O_2. Subsequently, catalase (CAT, EC 1.11.1.6) detoxifies the H_2O_2 producing water and molecular oxygen (Aguirre et al., 2005; Heller and Tudzynski, 2011). The thioltripeptide glutathione (GSH) is also involved in the ROS scavenging. In this antioxidant system, GSH is oxidized by H_2O_2 to water in the presence of glutathione peroxidases (GPx, EC 1.11.1.9), forming oxidized glutathione (GSSG). The NADPH-dependent glutathione reductase (GR, EC 1.6.4.2) reduces GSSG regenerating GSH (Pocsi et al., 2004).

Physical and chemical stressors such as UV radiation, extreme temperatures, desiccation, heavy metals and exogenous H_2O_2 can increase the intracellular levels of ROS and overcome the antioxidant mechanisms, resulting therefore in oxidative stress (Angelova et al., 2005). However, the relationship between exposure to VOCs and oxidative stress in microorganisms has not yet been studied. This knowledge is important to better understand the microbial interactions mediated by volatile metabolites, and to develop new strategies to control the citrus black spot and other postharvest diseases. Formulations based on microbial VOCs could be employed to fumigate fruits infected but asymptomatic. This procedure could avoid the development of symptoms during the transport or at the final destination.

The yeast *Saccharomyces cerevisiae* strain CR-1, isolated from fermentative processes for fuel ethanol production, exhibited fungistatic effect on *G. citricarpa*. The antagonism was attributed to production of a mixture of volatile alcohols and esters (Fialho et al., 2010). Therefore, this study aimed to investigate antioxidative response of *G. citricarpa* exposed to the artificial mixture of VOCs, originally identified from the yeast *S. cerevisiae*.

MATERIALS AND METHODS

Phytopathogenic fungus

Guignardia citricarpa was isolated from orange fruit lesions and maintained in Potato-Dextrose-Agar (PDA) (Difcolaboratories, Detroit, MI, USA) at 26ºC. The fungus is deposited as isolate IP-92 in the mycological culture collection of the Laboratory of Plant Pathology in the Department of Crop Protection at FCAV/UNESP in Jaboticabal, SP, Brazil. The origin and DNA sequence of the fungus was reported by Wickert et al. (2012).

Antifungal activity

From previous information obtained by gas chromatography coupled to mass spectrometric detection (GC-MS) analysis of the gaseous atmosphere was produced by *Saccharomyces cerevisiae* strain CR-1 (Fialho et al., 2010). It produced an artificial mixture of VOCs using authentic standard chemicals (Sigma/Aldrich, St. Louis, MO, USA). The mixture contained the six compounds positively identified, and the proportion of each compound was calculated in relation to all other components of the mixture (Table 1).

Table 1. VOCs produced by *Saccharomyces cerevisiae* strain CR-1 on PDA medium (Fialho et al., 2010).

Compound	% Relative (v/v)
Ethanol	85.3
Unidentified	1.5
Ethyl acetate	1.8
3-methyl-1-butanol	6.9
2-methyl-1-butanol	2.4
Phenylethyl alcohol	0.7
Ethyl octanoate	1.4

As previously described by Fialho et al. (2011), it was employed for the bioassays of a two section-divided polystyrene plate (BD Falcon, Franklin Lakes, NJ, USA). On one side of the plate, containing 10 ml of PDA, a 5 x 5 cm sterile semi-permeable membrane was placed. A pathogen mycelium plug (5 mm) was added onto the membrane, and the plate was incubated at 26°C. After five days of growth, on the opposite side of the plate, 24 µl (0.48 µl/ml of air space) of the artificial mixture of VOCs were added on a sterile cotton wool. The plates were sealed with parafilm and incubated at 26°C. The free headspace of the plate was 50 ml and this was used to calculate the concentration of VOCs. The control consisted of plates containing the pathogen in the absence of the artificial mixture.

After 24, 48 and 72 h of exposure to the VOCs the mycelia were harvested from the membranes, weighed and stored at -80°C. The radial mycelial growth was also evaluated daily. Three replicates were used for each treatment.

Determination of the respiratory rate

Five days old cultures of *G. citricarpa* were exposed to the artificial mixture of VOCs at 0.48 µl/ml of air space, as described above. After 72 h of exposure, atmosphere samples were collected with a gas-tight syringe through a rubber septum adapted to the plates and injected into a gas chromatographer Trace 2000 (Thermo Fisher Scientific,Austin, TX, USA), equipped with flame ionization detector (FID). The CO_2 concentration was expressed in ml CO_2/g biomass per day of growth. Five replicates were used for each treatment.

Protein extraction

Antioxidant enzymes were extracted as described by Monteiro et al. (2011) with minor modifications. The frozen mycelia were grounded in a mortar with liquid nitrogen and added to 100 mmol/L potassium phosphate buffer (pH 7.5) containing 1 mmol/L EDTA and 3 mmol/L dithiothreitol (DTT) in the proportion of 5 ml/g biomass. The homogenates were centrifuged at 15,000*g* for 20 min at 4°C and the supernatants were collected and kept at -80°C prior the enzyme analysis. The protein concentration was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as standard.

Enzymatic assays

All the enzymatic activities were performed as previously described by Monteiro et al. (2011) with modifications. For superoxide dismutase (SOD) activity non-denaturing PAGE was carried out in gels containing 10% polyacrylamide with a 4% polyacrylamide stacking gel (Laemmli, 1970). Equivalent to 20 µg total protein were loaded and the electrophoresis performed at constant current of 20 mA for 4 h at 4°C. The gels were washed with distilled water and incubated in the dark for 30 min in 100 mmol/L sodium phosphate buffer (pH 7.8), containing 1 mmol/L EDTA, 0.05 mmol/L riboflavin, 0.1 mmol/L nitrobluetetrazolium (NBT) and 0.3% (v/v) N,N,N´,N´-tetramethylethylenediamine (TEMED). The gels were washed in distilled water and exposed to fluorescent light until the development of colourless bands in a purple-stained gel and fixed with 7% (v/v) acetic acid. Bovine liver SOD (2 units) was used as standard. The densitometric analysis was performed using ImageJ 1.47v analysis software (National Institute of Health, Bethesda, MD, USA).

The catalase (CAT) activity was determined by using a reaction mixture containing 1.450 ml 0.036% (v/v) H_2O_2 in 100 mmol/L sodium phosphate buffer (pH 6.8). After addition of 0.05 ml of protein extract the H_2O_2 decomposition was monitored at 240 nm for 1 min at 25°C. The specific enzyme activity was expressed in U/mg protein, where one unit (U) corresponds to the decomposition of 1 µmol of H_2O_2 per min under the assay conditions.

The glutathione reductase (GR) activity was determined by using a reaction mixture consisting of 1 ml 100 mmol/L potassium phosphate buffer (pH 7.5) containing 1 mmol/L 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), 1 mmol/L oxidized glutathione (GSSG) and 0.1 mmol/L NADPH. After addition of 0.05 ml of protein extract the GSSG reduction was monitored at 412 nm for 1 min at 30°C. The specific enzyme activity was expressed in U/mg protein, where one unit (U) corresponded to 1 µmol of reduced glutathione (GSH) per min under the assay conditions.

Determination of the lipid peroxidation

Lipid peroxidation was determined by measuring the concentration of thiobarbituric acid (TBA) reactive substances, estimated as malondialdehyde (MDA) equivalents (Heath and Packer, 1968). Mycelial material (500 mg) was homogenized in 5 ml 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000*g* for 20 min. The supernatant (0.5 ml) was added to 1 ml 0.5% (w/v) TBA in 20% (w/v) TCA, heated in boiling water bath for 30 min, and cooled in ice. After centrifugation at 10,000 *g* for 5 min, the absorbance of the supernatant was determined at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA-TBA complex was calculated using the extinction coefficient 155 mM^1 cm^1 and expressed as mmol/L of MDA/g biomass.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using Tukey's test at $P < 0.01$ to examine significant differences between treatments. All results were expressed as means ±standard deviation (SD).

RESULTS

The synthetic mixture of VOCs was prepared and its effect on the mycelial growth of *G. citricarpa* was evaluated (Figure 1). The fungal development was paralyzed after the first 24 h of exposure and at the end of 72 h the mycelial growth was approximately 30% lower when compared to the control. In addition, the respiratory rate of *G. citricarpa* decreased 54% (Figure 2). However, the fungus resumed normal development when transferred to new medium in the absence of the VOCs.

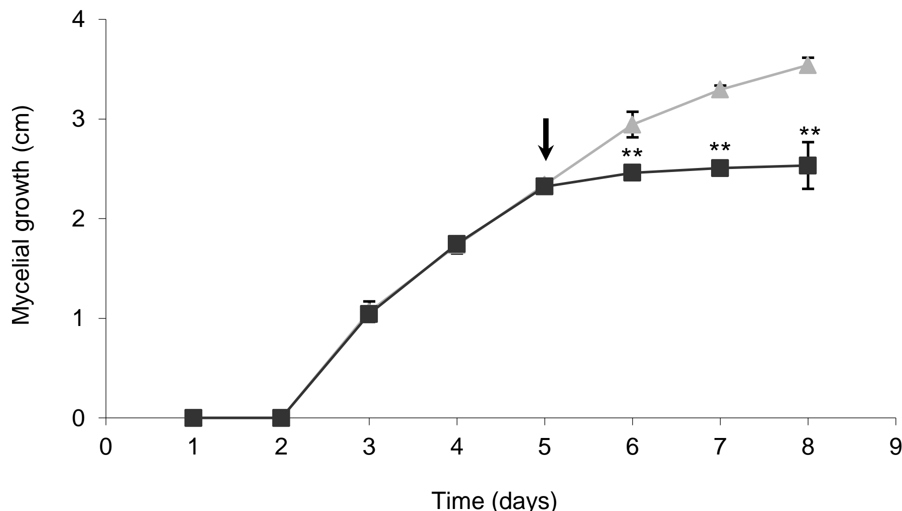

Figure 1. Effect of the artificial mixture of VOCs on mycelial growth of *Guignardia citricarpa*. The arrow indicates the addition of the VOCs. Values are means of three replicates (±SD). ** Indicates values that differ significantly from the corresponding control at $P < 0.01$, Tukey's test. Control (▲) and Volatiles (■).

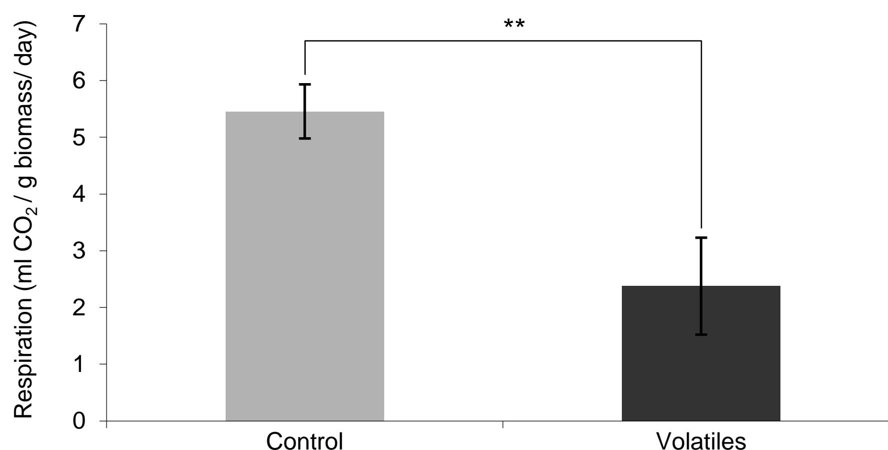

Figure 2. Effect of the artificial mixture of VOCs on respiratory rate of *Guignardia citricarpa* after 72 h of exposure. Values are means of five replicates (±SD). ** Indicates values that differ significantly from the corresponding control at $P < 0.01$, Tukey's test. Control (■) and Volatiles (■).

In order to study the antioxidative response of *G. citricarpa* to the antimicrobial VOCs, the activity of three key antioxidant enzymes (SOD, CAT and GR) were evaluated. As observed in the Figure 3, three SOD isoforms (I, II and III) were detected. The SOD isoform I did not show significant change, however, the activity of the SOD isoform II increased 37 and 110% after 24 and 48 h of exposure to the VOCs, respectively, while the activity of the SOD isoform III increased, 17 and 90%, respectively, in the same period. After 72 h of exposure both SOD isoforms were not significantly affected when compared to the controls. Furthermore, no other change, including appearance or disappearance of isoforms was observed.

Under normal physiological conditions, *G. citricarpa* exhibited gradual increase in CAT activity during the culture ageing. The exposure to the VOCs increased the CAT activity by 32% in the first 24 h; however, after 72 h the activity was reduced by 24% (Figure 4A). In the present study, despite the effects observed on the SOD and CAT activities, GR activity was not affected by the VOCs (Figure 4B). Differently from CAT, under normal

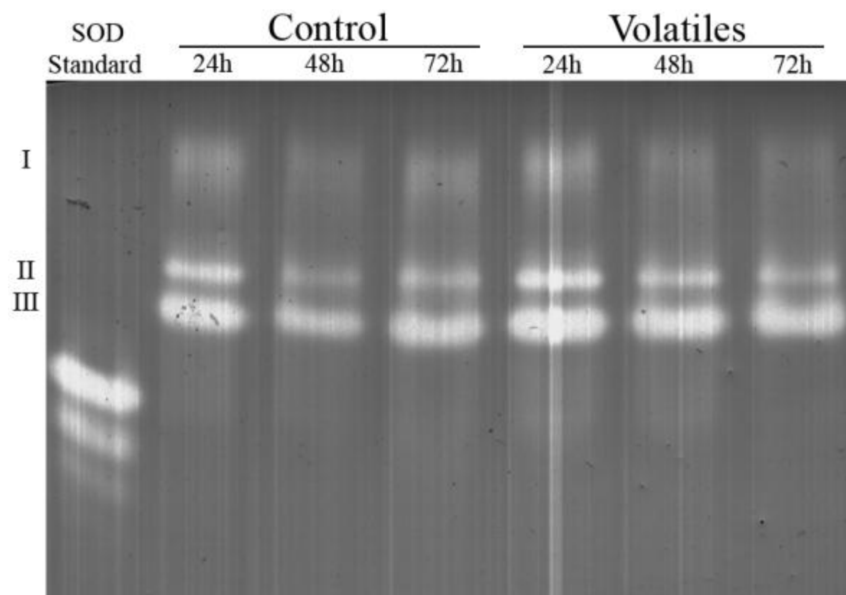

Figure 3. Effect of the artificial mixture of VOCs on superoxide dismutase activity of *Guignardia citricarpa* after 24, 48 and 72 h of exposure, determined by non-denaturing PAGE. The numbers I, II and III indicates the SOD isoforms detected. Bovine liver SOD (2 units) was used as standard.

conditions the GR activity remained stable during the fungal development.

The MDA content in *G. citricarpa* was quantified aiming to evaluate the lipid peroxidation triggered by the VOCs (Figure 4C). The lipid peroxidation increased approximately 3 and 1.5 times after 24 and 48 h of exposure to the VOCs, respectively, however, after 72 h the lipid peroxidation was reduced to normal levels.

DISCUSSION

The mycelial growth of *G. citricarpa* stopped when the artificial mixture was added to the plates, mimicking the fungistatic effect of the *S. cerevisiae* atmosphere on the phytopathogen as previously reported by Fialho et al. (2010, 2011). In addition, reduction in the respiratory rate was observed, probably as a result of the low metabolism. Reports on the influence of VOCs on the microbial respiration are limited and the results available have been shown to vary. Some volatiles such as acetaldehyde can increase the respiration of soil microorganisms (Owens et al., 1969), while others did not show any effect (Ko and Chow, 1977). Humphris et al. (2001) evaluated the respiratory rate of wood degrading basidiomycetes exposed to antifungal VOCs identified from *Trichoderma* spp. The compound 2-methyl-1-butanol inhibited between 77 and 100% the respiration of all fungi at 2500 µg/ml, however, stimulated the respiration of *Postia placenta* at low concentrations (2.5 and 24 µg/ml). The VOCs octanal and hexanal inhibited the respiration of the fungi while acetone produced a stimulating effect on *Trametes versicolor*.

The early response of SOD and CAT in *G. citricarpa* indicates that the VOCs triggered the formation of $O_2^{\bullet -}$ and H_2O_2, respectively. The exposure of bacteria, yeasts and filamentous fungi to the herbicide paraquat, which generates specifically $O_2^{\bullet -}$, increases the synthesis of SOD, but not CAT (Bussink and Oliver, 2001; Moradas-Ferreira et al., 1996; Amo et al., 2002). Angelova et al. (2005) studying 12 species of filamentous fungi reported that the SOD activity in the fungi was increased by 2 times when exposed to paraquat while the CAT activity was increased by 2.5 times when exposed to H_2O_2.

As expected, the results demonstrated that *G. citricarpa* contains antioxidant enzymes even under normal physiological conditions as ROS are generated during the respiratory metabolism. In addition, antioxidant enzymes are important for phytopathogens not only to neutralize their own ROS, but also to counteract the oxidative burst generated by their host cells during the initial phase of the infection process. The ROS produced by plants may have direct antimicrobial activity and/or may signal the activation of latent defense mechanisms. Therefore, antioxidant enzymes are considered an important virulence factor for fungal phytopathogens (Rolke et al., 2004; Heller and Tudzynski, 2011; Veluchamy et al., 2012). The citoplasmatic Cu/Zn-SODs, encoded by the genes *bcsod1* and *sssod1* in the plant pathogens *Botrytis cinerea* and *S. sclerotiorum*, respectively, were implicated in the $O_2^{\bullet -}$ detoxification and in host defense suppression (Rolke et al., 2004; Veluchamy

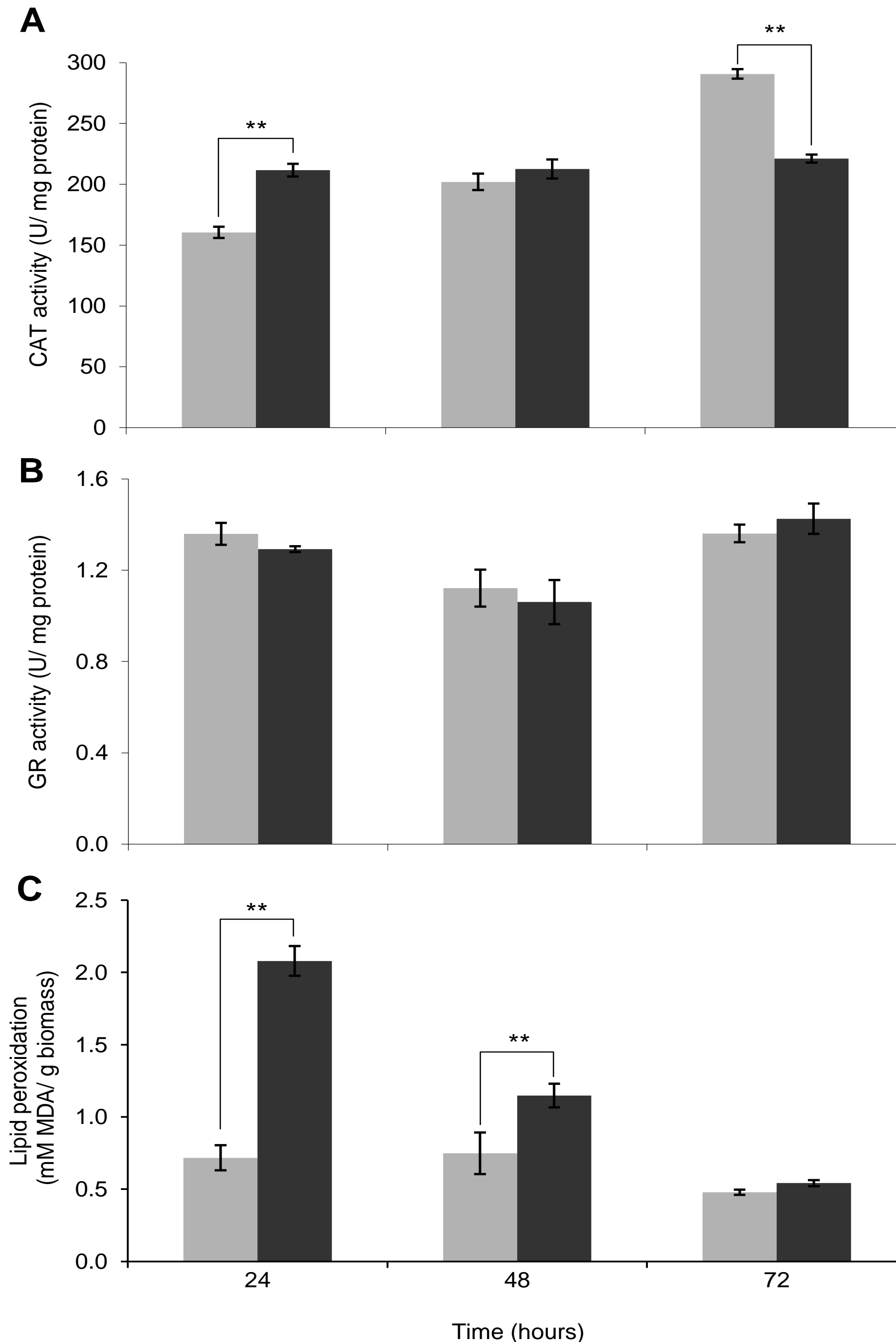

Figure 4. Effect of the artificial mixture of VOCs on catalase (A) and glutathione reductase (B) activities, and lipid peroxidation (C) of *Guignardia citricarpa* after 24, 48 and 72 h of exposure. Values are means of three replicates (±SD). ** Indicates values that differ significantly from the corresponding control at $P < 0.01$, Tukey's test. Control (■) and Volatiles (■).

et al., 2012). It is possible that SOD could also be a virulence factor in *G. citricarpa*.

The gradual increase in CAT levels during the culture ageing of *G. citricarpa* in normal conditions may constitute an adaptive response to the enhanced generation of H_2O_2 in entering into the stationary phase, as also observed in *S. cerevisiae* (Jakubowski et al., 2000). In *A. nidulans* the genes *catA* and *catB* are expressed during processes of spore formation and in vegetative mycelium, respectively. Both genes provide

protection at different stages of development against exogenous H_2O_2 and other stress conditions. Homologues of the genes *catA* and *catB* have been found in other *Aspergillus* species, and in *Neurospora crassa* (Li et al., 2009; Hansberg et al., 2012). Malolepsza (2004) demonstrated that CAT is an important virulence factor in *B. cinerea* infecting tomato plants.

The antioxidant glutathione pathway in *G. citricarpa* was not activated by the VOCs. Therefore, it is proposed that SOD and CAT may play more important roles in the detoxification of ROS triggered by VOCs. Moreover, an antioxidant system other than glutathione could have been employed by *G. citricarpa* exposed to the VOCs. A complex system consisting of thioredoxins, which operates in parallel to the glutathione system, can also be important to maintaining the GSH redox state by regenerating the GSH from GSSG (Tan et al., 2010). Studies related to the glutathione pathway in filamentous fungi are scarce. In *A. niger* and *Penicillium chrysogenum* CAT played a more important role in H_2O_2 detoxification than enzymes belonging to the glutathione pathway (Emri et al., 1999; Li et al., 2008). More recently, Bento et al. (2013) reported increased GR activity in the basidiomycete *Pycnoporus sanguineus* growing in PDA medium supplemented with antifungal plant extracts of two species of *Casearia*, however, this effect was less evident in *Trametes villosa*.

The VOCs increased the lipid peroxidation in *G. citricarpa* mainly in the first 24 h of exposure. Some of the consequences of the lipid peroxidation are decreased membrane fluidity, increased permeability to H^+ and other ions, and eventual cellular rupture. In addition, cytotoxic by-products, mainly MDA, are formed during the process, which has effects on DNA and proteins away from the area of their generation (Li et al., 2009). The lipid peroxidation is one of the most important parameters of oxidative stress, however, studies reporting lipid peroxidation in fungi exposed to antifungal compounds are limited, and the effect of VOCs on this oxidative stress biomarker had not been reported until now.

Although the mycelial development of *G. citricarpa* had been suppressed by the VOCs, the fungus was not killed (fungistatic effect). In part, the non-lethality may be due to the early response of SOD and CAT, enhancing the fungus ability to avoid continued exposure to high levels of ROS and associated deleterious intracellular events. This observation, associated to the decreased lipid peroxidation after prolonged exposure to the VOCs suggest that *G. citricarpa* was able to counteract at least in part the oxidative stress established by the VOCs. In addition to the initial unbalanced redox state, other inhibitory mechanisms may be involved. It was reported previously in *G. citricarpa* negative effect of the VOCs on the morphogenesis-related enzymes laccase, tyrosinase, β-1,3-glucanase and chitinase (Fialho et al., 2011).

Oxidative stress caused by several chemical and physical factors has been extensively studied in prokaryotes, animal and plant cells and yeasts (Lushchak, 2011). However, information about filamentous fungi, especially phytopathogens, is scarce and fragmented. Studies have shown that the activity of antimycotics such as amphotericin B and itraconazole are linked to oxidative stress (Kim et al., 2012); however, this is the first study reporting the relationship between exposure to VOCs and oxidative stress in microorganisms.

Conclusion

The results demonstrate that oxidative stress in microorganisms can be triggered not only by physical stress and water-diffusible substances, as already extensively reported, but also by VOCs. Information on the action mechanisms is important to better understand the microbial interactions mediated by volatile metabolites in nature, and to develop safer fumigants to control the citrus black spot caused by *G. citricarpa* and other postharvest diseases.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Faecal lactobacilli from common pheasants and their characterization

A. Kandričáková and A. Lauková*

Institute of Animal Physiology Slovak Academy of Sciences, Šoltésovej 4-6, 04001 Košice, Slovakia.

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Common pheasants have been most often breeded for sport hunting and for culinary meat. Lactic acid bacteria (LAB) are a part of the obligate intestinal microflora. They play important role in the development of the immune system. Concerning the common pheasants, only limited information exists in relation with LAB. The aim of our work was to study faecal lactobacilli isolated of 60 common pheasants (farm in west Slovakia) to the genus, *Lactobacillus* were allotted two strains of *Lactobacillus brevis* and two to *Lactobacillus plantarum* (50 isolates) identified by Maldi-Tof system expressed on the basis of high score values identification; confirmed by phenotypization and polymerase chain reaction. Undesirable enzymes β-glucuronidase and β-glucosidase showed negative reaction in *L. plantarum* LP13, *L. plantarum* LP14, *L. brevis* LB12. Amounts of lactic acid produced by strains were in accordance with typical representants of homofermentative lactobacilli. Strains inhibited the growth of at least of two out of 16 indicators; mostly listeriae. *L. plantarum* LP13 was the most active strain. LB12, LP13 and LP14 tolerate very low pH and 1% bile in growth medium. Results are contribution to the limited knowledge in this area; LP13 strain was selected for the further detailed antimicrobial studies.

Key words: Common pheasants, lactobacilli, identification, characterization.

INTRODUCTION

Common pheasants husbandry has been most often provided for sport hunting but also for culinary meat. Therefore, healthy pheasants are of paramount importance to farmers and consumers. Immune system plays an important role in health of animals; the common intestinal microflora plays an important role in development of the immune system. Lactic acid bacteria (LAB) are a part of the obligate intestinal microflora. Only limited information can be found in the literature on the microflora of common pheasants (including LAB). Some authors assert that the dominated LAB in pheasants are bifidobacteria, lactobacilli, peptococci and streptococci (Shulin and Xiuli, 1998). LAB especially those possessing probiotic properties have shown many beneficial effect on health of animals and humans; e.g. lactobacilli are known to transform hexoses to lactic acid which has antimicrobial effect (Jahreis et al., 2002; Strompfová and Lauková, 2013). They can also tolerate lower pH or bile in the

*Corresponding author. E-mail: laukova@saske.sk. Tel. +421557922964; Fax:+421 55 7287842.

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Abbreviations: LAB, Lactic acid bacteria; **CFU,** colony forming unit; **Maldi-Tof,** matrix assisted laser desorption/ionization-time of flight mass spectrometry; **mmo/l,** millimol per liter; mm-millimeter; **HSP,** feed mixtures for animals; **log10,** logarithmus 10; **nmol,** nannomol; **µg,** microgram.

intestinal tract which is an acceptable condition for their probable application (Vinderola and Reiheimer, 2003). Moreover, some lactobacilli can cause antimicrobial effect due to the production of bacteriocin substances (De Vuyst et al., 1996; Enan, 2006). Following these properties, the aim of our work was to characterize lactobacilli detected in faeces of common pheasants; the results will contribute to limited knowledge in microflora of pheasants; promising strain with beneficial properties can be selected.

MATERIALS AND METHODS

Sampling and identification

Pheasants were located in the aviaries of west Slovakia farm with free movement. They were 16-17 weeks old. Faeces from 60 pheasants were sampled. Six feacal mixture of randomly picked up faeces from 10 pheasants in each sample were provided. Pheasants were fed commercial feed mixture BZ3 (HSP, Bratislava, Slovakia) for pheasants broilers. They had access to water *ad libitum*. Sampling, birds handling and care were performed in relation to acceptance of Slovak Veterinary and Food Administration and farmer. Mixture samples (in each, faeces of 10 pheasants) were treated by the standard microbiological method. Briefly, 1 g of faecal mixture from each sample in Ringer solution (Merck, Germany) was treated using the Stomacher (Masticator, Spain) and then diluted in Ringer solution (ratio 1:9). To enumerate lactic acid bacteria (LAB), the appropriate dilutions were plated on MRS agar (Merck, Germany; MRS broth supplemented with 1.5% (v/w) agar, ISO 15214) and the plates were incubated at 37°C for 48 h (in CO_2 atmoshpaere). The total counts of lactic acid bacteria were enumerated as an average count of colonies grown in the highest dilution (per sample) and expressed in colony forming units per gram (CFU/g, log 10). Selected colonies were checked for their purity and prepared for the identification to the species level. The species identification was provided by the use of the Maldi-Tof BioTyper TM identification system (Bruker Daltonics, 2008) based on the analysis of bacterial proteins measured by Maldi-Tof mass spectrometry. Lysates of bacterial cells were prepared according to the instruction of producer prior to identification (Bruker Daltonics, 2008). Moreover, phenotypization using the commercial BBL Anaerobe kit (Becton and Dickinson, Cockeysville, USA) was performed. As control strain, *Lactobacillus plantarum* CCM 4000 (Dr. Nemcová, University of Veterinary Medicine and Pharmacy, Košice, Slovakia, deponed to Czech Culture Collection in Brno, Czech republic) was used. Bacterial DNA for the PCR reaction was extracted according to Klein-Lanhorst et al. (1991). The primers and protocol for PCR (Bethier and Ehrlich, 1998) were as follows: 5´-ATGAGGTATTCAACTTATG-3´ and 5´-GCTGGATCACCTCCTTTC-3´, initial denaturation at 95°C for 5 min, 30 cycles at 95°C for 1 min, temperature and time of annealing were 51°C 1 for min, 72°C for 1 min and elongation at 72°C for 7 min. Thermocycler Techgene KRD (the Great Britain) was used. The PCR product was separated by electrophoresis in 0.8% agarose gels (Sigma, Germany) containing ethidium bromide (1 µg/L Sigma). The molecular mass standard (100 bp DNA ladder, Invitrogen, USA) was used according to the manufacturers instruction. *L. plantarum* CCM 4000 (Dr. Nemcová, University of Veterinary Medicine and Pharmacy, Košice, Slovakia) was used as positive control.

Enzymes testing

The isolated strains were tested for enzyme production using the API-ZYM system (BioMérieux, France) following the manufacturers recommendations. The inocula (65 µl) of the McFarland standard 1 suspensions were pipetted into each well of the kit. Enzyme activities were evaluated after 4 h of incubation at 37°C and after the addition of Zym A and Zym B reagents. Color intensity values from 0 to 5 and their relevant value in nanomoles were assigned for each reaction according to the color chart with the kit.

Antibiotic phenotype and lactic acid production

Antibiotic phenotype of the strains was tested according to EFSA test (2012) using the antibiotic discs as follows: gentamicin (10 µg), erythromycin (15 µg), chloramphenicol (30 µg), kanamycin (30 µg), vancomycin (30 µg), rifampicin (30 µg) and tetracycline (30 µg). They were evaluated according to the recommendations of the suppliers (Becton and Dickinson, Cockeysville, USA or Lach-Ner, Czech republic) as sensitive or resistant reading of the zones of inhibition. As a control *L. plantarum* CCM 4000 was used (Dr. Nemcová, UVMP Košice, Slovakia). Briefly, the strains were incubated in MRS broth (Merck) up to the optical density-OD_{600} 1.0. Then aliquots (100 µl) were plated on MRS agar enriched with defibrinated sheep blood. The sterile discs were applied onto plates. The plates were cultivated at 37°C for 18 and/or 24 h in the atmosphaere with CO_2. The sizes of inhibitory zones were recorded in millimeter (mm).

Lactic acid was analyzed by the validated spectrophotometric method and expressed in mmol/L. This method is based on the conversion of lactic acid to acetaldehyde by heat from sulfuric acid. Acetaldehyde reacts with 4-hydroxybiphenyl to form a color complex. Prior to the analysis, the strains were cultivated in MRS broth (Merck, Germany) at 37°C (under CO_2 atmosphaere) for 18 h. Aliquotes volumes of these cultures were then analyzed (Pryce, 1986).

Antimicrobial activity

Antimicrobial activity of the strains was tested by the qualitative method. Briefly, the plates with MRS agar (1.5%) were inoculated by testing strains of lactobacilli (as stripes) and cultivated at 37°C overnight. Indicator bacteria were grown in Trypticase soy broth (Difco, USA) at 37°C up to the optical density 1.00 (OD_{600}). Plates with grown lactobacilli were then overlaid with Trypticase soy agar (0.4%) supplemented with 200 µl of broth cultures of indicators; then they were incubated again under the same conditions. The average of the clear inhibion zones were evaluated in mm; it means the inhibition of indicator strain by tested lactobacilli. The size of zones from 5 to 10 mm was evaluated as low antimicrobial activity; zones measuring over 10 mm in average were evaluated as high antimicrobial activity. The target of indicator strains were: *Enterococcus avium* EA5 (our isolate from faeces of piglets- the principal indicator), *Staphylococcus aureus* SA5 (our isolate from mastitis milk), 12 strains of *Listeria monocytogenes*: (2116, P3300, P2024, P5258, P6301, P6501, 7223, P7395, P7401, P7562, Ve405, TS10811, isolates from different meat products, State Veterinary Institute, Olomouc, Czech republic), *L. monocytogenes* CCM4699 (clinical isolate, Czech Culture Collection-CCM, Brno, Czech republic), *Listeria innocua* LMG13568 (University of Brussel, Belgium).

Resistance to low pH and bile tolerance

To check resistance to low pH, the method according to Arboleya et al. (2011) was used. Lactobacilli were grown in 5 ml of MRS broth (Merck, Germany) at 37°C overnight. After centrifuging, cells were resuspended in Ringer solution (Oxoid). Bacterial suspension was

added to gastric juice stimulated solution (1:9, pH 2.5 and 7) and incubated for 90 and 180 min. To check surviving of cells, samples of 0, 90 and 180 min were spread on MRS agar (Merck, Germany) to count cells (CFU/mL). The test was performed in duplicate.

To check bile tolerance, the tubes were inoculated with 0.1% of tested strain grown overnight in MRS (Merck, Germany) and enriched with 1% of bile (at 37°C). The control tubes contained MRS broth with strain and no bile). They were cultivated for 12 and 24 h and the surviving growth of strains was checked by samples plating on MRS agar (Merck, Germany). Plates were kept at 37°C for 48 h and the counts were expressed in CFU/mL.

RESULTS

The counts of lactic acid bacteria reached up to 2.72 ± 0.64 CFU/g. Among 50 randomly picked up colonies, 4 strains were taxonomically allotted to the genus *Lactobacillus* by the identification method used. The other strains were not lactobacilli. According to the Maldi-Tof analysis, the species detected in faecal samples of pheasants were *L. brevis* (2) and *L. plantarum* (2). This taxonomic identification was expressed on the basis of high score values (*L. brevis* LB11- 2. 401; *L. brevis* LB12- 2. 383; *L. plantarum* LP13-2. 292; *L. plantarum* LP14-2. 104). Moreover, phenotypic properties confirmed this identification; NH3 formation from arginine and fermentation of disacharides were in accordance with control strain reported by Vos et al. (2009). Identity of *L. plantarum* strains was also confirmed by PCR; 256 bp band was visualized by electrophoresis.

Lactobacilli were sensitive to erythromycin, rifampicin, chloramphenicol and tetracycline. Resistance to vancomycin and kanamycin was found in the strains LB12 and LP13. In addition, LP14 strain was resistant to gentamicin.

Concerning the enzymes, lactobacilli showed negative tests for lipase, α-fucosidase. Negative reaction was also found for trypsin in both strains of *L. brevis*, but low value (5 nmol) for trypsin was evaluated in *L. plantarum* strains (Table 1). *L. brevis* LB11 and *L. plantarum* LP14 showed no α-chymotrypsin activity; LP13 and LB11 showed weak reaction concerning α-chymotrypsin (5 nmol). *L. plantarum* LP13, LP14 and *L. brevis* LB12 were negative for β-glucuronidase, while *L. brevis* LB11 was positive (30 nmol). Similarly, LB11 was positive for β-glucosidase (40 nmol) and LP14 showed moderate reaction (20 nmol) for β-glucosidase; the strains LP13 and LB12 were negative for β-glucosidase. N-acetyl-β-glucosaminidase was negative in the strains LB11, LB12 and weak (10 nmol) in LP14. The enzymes α-chymotrypsin, β-glucuronidase, β-glucosidase and N-acetyl-β-glucosaminidase are related to some diseases for example, cancer; therefore tolerance to bile and low pH (probiotic properties) were tested only in the strains LP13, LB12 and LP14. The strain LB11 was excluded from this test.

The values of lactic acid reached from 2.82 to 4.86 mmol/L; average value was 3.73 mmol/L. *L. plantarum* strains showed higher lactic acid production (Table 2) as compared to *L. brevis* strains.

Table 1. Enzymatic activities of lactobacilli from common pheasants assayed by the API ZYM system.

Enzymatic activity (nmol/4h)	Strains			
	LB11	LB12	LP13	LP14
Alkaline phosphatase	-	-	-	5
Esterase (C4)	20	10	10	10
Esterase Lipase (C8)	10	10	5	10
Lipase (C14)	-	-	-	-
Leucin arylamidase	20	10	20	30
Valin arylamidase	30	5	10	30
Cystin arylamidase	20	5	10	20
Trypsin	-	-	5	5
α-chymotrypsin	-	5	5	-
Acid phosphatase	30	20	10	30
Naphtol-AS-Bi-phosphohydrolase	10	5	5	10
α-Galactosidase	40	-	-	-
β-Galactosidase	30	-	-	10
β-Glucuronidase	30	-	-	-
α-Glucosidase	40	-	-	20
β-Glucosidase	-	-	-	10
N-Acetyl β-glucosaminidase	-	-	-	10

α-Manosidase, α-fucosidase were negative in all lactobacilli; -negative, 5-negative (weak), 10-moderate, 20-40-positive; LB 11, LB12-*Lactobacillus brevis*, LP13, LP14-*Lactobacillus plantarum*

Table 2. Antimicrobial activity of lactobacilli against indicator bacteria (in mm) and lactic acid (in mmol/L).

Indicator	LB11	LB12	LP13	LP14
L. monocytogenes				
P2116	-	-	20	-
P3300	-	-	21	-
P5258	-	-	21	10
P6301	-	-	19	6
P6501	-	-	21	-
P2024	-	-	17	-
P7401	-	-	19	-
P7562	-	-	20	-
P7223	-	-	18	-
P7395	-	-	20	-
Ve405	-	-	20	-
TS10811	-	-	18	-
CCM4699	7	6	12	17
L. innocua LMG13568	5	9	10	13
Lactic acid	2.82±0.16	3.11±0.17	4.14±0.03	4.86±0.09

Antimicrobial activity from 5-10 mm- very slight; more than 10-bacteriocin active strains; *Listeria monocytogenes*- strains from meat products (SVA Olomouc, Czech republic; CCM- Czech Culture Collection, LMG 13568-University of Brussel, Belgium; The growth of the strains *Enterococcus avium* EA5, *Staphylococcus aureus* SA5 was not inhibited.

Table 3. Tolerance of lactobacilli to low pH (counts in CFU/mL).

Strain	pH 2.5	pH 7.0
LB12		
0 min	9.59 ± 0.39	9.96 ± 0.31
90 min	3.36 ± 0.83	8. 91 ± 0.29
180 min	<1.0	9.59 ± 0.30
LP13		
0 min	9.87 ± 0.31	9.96 ± 0.32
90 min	6.16 ± 0.48	9.50 ± 0.80
180 min	4.64 ± 0.15	> 9.0
LP14		
0 min	9.91 ± 0.15	9.92 ± 0.31
90 min	5.16 ± 0.27	9.42 ± 0.06
180 min	5.06 ± 0.24	> 9.0

L. brevis LB11 and LB12 possessed antimicrobial activity only against *L. monocytogenes* CCM4699 and *L. innocua* LMG13568 with slight inhibition (Table 2). *L. plantarum* LP13 was the most active strain; LP13 strain showed antimicrobial activity against 14 from 16 indicators; it was not active against the strains EA5 and SA5. LP14 strain showed inhibitory activity against 3 strains *L. monocytogenes* and 1 strain of *L. innocua*. In summary, the tested lactobacilli showed inhibitory (antimicrobial) activity against at least of 2 indicator bacteria from 16 used.

Selected strains LB12, LP13 and LP14 tolerate very well pH 2.5 (Table 3). As compared to physiological pH 7.0, the difference in enumerating counts at 90 min was 5.55 log cycle for LB12, 3.34 log cycle for LP13 and 4.26 log cycle for LP14. LP14 survived in the highest counts at incubation time of 180 min at pH 2.5 and when compared with pH 7.0, the lowest difference in counts was found at time 180 min (Table 3).

The strains were able to survive in the the presence of 1.0% (w/v) oxgall-bile with the different 1.0 log cycle as compared to the control tubes and experimental tubes after 24 h of growth; B12, 10.23:9.69 log CFU/mL; LP14, 10.25:9.1 log CFU/mL. In LP13, even the same count was enumerated (10.38 log CFU/mL) in control as in experimental tubes (10.38 log CFU/mL).

DISCUSSION

Lactobacilli belong to *Firmicutes,* class *Bacilli,* order *Lactobacillales,* family *Lactobacillaceae* (Vos et al., 2009). Detection of the species *L. plantarum* and *L. brevis* presented here are in agreement with highly probable species identification of Maldi-Tof identification system (value score 2.300-3.000) and/or secure probable species identification-probable species identification (value score 2.000-2.299; Bruker Daltonics, 2008). Only limited information exists in relation to the species of lactobacilli detected in pheasants and/or poultry, in general. For instance Heravi et al. (2011) detected two species of lactobacilli in chicken; *Lactobacillus crispatus* and *Lactobacillus salivarius;* moreover, they possessed probiotic properties. In our study, different species were identified. It seems, microbiota associated directly with pheasants are still open field for the study. However, up till now, reported studies are mostly connected with probiotic application of lactobacilli in chickens (Audisio et al., 2000).

Bacterial β-glucuronidase is known to be associated with initiation of some diseases, e. g. in human it is colon cancer (Lidbeck et al., 1992). Having in the mind probable probiotic potential of the isolated lactobacilli, it means to assess safety of the strains, the strains positive for enzymes associated with the intestinal diseases such as α-chymotrypsin, β-glucuronidase, β-glucosidase and N-acetyl-β-glucosaminidase should be eliminated for further probiotic studies. Lactobacilli presented here were mostly negative or with weak reaction with regards to the enzymes α-chymotrypsin, β-glucuronidase, β-glucosidase and N-acetyl-β-glucosaminidase. It is one of parameters which indicates their probable use as probiotic bacteria in the future. Positive strain *L. brevis* LB11 for former mentioned enzymes was excluded from further testing.

Different susceptibility or resistance of lactobacilli to vancomycin was described as one of the identification characteristic (Swenson et al., 1990; Hamilton-Miller and Shah, 1998). Likewise, Swenson et al. (1990) described susceptibility of vancomycin-resistant lactobacilli to other antimicrobials. Lactic acid values presented here are in accordance with typical representants of homofermentative lactobacilli (Gereková et al., 2011).

Lactobacilli showed antimicrobial activity in at least of 2 indicator bacteria from the 16 used. Antimicrobial activity in lactobacilli e.g. those due to bacteriocins have been reported as good in inhibiting the growth of listaerie which are common contaminants in feed/food industry. Those substances were for example, sakacins or plantaricin 423 (Drider et al., 2006). Therefore, we also plan to continue detail studies of antimicrobial activity of identified lactobacilli which seems done due to bioactive susbtances produced by *L. plantarum* LP13 and LP14; it is good that these strains should did not produce biogennic amines (Greif and Greifová, 2013).

Generally, LAB are commonly more resistant against acidic environment and low pH (Piard and Desmazeud, 1991). This fact was confirmed also by Strompfová and Lauková (2013) in lactobacilli from dogs and primates. Tolerance to low pH and bile are properties which lead to selection of strains for their application, although it can be strain-dependent. Although only 4 taxonomically allotted lactobacilli from fececes of pheasants were isolated, these results are important contribution to the microbiology of

pheasants. In conclusion, *L. plantarum* LP13 and LP14 represent promising probiotic candidates; moreover, detail studies of their antimicrobial activity are in process.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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