ISSN Print: 2518-4261
ISSN Online: 2518-427X
Vol. 59(4), December 2022

# PROCEEDINGS
## OF THE PAKISTAN ACADEMY OF SCIENCES:
## B. Life and Environmental Sciences


PAKISTAN ACADEMY OF SCIENCES
ISLAMABAD, PAKISTAN

# Proceedings of the Pakistan Academy of Sciences: Part B Life and Environmental Sciences




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**Annual Subscription:** **Pakistan:** Institutions, Rupees 4000/- ; Individuals, Rupees 2000/- (Delivery Charges: Rupees 150/-)
**Other Countries:** US$ 200.00 (includes air-lifted overseas delivery)





**HEC Recognized, Category X; Scopus Indexed**

*Published by* **Pakistan Academy of Sciences**, 3 Constitution Avenue, G-5/2, Islamabad, Pakistan
**Email:** editor@paspk.org; **Tel:** 92-51-9207140; 92-51-920 6770; **Websites:** www.paspk.org/proceedings/; www.ppaspk.org

*Printed at* **Graphics Point.,** Office 3-A, Wasal Plaza, Fazal-e-Haq Road, Blue Area, Islamabad
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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 1-13 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)780

Pakistan Academy of Sciences

Review Article

# Endophytes: Potential Source of Bioactive Compounds of Pharmaceutical Importance

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**Abstract:** Microbes exists as mutualists, parasite, and symbiont or as pathogens in nature. In plant microbiota, plant immunity determines whether the interaction with microbes is friendly or hostile. Friendly interaction may have an eccentric way of mutual interrelations for a resource contribution. This interaction is called plant-endophyte mutualistic or symbiotic relation in which microorganisms (fungi, bacteria and actinomycetes) live within robust plant tissues. It has been discovered that almost all plant species investigated by various researchers harbor one or more endophytes. They benefit their host by producing various secondary metabolites that can be employed in agriculture and medicine. Endophytes are a treasure house of many novel bioactive compounds such as steroids, tannins, terpenoids, quinones, alkaloids, saponins and phenolic acids which makes them a potential candidate for anticancer, antibiotic, antioxidant, anti-inflammatory, antiviral, antidiabetic properties, etc. Endophytes continue to be the peculiar source of various potential drugs. This review intends to shed light on the function and potential applications of endophytes as a forthcoming source of medications for a range of illnesses/diseases as well as other potential medical uses.

**Keywords:** Endophytes, Antibiotics, Antimicrobial, Medicinal Plants, Secondary Metabolites, Pharmacology

## 1. INTRODUCTION

Phytomicrobiome associated with the different plant structures plays an essential role in which microorganisms in the microbiome provide different beneficial services to the plants causing without any immediate, overt and adverse effect on the host plant [1]. These plant growth-promoting endophytes act as a valuable agricultural resource as they form symbiotic associations with their host plant by penetrating internal tissues. The host plant provides protection and nutrients to the endophytes and these endophytes produce the bioactive compounds that add to the protection against herbivores and plant diseases, as well as boost resilience to a variety of stresses [2]. Endophytes, particularly endophytic fungi, are found to have a wide spectrum of bioactive compounds, and hence Owen and Hundley [3] referred to them as "the chemical synthesizer inside the plant". Various endophytic microbes have been identified and studied over the last 50 years, leading to the biological and chemical characterization of many natural products with distinctive structures and biological activity [4]. According to recent research, secondary metabolites produced by endophytes may be the primary source of protection against diseases [2]. Endophytes are gaining industrial and biotechnological relevance due to their potential to produce various bioactive compounds which act as antitumor agents, biocontrol agents, antimicrobial agents, immunosuppressants and release antiviral compounds, as well as the production of natural antibiotics, antioxidants, insecticidal and antidiabetic products [5].

Plants are being widely investigated for novel chemical entities that may exhibit diverse therapeutic properties and endophytes play a significant role in the search for compounds with potential applications in health and medicine [6]. Endophytes have produced a large number of

Received: September 2022; Accepted: December 2022
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bioactive substances that have been identified and characterized by many researchers like vincristine, taxol, podophyllotoxin, hypericin and many others given in the tables below. With an emphasis on endophytic bacteria and fungi, this study elucidates the endophytic bioactive compounds and their pharmacological applications.

## 2. AN OVERVIEW OF ENDOPHYTES

Endophyte was initially defined by De Bary [7] who stated that "any organism that resides inside plant tissues is referred to as an endophyte." However, the definition continues to evolve based on the findings of other researchers [8, 9]. Depending on the colonization's nature and their potential roles, plant associations with microbes can be characterized as mycorrhizal, pathogenic, epiphytic, saprotrophic, and endophytic [10]. Endophytes can penetrate the internal tissues of the plant. Endophytes like fungi, bacteria, eukaryotes, and archaea grow within plant tissues and are recognized to cause no harm to plants. Endophytes show symbiosis with the tissues of plants that are occasionally harmful. Wheat, rice, maize, mustard, chili, soybean, tomato, citrus, and sunflower are just a few of the plants that have been found to have endophytic microorganisms [11, 12].

They have grabbed the attention of many scientists due to their ability to promote plant growth and plant survival in stressful conditions [13]. Natural compounds produced by endophytes are beneficial in agriculture, medicine and in industries (Figure 1). The type of microbe that can be associated with a plant strongly relies on the composition of its root exudates. [14]. Endophytes utilize the root exudates as a source of energy which is essential for their association with host plants [15,16].

## 3. ENDOPHYTES: SOURCE OF POTENTIAL BIOACTIVE COMPOUNDS

At present, the entire world's population is tormented by deadly chronic diseases. The therapeutic potential and efficacy of antibiotics are being constrained by the rise in bacterial resistance to commercial antibiotics. Therefore, it is crucial to look for innovative, affordable, and non-toxic natural bioactive chemicals from endophytes for producing novel drugs with diverse mechanisms to fulfill people's needs. Endophytes have been mentioned in several publications as excellent sources of bioactive compounds and their beneficial role in the cosmetic and drug industries. Numerous bioactive compounds isolated from different endophytic fungi of medicinal plants are now used in both pharmaceutical and agricultural applications e.g. Paclitaxel is a well-known and highly functionalized tetracyclic diterpenoid bioactive compound was discovered from the fungus Taxomyces andreanae. It has proved to exhibit efficient activity against prostate, ovarian, breast, and lung cancers [17].

### 3.1 Antibiotics

Bioactive compounds that are active against pathogenic microbes at low concentrations are characterized as antibiotics [18]. Strobel and Daisy [19], summarized the antibiotics identified from endophytes, the majority of which were proven to

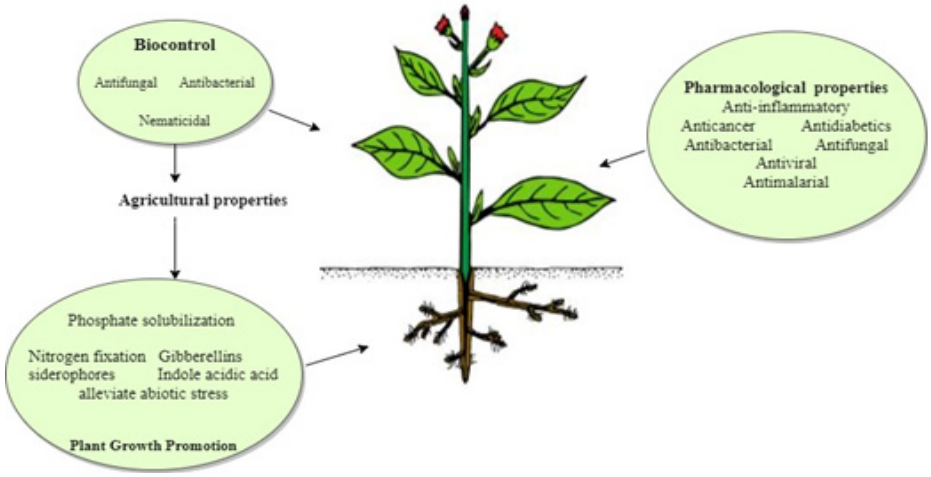


**Fig. 1.** Metabolites and functions of beneficial endophytes

be relevant. During the last 10 years, significant progress has been made and prior work has been evaluated and updated in Table 1.

### 3.2 Anticancer Compounds

Over six million new cases of cancer are reported each year, making it one of the most fatal diseases in the world. Many bioactive compounds from microbes, plants, and marine sources have been investigated as anticancer medicines; there is some indication that some endophytes produce natural compounds that can be used in treating different types of cancer [53]. Several secondary metabolites extracted from endophytes that have lately been studied for their anticancer effects are mentioned (Table 2).

### 3.3 Antioxidant Compounds

These are compounds that can shield cells from the damage caused by reactive oxygen species (ROSs),

**Table 1.** Antibiotics produced by endophytes.

| Endophytes | Host Plant | Activity | Bioactive Compound | References |
|---|---|---|---|---|
| **Endophytic Bacteria** | | | | |
| ***Bacillus subtilis*** | *Allanmandas cathartica* L. | Antifungal | Terpenoids | [20] |
| ***B. subtilis, B. licheniformis and B. pumilus*** | *Moringa peregrina* (Forssk.) | Antifungal and Antibacterial | - | [21] |
| ***B. subtilis*** | - | Antifungal | Phospholipids | [22] |
| ***Pseudomonas, Enterobacter, Staphylococcus, Lysinibacillus*** | *Combretum molle* | Antibacterial and Antifungal | - | [23] |
| ***B. subtilis*** | - | Antibacterial | Peptides | [24] |
| ***B. mojavensis, B. atrophaeus*** | *Glycyrrhiza uralensis* Fisch.ex DC. | Antifungal | Polyketides | [25] |
| ***B. thuringiensis*** | *Physalis alkekengi* L. | Antibacterial | - | [26] |
| ***Amycolatopsis tolypophora*** | *Stachys lavandulifolia* Vahl. | Antibacterial | - | [27] |
| **Endophytic Fungi** | | | | |
| ***Acremonium zeae*** | *Zea mays* L. | Antifungal | Pyrrocidines A, B | [27] |
| ***Nodulisporium* sp.** | *Juniperus cedre* L. | Antibacterial | Flavonoids | [28] |
| ***Phomopsis* sp.** | *Ginko biloba* L. | Antifungal and Antibacterial | Alkaloids | [29,30, 27] |
| ***Acremonium zeas*** | *Zea mays* L. | | | |
| ***Chaetomium globosum*** | *Garcinia dulcis* (Roxb.) Kurz | | | |
| ***Cryptosporiopsis* sp.** | *Pinus sylvestris* L. | Antifungal and Antibacterial | Peptides | [31, 32] |
| ***Pezicula* sp.** | *Fagus sylvatica* L. | | | |
| ***Chaetomium globosum*** | *Ginko biloba* L. | Antifungal | Azaphilone derivative | [30] |
| ***Pestalotiopsis mangiferae*** | *Magnifera indica* L. | Antibacterial | Phenols | [33] |
| ***Aspergillus* sp.** | *Bauhinia guianensis* Aubl. | Antibacterial | Alkaloids | [34] |
| ***Xylaria* sp.** | *Abies holophylia* Maxim. | Antifungal | - | [35] |
| ***Phompsis* sp.** | *Aconitum carmichaelii Debeaux.* | Antifungal | Steroids | [36] |
| ***Phoma* sp.** | *Cinnamomum* Schaeff. | Antibacterial and Antifungal | Polyketides | [37] |

| Endophytes | Host Plant | Activity | Bioactive Compound | References |
|---|---|---|---|---|
| ***Phomopsis* sp.** | *Gracinia* L. | Antifungal and Antibacterial | - | [38] |
| ***Botryosphaeria* sp.** | | | | |
| ***Geotrichum candidum*** | *Phyllanthus reticulatus* Poir. | Antifungal and antibacterial | - | [39] |
| ***Nigrospora sphaerica*** | *Indigofera suffruticosa* Mill. | Antibacterial | - | [40] |
| **Endophytic Actinomycetes** | | | | |
| ***Streptomyces sp.* DSM 1175** | *Alnus glutinosa* L. | Antibacterial | Quinones | [41] |
| ***Streptomyces sp.*** | *Grevillea pterdifolia* Knight. | Antibacterial | Peptides | [42] |
| ***Streptomyces noursei*** | - | Antifungal | Steroids | [43] |
| ***Dactylosporangium* sp.** | *Cucubalus* L. | Antifungal | Tannins | [41] |
| ***Streptomyces auereofaciens* CMUAc130** | *Zingiber officinale* Rosc. | Antifungal | Coumarins | [44] |
| ***Aeromicrobium poni*** | *Vochysia divergens* Pohl | Antibacterial | Alkaloids | [45] |
| ***Streptomyces sp.*** | *Aucuba japonica* Thunb. | Antibacterial | Terpenoids and terpenes | [46] |
| ***Streptomyces* sp.** | *Boesenbergia rotunda* (L.) Mansf. | Antibacterial | Flavonoids | [47] |
| ***Streptomyces* sp.** | - | Antibacterial | Peptides derivatives | [48] |
| ***Actinosynema pretiosum*** | *Maytenus serrata* | Antibacterial | polyketides | [49] |
| ***Verrucosispora maris*** | *Sonchus oleraceus* L. | Antibacterial | Peptides | [50] |
| ***Streptomyces* sp.** | *Glycin max* (L.) Merr. | Antifungal | Alkaloids | [51] |
| ***Streptomyces remosus*** | - | Antifungal | Steroids | [52] |

which are responsible for several pathological effects including cellular aging, carcinogenesis and DNA damage [87]. Cancer, atherosclerosis, rheumatoid arthritis, cardiovascular disease, ischemia/reperfusion injury, hypertension, neurological illnesses (Parkinson's and Alzheimer's diseases), diabetes mellitus, and aging have all been associated with ROS [88]. A novel compound sesquiterpene, 3,5-dihydroxy-2,5-dimethyltrideca-2,9,11-triene 4,8-dione has strong antioxidant activity isolated from *Acremonium* sp. [89]. A detailed list of antioxidant compounds isolated from endophytes and their uses against various diseases is presented in (Table 3).

### 3.4 Antiviral Compounds

The isolation of antiviral agents from endophytes is still new. The lack of antiviral screening mechanisms is the limiting factor in the endophyte synthesis of antiviral compounds. Some antiviral compounds isolated from endophytes are mentioned in (Table 4).

### 3.5 Antidiabetic Compounds

Nature has provided us with a plethora of natural compounds that can be used to treat various diseases. Glucose levels in the rats' blood had successfully reduced by endophytic fungi like *Aspergillus* sp. and *Phoma*. Kaur [108], also investigated endophytic fungi that could behave as alpha-glucosidase inhibitors such as *Fusarium* sp. and *Alternaria* sp. was also identified to secrete gancidinW (GW) that is active against α-glycosidase [109]. Extracts of endophytes isolated from the two most common medicinal plants Leucas ciliate and Rauwolfia

**Table 2.** Anticancer compounds isolated from endophytes

| Endophytes | Anticancerous Compound | Activity | Cell lines used | References |
|---|---|---|---|---|
| **Endophytic Fungi** | | | | |
| ***Fusarium oxysporum*** | Vincristine | Anticancer | - | [54] |
| ***Acremonium* sp.** | Leucinostatins | Anticancer | - | [55] |
| ***Taxomyces andreanea*** | Paclitaxel | Anticancer | - | [56] |
| ***Lasidiplodia theobromae*** | Taxol | Anticancer | MCF-7 | [57] |
| ***Cephalotheca faveolata*** | sclerotiorin | Anticancer | Colon cancer (HCT-116) | [58] |
| ***Enthrophospora infrequens*** | camptothecin | Anticancer | - | [59] |
| ***Trametes hirsute*** | Podophyllotoxin | Anticancer | - | [60] |
| ***Fusarium oxysporum*** | Podophyllotoxin | Anticancer | - | [61] |
| ***Aspergillus fumigatus*** | Cytotoxic alkaloids | Cytotoxicity | Leukemia cancer cells | [62] |
| ***Garcinia* sp.** | Ethyl acetate extract | Antiproliferative and cytotoxicity | Vero cell lines | [63] |
| ***Penicillium sp.*** | Penicillenone | Anticancer | - | [64] |
| ***Colletotrichum gloesporiodes*** | Taxol | Cytotoxicity | Human cancer cell lines BT220, int407,H116 and HLK210 | [65] |
| ***Mycellia strerilia*** | Vincristine | anticancer | - | [66] |
| ***Alternaria alternata*** | Ethyl Acetate extract | cytotoxicity | HeLa cells | [67] |
| ***C. gloesporiodes*** | Taxol | Anticancer | - | [68] |
| ***Phomopsis cassiae*** | 3,12- dihydroxydalene 2,3,12- trihydroxycadalene | Antiproliferative | HeLa cervical cells | [69] |
| ***Alternaria* sp.** | Xanalteric acid | Cytotoxicity | - | [70] |
| ***Fusarium solani*** | Camptothecin | Anticancer | - | [71] |
| ***Alternaria* sp.** ***Colletotrichum* sp.** ***Chaetomium* sp.** | Ethyl acetate extract | Cytotoxicity | MCF-7 cells lines and HeLa | [72] |
| ***Phoma* sp.** | *5- hydroxyramulosin* | Anticancer | - | [37] |
| ***Penicillium* sp.** | *Arisugacin* | Anticancer | HeLa and K562 | [73] |
| ***A. flavus*** | *Solamargine* | Cytotoxicity | - | [74] |
| ***Emericella variecolor*** | *Tajixanthone hydrate* | Anticancer | - | [75] |
| ***Pestalotiopsis* sp.** | Pestalotiopsone A,B,C,D,E,F | Anticancer | L5178Y | [76] |
| ***Guignardia* sp.** ***Halorosellinia* sp.** | Anthracene-9,10-dione 1-hydroxy-3-methyl | Anticancer | KB KBv200 | [77] |
| ***Fusarium* sp.** | 5-O-methyl-2′-methoxy-3′-methylalpinumisoflavone | Anticancer | HEp2 HepG2 | [78] |
| ***Paecilomyces* sp** | Paeciloxocins A Paeciloxocins B | Anticancer | HepG2 | [79] |

| Endophytes | Anticancerous Compound | Activity | Cell lines used | References |
|---|---|---|---|---|
| **Endophytic Bacteria** | | | | |
| ***Pantoea* sp.** | Ethyl acetate | Anticancer | A549 LUNG CARCINOMA and UMG87 glioblastoma | [80] |
| ***Acinetobacter guillouiae*** | Ethyl acetate extract | Anticancer | A549 lung carcinoma cells | [81] |
| ***Bacillus subtilis*** | Camptothecine | Anticancer | - | [82] |
| **Endophytic Actinomycetes** | | | | |
| ***Streptomyces laceyi* MS53** | 6-alkalysalicilic acids, salaceyins Aand B | Anticancer | - | [41] |
| ***Actinosynnema pretiosum*** | Ansamitocin | Antitumor | - | [49] |
| ***Streptomyces thermoviolaceus*** | Anicemycin | Anticancer | - | [83] |
| ***Streptomyces* sp. SUC1** | Lansai A-D | Antitumor | - | [84] |
| ***Streptomyces* sp. CS** | Naphtomycin | Antitumor | - | [85] |
| ***Micromonospora lupine Lupac* 08** | Lupinacidin C | Antitumor | Murine colon | [86] |

**Table 3.** Antioxidant compounds from endophytes.

| Endophytes | Host Plant | Bioactive Compounds | Class of Compounds | References |
|---|---|---|---|---|
| **Endophytic Bacteria** | | | | |
| ***Methylobecterium radiotolerans*** | *Combret eryhrophyllum* (Bruch.) Sond. | Chloroform EtOAc | Alkaloids, flavonoids | [90] |
| ***Pseudomonas hibiscicola, Micrococcus caseolyticus, Enterobacter ludwigi*** | *Aloe vera* (L.) Burm.f. | EtOAc | Flavonoids Alkaloids | [91] |
| ***Pseudocercospora* sp.** | *Elaeocarpus Sylvestris* (Lour) Poir. | Terric acid and 6-methylsalicyclic acid | - | [92] |
| ***Enterobactor* sp. EC3** | *Carica papaya* L. | Gallic acid | Phenolic compounds | [93] |
| ***Lactobacillus sp.*** | *Adhathoda beddomei* | EtOAc | Phenolic compounds | [94] |
| **Endophytic Fungi** | | | | |
| ***Phomopsis loropetali* AcapF3** | *Tapernaemontana divaricate* L. *Rauvolfia verticillate* (Lour.) Baill. | - | Phenolic compounds | [79] |
| ***Phyllosticta* sp.** | *Guazuma tomentosa* | EtOH | phenol | [95] |
| ***Phoma* sp., *Colletotrichum spiralis*** | - | MeOH | phenol | [96] |
| ***Alternaria alternata Aspergillus flavus, A. niger*** | *Lannea coromendalica* | EtOAc | Phenolic compound | [97] |
| ***Aspergillus minisclerotigens* AKF1 and *Aspergillus oryzae*** | *Mangifera casturi* | 4H-Pyran-4-one and dihydropyran | - | [98] |
| ***Aspergillus* sp.** | *Euphorbia prostrate* Aiton. | Gallic acid | Phenol | [99] |
| ***Chaetomium globosum*** | *Adiantum capillus* L. | EtOAc | Phenol | [100] |

| Endophytes | Host Plant | Bioactive Compounds | Class of Compounds | References |
|---|---|---|---|---|
| **Endophytic Actinomycetes** | | | | |
| ***Streptomyces aureofaciens* CMUAc130** | *Zingiber officinale* Rosc. | 5,7- dimethyl -4- p-methoxyl phenyl coumarin | Coumarins (alpha benzopyrones) | [44] |
| ***Streptomyces* sp. MS1/7** | - | 2- Allyloxyphenol | phenol | [101] |
| ***Micromonospora* sp. PC1052** | *Puereria candoliei* | S-adenosyl-nacetylhomocysteine | peptides | [102] |

**Table 4.** Antiviral compounds from endophytes.

| Endophyte | Host Plant | Antiviral Compound | Reference |
|---|---|---|---|
| ***Pestalotiopsis theae*** | Unidentified tree on Jianfeng Mountain, China | Pestalotheol C | [103] |
| ***Paecilomyces* sp.** | *Taxus mairei* | Brefeldin A | [104] |
| **Fungal isolate** | *Quercus coccifera* L. | Hinnuliquinone, | [105] |
| ***Cytonaema* sp.** | *Quercus* L. | Cytonic acids A and B | [106] |
| ***Pullularia* sp. BCC 8613** | Unidentified tree | Pullularin A | [107] |

densiflora were evaluated by Akshatha *et al*. [110] for their anti-diabetic potential.

### 3.6 Antiarthritis and Anti-Inflammatory Compounds

Rheumatoid arthritis (RA) is a systemic, autoimmune and inflammatory disease that causes swelling, discomfort, bone and cartilage degradation, and can eventually cause permanent disability. Surprisingly, the disease's actual causal agent remains unknown. Many researchers are currently hunting for additional therapeutic compounds from microorganisms because synthetic medications are currently quite expensive and have a lot of drawbacks [111]. Methanolic extract of endophytic fungi Talaromyces wortmannii from *Aloe vera* produced compounds with anti-inflammatory properties [112]. Endophytic fungi *Lepidosphaeria* sp. also has anti-inflammatory action, suggesting that inflammatory diseases such as rheumatoid arthritis can be treated [113].

## 4. CONCLUSION AND FUTURE PERSPECTIVE

The present review summarized the facts of endophytes-mediated bioactive compounds that are symbiotically associated with different plant species and are an interesting source of novel therapeutic compounds. Different plant species have different endophytes that serve as a vital source of significant natural compounds that are beneficial in agriculture, medicine and in industries. These bioactive compounds are produced under particular-environmental conditions, stressful conditions, nutrient availability, or during a particular developmental stage. They possess different therapeutic activities such as antibacterial, antioxidant, antidiabetic anti-inflammatory, anticancer, antiviral, and antimalarial, etc. Therefore, there is a further need to isolate endophytes and their secondary metabolites from medicinal plants to discover novel compounds for therapeutic/pharmacological purposes. Towards this aim, further insights into the origin of endophytic genes and dynamic endophyte-host plant interactions would be of utmost importance.

## 5. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 15-23 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)722

Pakistan Academy of Sciences

Research Article

# Microbiocenosis of Anthropogenically Transformed Soils

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**Abstract:** A microbiological examination of the soils, polluted with different types of urban wastewater (Tashkent city), aiming determination of the microbial diversity and characterization of the bacterial community was carried out. The examination was conducted with use of classical microbiological methods with cultivation of samples on elective nutrient media. The soil sampling was carried out during winter season and period of plants' vegetation. As result of examination the qualitative and quantitative characteristics of the bacterial community were determined and the microbial diversity was established. The predominant microorganisms of this community, capable to active functioning at chloride concentrations of the environment for up to 10 % and possessing high remediation potential towards biological and chemical pollutants, have been isolated. Rare strains belonging to the genus Amycolatopsis, which, in contrast to typical representatives of this genus, have the ability to form a water-soluble blue pigment, have been isolated. It was established that typical representatives of microbial biota, such as heterotrophic microorganisms Bacillus, Pseudomonas and actinomycetes, possess significant remediation potential towards biological and chemical pollution. It was determined that pollution of the soil caused by anthropogenic factors at the end of the day leads to decrease in species diversity and changes in composition of the soil microbiocenosis. The results obtained convincingly testify perspectives of biomonitoring and possible use of microorganisms in the processes of soil rehabilitation. The introduction of pollutant-resistant microorganisms, which are capable to degrade them, may become a practical approach for soil cleansing in the future.

**Keywords:** Microbial Diversity, Microbiocenosis, Pollution, Anthropogenic Impact, Reflecting Soil Stability.

## 1. INTRODUCTION

The growth of cities and their increasing population density are inextricably bound to heavy anthropogenic impact on the environment and ecological situation. Growing population density contributes to the generation of more pollutants and waste, which promotes to the likelihood of increased impact of pollutants on the ecosystem as a whole. Globally, it is estimated that by 2050, approximately 68 % of the world's population will live in urban areas [1]. The importance of natural processes within the ecosystem, the necessity to study microbiocenoses with aim of possible impact and treatment of excess urban runoff, the study of possibility of using microorganisms to improve air and water quality are often overlooked within the context of urbanization [2].

The expansion of urban areas globally increases anthropogenic impacts on soil and underlines the important role of urban areas in securing the sustainable future. Thus, urban soil becomes increasingly important in providing a wide range of ecosystem conditions for life [3]. Cities around the world have begun to improve existing infrastructure with the latest technologies to manage storm water flow, to improve air quality and to provide additional social and economic benefits, at same time increasing levels of all kinds of waste. First of all, an increase in the level of pollutants affects soil microbiocenoses, since microorganisms are very sensitive indicators and immediately react to various changes in the environment, resulting in a high dynamic of microbiological indices [4-7].

The heavy anthropogenic impact and the high velocity of urbanization lead to a decrease in the

Received: June 2022; Accepted: December 2022
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ability of urban soil to recover from negative impacts. Morphological and structural changes in microbial populations and changes in their biochemical activity serve as a reflection of the anthropogenic impact on the ecosystem. Microbial reactions to the impact of anthropogenic factors are manifested quickly and quite clearly, which makes it possible to quickly identify the most vulnerable ecological zones, to predict their state while maintaining anthropogenic impact and to arrange the measures necessary to mitigate this impact [8].

Soil pollution leads to a deterioration of their properties, causes a decrease in biological activity, therefore, the identification of individual groups of microorganisms that can withstand toxic effects and the use of their adaptive capabilities can help cleanse soil from the products of technogenic pollution [9]. It is supposed that, even with a very strong bacterial presence, the process of self-cleaning of soil can take several months [10]. In a difficult ecological situation in the city, including cases of disturbed soil properties, saprotrophic microbial communities continue to function due to their high biological and ecological plasticity [11]. It was also established that humidity affects the quantity and activity of microbial biomass, controls the availability of oxygen for microorganisms, causes periods of microbial stress in water, and may also contribute to the destruction of organic matter, which leads to increased availability of carbon for soil microorganisms [8]. Thus, an attention should be focused on supporting and restorative processes that stimulate the functioning of soil and soil ecosystems [12, 13]. It is also possible that in order to assess the microbial community in the future, it would be necessary to determine the source of organic matter and to establish the relationship between vegetation, contaminated soil and microbial community.

In this regard, the purpose of this work was to identify the microbial diversity and to characterize the bacterial community inhabiting soils of the contaminated areas located at different distances from the Bozsu wastewater treatment plant (Tashkent).

## 2. MATERIALS AND METHODS

### 2.1 Sampling

The object of research were samples of serozem soil from zones of different levels of pollution, located both on the territory of the Bozsu wastewater treatment plant (BWTP) itself and directly adjacent to it. Five soil samples were collected and analyzed in total (Table 1). Upper soil layer (0 - 20 cm) was subjected to microbiological analysis. Soil samples were collected according to "the envelope" method from 25-point samples. The soil sampling was carried out in the winter and spring.

### 2.2 Chemical Analysis

The soil samples were air dried, ground and sieved through a sieve (1 mm) before analysis. The samples were dried to a constant weight in an oven at 105 °C. Water extracts were prepared for the analysis: 30 g of soil + 150 ml of distilled water. Acidity and electrical conductivity were measured with a pH-EC meter (Hannah, Germany) directly in aqueous extracts (suspensions) after 1 min stirring with a glass rod.

Anions and cations were measured in filtered samples, filtered through "blue ribbon" filter. Nitrates and phosphates were measured photometrically

**Table 1.** Collecting sites and characteristics of samples

| Samples | Sampling coordinates | Characteristics of the sampling site |
|---|---|---|
| 2 sample | 41°15′44.07″N<br>69°07.44′57″E | 50 m from sump (zone of high contamination level) |
| 3 sample | 41°15′45.13″N<br>69°07.49′01″E | 100 m from sump (sludge) |
| 5 sample | 41°15′42.41″N<br>69°08.17′7″E | 200 m from sump (zone of medium contamination level) |
| 6 sample | 41°15′42.98″N<br>69°08.21′18″E | 250 m from sump (fertilizer) |
| 7 sample | 41°16′12.66″N<br>69°08.11′15″E | 500 m from sump (zone of low contamination level) |

(Riele 520). Mobile sodium and potassium, as well as the chlorides content, were measured using ion-selective electrodes (Elite-031 ($K^+$); Alice-112Na ($Na^+$); Elite-261 ($Cl^-$)) potentiometrically on an ion meter (Expert-001.3, Russia) (Table 2).

### 2.3 Nutrient Media

To isolate microorganisms and determine the microbial landscape in the processes of destruction of pesticides, the standard Beef Extract Peptone, Czapek-Dox, Sabouraud, Endo, Giltai, Ashby, Postgate nutrient media were used (HiMedia, India).

### 2.4 Microorganisms

The structure of bacterial complex was characterized using physiological, biochemical and morphological indicators of individual cultures [14]. Taxonomic identification of bacteria was carried out according to the Bergey's manual of determinative bacteriology [15]. Taxonomic identification of micromycetes was conducted according to morphological and cultural features [16, 17].

## 3. RESULTS AND DISCUSSIONS

Soil-borne microorganisms (bacteria, actinomycetes, microscopic fungi) play a leading role in the processes of self-regulation of the natural ecosystem. It is a well-known fact that the number of microorganisms in the soil is constantly changing. But, in any soil layer there is a certain natural level of microbiota, which can be considered as a pool, in other words, the reserve of soil-borne microorganisms, which is not provided with the energy substance necessary for continuous reproduction, but is in a state of maintenance. The size of such stock is not affected by seasonality; the pool is determined by the characteristics of the soil itself and environmental factors that affect the soil properties [18].

The study of the quantitative and qualitative composition of microbiocenoses is of particular interest in such studies. In qualitative terms, the microbial communities of the studied sites are distinguished by high biodiversity (Figure 1).

Soil intensively accumulates a significant part of the pollutants that enter it and retains them for a long time. Heavy metals are fixed most strongly in the upper humus-containing horizons, that is, these toxicants are accumulated in the most fertile layer. The soil cover in the city carries out different ecological functions. The main and most important features of the urban soil are fertility and its suitability. Microbiological activity of

**Table 2.** Chemical indices of soil samples

| Sample | pH | EC (mS/cm) | Mineral composition, % of dry weight | Losses during combustion (900 °C) (humus, carbonates, $CO_2$ and others), % of dry weight | $NO_3^-$ (mg/kg dry weight) | $PO_4^{3-}$(mg/kg dry weight) | $Cl^-$ (mg/kg dry weight) | $Na^+$, mobile forms (mg/kg dry weight) | $K^+$, mobile forms (mg/kg dry weight) |
|---|---|---|---|---|---|---|---|---|---|
| 2 | 7.8 | 0.27 | 86.00+0.010 | 14.00+0.010 | 14.92±0.83 | 8.21±0.07 | 1.08±0.12 | 45.80±0.26 | 651.67±12.58 |
| 3 | 7.9 | 0.33 | 85.76+0.039 | 14.24+0.039 | 5.23±0.19 | 8.13±0.02 | 11.22±0.58 | 26.33±0.35 | 72.50±0.50 |
| 5 | 7.9 | 0.13 | 86.29+0.050 | 13.71+0.050 | 6.11±0.28 | 2.08±0.01 | 3.35±0.10 | 7.92±0.13 | 225.00±0.50 |
| 6 | 7.8 | 0.2 | 84.37+0.019 | 15.63+0.019 | 5.61±0.02 | 8.31±0.03 | 11.32±0.43 | 12.38±0.03 | 44.43±0.47 |
| 7 | 7.9 | 0.13 | 86.68+0.034 | 13.32+0.034 | 5.48±0.26 | 4.77±0.01 | 4.80±0.21 | 13.52±0.23 | 327.00±1.00 |

soil determines the transformation, migration and accumulation of matter, energy and formation in the soil.

The results of studies reveal that bioremediation is usually associated with several key genera: Pseudomonadales, Actinomycetales, Flavobacteriales, Bacilleas and Clostridiales. It was established that in the soil microbiocenoses of all studied samples, the dominant position is occupied by the bacterial complex, among which prevail bacteria of the genera *Bacillus* and *Pseudomonas.* Coccal forms are rare, mainly representatives of genera Sarcina and Micrococcus. Thus, the carried-out survey revealed that diversity of microorganisms in the studied soil samples is somewhat reduced.

According to the results of the chemical analysis of the soil, these samples have a fairly high chloride and phosphate salinity (table 2). The carried-out studies made it possible to identify microorganisms that can grow at the salt concentration for up to 10 % and obtain salt-tolerant forms of bacteria exhibiting physiological activity at 5-7 % chloride; based on physiological and biochemical characteristics and MALDI-TOF analysis the isolated strains were identified as *Bacillus cereus* and *Pseudomonas aeruginosa* (Figure 2).

The survey of the contaminated areas revealed the presence of a high titer of nitrogen-fixing microorganisms, which were attributed to the genus Azotobacter (especially during spring) (Figures 3 and 4). It is necessary to note that oligonitrophils are quite common microorganisms in soils, which are capable to grow on a substrate with a low concentration of nitrogenous compounds and fix free atmospheric nitrogen (they take part in all the most important biological stages of organic decomposition).

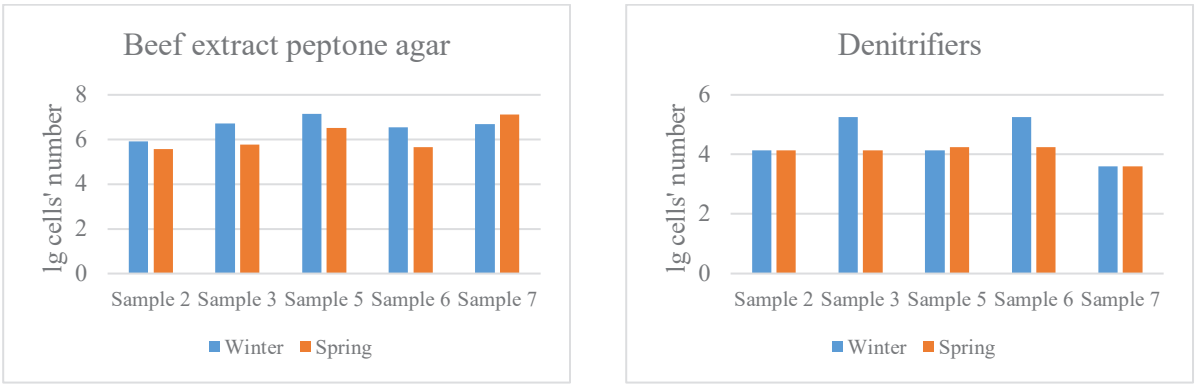


**Fig. 1.** Quantitative analysis of soil samples (total number of saprophytic and denitrifying microorganisms)

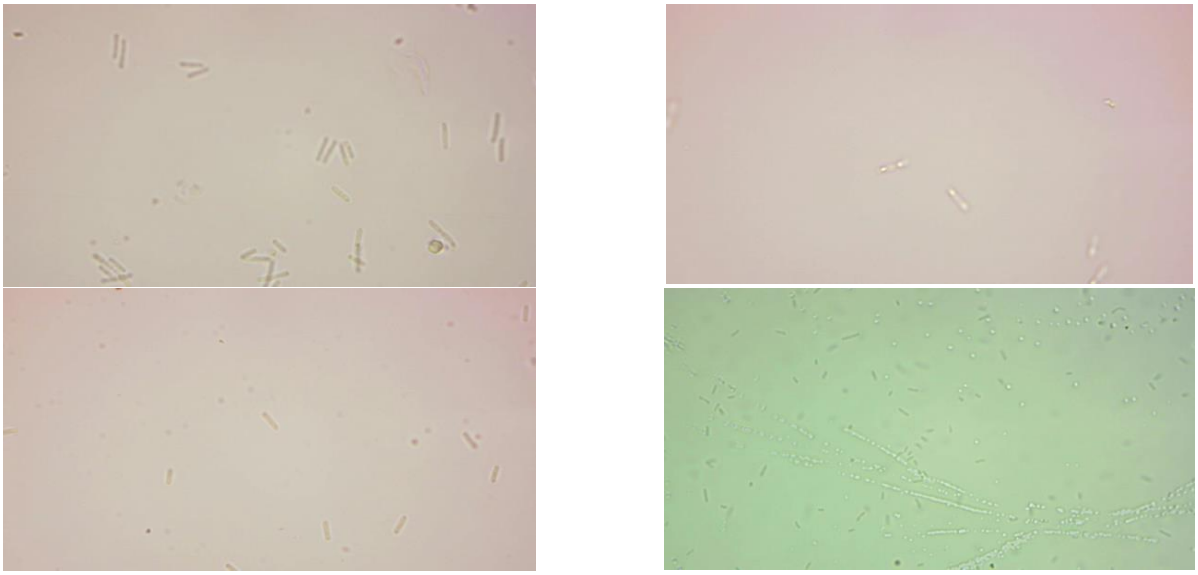

**Fig. 2.** Salt resistant strains *Bacillus cereus* and *Pseudomonas aeruginosa*

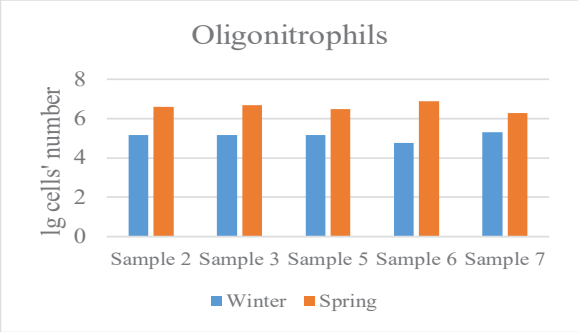


**Fig. 3.** Quantitative analysis of soil samples (oligonitrophils)

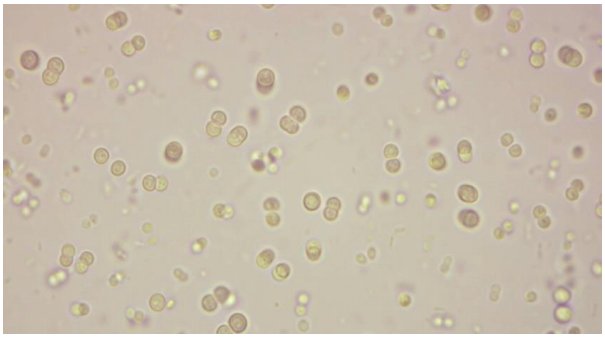

**Fig. 4.** *Azotobacter chroococcum* (x1000)

It should be noted that in polluted urban soil a significant change in the structure of microbial communities is noted, characterized by a decrease in the proportion of physiologically active bacterial cells, in comparison with background soils. It was established that micromycetes composes a significant part of the identified biocenoses; among fungi there were active forms of Trichoderma genus, as well as representatives of the genera Aspergillus and Penicillium, an increase in the titer of which, as a rule, is a specific reaction of the microbiota to urbanization. It is known that representatives of the genus Penicillium, as well as dark-colored populations of Azotobacter chroococcum, quite active in the soils of polluted urban zones, which is a specific response of the microbiota to urbanization [19].

Analysis of the development of sulfate-reducing microorganisms showed their presence in the soil samples in an insignificant amount. Only a slight increase was observed during the spring in the sample 5 (Figure 5).

Actinomycetes represent an essential link in the trophic chain of the ecosystem, carrying out the functions of microbes-decomposers. At the initial stage of the intake of organic matter actinomycetes destroy the hard-to-reach structural components of plant and animal origin and, together with bacteria, perform in the ecosystem the function of processing organic matter that has entered the soil. The main role of mycelial prokaryotes is the decomposition of complex polymers (lignin, chitin, xylan, cellulose, humic compounds) [20-23]. An increase in the number of soil-borne actinomycetes occurs at the late stages of microbial succession, when the biomass of fungi begins to decrease [24].

The participation of actinomycetes in the decomposition and synthesis of humic substances in the soil has been repeatedly noted [25, 26]. There is information about the use of humic acid polyphenols by actinomycetes in the presence of available carbon sources. Some representatives of the genera Nocardia, Micromonospora are able to oxidize humates, taking part in the mineralization of humic substances in the soil. Actinomycetes are involved in the accumulation of biologically active substances in the soil [27] and the formation of the nitrogen balance of soils [28]. It was established that many representatives of actinomycetes inhabit the studied soil samples, majority belonging to the genus Streptomyces (Figure 6).

The presence of various pigmented forms of actinomycetes, which is typical for soils with severe pollution, including heavy metal ions, should be noted separately (Figure 7).

There also were identified rare forms belonging to the genus Amycolatopsis, which possess the ability, in contrast to typical representatives of this genus, to form a water-soluble blue pigment (Figure 8).

It is known that sanitary and hygienic monitoring is directly related to the microbiological properties of the soil, since spores of fungi, actinomycetes, bacteria, as well as other life forms of microorganisms' resistant to insolation, are transported by air with soil dust. A significant change in the structure of microbial communities occurs, the ratio of taxa changes and new dominants appear in heavily polluted urban soils. Especially, in case of the complex household pollution, enterobacteria are detected in significant quantities (genera *Escherichia,* and *Enterobacter*).

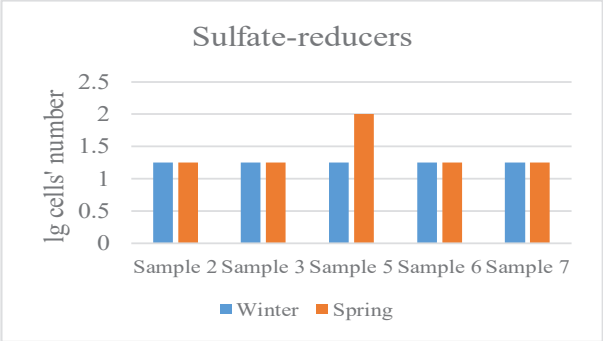


**Fig. 5.** Quantitative analysis of soil samples (sulfate-reducing microorganisms)

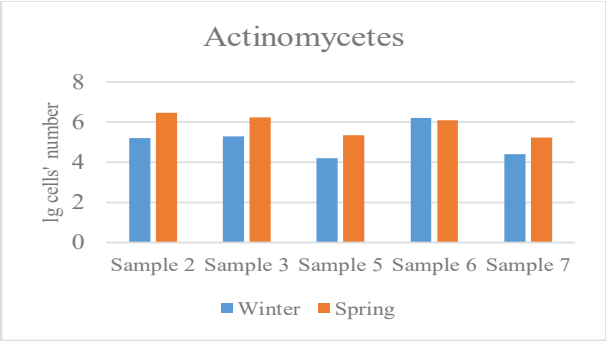


**Fig. 6.** Quantitative analysis of soil samples (actinomycetes)

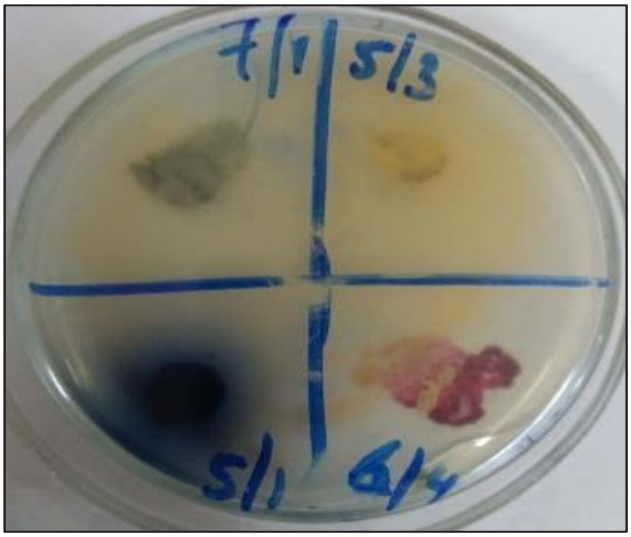

**Fig. 7.** Pigmented forms of actinomycetes isolated from the soils of BWTP contaminated with heavy metals

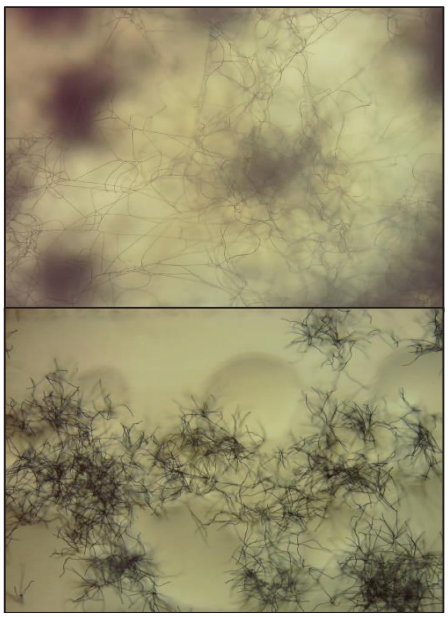

**Fig. 8.** Representative picture of *Amycolatopsis* genus (x400)

It is very important in microbiological studies to identify such a sanitary-indicative microorganism as Escherichia coli and other species belonging to *E. coli* bacteria group. Our study revealed that such bacteria were present in significant quantities in soil samples, especially sample 3 and 6; their titer reached 5.2 x10³ and 1.5 x10⁴ CFU/g soil in winter and increased up to 2.36x10⁴ CFU/g soil during spring (Figures 9 and 10).

The presence of *E. coli* bacteria is an indication of possible contamination by pathogenic microorganisms. The source of origin of coliform bacteria may be not only the excrement of warm-blooded animals, but also vegetation and soil.

In many cases, the soil under study is a kind of depot and accumulator that ensures long-term preservation of both pathogens and soil-borne microorganisms. The duration of the preservation of pathogens in a viable state is determined by the self-cleaning ability of the soil: its combined biological, chemical, physical, suppressive and other properties. The untreated sewage, non-composted animal waste and human waste products used as local organic fertilizers pose a constant threat to soils. Their widespread disinfection and disposal should be an obligatory part of the regional social and environmental policy.

## 4. CONCLUSION

Anthropogenic changes in the soil can be traced based on the results of environmental monitoring of the complex of soil-borne microorganisms. The state of the soil of urban areas requires special attention, since the influence of anthropogenic processes on the soil system leads to a change in almost all of its components, from agrochemical and physical properties to microbiological and biochemical

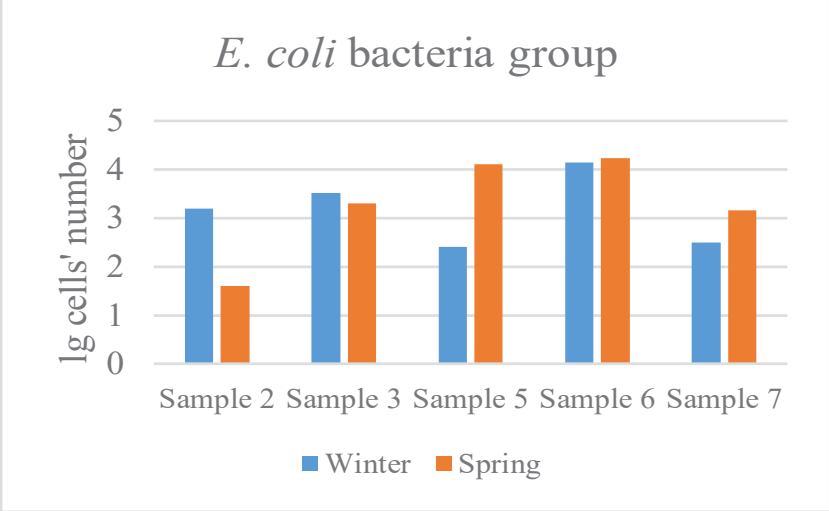


**Fig. 9.** Quantitative analysis of soil samples (*E. coli* bacteria group)

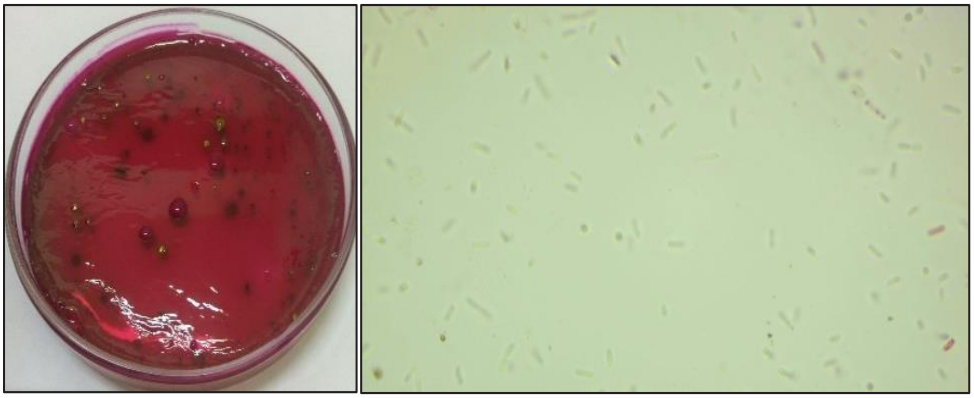

**Fig. 10.** Colonies of *E. coli* bacteria group and *E.coli* (x1000)

indicators, depriving the soil cover of the ability to perform important ecological functions.

As a result of the studies, it has been established that such typical representatives of microbiota as heterotrophic microorganisms related to genera *Bacillus* and *Pseudomonas*, and actinomycetes possess significant remediation potential. Moreover, soil contamination of the studied remediation areas caused by anthropogenic reasons ultimately leads to a decrease in species diversity and a change in the species composition of soil microbocenosis. A stable and resilient microbial community is of great importance for the restoration and functioning of the ecosystem, in this regard, it is necessary to pay special attention to the effective conservation of endangered ecological communities [29]. The results obtained undoubtedly reveal the prospects for biomonitoring and the possible use of microorganisms in soil cleaning processes. A practical approach in the future for cleaning soils from various kinds of contaminants may be the introduction of contamination-resistant microorganisms that can decompose them.

## 5. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 25-26 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)722A

Pakistan Academy of Sciences

# Analysis of the main provisions of the article LS-722 ‘‘Microbiocenosis of Anthropogenically Transformed Soils”

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State University of Management (SUM), Moscow, Russia
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**Relevance of the topic:** Soils are complex bio-inert systems. The biological component in them is a factor of resource value and stability of soil ecosystems. The authors have chosen interesting objects for research from the point of view of soil science, soil biology and toxicology. The study of microbiocenoses in soils is always relevant. Since it is a complex biological marker of geochemical processes and stability. In view of the significant variability of the pedosphere and directly soil biota, the study of representatives of the microbiota is an important area in soil science. And this is at any time. In addition, this study can be a scientific basis for soil bioremediation directly within the framework of environmental design in the territories of treatment facilities and other technospheric facilities.

**Scientific novelty:** New information about the composition of microorganism strains in the soils of Tashkent has been obtained. It is important that sampling was carried out in the zone of influence of the treatment facilities. The authors revealed the issues of the formation of microbiocenoses and some aspects of the stability of these organisms. A number of representatives of new representatives of microorganisms have been identified. The limits of resistance to the environment are determined.

**Content and merit of the work:** The identification of microorganisms was carried out according to the methodological guidelines generally accepted in international practice. Identification of taxa is of great importance in understanding trends in the formation of the composition and structure of microcenoses. The quantitative and qualitative composition of microorganisms was determined. This is a certain fundamental basis for further biological research of soils and soils, both in Uzbekistan and in other territories. The authors also established the bacterial complex dominating in terms of remediation ability. These are representatives of *Bacillus* and *Pseudomonas*.

Studies have also been carried out on the subject of tolerance of microorganisms in relation to the salinity of the pedosphere. A strong point is that salt-tolerant forms of bacteria have been obtained. They show vital activity at a chloride content of up to 5-7 %. Other biogeochemical properties of bacteria have also been obtained, including those with respect to nitrogen and phosphorus. This is important for future planning of environmental management when cultivating pollution-resistant plants in the zones of influence of wastewater treatment and other technospheric facilities in Asia. Data have also been obtained on the degradation of a number of pollutants,

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including organic composition.

**Weaknesses and wishes:** One of the most important issues of modern ecology and soil science is the detoxification of pollutants. It was necessary to dwell in more detail on the issue of bacterial resistance to various aggressive environmental conditions. It is the resistant forms of bacteria that are useful to recommend for the biodegradation of pollutants. The authors are encouraged to continue their research. They have relevance and practical necessity for many Asian cities and towns. It is expedient and very useful to carry out collaboration work between different countries: Uzbekistan, Pakistan, Russia and other neighboring states.

*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 27-34 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)736

Pakistan Academy of Sciences

Research Article

# Monitoring of the Fruit Flies (*Bactrocera* spp.) Infesting Jujube Orchard using Static Spinosad Traps

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**Abstract:** Fruit flies (*Bactrocera* spp.) are regarded as serious insect pests of fruits and vegetables in the world. The goal of this study was to examine the effect of spinosad traps on *Bactrocera* spp. at different heights 0, 1, 2, and 3 m on jujube tree during 2020-2021. Flies' populations were counted weekly. The results revealed that the highest population of *B. zonata* (225.6 flies) were recorded at 2 m height on (22 October, 2020) and the lowest ones (21.6 flies) were recorded at the ground level (0 m height) during (4 February, 2021). However, the overall maximum catches were 158.95 at 2 m height and minimum was 68.72 at the ground level. Similarly, the maximum population of *B. dorsalis* was (50.5 flies) at 2 m height during (9 October, 2020), but the minimum (2.5 flies) was in the ground level during (4 February, 2021). The overall highest *B. dorsalis* catches were (43.50 flies) at 2 m height and the lowest was (3.55 flies) at ground level. The population of *B. zonata* correlated positively (r= 0.2939**) with temperature, but negatively (r= -0.0223$^{NS}$) with relative humidity. However, *B. dorsalis* populations was positive correlated with both of the temperature and relative humidity (r= 0.0261** and r= 0.0091$^{NS}$, respectively). Ultimately, pheromone traps (Spinosad+Methyl eugenol) at 2 m height are highly recommended to catches both fruit flies (*B. zonata* and *B. dorsalis*) in Jujube Orchards.

**Keywords:** *Bactrocera zonata, Bactrocera dorsalis*, Jujube, Methyl eugenol, Spinosad.

## 1. INTRODUCTION

Jujube (*Ziziphus mauritiana* Lamk.), known as "Ber or apple of the desert", is a member of the Rhamnaceae (Buckthorn) family native to China and the Indo-Pak Subcontinent. It thrives in the semi-arid and arid zones marginal ecosystems. Ber's xerophytic characteristics such as its tap root system, the presence of scales on buds, and its deciduous nature in the heat of summer, have made it a profitable crop [1]. Besides, Jujube is widely grown in Pakistan, although it thrives best in the ecological zones of Hyderabad, Khairpur, Multan, Sargodha, and Lahore Divisions. Hyderabad is well-known for its high-quality fruit exports to the Middle East. The tree is tough, drought-resistant, and can grow on poor alkaline soils without a lot of water, as well as on soils where other fruit trees can't grow [2]. Ber is grown on 5425 hectares in Pakistan, with an annual output of 27950 tonnes [3].

Received: August 2022; Accepted: December 2022
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Fruit flies are considered as one of the most damaging agricultural pests around the globe and cause huge threats to horticultural crops, both fruits and vegetables [4-6]. There are about 4000 species of fruit flies in the family of Tephritidae throughout the world, out of which around 350 species have great importance [7]. Tephritid fruit flies cause 90 to 100 % yield loss in fruits and vegetables depending upon several factors such as area season, variety and their population [8]. Fruit flies caused direct loss in the form of yield and indirect loss such as reduction in trade and export prospect [9].

Fruit flies, *Bactrocera* spp. (Diptera: Tephritidae) are frequently found in mango, citrus, and guava plantations [10] and they are often regarded as the world's most damaging insect pests of fruits and vegetables. Many important commercial crops are among the flies' hosts, which come from a broad range of plant groups [11-12]. Their direct damage ranging from 30 to 80 percent based on the fruit host, type, location, and season [13]; decreasing crop output either numerically or qualitatively [14-15].

*Bactrocera zonata* (Sunder) and *B. dorsalis* (Hendel) are the most damaging fruit flies among 400 species found throughout the globe [16]. They overwinter as adults and cause harm to fruits by infesting them. Their maggots feed within the host fruit after female flies' deposit eggs in fragile and sensitive fruit tissues [17]. In Pakistan, 11 species of the genus *Bactrocera* have been identified, out of a total of 43 species. The most common flies are *B. zonata, B. dorsalis* and *B. cucurbitae,* which infest apple (*Malus domestica*), bitter ground *(Mongifera indica*), muskmelon (*Cucumis melon*) and snack ground (*Trichosanthes cucumerina*) [18-19].

Monitoring accumulated degree days are available tool for predicting insect activity and timing pest management practices. Temperature and relative humidity are important abiotic factors affecting the survival and developmental rates of fruit flies [20]. This research work was undertaken to study the fruit flies' species diversity, incidence pattern and their relationship with different weather parameters in relation to static spinosad traps at different heights in Jujube orchards.

## 2. MATERIALS AND METHODS

### 2.1 Experimental Site

The experiment was conducted at Jujube orchard farm, Agriculture Research Institute (ARI), Tandojam for the monitoring of fruit flies, *Bactrocera* spp. during the year 2020-21. The orchard size was 8 acres.

### 2.2 Experimental Design and Treatments

The experiment was laid out in Randomized Complete Block Design (RCBD) was used with five replications for each tested height. The pheromone trap baited with static spinosad (Spinosad+Methyl eugenol) was used for catching the male fruit flies (*B. zonata* and *B. dorsalis*) at different heights on jujube trees. Four treatments were assessed in this study:

$T_1$ = Pheromone traps installed on a ground surface
$T_2$ = Pheromone traps installed at 1 m height
$T_3$ = Pheromone traps installed at 2 m height
$T_4$ = Pheromone traps installed at 3 m height

### 2.3 Procedure of Experiment

The male adult population of fruit flies was counted weekly basis. The pheromone traps were (36 x 11 x 16 cm) in size, cylindrical in form, with a top cover and two openings spaced evenly in opposing directions. Cotton wicks were utilized to absorb 3 g of static spinosad therapy and were wrapped in wire to connect with the trap. These traps were replaced every 35 days to ensure that the chemical used to attract the fruit flies was fresh. At weekly intervals, the number of attracted male flies in traps was tallied, and the species was recognized.

### 2.4 Statistical Analysis

The collected data were statistically analyzed using Statistix 8.1 software. Means treatments were compared with LSD test at $P< 0.05$ level.

## 3. RESULTS

The weekly population of *B. zonata* on different heights is presented in (Table 1). The highest

**Table 1.** Weekly mean population of *B. zonata* on different trapping heights at Jujube orchard

| Weeks | Trapping height | | | |
|---|---|---|---|---|
| | 0 m (Ground surface) | 1 m | 2 m | 3 m |
| 15/10/2020 | 131.6 ± 3.7 $^{ab}$ | 154.1 ± 7.5 $^{a}$ | 221.3 ± 10.9 $^{ab}$ | 197.6 ± 11.7 $^{ab}$ |
| 22/10/2020 | 138.8 ± 5.6 $^{ab}$ | 157.3 ± 8.9 $^{a}$ | 225.6 ± 15.6 $^{a}$ | 206.6 ± 14.3 $^{a}$ |
| 29/10/2020 | 142.6 ± 4.5 $^{a}$ | 153.3 ± 9.7 $^{a}$ | 220.8 ± 14.1 $^{ab}$ | 194.6 ± 14.4 $^{ab}$ |
| 05/11/2020 | 134.2 ± 5.6 $^{ab}$ | 144.6 ± 2.0 $^{a}$ | 208.2 ± 17.1 $^{a-c}$ | 189.8 ± 12.3 $^{ab}$ |
| 12/11/2020 | 125.6 ± 7.5 $^{bc}$ | 140.2 ± 7.0 $^{ab}$ | 201.8 ± 15.1 $^{a-d}$ | 177.4 ± 15.2 $^{a-c}$ |
| 19/11/2020 | 115.6 ± 5.1 $^{cd}$ | 122.7 ± 9.6 $^{bc}$ | 183.8 ± 10.6 $^{a-e}$ | 166.4 ± 15.6 $^{b-d}$ |
| 26/11/2020 | 108.7 ± 5.6 $^{d}$ | 111.5 ± 8.7 $^{cd}$ | 174.8 ± 21.4 $^{a-f}$ | 153.2 ± 18.6 $^{c-e}$ |
| 03/12/2020 | 87.7 ± 6.2 $^{e}$ | 102.2 ± 4.5 $^{d-f}$ | 163.4 ± 20.3 $^{c-g}$ | 144.6 ± 8.8 $^{d-f}$ |
| 10/12/2020 | 80.2 ± 8.5 $^{ef}$ | 91.6 ± 7.2 $^{e-h}$ | 158.2 ± 23.4 $^{c-g}$ | 135.4 ± 8.9 $^{d-g}$ |
| 17/12/2020 | 67.0 ± 5.5 $^{f-h}$ | 86.2 ± 7.2 $^{f-i}$ | 149.6 ± 18.3 $^{d-g}$ | 124.6 ± 7.9 $^{e-i}$ |
| 24/12/2020 | 62.4 ± 4.9 $^{gh}$ | 81.4 ± 8.3 $^{g-k}$ | 127.0 ± 20.7 $^{fg}$ | 118.2 ± 8.9 $^{f-k}$ |
| 31/12/2020 | 60.9 ± 4.6 $^{h}$ | 77.8 ± 5.7 $^{h-k}$ | 133.8 ± 30.6 $^{e-g}$ | 83.4 ± 2.9 $^{l-n}$ |
| 07/01/2021 | 39.8 ± 4.6 $^{jk}$ | 63.6 ± 4.8 $^{k}$ | 119.4 ± 18.1 $^{g}$ | 89.8 ± 9.1 $^{j-n}$ |
| 14/01/2021 | 35.8 ± 6.1 $^{j-l}$ | 66.6 ± 5.3 $^{k}$ | 122.8 ± 23.9 $^{fg}$ | 79.8 ± 15.8 $^{mn}$ |
| 21/01/2021 | 24.2 ± 3.5 $^{lm}$ | 63.2 ± 3.5 $^{k}$ | 117.3 ± 20.8 $^{g}$ | 58.0 ± 7.4 $^{n}$ |
| 28/01/2021 | 23.6 ± 4.0 $^{lm}$ | 64.4 ± 3.6 $^{k}$ | 123.1 ± 14.4 $^{fg}$ | 86.4 ± 13.4 $^{k-n}$ |
| 04/02/2021 | 21.6 ± 3.9 $^{m}$ | 67.9 ± 4.2 $^{jk}$ | 126.4 ± 18.8 $^{fg}$ | 93.4 ± 10.9 $^{i-m}$ |
| 11/02/2021 | 26.4 ± 3.9 $^{k-m}$ | 76.8 ± 4.2 $^{h-k}$ | 141.7 ± 18.5 $^{e-g}$ | 98.8 ± 10.0 $^{h-m}$ |
| 18/02/2021 | 24.4 ± 3.2 $^{lm}$ | 71.8 ± 5.7 $^{i-k}$ | 134.1 ± 9.1 $^{e-g}$ | 105.8 ± 6.4 $^{g-m}$ |
| 25/02/2021 | 25.0 ± 3.3 $^{lm}$ | 75.4 ± 8.2 $^{h-k}$ | 141.6 ± 22.5 $^{e-g}$ | 112.8 ± 10.2 $^{f-l}$ |
| 04/03/2021 | 29.6 ± 2.1 $^{k-m}$ | 85.6 ± 7.5 $^{f-j}$ | 143.2 ± 17.9 $^{e-g}$ | 116.4 ± 8.3 $^{f-k}$ |
| 11/03/2021 | 36.8 ± 4.1 $^{j-l}$ | 92.6 ± 5.1 $^{e-h}$ | 145.0 ± 18.8 $^{e-g}$ | 122.6 ± 15.0 $^{e-i}$ |
| 18/03/2021 | 44.6 ± 4.6 $^{ij}$ | 93.2 ± 5.8 $^{d-h}$ | 156.4 ± 16.1 $^{c-g}$ | 118.8 ± 9.8 $^{f-k}$ |
| 25/03/2021 | 55.4 ± 3.7 $^{hi}$ | 97.8 ± 6.1 $^{d-g}$ | 165.8 ± 19.2 $^{c-g}$ | 119.6 ± 11.3 $^{f-j}$ |
| 01/04/2021 | 75.6 ± 4.8 $^{e-g}$ | 106.5 ± 5.2 $^{c-e}$ | 168.6 ± 21.0 $^{b-g}$ | 131.0 ± 11.7 $^{e-h}$ |

Different letters within a column indicate significant difference (Fisher's Protected LSD test: P<0.05)

trapping was observed (206.6 ± 14.3 flies) at 3 m height on 22 October, 2020, while the lowest population was (58.0 ± 7.4 flies) on 21 January, 2021. Similarly, at the height of 1 and 2 m, the same trapping trend of peach fruit fly population was noted (157.3 ± 8.9 and 225.6 ± 15.6 flies, respectively) on 22 October, 2020. However, the least catching of male fruit flies was (63.2 ± 3.5 flies) at 1 m height, as well as (117.3 ± 20.8 flies) at 2 m on 21 January, 2021. Furthermore, the maximum weekly population (142.6 ± 4.5 flies) was recorded in ground level (0 m) during 29 October, 2020, but the minimum catch was (21.6 ± 3.9 flies) on 4 February, 2021. The analysis of variance (ANOVA) shows a significant difference (P<0.05) among all treatments during whole weeks. The overall trapping of male fruit flies at different heights are shown in (Figure 1). In this regard, the maximum male catches were recorded at 2 m trapping height, followed by 3 m and the lowest was on a ground level (0 m).

On the other hand, the weekly population of *B. dorsalis* on various trapping heights is shown in (Table 2). The maximum catching of male oriental fruit flies was counted (50.5 ± 3.3 flies) on 29 October, 2020 at 2 m height, but the minimum mean trapping was found (36.1 ± 3.9 flies) on 18 February, 2021. The same highly population trapping trend was (27.2 ± 2.6 flies) during 5 November, 2020, but the least population was (15.8 ± 2.9 flies) on 11 February, 2021 at 3 m height. At 1 m trapping height, the maximum mean population was (17.6 ± 3.1 flies) during 22 October, 2020, but the least one was (8.7 ± 0.7 flies) on 14 January, 2021.

At the ground level (0 m), the maximum

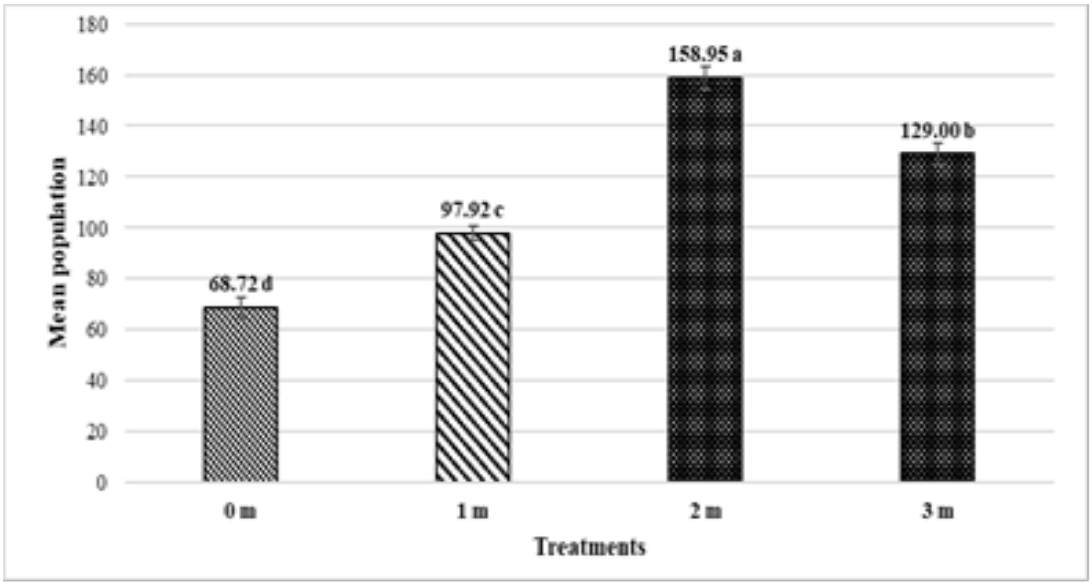


**Fig. 1.** Overall mean population of *B. zonata* at different trapping heights at Jujube orchard

**Table 2.** Weekly mean population of *B. dorsalis* on different trapping heights at Jujube orchard

| Weeks | Trapping height | | | |
|---|---|---|---|---|
| | 0 m (Ground surface) | 1 m | 2 m | 3 m |
| 15/10/2020 | 4.2 ± 0.7 $^{a-c}$ | 14.5 ± 1.4 $^{a-g}$ | 47.5 ± 3.7 $^{ab}$ | 24.0 ± 2.9 $^{ab}$ |
| 22/10/2020 | 3.9 ± 0.6 $^{a-d}$ | 17.6 ± 3.1 $^{a}$ | 48.6 ± 4.2 $^{a}$ | 25.4 ± 2.7 $^{ab}$ |
| 29/10/2020 | 3.8 ± 0.6 $^{a-e}$ | 16.8 ± 1.9 $^{ab}$ | 50.5 ± 3.3 $^{a}$ | 26.2 ± 3.1 $^{ab}$ |
| 05/11/2020 | 4.0 ± 0.5 $^{a-d}$ | 15.3 ± 1.9 $^{a-e}$ | 49.1 ± 4.9 $^{a}$ | 27.2 ± 2.6 $^{a}$ |
| 12/11/2020 | 3.9 ± 0.6 $^{a-e}$ | 12.3 ± 1.2 $^{b-i}$ | 45.7 ± 4.6 $^{ab}$ | 25.8 ± 2.9 $^{ab}$ |
| 19/11/2020 | 4.3 ± 0.5 $^{ab}$ | 15.0 ± 2.0 $^{a-f}$ | 46.6 ± 6.0 $^{ab}$ | 25.2 ± 2.7 $^{ab}$ |
| 26/11/2020 | 4.6 ± 0.3 $^{a}$ | 14.6 ± 2.8 $^{a-g}$ | 44.5 ± 4.6 $^{ab}$ | 24.2 ± 2.3 $^{ab}$ |
| 03/12/2020 | 3.4 ± 0.5 $^{a-f}$ | 12.7 ± 1.6 $^{b-i}$ | 45.4 ± 5.6 $^{ab}$ | 23.2 ± 2.5 $^{a-c}$ |
| 10/12/2020 | 3.2 ± 0.4 $^{b-f}$ | 12.3 ± 1.2 $^{b-i}$ | 44.9 ± 4.9 $^{ab}$ | 22.6 ± 2.2 $^{a-c}$ |
| 17/12/2020 | 3.9 ± 0.6 $^{a-d}$ | 10.1 ± 0.6 $^{g-i}$ | 42.3 ± 4.1 $^{ab}$ | 24.0 ± 3.0 $^{ab}$ |
| 24/12/2020 | 3.9 ± 0.3 $^{a-d}$ | 14.0 ± 2.5 $^{d-i}$ | 40.6 ± 5.0 $^{ab}$ | 20.2 ± 3.5 $^{ab}$ |
| 31/12/2020 | 3.6 ± 0.3 $^{a-e}$ | 10.3 ± 1.1 $^{g-i}$ | 42.4 ± 3.6 $^{ab}$ | 20.0 ± 3.1 $^{a-c}$ |
| 07/01/2021 | 2.9 ± 0.4 $^{d-f}$ | 11.3 ± 1.0 $^{d-i}$ | 41.2 ± 4.4 $^{ab}$ | 22.8 ± 2.6 $^{a-c}$ |
| 14/01/2021 | 3.4 ± 0.2 $^{a-f}$ | 8.7 ± 0.7 $^{i}$ | 41.9 ± 2.8 $^{ab}$ | 24.4 ± 2.5 $^{ab}$ |
| 21/01/2021 | 2.7 ± 0.4 $^{ef}$ | 10.5 ± 1.7 $^{f-i}$ | 39.9 ± 3.6 $^{ab}$ | 20.8 ± 1.5 $^{a-c}$ |
| 28/01/2021 | 2.9 ± 0.1 $^{c-f}$ | 9.9 ± 1.1 $^{hi}$ | 38.6 ± 3.9 $^{ab}$ | 23.2 ± 2.5 $^{a-c}$ |
| 04/02/2021 | 2.5 ± 0.3 $^{f}$ | 12.3 ± 0.5 $^{b-i}$ | 40.3 ± 3.0 $^{ab}$ | 24.0 ± 3.4 $^{ab}$ |
| 11/02/2021 | 2.9 ± 0.4 $^{c-f}$ | 13.2 ± 1.3 $^{a-i}$ | 38.6 ± 3.9 $^{ab}$ | 15.8 ± 2.9 $^{c}$ |
| 18/02/2021 | 2.9 ± 0.3 $^{d-f}$ | 10.6 ± 1.1 $^{e-i}$ | 36.1 ± 3.9 $^{b}$ | 18.8 ± 2.4 $^{bc}$ |
| 25/02/2021 | 3.5 ± 0.2 $^{a-f}$ | 11.9 ±1.7 $^{c-i}$ | 41.8 ± 3.8 $^{ab}$ | 19.0 ± 2.3 $^{bc}$ |
| 04/03/2021 | 3.4 ± 0.3 $^{a-f}$ | 13.9 ± 1.2 $^{a-h}$ | 43.3 ± 2.6 $^{ab}$ | 19.2 ± 3.2 $^{bc}$ |
| 11/03/2021 | 3.8 ± 0.3 $^{a-e}$ | 14.6 ± 1.7 $^{a-g}$ | 46.2 ± 6.1 $^{ab}$ | 19.0 ± 2.4 $^{bc}$ |
| 18/03/2021 | 3.7 ± 0.4 $^{a-f}$ | 16.2 ± 2.4 $^{abc}$ | 41.0 ±4.2 $^{ab}$ | 22.1 ± 1.9$^{a-c}$ |
| 25/03/2021 | 3.5 ± 0.5 $^{a-f}$ | 13.8 ± 1.2 $^{a-h}$ | 45.8 ± 5.9 $^{ab}$ | 22.5 ± 3.3 $^{a-c}$ |
| 01/04/2021 | 3.8 ± 0.5 $^{a-e}$ | 15.6 ± 1.2 $^{a-d}$ | 44.7 ± 2.6 $^{ab}$ | 21.6 ± 4.0 $^{a-c}$ |

Different letters within a column indicate significant difference (Fisher's Protected LSD test: P<0.05)

population of *B. dorsalis* was (4.6 ± 0.3 flies) on 26 November, 2020, but the minimum population was (2.5 ± 0.3 flies) during 4 February, 2021. The overall catching of *B. dorsalis* on different heights is presented in (Figure 2). In this regard, the highest male catches were counted at 2 m trapping height, followed by 3 m while the lowest catching was on a ground level (0 m).

Similarly, the result regarding trapping population of both fruit flies correlated with abiotic factors (temperature and relative humidity) is mentioned in (Table 3). A positive significantly relationship (0.2939**) was noted between the population of *B. zonata* and temperature but was negatively non-significant (-0.0223NS) with relative humidity %. For *B. dorsalis*, A positive relationship (0.0261** and $0.0091^{NS}$) was with temperature and relative humidity, respectively.

**Table 3.** Pearson's correlation among *B. zonata* and *B. dorsalis* population with abiotic factors

| Variables | *B. zonata* | *B. dorsalis* |
|---|---|---|
| **Temperature (˚C)** | r = 0.2939** | r = 0.0261** |
| **Relative humidity (%)** | r = $-0.0223^{NS}$ | r = 0.0091** |

**Significant; $^{NS}$= Non-significant.

## 4. DISCUSSION

The present results revealed that the baited static spinosad (Spinosad+Methyl eugenol) trap placed at 2 m height caught the most fruit flies (*B. zonata* and *B. dorsalis*) in the Jujube Orchard. The population of *B. zonata* was found to be greater than *B. dorsalis*, during seasonal fruit fly infestation monitoring. Our findings were corroborated with those reported previously by Vistro *et al.* [21], who claimed that *B. zonata* was measured (61.38 flies) at a height of 2 m. On the other hand, weekly trap catches were recorded at 3 m (51.35), 1 m (43.03), and 0 m (38.09), respectively. In the same way, the highest weekly *B. dorsalis* at a height of 2 metres were 0.49, and 0.43, 0.36, and 0.29, respectively for 3 m, 1 m, and ground surface. Although, this work found the maximum population of *B. zonata* was observed (206.6 flies) at 3 m height on 22 October, 2020, but the minimum catches flies were (21.6 ± 3.9 flies) at 0 m (ground level) on 4 February, 2021. On the other hand, highest *B. dorsalis* was counted (50.5 ± 3.3 flies) on 29 October, 2020 at 2 m height, but the minimum population was (2.5 ± 0.3 flies) during 4 February, 2021 at 0 m (ground level). According to the findings of Solangi *et al.* [22], the maximum *B. zonata* was captures at 2 m height with (1428.4 flies), followed by 1 m, 3 m and 0 m (ground surface) with (1340.5, 1185.4, and 1177.3, respectively). However, the highest *B. dorsalis* 7.34 flies were counted at 2 m height, while lowest was noted 4.67 flies at 3 m height, followed by 0 m (ground surface) and 1 m with (6.29 and 4.96 flies). Similarly, Hasnain *et al.* [23] also observed that the maximum male fruit flies 515 was counted at 5 m height, while

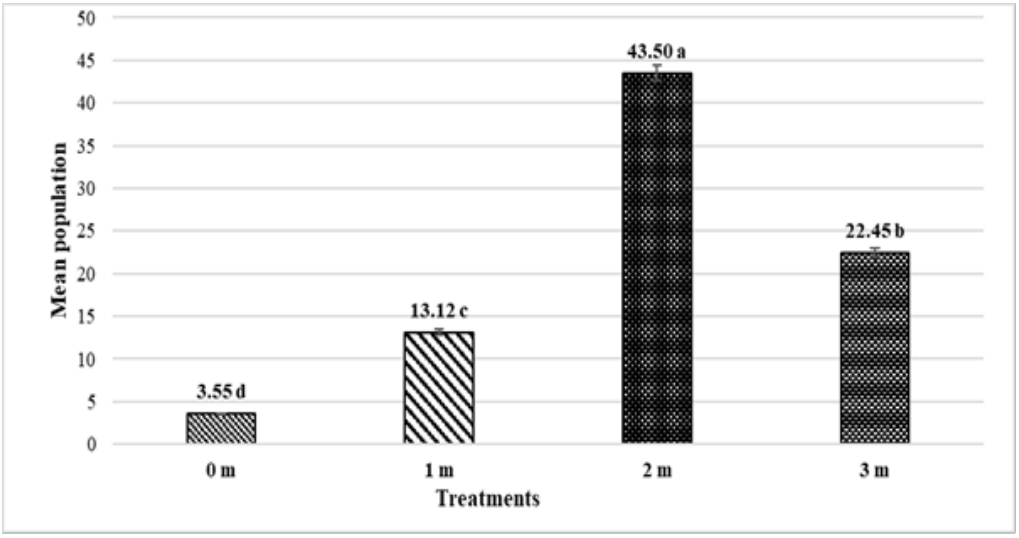


**Fig. 2.** Overall mean population of *B. dorsalis* on different trapping heights at Jujube orchard

minimum 315 flies were noted at 3 m height.

In addition, as reported by Wazir *et al.* [24], the highest population of fruit fly was recorded in July, while the lowest number was in January. However, as observed previously by Ahmad and Begum [25], the highest population density for fruit flies was discovered in methyl eugenol (382) in comparable to that of Gf 120 (197.2), while the lowest was found in Raspberry essence (23.6). Moreover, Darwish *et al.* [26] found that when methyl eugenol traps were placed at heights of 1 and 2 m, the guava fruit fly was highly captured. According to the observation by Singh and Sharma [27], the trap captures varied from 76.3 flies in the first week of June to 326.3 flies in the late week of July. According to the previous results noted by Khan *et al.* [28], Methyl eugenol traps catchs the most important fruit flies such as *B. dorsalis*, *B. zonata* and *C. vesuviana.*

Similarly, the result regarding trapping population of both fruit flies correlated with abiotic factors (temperature and relative humidity), a positive significantly relationship (0.2939**) was noted between the population of *B. zonata* and temperature but was negatively non-significant ($-0.0223^{NS}$) with relative humidity %. For *B. dorsalis*, a positive relationship (0.0261** and $0.0091^{NS}$) was with temperature and relative humidity, respectively. According to the observation by Ye and Liu [29], to record the effect of abiotic factors on population of fruit flies three peaks, during the 27, 45, and 48$^{th}$ standard weeks, *B. dorsalis* was found in a guava orchard, whereas *B. correcta* reached its highest point during the 27-standard week, although it also reached two more high points during the 11 and 18 standard weeks, respectively.

According to Khoso *et al.* [30], the population of *Bactrocera* spp. (*B. dorsalis* and *B. zonata*) had a positive relation with wind velocity and temperature, while with mean relative humidity had a negative association. Besides, Khan and Naveed [31] exhibited that populations and temperature have a positive connection, whereas relative humidity has a slightly negatively. Das *et al.* [32] recorded the *B. dorsalis* had a significant positive correlation coefficient with the seasonal average maximum temperature (0.187), and a significant negative correlation coefficient with the lowest temperature (-0.087), morning relative humidity (-0.257), afternoon relative humidity (-0.511), and rainfall (-0.329). In a similar concept was noted in *B. zonata* with maximum temperature (0.543), minimum temperature (0.192), and rainfall (0.017), all had substantial positive correlations, whereas morning relative humidity (-0.241) and afternoon relative humidity (-0.215) had significant negative correlations. During the dry season, Vayssieres *et al.* [33] found the greatest influence on the population of Ceratitis cosyrawas, but the least impact was on the population of *B. invadancs*. The effect of daily rainfall on the population of *B. invadens* has been shown to be beneficial. Invaders, to be precise.

## 5. CONCLUSION & RECOMMENDATIONS

The present findings concluded that 2 m height showed maximum catches of both fruit flies (*B. zonata* and *B. dorsalis*) when installed pheromone traps (Spinosad+Methyl eugenol) in Jujube orchard. However, the minimum catches of both fruit flies were counted at 3 m, 1 m and 0 m (ground level) heights. Ultimately, the trap should be installed at 2 m height from the ground level for capturing the optimum fruit flies and highly recommended to control fruit fly males in the Jujube orchard. Further study is much needed to observe the different heights of traps based on various distance against different species of fruit flies.

## 6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 35-42 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)742

Pakistan Academy of Sciences

Research Article

# Molecular Analysis of *phe* Operon Genes determining Phenol-Degrading *Pseudomonas* sp. from Polluted Sites in Baghdad City

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**Abstract:** Phenolic compounds are toxic to plants, animals and even for microorganisms at low concentrations. Because of this toxicity, it is important that soils polluted with these compounds to be remediated immediately. *Pseudomonas aeruginosa* and *Pseudomonas putida*, as well as their both intra- and extradiol enzymes, were the targets of this study, which aimed to detect the enzymes responsible for phenol degradation capability in bacteria and the genetic variation of the catabolic genes related to the phe operon among the positive isolates. In this study one hundred twenty five samples of contaminated soils have been collected from different sources at Baghdad city (89 samples from Daura refinery, 21 samples from private electricity generators and 15 samples from different farm lands). Collected samples have cultured on mineral salt medium as well as using differential and selective media, then diagnosed by classical biochemical tests and VITEK system beside using Housekeeping gene 16s rDNA for molecular diagnosis. The results of VITEK system revealed that 29/89 (32.5 %) of samples from Daura refinery had *P. aeroginosa* isolates and only one sample 1/89 (1.1 %) of *P. putida*. On the other hand, none of the samples from generators (0 %) were *P. aeroginosa* and 5/21(23.8 %) were *P. putida* while 5/15 (33.3 %) samples of farm lands were *P. aeroginosa* and (0 %) were *P. putida*. Molecular diagnosis using 16S rDNA detected 40/125 (32 %) positive isolates for *Pseudomonas* sp.; 34 (85 %) isolates for *P. aeruginosa* and 6 (15 %) isolates for *P. putida*. Phenol degradation capability of the forty isolates has been tested on mineral salt medium using different concentrations of phenol (100 ppm to 1500 ppm) and all of them (100 %) were able to degrade phenol to 600 ppm but a number of 4 isolates (10 %) have exceeded this concentration to 1200 ppm and only one isolate (2.5 %) tolerated phenol to the maximum level which is 1500 ppm. Phenol degrading isolates were subjected to PCR technique to detect the phe-like genes: catechol 1, 2 dioxygenase (*cat*1), and catechol 2, 3 dioxygenase (*cat*2). As a result, this set of enzymes were found in the whole five (12.5 %) isolates that effectively degraded phenol to the concentration of 1200 ppm and 1500 ppm.

**Keywords:** Pollution, Phenol, Phenol-Degradation, Biodegradation, *phe* genes, *Pseudomonas aeruginosa*, *Pseudomonas putida*, Catechol Dioxygenase.

## 1. INTRODUCTION

Toxic environmental pollutants such as phenol originate primarily from industrial processes and it has been listed among the most common pollutants. In order to protect the environment, the U.S. Environmental Protection Agency has set a limit of 0.1 mg/L of phenol in the water supply. Removal from the environment is necessary. For the treatment of phenol-contaminated wastewater, more effective and less expensive biodegradation methods are available. More and more microbes have been discovered to coexist in almost all natural environments in the last three decades, particularly in soils, where microbial catalysts have been used extensively [1]. Oil production stations and refineries serve as the major contributors in the environmental problems especially in soil and water [2]. Oily wastewater and soils contains hazardous and toxic substances that have inhibitory effects on animal and plant growth [3] as well as their mutagenic and carcinogenic effects on humans around the world [4, 5].

Numerous studies suggested that biodegradation has become a priority for scientific

Received: September 2022; Accepted: December 2022
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research as it aims to safely and quickly remove for these contaminants from soils [6] and confirmed that the final success of biodegradation depends on the nature of microorganisms which are in direct contact with the biodegradable substance, and that the bio-treatment of soils contaminated with hydrocarbons from crude oil linked to the ability of organisms to consume hydrocarbons [7].

Treatment of oil- contaminated soils is done by the aid of common microorganisms or ones isolated from oil- contaminated sites. The ability of these microorganisms for biodegradation was tested *in vitro* [8]. Many types of bacteria have been isolated from contaminated soil and one of these are *Pseudomonas* spp. These microorganisms gradually reduced the concentrations of polycyclic hydrocarbon compounds due to their ability to survive in such soils by developing a certain enzymatic and physiological response allowing them to use hydrocarbonic compounds present in oil as an alternative source of carbon [9]. Thus, when they consume carbon, they had already broken down these long bonds and converted them into simpler form that can be easily degraded [10].

The Materials that have been depolymerized and contain phenol can be further degraded by hydroxylation using a single or multicomponent hydroxylases (MPH). Ring cleaving enzymes like catechol-1,2-dioxygenase are used also to catalyze the ortho-cleavage route for catechol degradation [11]. Catechol is an important intermediary not only in the breakdown of aromatic compounds derived from plant-based materials, but also in the degradation of pollutants [12]. The current study aims to detect the prevalence of *Pseudomonas* sp. in three potentially polluted spots and the enzymes responsible for phenol degradation capability in bacteria and the genetic variation of the catabolic genes related to the *phe* operon among the positive isolates.

## 2. MATERIALS AND METHODS

### 2.1 Bacterial Isolation and Identification

One hundred twenty-five soil samples have been collected from different polluted sites at Baghdad city for the isolation of the most effective *Pseudomonas* sp. isolates in phenol-degradation. Eighty-nine (89) samples were collected from Midland refineries company (MRC)/ Daura refinery, twenty-one samples were collected from private electricity generators and fifteen samples were collected from different farm lands during the period from April 2021 to August 2021. Mineral salt medium (a liquid enriched medium) [1] and a number of differential and selective media like macConkey agar and *Pseudomonas* agar base media which were used to grow and diagnose the collected samples subsequently. VITEK system (BioMérieux, France) has been used for diagnosing samples by using the card VITEK® 2 GN ID Card.

The broth of the bacterial isolates was cultured overnight in nutrient broth medium (N. B), then they were subjected for DNA extraction, by using ABIO pure TM kit (Alliance Bio, USA). The concentration and purity of the extracted DNA were measured using Nanodrop. All the previously diagnosed isolates were subjected to DNA extraction and samples have been additionally diagnosed by PCR using the housekeeping gene 16S rDNA to confirm their identities [13] (Table 1). The reaction components included; 12.5 µl of GoTaq®Green Master Mix (Promega, USA), 1 µl of each sense and anti-sense primer (Macrogen), 2 µl of DNA templates of isolated bacteria, then the volume was completed to 25 µl by adding 8.5 µl of nuclease free water. PCR reaction was then carried out using the following program: 2 minutes of initial denaturation at 95 °C, followed by 25 cycles of three stages: denaturation at 94 °C for 20 seconds, annealing at 58 °C for 20 seconds, and extension at 72 °C for

**Table 1.** Primer sequences used in the current study

| Gene name | Primer Name | Sequence 5′→3′ | Product Size | Reference |
|---|---|---|---|---|
| **16s rDNA** | F | AGAGTTTGATCCTGGCTCAG | 956 bp | [13] |
| | R | CTTGTGCGGGCCCCCGTCAATTC | | |
| ***cat*1** | F | AAACCCGCGCTTCAAGCAGA | 650 bp | Present study designated |
| | R | AAGTGGATCTGCGTGGTCAGG | | |
| ***cat*2** | F | TGATCGAGATGGACCGTGAC | 821 bp | Present study designated |
| | R | TCAGGTCAGCACGGTCATGAA | | |

40 seconds. Finally, final extension temperature was adjusted to 72 °C for one minute. PCR results were visualized on gel electrophoresis of 1 % agarose and showed sharp single bands on the gel. PCR products for the amplified gene (stored at -20 ºC) was sequenced by sending 20 µl of the amplified product to Macrogen, Korea. Data were analyzed using Geneious software and the results were read by comparing them with the NCBI control standard strains. Query, pairwise alignment and identity, were anatomized with same software.

As for phenol treatment, the positive isolates for HKG have been tested for phenol degradation capability by adding different concentrations of phenol (100 ppm to 1500 ppm) to the mineral salt medium to examine their tolerance to phenol.

### 2.2 Detection of Catechol Dioxygenases using Conventional PCR

DNA templates of the targeted isolates were used to amplify the catechol dioxygenase enzymes using specific primers: catechol 1,2 dioxygenase (*cat*1), and catechol 2,3 dioxygenase (*cat*2) genes by conventional PCR. After many optimization trials, the two set primers were subjected to the following conditions: Initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of three stages, including denaturation at 94 °C for 30 seconds, annealing at (56 °C to 60.2 °C) for one minute, extension at 72 °C for two minutes. Then final extension was set for 7 minutes at 72 °C.

The primer sequences that used are summarized in (Table 1). After sending those PCR products to Macrogen, Korea. Data were analyzed using Geneious software and the results were read by comparing them with the NCBI control standard strains. Query, pairwise alignment and identity, were anatomized with same software.

## 3. RESULTS AND DISCUSSION

### 3.1 Bacterial Isolation and Identification

After the collection of samples, they have been classified according to the source of collection (89 samples from Daura refinery, 21 samples from the soils of private electricity generators, and 15 samples from farm lands at Baghdad city, these sites are mostly exposed to pollution due to factories in that area with the farm land as least polluted site (Table 2).

The diagnosis of soil samples using enriched and selective media and VITEK system revealed that 29/89 (32.5 %) of Daura refinery were *Pseudomonas aeroginosa* and only one sample was *Pseudomonas putida* 1/89 (1.1 %). on the other hand, none of the samples from generators were *P. aeroginosa* (0 %) and 5/21 (23.8 %) samples were *P. putida*; while 5/15 (33.4 %) samples of farm lands were *P. aeroginosa* 5/15 (3.33 %) and no sample were detected for *P. putida* (0 %). That means that this step detected forty (32 %) phenol-degrading isolates from the total number of isolates (Table 2).

Phenol degradation capability of the targeted isolates have been tested and the results revealed that all the forty isolates that previously diagnosed as *Pseudomonas* sp. (100 %) were able to degrade phenol till the concentration of 600 ppm.

Only four isolates (10 %) of the total number were characterized to tolerate phenol to a concentration exceeded 1200 ppm and only one isolate (2.5 %) tolerated phenol to the maximum concentration (1500 ppm).

A housekeeping gene 16S rDNA was used for genotypic identification of *Pseudomonas* spp. isolates and all the isolates (100 %) showed a

**Table 2.** Distribution of *Pseudomonas* species according to the collection source

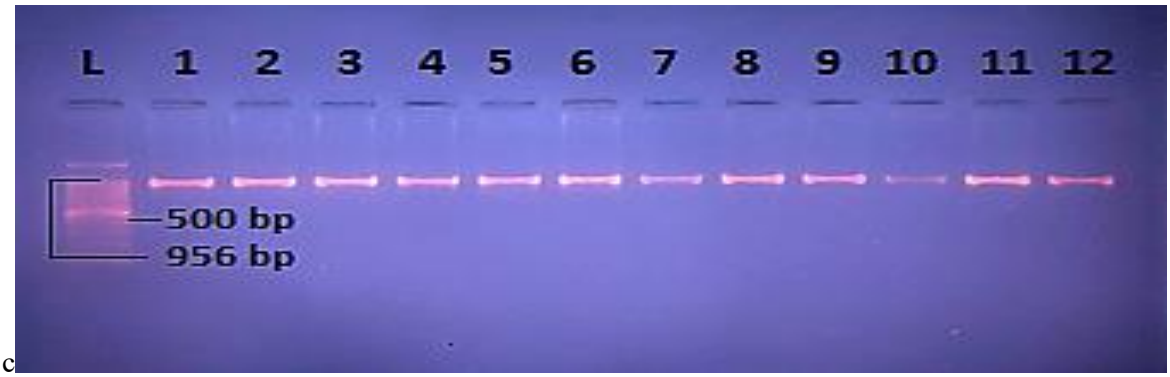
| Source of sample | Total no. Pre-Diagnosis | *Pseudomonas aeruginosa* | *Pseudomonas putida* | Total number |
|---|---|---|---|---|
| **Daura refinery** | 89 | 29 (32.5 %) | 1 (1.1 %) | 30 (2.37 %) |
| **Private Generators** | 21 | 0 (0 %) | 5 (23.8 %) | 5 (23.8 %) |
| **Farm lands (not polluted)** | 15 | 5 (3.33 %) | 0 (0 %) | 5 (3.33 %) |
| **Total number** | 125 | 34 (27.2 %) | 6 (4.8 %) | 40 (32 %) |

positive result. Clear bands were shown on agarose gel and expected size was 956 bp (Figure 1A) and they were matched with 100 bp DNA ladder. The Blast hit is presented in figure (1B) clarifies the amplified size from the part of the gene size. The pairwise identity was 99.89 %, which represents the residue percentages that were identical to gaps versus non-gap residue. Some of the differences appeared between local isolate and recorded NCBI strain as compared with WHO stander strain MH114980 (Figure 1C).

### 3.2 Detection of Catechol Dioxygenases Using Conventional PCR

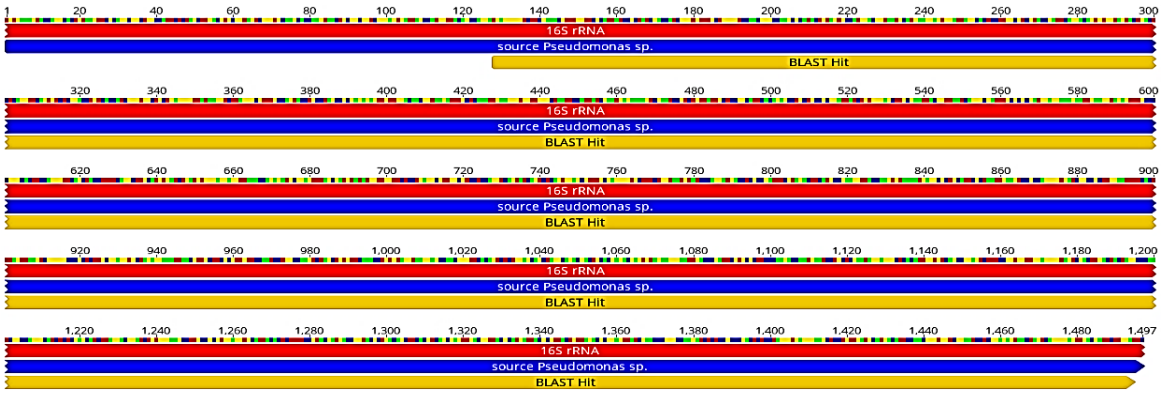
The results of detecting *cat*1 gene showed that all

A.




c

B.




C.

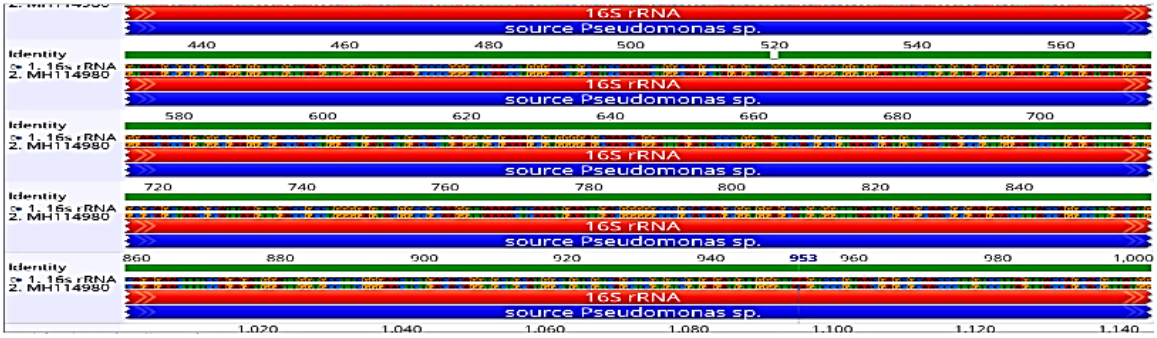



**Fig 1.** A. Agarose gel electrophoresis (1 % percent agarose, 5 V/cm for 90 minutes) for 16S rDNA gene (amplified size of 956 bp) vs. DNA ladder B. 16S *Pseudomonas* spp: precise molecular size 1368 bp, Blast hit: interval: 128 ->1495 from the original 16s DNA related to the NCBI standard strain MH114980 C. Pairwise identity 99.89 % as compared with WHO stander strain MH114980.

the 40 (100 %) isolates were positive for this gene (Table 3). Clear bands appeared in agarose gel and expected size was 650 bp as in (Figure 2A). The blast hit is presented in (Figure 2B) which clarifies the amplified size from the part of the gene size. The sequences of *cat*1 gene for *P. putida* local isolate was displayed in (Figure 2C). Sequence comparison was done between DNA segment of the current study and the standard strain CP016212 in which pairwise identity reached the percentage of residues that were similar in alignments with gap vs. no gap residues was 98 % percent. There were just a few discrepancies between the local isolates and the reference strain.

The results of *cat*2 gene amplification showed that this gene was located in six isolates (15 %) of the total number as shown in (Table 3) and Figure (3A). The result was represented with sharp single bands with expected size of (821bp). Blast hit is presented in figure (3B) which illustrated the precise amplified region from the complete gene. The resulted sequence has aligned with a sequence from NCBI database under the reference ID APO15030, and the results of sequence comparison represented in Figure (3C).

This study was conducted for the isolation of *Pseudomonas* sp. from the soils contaminated with phenolic compounds at Baghdad city and the evaluation of their capability to degrade phenol. Polluted soil samples were collected from different regions at Baghdad city, Iraq. These samples were cultured on the mineral salt medium [1] with the presence of phenol in this enriched medium for the isolation of phenol degrading bacteria. Further culturing was done using differential and selective media for the diagnosis of these isolates, beside diagnosing them by the VITEK system and the HKG using 16S rDNA gene at the molecular level. Soil indigenous microbial communities have a flexible capability in biodegrading hydrocarbonic compounds in oil-polluted soils as previously demonstrated [1,14]. The results of this study showed higher frequency of *P. aeruginosa* among the isolates harvested from Daura refinery. This results agreed with a previous study done by Khatoon and Malik [15].

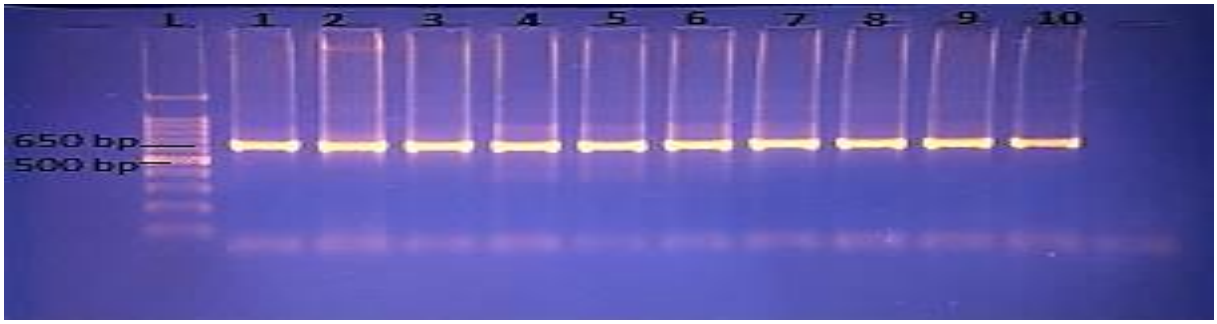
Many researchers have investigated the degradation ability of phenol by various *Pseudomonas* species under aerobic and anaerobic conditions. A previous study suggested that the isolated *Pseudomona*s degrades phenol aerobically via the ortho-cleavage route [16]. The degradation process begins with the creation of catechol, which occurs when phenol hydroxylase (monooxygenases) attaches a hydroxyl group to the benzene ring in the ortho position. In addition to monooxygenases, dioxygenases which are classified into two families, intradiol and extradiol dioxygenases can degrade catechol rapidly by catalyzing the oxidative cleavage of catechol by any pathway resulting in the rapid elimination of the intermediate metabolites [17]. The primary intermediate generated during the biodegradation of phenol by various microbial strains is catechol. Catechol 1,2-dioxygenases catalyze the ortho-cleavage pathway [18] while catechol 2,3-dioxygenases catalyze the meta-cleavage pathway [19]. Both *P. aeruginosa* and *P. putida* isolates were found in this investigation and they were efficient as phenol degrading isolates because they have the enzymes responsible for this ability and also all of them were positive for the catechol 1,2 dioxygenase gene (*cat*1) targeted in the current study.

The catechol dioxygenases serve as part of nature's strategy for degrading aromatic molecules in the environment. They are found in the soil bacteria and involved in the transformation of aromatic precursors into aliphatic products. The

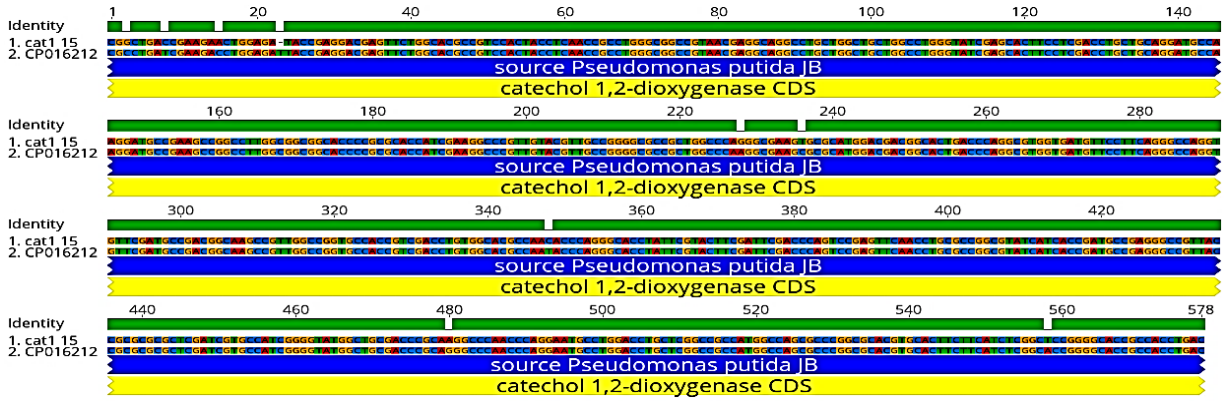
**Table 3.** Detection of Catechol Dioxygenases in *Pseudomonas sp*. isolates

| ***Pseudomonas sp*. isolates** | **Catechol Dioxygenase genes** | |
|---|---|---|
| | ***cat*1** | ***cat*2** |
| *Pseudomonas aeruginosa* isolates | 34 ( 85 %) | 4 (10 %) |
| *Pseudomonas putida* isolates | 6 (15 %) | 2 (5 %) |
| Total | 40 (100 %) | 6 (15 %) |

A.




B.




C.

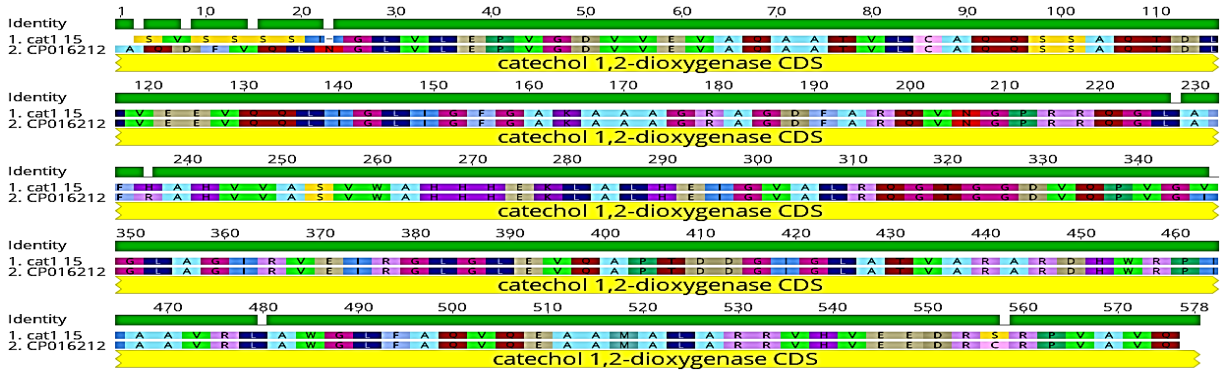


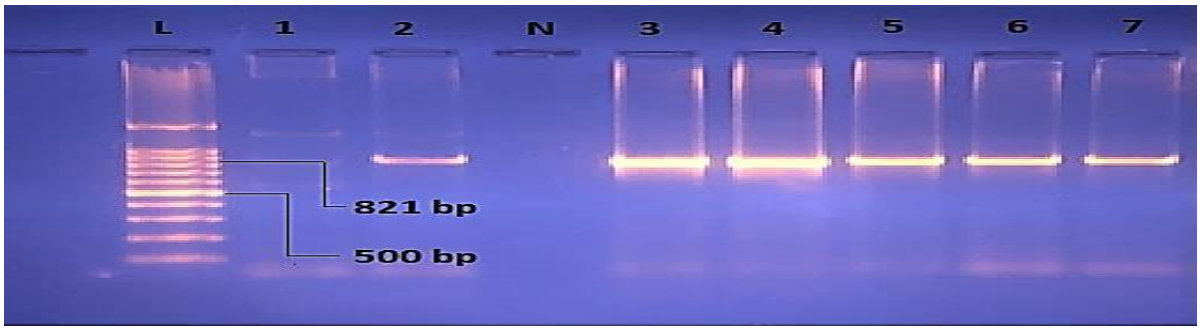

**Fig 2.** A. Agarose gel electrophoresis (1% percent agarose, 5 V/cm for 90 minutes) for *cat*1 gene (amplified size of 650 bp) vs. DNA ladder lane B: In the current investigation, a blast hit of the amplified gene. C: Pairwise identification CP016212 and DNA sequencing for the *cat*1 gene shows that the local isolate has few gaps.

intradiol cleaving enzymes utilize Fe (III), while the extradiol cleaving enzymes utilize Fe (II) and Mn (II) in few cases [20]. In *Pseudomonads*, many of its induced enzymes are nonspecific and its metabolic pathway contains a high degree of convergence. This convergence of catabolic pathways allows them efficiently utilizing a wide range of growth substrates, while the non-specificity of these induced enzymes allows for the simultaneous utilization of many similar substrates without an extra redundant genetic coding for enzyme induction [21].

## 4. CONCLUSION

In the present study, two species of *Pseudomonas,*

A.

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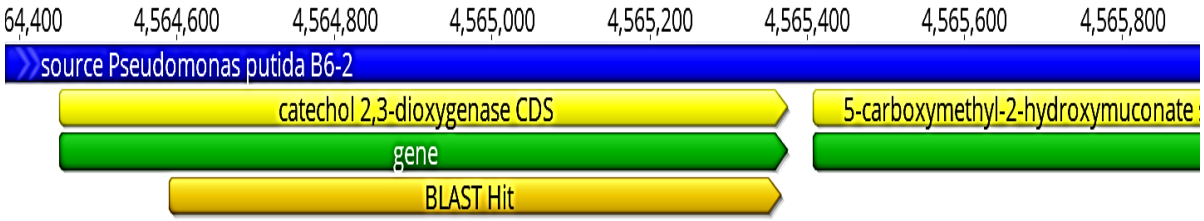


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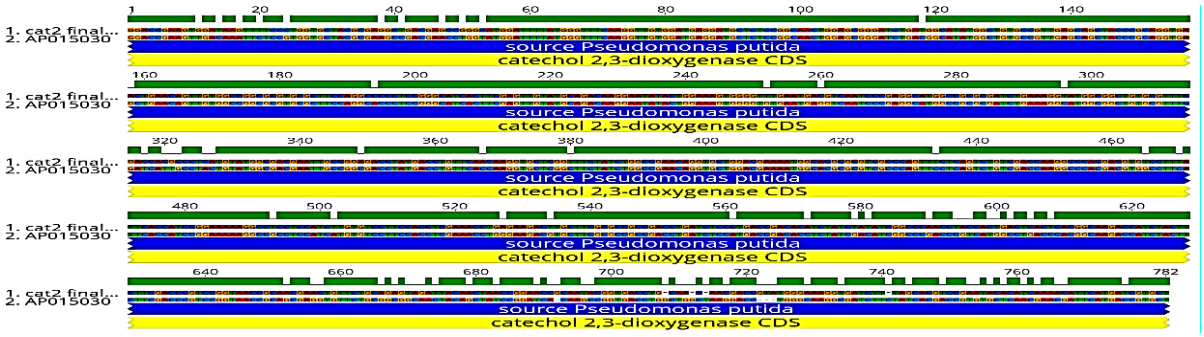


**Fig 3.** A. Agarose gel electrophoresis (1 percent agarose, 5 V/cm for 90 minutes) of the *cat*2 gene (amplified size of 821 bp) vs. DNA ladder lane. B: The magnified Blast Hit gene in the current study. C: Pairwise identity APO15030 and *cat*2 gene DNA sequencing, which revealed a few gaps in the local isolate.

*P. aeruginosa* and *P. putida* were isolated from soils contaminated with phenolic compounds. Phenol degradation capability of the targeted isolates have been tested and vast majority of them were effectively capable of degrading phenol to the concentration of 1200 ppm and only one isolate tolerated phenol to the maximum concentration (1500 ppm) which is the isolate number 15 which is considered the most effective isolate in phenol degradation in or study. The previous findings can be seen as an important tool in the treatment of soils and water that polluted with phenol. The existence of such enzymes in the tested isolates reflects their catalytic potential in degrading such pollutants.

## 5. ACKNOWLEDGMENTS

The authors would like to thank the Department of Biology/College of Science, Mustansiriyah University/ Iraq (https://uomustansiriyah.edu.iq/) for their support to complete this work.

## 6. CONFLICT OF INTEREST

The authors declared no conflict at interest.

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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 43-51 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)752

Pakistan Academy of Sciences

Research Article

# Synergistic Effect of Yemeni Sesame Oil and Squalene on Hyperlipidemia-induced Reproductive Damage in Male Rats

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**Abstract:** This study was purposed to explore the synergistic amelioration effect and optimal feeding time of sesame oil and squalene on hyperlipemia-induced sexual dysfunction rats. We established the hyperlipidemia-induced reproductive damage model, the three groups of test substances (sesame oil, a mixture of sesame oil and squalene, and sildenafil) were orally administrated to those hyperlipidemic rats on day 30 and day 60. The results showed that compared with the pure sesame oil, the mixture of sesame oil and squalene can synergistically decrease concentration levels of TG, TC, and LDL-C, significantly increasing the serum testosterone level and sperm count of the epididymal tail, which the 30 days' effect was better than the day 60. Compared with the model control (MC) group, the Organ Coefficient of penile increased significantly in the sesame oil (SO), sesame oil+ Squalene (SOS), and Sildenafil (SN) group, and no pathological changes were found in the penile and testis in above three groups at the day 30 and the day 60. In conclusion, the present results demonstrated that sesame oil and squalene have a synergistic amelioration effect on lowering blood lipid and promoting the recovery of erectile and sexual function on hyperlipemia-induced reproductive damage rats at day 30. However, further studies should be carried out to deeply elucidate the molecular mechanisms of Sesame oil and squalene in lowering blood lipids and improving sexual function *in vivo*.

**Keywords:** Sesame oil, squalene, Ameliorating effect, hyperlipidemia model, sexual dysfunction rats

## 1. INTRODUCTION

Hyperlipidemia, one of the common metabolic syndromes [1], is usually expressed as the abnormal elevation of any or all lipids or lipoproteins in the blood [2]. Hyperlipidemia is a critical damage-inducing factor for cardiovascular disease and frequently brings about many complications, such as cardiac damage [3], sexual dysfunction [4], cognitive impairment [5], inflammation, and insulin resistance [6]. Strong associations are seen between hyperlipidemia and sexual dysfunction especially erectile dysfunction (ED). Experimental studies have shown that the reduction of arterial blood flow induced by hyperlipidemia directly can affect the organ functions of the cortical center, pituitary-testis axis, and corpus cavernosum of the penis [7], and inhibit the production of testosterone [8], which all caused a decline of sexual function. Nowadays, many synthetic pharmaceuticals like sildenafil are widely used for the management of ED. However, their long-term use always causes many serious side effects, including vasodilatation, dizziness, indigestion, stuffy nose, heartburn, and headaches, indigestion, stuffy nose, heartburn, and headaches. For these reasons, investigating efficient

Received: September 2022; Accepted: December 2022
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and safe candidates is of great value.

Medicinal plants are promising sources in the regulation and management of ED [9]. sesame oil is a common edible oil in Yemen and contains a variety of active ingredients [10,11], such as tocopherols, polyphenols, flavonoids, phenolic ligands, Squalene, sesamol, sesamin, sesamolin, which can raise the activity of internal Superoxide Dismutase (SOD) and blood oxygen content [12], stimulate blood circulation [13], improve sexual function [14]. Vitamin E can promote the secretion of sex hormones and maintain the normal function of the Genital organs [8]. Sesame tocopherols and polyphenols can reduce serum cholesterol and prevent cardiovascular diseases [15]. The research group observed in the previous study that the proper amount of sesame oil can decrease blood lipids, promote the secretion of the sex hormone testosterone and improve sexual function [14]. However, It's not effective enough. Based on this research, Squalene, which is one of the active ingredients of sesame oil, was proposed to be added to feed the hyperlipemia-induced sexual dysfunction rats as a combining substance in this research, and then observe the changes in blood lipid and testosterone levels, epididymal sperm count, testis and corpus cavernosum tissue structure. This study aims to explore the synergistic amelioration effect and optimal feeding time of sesame oil and squalene on hyperlipemia-induced sexual dysfunction rats and then provide a basic experimental basis for the later development of sesame oil health products.

## 2. MATERIALS AND METHODS

### 2.1 Materials and chemicals

High-purity sesame oil was purchased from Yassin laboratories for Yemeni oils and spices (Sana'a, Yemen). Its nutritional ingredients contain 39.7 g/100 g of monounsaturated fatty acid, 6.72.0 g/100 g of linoleic acid, 1.9 g/100 g of linolenic acid, 5 g/100 g of stearic acid, 8g/100g of palmitic acid, 1.40 mg of vitamin E, 13.6 μg of vitamin K, 0.0 g/100 g of protein, 0.0 g/100 g of cholesterol and 0.0 g/100 g of sugar.

Basal feed and high-fat feed were provided by Al Shamel Trading Co., Ltd., Al Qasr Street (Sana'a, Yemen). 100 g of high-fat feed consists of 78.8 g basal feed, 10.0 g animal fats, 10.0 g yolk powder, 1g cholesterol, and 0.2 g cholate.

Sildenafil was bought from Shaphaco Pharmaceutical Ind. Attan, (Sanaa- Yemen). Squalene was bought from Beijing InnoChem Science and Technology Co., Ltd (Beijing, China). Total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) test kits were obtained from Spinreact (Spain). A testosterone ELISA kit was purchased from Roche-Diagnostic LTD (Germany). All other used reagents were of analytical grade.

### 2.2 Animals

Pathogen-free male albino rats weighing 160-190 g were purchased from the Sana'a Zoo in Yemen. Prior to the trials, the animals were housed in cages in a pathogen-free room with controlled temperature (18-26 °C), relative humidity (40-60 percent), and 12/12 h of light-dark intervals with ad libitum food and water. All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH, 1978). Animal handling and all related procedures were carried out by the procedures approved by the Animal Experiment Ethics Committee of the faculty of medicine and health science, Al-Razi University, Yemen (021/FMHS/2022).

### 2.3 Animal experiment design

Rats were fed a high-fat diet to establish the hyperlipidemia-induced reproductive damage model. Rats were randomly divided into the normal control (NC) group (12 rats) and model control (MC) group (48 rats), and were respectively supplied with basal feed high-fat fat feed for 4 weeks. The serum TC, TG, LDL-C, and testosterone levels of rats were measured by the corresponding kits, according to the manufacturer's instructions. Compared with the NC group, rats showed significant increments in serum TC, TG, and LDL-C levels and a prominent reduction in serum testosterone content in the MC group were identified to be successfully modeled.
Then, the successfully modeled rats were randomly classified into four groups (12 rats per group): Converted by recommended intake of human oils and fats, squalene, sildenafil and "equivalent dose ratio table of human and animal body surface area conversion", MC group received 3 mL/

kg•bw•d of 0.9 % normal saline; sesame oil (SO) group treated with 3mL/kg•bw•d of sesame oil; sesameoil+Squalene (SOS) group supplemented with 3mL/kg•bw•d of suspension solution (sesame oil: squalene=30:1) composed by sesame oil and squalene; The Sildenafil (SN) group was supplemented with 3 mL/kg·bw·day suspension solution (SO: sildenafil = 3:1) containing SO and sildenafil. Meanwhile, rats in the NC group were orally administrated with 3 mL/kg•bw•d of 0.9 % normal saline. During the experimental period, the blood of rats was collected on the 30th day (Day 30) and the 60$^{th}$ day (Day 60). Half of the rats in each group were sacrificed on day 30 and day 60 respectively, and the testis, epididymis, and penis of rats were gained.

### 2.4 Sexual organ index determination

The obtained testis, epididymis and penis were rinsed with 0.9 % normal saline and blotted with filter paper. Then, they were weighed and the organ indexes were calculated as the weight of organ/ the weight of the rat.

### 2.5 Serum lipid and testosterone levels measurement

The blood of rats was centrifuged at 3000 rpm/min for 10 min under 4 °C to collect the serum. TC, TG, LDL-C, and testosterone levels of serum were detected by assay kits referring to the instructions provided by the manufacturer.

### 2.6 Sperm count metering in epididymitis

The cauda of epididymitis was cut off and put into 4 mL of 0.9 % normal saline, followed by a cut up. It was incubated at 37 °C for 20 min to allow the running out of sperm. After that, the supernate was obtained by filtration using a 100 mesh strainer. The supernatant 10 μL was transferred into the sperm count board, and the sperm number was counted under microscopy.

### 2.7 HE staining observation of testis and penis

A part of the testis and penis was respectively fixed in 10 % formalin for over 24 h and embedded in paraffin. Then, paraffin sections (5 μm thick) were stained with HE dye and followed by histopathological observation under light microscope.

### 2.8 Statistical analysis

The mean and standard deviation were used to represent all data values (SD). Software called SPSS 23.0 was used to do the statistical analysis. To compare the significant differences across all the groups using Tukey's technique, one-way analysis of variance (ANOVA) was used. At p 0.05, differences were deemed significant.

## 3. RESULTS

### 3.1 Hyperlipidemia-induced reproductive damage in high-fat diet model

As shown in Figure 1(a), the TC, TG, and LDL-C levels of rats were significantly elevated in high-fat diet treatment groups, including MC, SO, SOS, and SN groups, compared with the NC group. It indicated that the hyperlipidemia model was successfully established. The serum testosterone level of rats was determined to evaluate whether hyperlipidemia induces reproductive damage, as illustrated in Figure 1(b). Compared with the NC group, the serum testosterone level was prominently decreased in hyperlipidemia rats.

### 3.2 Synergistic effect of Sesame oil and squalene on hyperlipidemia-induced reproductive damage in male rats

The sexual organ (testis, epididymis, and penis) indexes of rats were revealed in Table 1. There were no significant differences in testis and epididymis organ indexes of rats among the NC, MC, SO, SOS, and SN groups. While compared with the MC group, the penis organ index of rats increased significantly in the NC, SO, SOS, and SN groups on day 30 and day 60, suggesting that the SO, SOS, and SN groups can improve the relaxation and atrophy of the Corpus cavernosum penis in rats.

The serum lipid (TG, TC, and LDL-C) levels of normal and hyperlipidemia rats were shown in Figure 2. Compared with the NC group, the serum lipid levels of rats were notably increased in the MC group on day 30 and day 60. Treatments of test substances (Sesame oil, mixture of Sesame oil and squalene, and sildenafil) could reverse this phenomenon. As illustrated in Figure 2(a-b), on day 30, the serum TG and TC levels of rats in the SOS and SN groups observably declined in comparison with that in the MC group. Whilst, no remarkable

**Table 1**. Sexual organ indexes of hyperlipidemia-induced reproductive damage rats

| Group | Testis index (g/100 g) | | Epididymis index (g/100 g) | | Penis index (g/100 g) | |
|---|---|---|---|---|---|---|
| | Day 30 | Day 60 | Day 30 | Day 60 | Day 30 | Day 60 |
| **NC** | 0.79±0.08[a] | 0.76±0.03[a] | 0.24±0.02[a] | 0.22±0.02[a] | 0.08±0.010[a] | 0.08±0.02[a] |
| **MC** | 0.80±0.08[a] | 0.75±0.09[a] | 0.25±0.02[a] | 0.22±0.04[a] | 0.07±0.011[b] | 0.06±0.01[b] |
| **SO** | 0.78±0.08[a] | 0.73±0.08[a] | 0.25±0.01[a] | 0.22±0.03[a] | 0.08±0.008[a] | 0.07±0.01[a] |
| **SOS** | 0.80±0.10[a] | 0.75±0.08[a] | 0.24±0.02[a] | 0.23±0.03[a] | 0.08±0.008[a] | 0.08±0.01[a] |
| **SN** | 0.76±0.07[a] | 0.73±0.05[a] | 0.25±0.02[a] | 0.23±0.03[a] | 0.08±0.011[a] | 0.07±0.01[a] |

NC, normal control; MC, model control; SO, *Sesame* oil; SOS, *Sesame* oil+ squalene; SN, sildenafil. Values in the same column or row with different letters(a-b) represent significantly different ($p$<0.05) from each other.

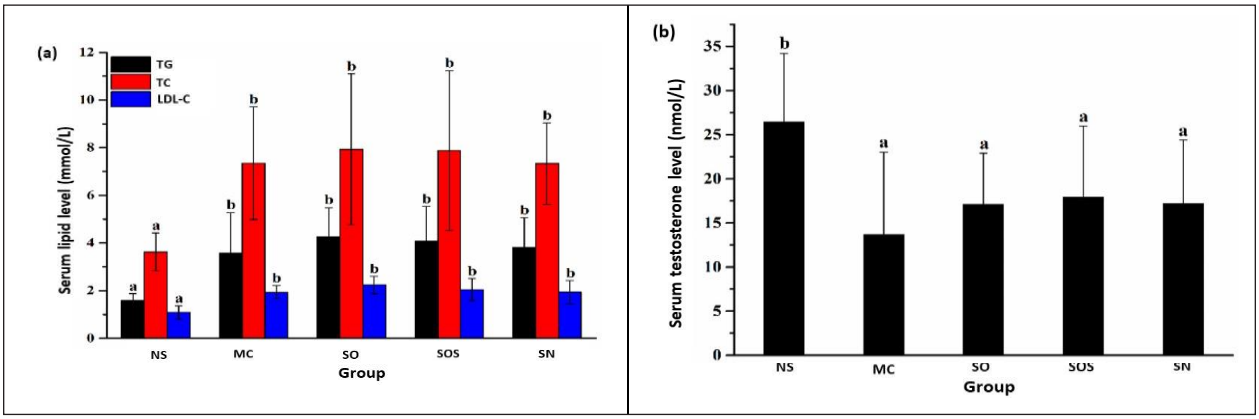


**Fig. 1.** Serum lipid (a) and testosterone (b) levels of rats in hyperlipidemia-induced reproductive damage model. TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol. NC, normal control; MC, model control; SO, *Sesame* oil; SOS, *Sesame* oil+ squalene; SN, sildenafil. The mean±SD was used to express all values. Different letters (a-c) indicated statistically significant differences (p<0.05).

distinction was observed between the SO group and the MC group, indicating Sesame oil and squalene could synergistically decrease the serum TG and TC levels of hyperlipidemia rats. On day 60, the serum TG and TC levels of rats were notably decreased in the CO, COS, and SN groups. Figure 2(c) reveals that the LDL-C level of rats supplemented with the above test substances was markedly lower than that in the MC group, on day 30 and day 60.

The serum testosterone levels of rats were detected in Figure 3. Compared with the NC group, the serum testosterone level of rats was significantly decreased in the MC group on day 30 and day 60. On day 30, the serum testosterone level of rats was dramatically increased in the SOS and SN groups, while no outstanding increment was seen in the SO group. It suggested that Sesame oil and squalene exerted a synergistic effect against hyperlipidemia-induced reduction in serum testosterone levels of rats. On day 60, rats in the SO, SOS, and SN groups showed significant elevation in serum testosterone levels in comparison with those in the MC group. While, compared with day 30, the serum testosterone level showed varying degrees of decrease in the SOS and SN group.

The sperm counts in the epididymitis of rats were revealed in Figure 4. Compared with the NC group, the sperm counts in the epididymitis of rats were prominently reduced in the MC group. Oral administration of the test substances (Sesame oil, mixture of Sesame oil and squalene, and sildenafil) could significantly increase the sperm counts in epididymitis of rats on day 30 and day 60, as compared to the MC group. On day 60, there was no significant difference in the sperm counts in epididymitis of rats in the SO, SOS, and SN groups, as compared to day 30.

The histomorphological structure of the testis and penis is shown in Figure 5-6. Compared with the NC group, no pathological changes were found in the Corpus cavernosum penis and testes of rats

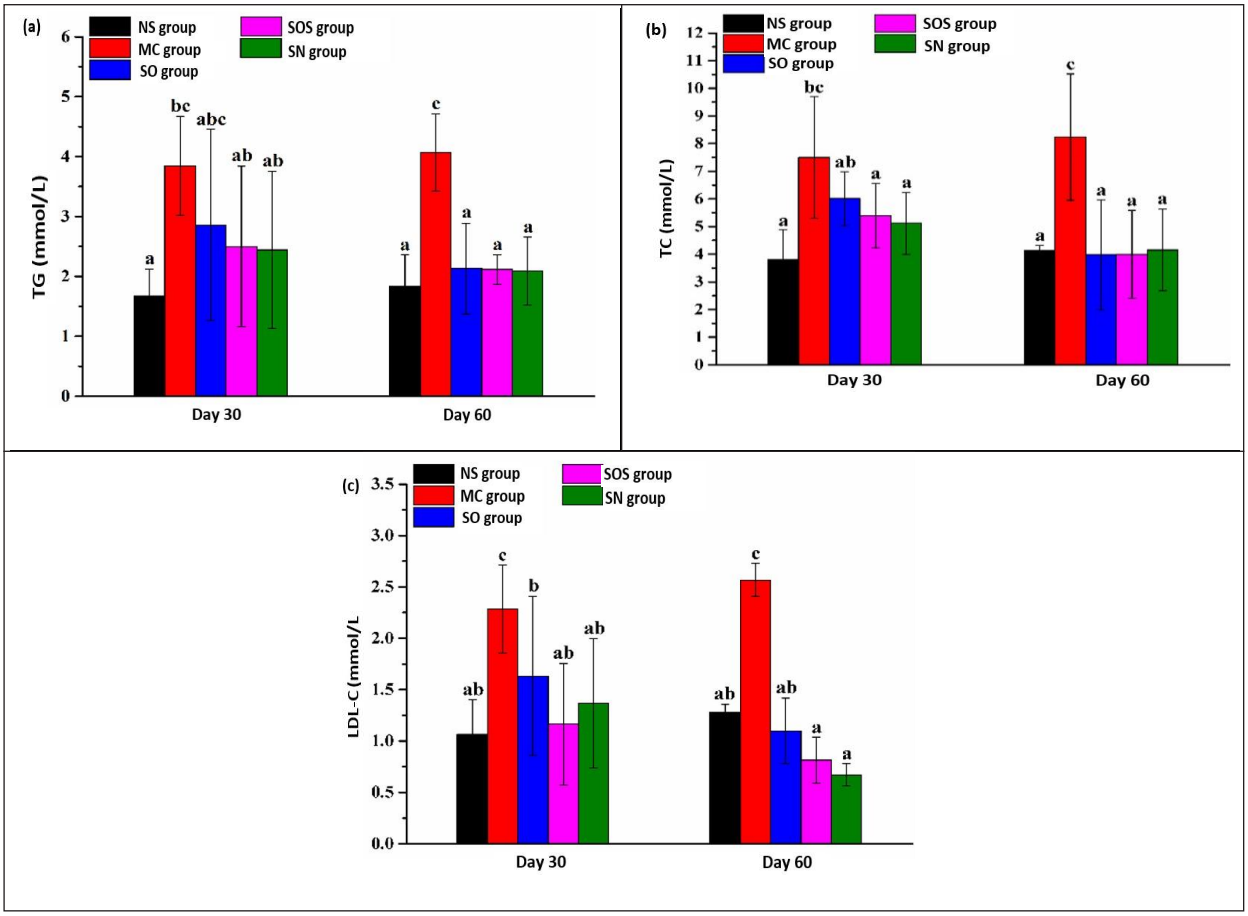


**Fig. 2.** Serum lipid levels of hyperlipidemia-induced reproductively damaged rats. (a) TG level; (b) TC level; (c) LDL-C level. TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol. NC, normal control; MC, model control; SO, *Sesame* oil; SOS, *Sesame*oil+squalene; SN, sildenafil. The mean±SD was used to express all values. Different letters (a-c) indicated statistically significant differences ($p<0.05$).

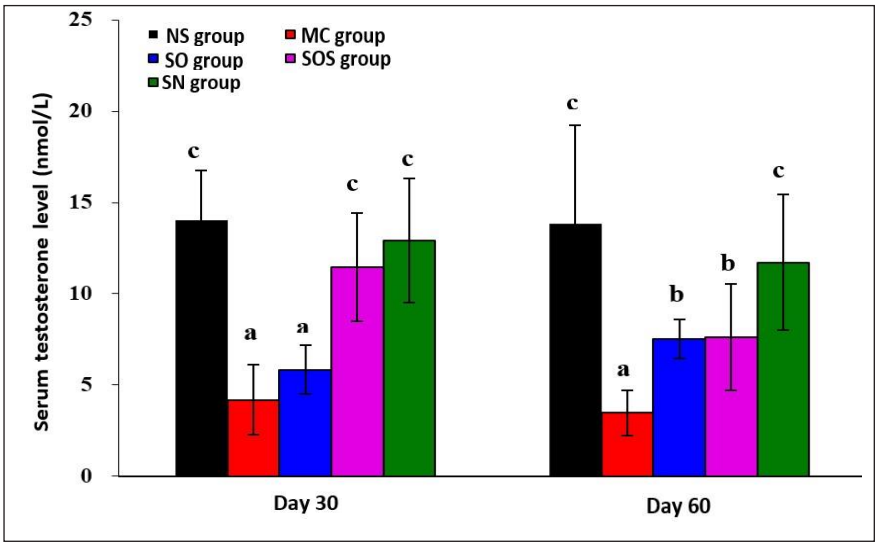


**Fig. 3.** Serum testosterone level of rats with reproductive damage due to hyperlipidemia. NC, normal control; MC, model control; SO, Sesame oil; SOS, Sesame oil+squalene; SN, sildenafil.Themean±SD was used to express all values. Different letters (a-c) indicated statistically significant differences ($p<0.05$).

in the SO, SOS and SN groups. While in the MC group, the number of smooth muscle cells and cavernous sinus decreased significantly in the Corpus Cavernosum of the Penis, and the smooth muscle of the Cavernous Body was distributed unevenly, arranged disorderly, and loosely. At the same time, all levels of spermatogenic cells in the testis were arranged disorderly, some spermatogenic cells showed necrosis, apoptosis, and nuclear pyknosis on day 30 and day 60.

## 4. DISCUSSION

The present study was designed to explore the amelioration effect and optimal feeding time of sesame oil and synergists squalene in hyperlipemia-induced sexual dysfunction rats. As expected, the hyperlipemia rat model was successfully induced by feeding a high-fat diet for 4 weeks [16]. Then, when the three groups of test substances (sesame oil, mixture of sesame oil and squalene, and sildenafil) were orally administrated to those hyperlipidemic rats, Serum levels of TC, TG and LDL-C were significantly decreased compared with the MC group, at the day 60. The above results indicate that sesame oil can reduce blood lipids significantly with rich in monounsaturated fatty acids, oleic acid, linoleic acid, linolenic acid, etc. [10]. However, only SOS and SN groups significantly reduced the level of blood lipids at day 30, suggesting that sesame oil mixed with squalene could play a synergistic role in enhancing the effect of reducing blood lipids, which the effect was better than pure sesame oil. According to studies, squalene likely contributed to the inhibition of intestinal cholesterol absorption or the activity regulation of key enzymes involved in the production of endogenous cholesterol, including hepatic acyl-CoA oxidase, fatty acid synthase, and hydroxyl-3-methylglutarylcoenzyme A reductase (HMG-CoA) [17,18].

Testosterone is the main male hormone in the body, which can promote the development of male reproductive organs and sperm, and maintain sexual function [19]. This study indicated that the level of testosterone and sperm count of the epididymal tail in SO, SOS, and SN groups were significantly higher than those in the MC group at day 60. We infer that the three groups of test substances significantly increase testosterone levels and sperm counts in the epididymal tail of male rats with hyperlipidemia. However, only SOS and SN groups significantly increased testosterone levels at day 30, suggesting that sesame oil mixed with squalene also could play a synergistic role in enhancing the effect of promoting the level of testosterone and the number of epididymal tail sperm, which the effect was better than pure sesame oil. It may be because squalene can participate in cholesterol biosynthesis and various biochemical reactions in the body, accelerate the synthesis of steroid hormones such as testosterone [20], increase the activity of superoxide dismutase (SOD) and blood oxygen content [21], promote blood circulation and improve sexual function. Meanwhile, on day 60, the testosterone level and sperm count in the epididymis tail did not increase compared with day 30, suggesting that the testosterone level and sperm count in the epididymis did not increase with feeding time. Therefore, we consider that the optimal feeding time of sesame oil and squalene on hyperlipemia-induced sexual dysfunction rats is day 30.

Current animal studies have shown that hyperlipidemia could impair erectile function by changing morphological structures of sexual organs, for example, penile corpus cavernosum lesions [22, 23]. The Organ Coefficient and pathological results of this study showed that the Organ Coefficient of the penile decreased significantly, and the penile and testis appeared pathological changes to different degrees in the MC group, which was consistent with the findings previously reported [22, 24]. Compared with the MC group, the organ coefficient of the penile increased significantly and no pathological changes were found in the penile and testis in the SO, SOS, and SN groups, suggesting that the three groups of test substances could improve the damage to the penis and testis, and promote the recovery of erectile function.

## 5. CONCLUSION

The present results demonstrated that the addition of squalene to sesame as a combining substance to feed hyperlipidemia-induced reproductive damage rats for 30 days could play an important role in combining the effect of lowering blood lipids, promoting the level of testosterone and the number of epididymal tail sperm, improving the damage of penis and testis, and promoting the recovery of erectile and sexual function. However, further studies should be carried out to deeply elucidate the molecular mechanisms of Sesame oil and squalene in lowering blood lipids and improving sexual function *in vivo*.

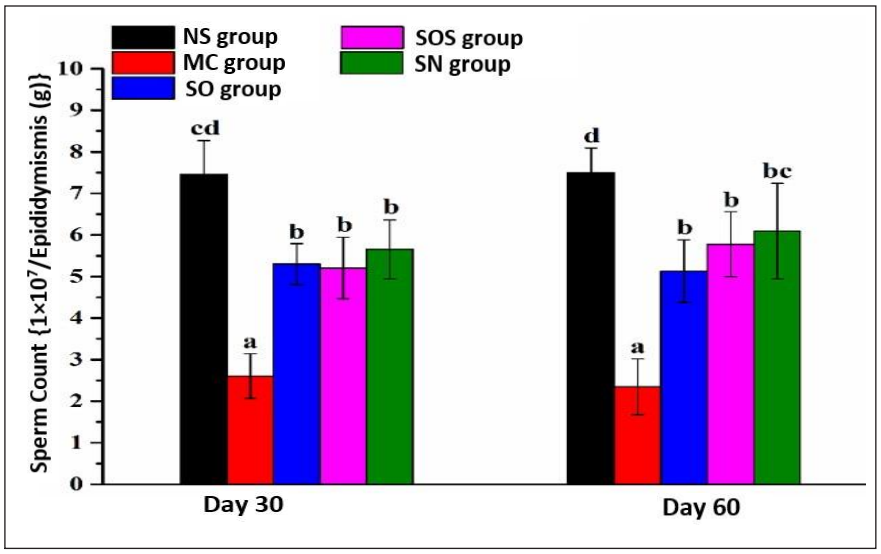


**Fig. 4.** Sperm count in epididymitis of rats with reproductive damage due to hyperlipidemia. NC, normal control; MC, model control; SO, Sesame oil; SOS, Sesame oil+ squalene; SN, sildenafil. The mean±SD was used to express all values. Different letters (a-c) indicated statistically significant differences ($p<0.05$).

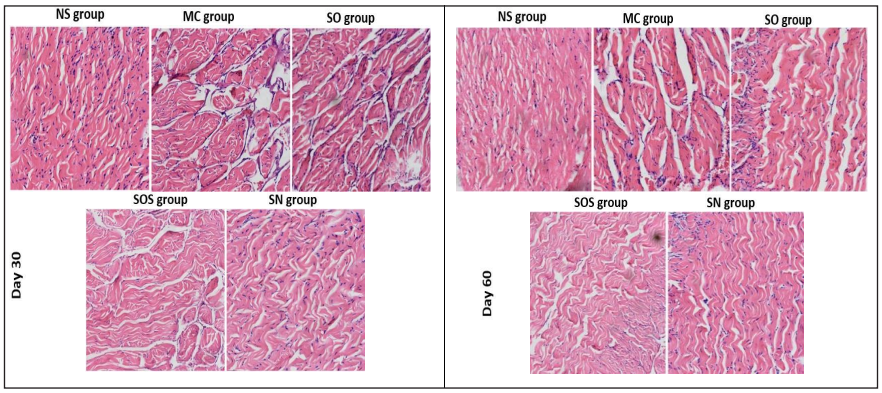


**Fig. 5.** Penis histomorphology of hyperlipidemia-induced reproductive damage rats. NC, normal control; MC, model control; SO, *Sesame* oil; SOS, *Sesame*oil+squalene; SN, sildenafil.

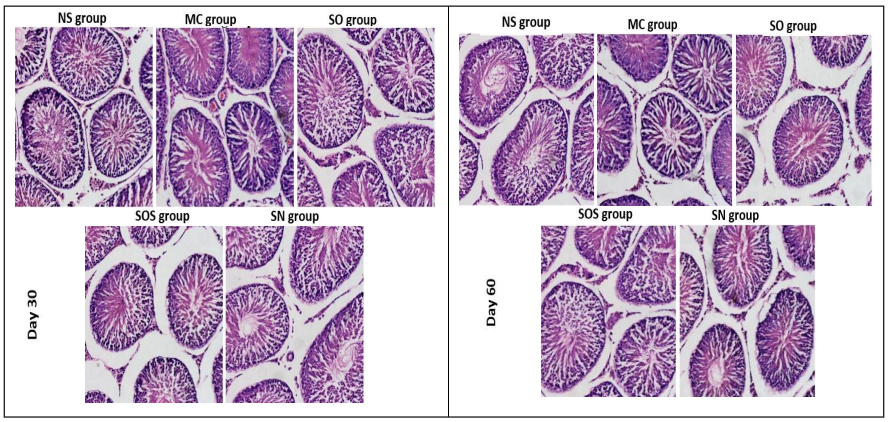


**Fig. 6.** Testis histomorphology of hyperlipidemia-induced reproductive damage rats. NC, normal control; MC, model control; SO, *Sesame* oil; SOS, *Sesame*oil+squalene; SN, sildenafil

## 6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 53-59 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)753

Pakistan Academy of Sciences

Research Article

# Isolation of Monochloroacetic Acid Biodegrading Bacteria from Tigris River

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**Abstract:** Organic compounds containing halogens are widely dispersed throughout the world, resulting in different types of pollution. One of the most common xenobiotics used in agricultural activities is monochloroacetic (MCA) which was Isolated from Iraqi mud in the Tigris River. This bacterial strain was known as SW2. Both standard universal primers Fd1 and rP1 were used with the colony PCR method for bacterial identification before being sent out for sequencing. Basic Local Alignment Search Tool nucleotide sequences and information were analyzed (BLASTn). The phylogenetic tree was constructed using the 16S rRNA sequence to determine their evolutionary distance. The Neighbor-Joining method was used to infer the evolutionary history. The Neighbor-Joining method was used to infer the evolutionary history. There is a 96 percent match between the SW2 bacterium and another type of aerobic Gram-Negative Bacteria. Strain SW2 (*Pseudomonas* sp.) was inoculated for two days and yielded colonies that were small, non-spore-forming, and rod-shaped. Growth slowed slightly after 48 hours. The release of chloride ions as a result of the degradation of MCA was seen using a halide ion test. Biochemical tests backed up the choice of the genus's name as well. As a result, bacteria found in the river are capable of utilizing and degrading the MCA compound. In conclusion, this study confirmed the potential ability of the isolated bacterial strain of utilizing MCA, especially from contaminated environments with the pragmatic application of the bacterial strains to degrade residual herbicide.

**Keywords:** *Pseudomonas* sp SW2, Dehalogenase, Monochloroacetic (MCA), Degradation xenobiotic.

## 1. INTRODUCTION

The use of haloaliphatic compounds has increased over the last century as a result of the widespread human industrial in addition to all agricultural activity [1]. Environmental contamination and toxic chemicals accumulation in our ecosystem especially Pesticides and fungicides, insecticides, solvents and herbicides result from the widespread use of these hazards [2]. Natural sources are also a source of chlorinated compounds entering the environment. Natural organic halogen compounds numbering in the thousands, according to research [3].

When it comes to halogens, the most common one is chlorine, which is responsible for the toxicity of these compounds. There are many examples, such as the production of Monochloroacetic acid (MCA) for agricultural use especially herbicides, which will lead to an increase in pollution and health issues [4].

Biodegradation is a natural process by which Microorganisms have the ability to eliminate xenobiotic chemicals from the environment, such as chloroaliphatic substances [5, 6]. A wide range of microorganisms capable of degrading these chemicals have been discovered, and studies have helped to clarify the workings of various microbial biodegradation mechanisms [7]. One of the major issues is the inability of these compounds to be

Received: September 2022; Accepted: December 2022
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degraded by microorganisms because they are barely water-soluble [8, 9].

For as long as the pollutant serves as both a carbon source and an electron donor, microorganisms will naturally degrade the pollutant. Halo-respiration, on the other hand, uses chlorinated compounds as electron acceptors [10].

It was possible to isolate bacteria that utilize and degrade monochloroacetic acid, identify the isolated bacteria using molecular analysis utilizing the 16S rRNA gene amplification and characterize the bacterium through biochemical testing, all of which were accomplished through this study. One of the most commonly used techniques for the identification and characterization of microorganisms is the Polymerase Chain Reaction (PCR) amplification of the bacterial small subunit ribosomal RNA gene (16S rRNA) [11].

Many bacteria use hydrolytic mechanisms to break down halogenated compounds. Cleavage of the halogen-carbon bond and substitution of hydroxyl for the halogen group are the methods used in this procedure. The primary objective of the study was to determine how to degrade environmental toxins to lessen the environmental pollution. Our ecosystem will continue to function normally and even improve as long as there exist microorganisms that can digest these toxins.

## 2. MATERIALS AND METHODS

### 2.1 Preparation of Samples and Minimal Media

Mud samples were collected from five separate locations. In 15 ml of sterile, distilled water, 0.5 g of soil sample was suspended. After adding the mixture, the soil fragments were let to settle. In a 250 ml flask containing soil, 5 mM monochloroacetic acid (MCA) was added as a carbon and energy source to 100 ml of the minimum media (10 g). An incubator at 37 $^{o}$C and 200 rpm rotary shakers were used to culture the culture for two days. The experiment began after a few drops of soil solution were applied to solid media. To obtain pure colonies, the sub-culture process had to be repeated several times. To prepare the bare minimum of media for use, we needed two stock solutions. The amount of trace metal salt and basal salt produced increased tenfold. The base salt and metal salt compositions in the minimal medium are shown in the following [12].

### 2.2 Growth Profile

The growth profile or "growth curve" was plotted to examine the growth of the microorganisms in the described medium. This experiment allowed researchers to distinguish between different stages of bacterial growth. Nylon filters of 0.22 microns were used to sterilize the MCA, which was then aseptically added to the mixture of basal and metal salts. It was incubated at 30 $^{o}$C and 200 rpm for 24 hours with a pure colony in the liquid medium. In the following six-hour intervals, a new 1mL cuvette was filled with the sample, and the procedure was repeated. At A600 nm, the BUCK SCIENTIFIC VIS 100 spectrophotometer was used to take the measurements [13].

### 2.3 Halide Ion Assay

An assay known as the halide ion assay measures the amount of halide ions that can be released from halogen compounds. Fluoride, Chloride, Bromide, and Iodine are all examples of halide ions, and each has a unique set of properties. Monitoring the release of chloride ions was done using the spectrophotometer at A460 nm, as described by Bergmann and Sanik [14]. Ferric ammonium sulfate and mercury thiocyanate were used as reagents in this experiment.

### 2.4 Preparation of Samples for PCR Analysis

PCR was amplified using the 16S rRNA gene and then sequenced to detect the bacterium isolated from Mud samples. In this experiment, universal primers have been utilized to amplify the 16S rRNA gene. DNA extraction is a standard procedure for isolating DNA. Using Promega Wizard® Genomic DNA Purification Kit, the procedure was carried out [15]. Before the extraction process, some materials and chemicals were prepared, and the standard procedure was strictly adhered to. Evaluation of Quantity and Purity of Extracted DNA, the extracted DNA samples were quantified using a NanoDrop spectrophotometer. The 260/280 nm absorbance ratio and DNA yield (μg) = DNA concentration (μg/μl) × total sample volume (ml) were used to

measure DNA purity and concentration as described by Sambroole and Russell [16]. Moreover, the quality of the isolated DNA was evaluated by 1.5 % Agarose gel electrophoresis. The approximate size of the isolated DNA was calculated using a molecular weight marker, a 100 bp plus DNA ladder (Bioneer, Korea).

### 2.5 Polymerase Chain Reaction (PCR)

In genetic studies, PCR was used to determine a specific gene sequence in the DNA using a polymerase chain reaction. Universal primers fD1 and rP1 were used for the general amplification of procaryotic 16S rRNA. A conserved region of the 16S rRNA gene was used by scientists in this case for molecular identification of the unknown bacteria and for comparison and species determination among the several kinds of prokaryotic microorganisms that are listed in the database. 16S rRNA gene region has the lowest mutation rate of any gene in bacterial DNA, so it can be used to identify bacterial species. Using universal PCR primers, the 16S rRNA gene was amplified. Phylogenetic information about the isolated bacteria can be obtained through amplification. AGA GTT TGA TCC TGG CTC AG-3' and 5'-ACG GCT ACC TTG TTA CGA CTT-3' are the forward and reverse primer sequences, respectively. PCR requires the following components: a Promega master mix, universal forward and reverse primers for 16S rRNA, and deionized water. PCR master mix (25 mL) was added to a 100 mL microtube, followed by 1 mL of each of the forward and reverse primers. Afterward, 5 L of the rehydrated DNA sample and 50 L of deionized water were added to the mixture. Once the mixture was ready, it was fed into the PCR machine for further analysis.

### 2.6 Sequencing and Analysis of PCR Products and Phylogenetic Analysis of 16S rRNA gene

The 1st Base® Company received the PCR products used in the "DNA sequencing" process. Eppendorf tubes containing 25 L of 16S rRNA gene amplification products were used to store the PCR products. They were not purified. Separate sterile tubes held 5 L of each primer's forward and reverse primer. For sequencing, the tubes were sealed and labeled. Blastn analysis was used to perform an alignment and comparison of our 16S rRNA sequence with sequences from the Gene Bank at NCBI. The command CULSTAL-W was utilized to align the sequence with the first 5 sequences. MEGA version 5.2.2 used a neighbor-joining method [17] to create phylogenetic tree [18].

## 3. RESULTS AND DISCUSSION

### 3.1 Isolation of Bacteria from Water-Capable Degrade MCA

Five different locations in the Tigris River, Baghdad / Iraq were used to collect mud from water samples. At 37 °C, 10 mM monochloroacetic acid was added to the broth minimal media supported by the Microbiology lab to find the bacteria capable of degrading this substrate. Incubation at 37 °C for 2 days resulted in the formation of tiny creamy circular colonies on the plate of the agar master after a few drops were transferred and separated.

To isolate the two colonies, we used the streaking plate method and then incubated them at 37oC for two days. The morphology of one of them had been grown, and it was found to be similar to the other 3 times, the bacteria were re-suspended in the same way. Eventually, the SW2 colonies were found and renamed. There was no sign of growth on the control plate, which was incubated in similar conditions. Figure 1 depicts the outcome.

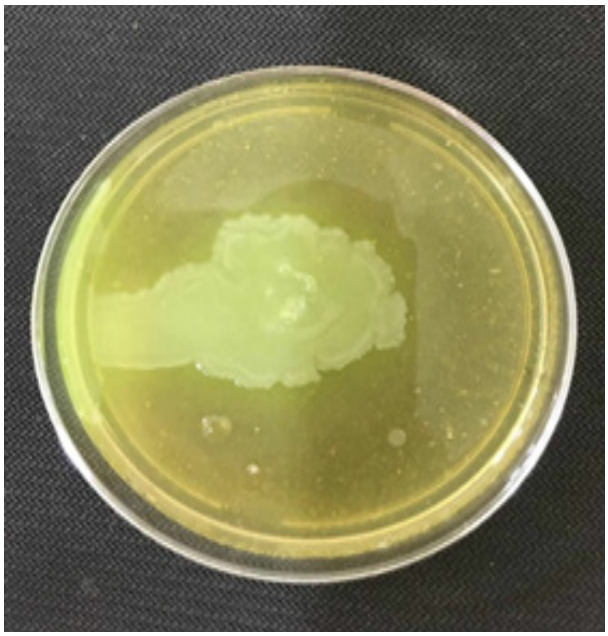

**Fig. 1.** SW2 strain grown in solid minimal media with 10 mM MCA.

### 3.2 Halide Ione Assay and Growth Profile

A growth profile had been performed on the isolated bacterium to ascertain its capacity for growth. MCA concentrations of 10 mM, 20 mM, 30 mM, and 40 mM were used to grow SW2 bacteria in liquid minimum at 30 °C in the shaker incubator at 200 °C. Every six hours, following a 24-hour adaptation period, the rate of growth was monitored with a spectrophotometer equipped with an A600 nm wavelength. Figure 2 depicts the result of the experiment.

Different stages of growth were discovered during this experiment by using a growth profile, also known as a "growth curve," to track the growth of the bacteria in the specified medium. There are three different concentrations of MCA that the bacteria can grow in. 10 mM, 20 mM, and 30 mM, whereas the growth was not detected in the medium containing 40 mM of MCA. Bacterial growth may have been stifled by the high concentrations of MCA that had been used. As a result, the halide ion assay for MCA concentrations of 10 mM, 20 mM, and 30 mM will be described in further detail in order to examine the relationship between the growth profile and the released chloride ions. The concentration of released chloride ions was determined by converting the absorbance readings to mM concentration using a standard curve built using sodium chloride as a reference measurement of soluble chloride.

By comparing the absorbance of the sample with a standard curve constructed using sodium chloride, where strain sw2 showed a value of 0.192 mol Cl/ml, the maximum chloride ion liberation is further determined. This finding is in line with that of Wong and Huyop [13], who found that bacteria using halogenated compounds released chloride ions. In addition, it has been demonstrated that the SW2 bacterial strain is capable of using MCA as its sole carbon and energy supply. By producing dehalogenase, microorganisms are capable of breaking down the substance.

In general, the bacteria grow well in MCA minimum media. The growth rate is influenced by the MCA concentration in the media, as shown in Figure 3. The medium with 10 mM MCA had the best growth rate, while media containing 20 mM and 30 mM had lower growth rates. After 24 hours, the bacteria entered the stationary phase in the solution containing 10 mM of carbon and energy. However, after 42 hours bacteria had reached the death phase, indicating an accumulation of the toxin.

### 3.3 16S rRNA Polymerase Chain Reaction

Figure 4 of the Promega® 1Kb DNA ladder shows the varied sizes of the ladders that were used. Extraction of bacterial DNA and universal primers were used to perform PCR. We used "Forward Primers" and "Reverse Primers" from 1st Base Company in this process. Figure 4 shows the results of gel electrophoresis, which was used to track the progress of the PCR reaction. According to this Figure, the DNA ladder in lane 1 had a band of

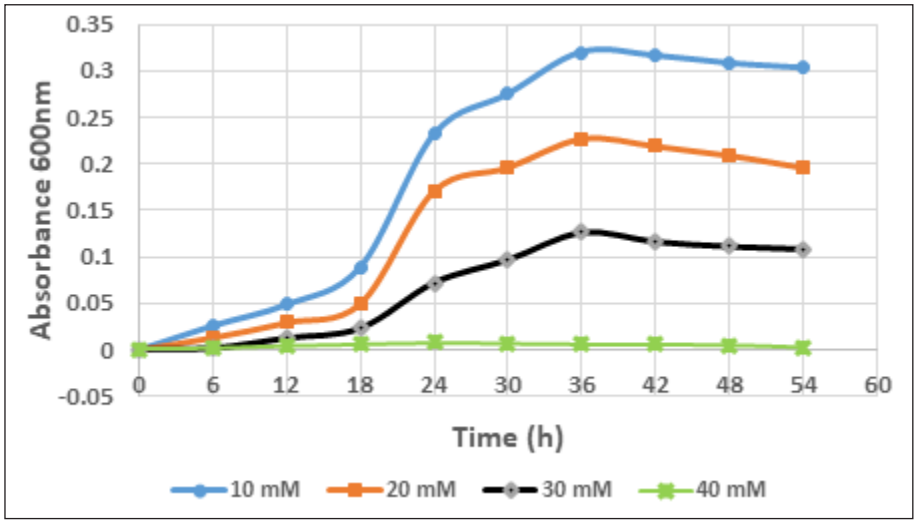


**Fig. 2.** SW2 strain growth profile at various concentrations.

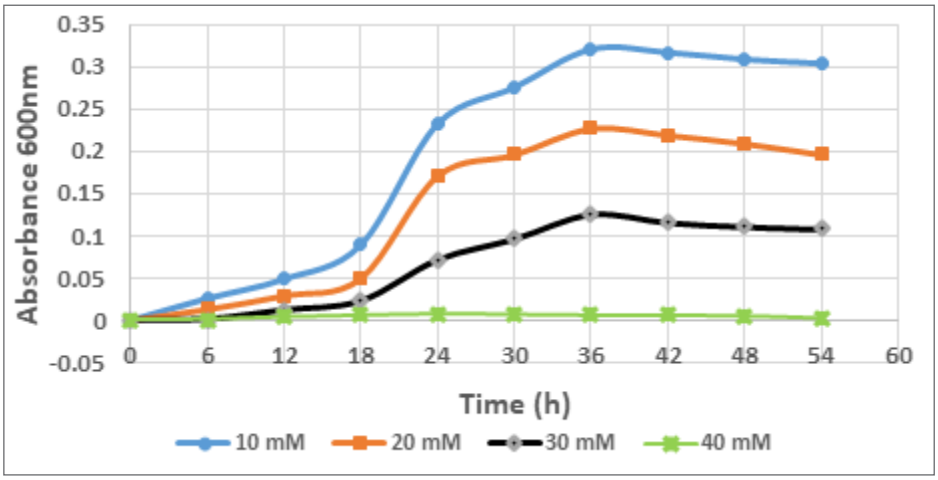


**Fig. 3.** Correlation between SW2 growth and chloride ion in 10 mM MCA.

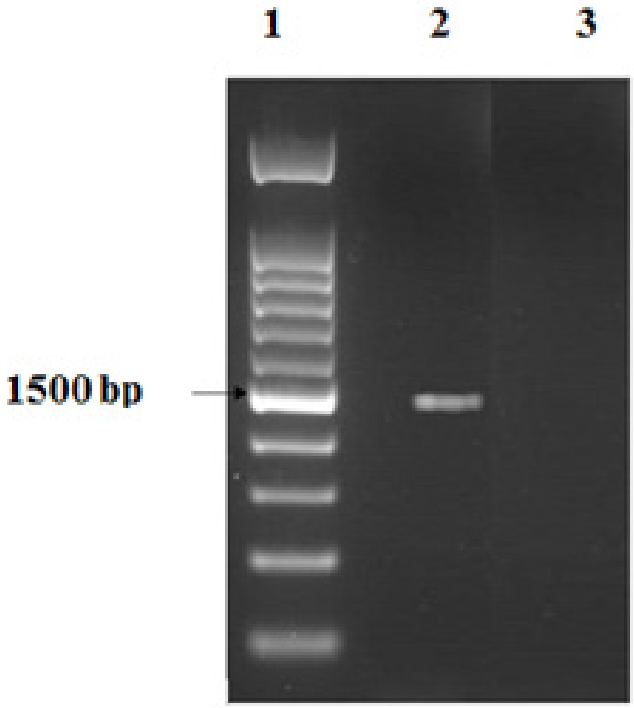


**Fig. 4.** Electrophoresis on 1 % agarose gel of DNA amplified with 16S rDNA universal primers. Lane 1: M, 1kb ladder, Lane 2: sample of SW2, and Lane 3: Negative control.

around 1500bp while the controls (PCR reactions without the use of forward or reverse primers) were found in lane 5 and 6.

### 3.4. 16S rRNA Gene Sequencing and Analysis

To sequence our gene of interest, the PCR product had been submitted to the 1st base firm. It was received by email in ".ab1" format for both the forward and reverse sequences, with the results. The signal quality of the sequence result was determined and analyzed using a chromatogram viewer. The low-quality bases were eliminated from both forward and reverse sequences.

It was then used to align forward and reverse sequences, removing the overlapping region between them, in order to retrieve the complete length of the 16S rRNA gene. It was at this point that the total length of our sequence was 1433 base

pairs, which we then used in NCBI's BLAST to compare it with other sequences and determine the similarity percentage. The percentage of similarity between our sequence and the BLAST result is shown in Table 1.

The 16S rRNA gene sequence is conserved in all studied bacterial species. In addition, this served as a target for identifying the bacteria. Before the PCR reaction, the DNA of the direct wild-type bacteria is extracted. Based on the BLASTn results, it was determined that *Pseudomonas* sp. and bacterium SW2 share 96 % sequence identity. As shown in Table 1, the NCBI database's top five species are compared to the BLASTn search results with significant alignments.

### 3. 5 Phylogenetic Analysis

ClustalW (version 5.2.2) is used to align the 16S rRNA gene sequences of strain SW2 with the nucleotide sequences from the Genbank database (NCBI) [17]. When using BLASTn, all of the identical bacteria are used to build the Neighbor-joining phylogenetic tree. *Pseudomonas* sp. SW2 has been identified as the bacterium SW2.

It was acknowledged by Towner and Cockayne [19] that the molecular approach has long been used to discover new species. Because it is found in all bacteria, the 16S rRNA gene sequence is an excellent tool for identifying bacteria. Aside from that, it is an extremely accurate procedure that is both reliable and reproducible [20]. Nucleotide sequence variations can be used to identify the most specific type of microorganism.

For phylogenetic analysis, bacterial species from the same isolate family were chosen. The 16S rRNA sequence for this species has been gotten from the database found in NCBI and aligned using the CLUSTAL W tool in MEGA version 5.2.2 software. A phylogeny tree of neighbor-junction with bootstrap value has been observed (Figure 5), and it suggests that the bacterial individual SW2 is closely designated as *Pseudomonas.*

**Table 1.** Sequences similarity with SW2 strain from the NCBI database.

| Microorganisms Description | Accession Number | Maximum Score | Maximum Identity (%) |
|---|---|---|---|
| *Pseudomonas aeruginosa strain MS14403* | CP049161.1 | 1088 | 96 % |
| *Pseudomonas aeruginosa strain WG-36* | MN793065.1 | 1088 | 90 % |
| *Pseudomonas aeruginosa strain SP4527* | CP034409.1 | 1088 | 96 % |
| *Pseudomonas aeruginosa strain FZD1* | MK493327.1 | 1088 | 92 % |
| *Pseudomonas aeruginosa strain TO4* | MH458773.1 | 1088 | 92 % |

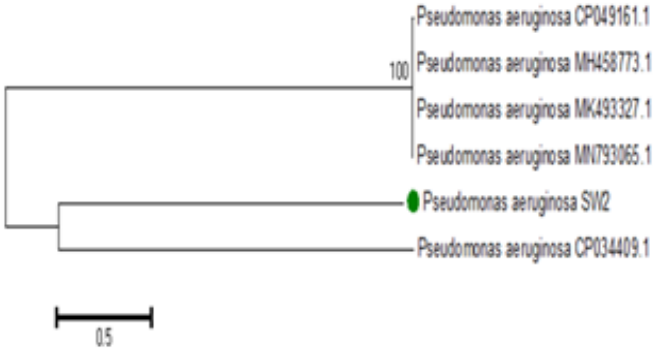


**Fig. 5.** Phylogenetic tree of the SW2 strain created using the neighbor-joining method and bootstrap values using MEGA software.

## 4. CONCLUSION

According to the study's findings, *Pseudomonas* sp. SW2, a soil-isolated bacterium that thrived in the minimum medium of 10, 20, and 30 mM, had a high capacity for degrading MCA. As an environmentally benign bacterial strain that produces the dehalogenase enzyme, it can be helpful as a bioremediation agent for detoxifying xenobiotic chemicals by MCA degradation.

## 5. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 61-70 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)754

Pakistan Academy of Sciences

Research Article

# Emissions Reduction by Combustion Modeling in the Riser of Fluidized Bed Combustor for Thar Coal Pakistan

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**Abstract:** Pakistan has experienced a protracted electricity shortage for the past few years. However, despite Pakistan's abundant coal deposits, modern coal combustion technology is still required to reduce emissions. Pakistan is struggling to utilize its energy resources and currently experiencing an electrical shortage of more than 8000 MW. The research study models the combustion performance in a fluidized bed riser using ANSYS FLUENT software to understand the combustion behavior of low-rank Thar coal. A simple circulating fluidized bed (CFB) combustion riser was modeled for computational fluid dynamics (CFD) to study the hydrodynamics of gas-solid flow in a circulating fluidized bed riser to reduce emissions and operating costs. Three different types of risers/combustors geometries were used center flow, counter flow, and parallel flow. The CFD model for the solids segment with a k-e turbulence model and the viscosity of static particles in the gas segment both showed excellent mixing performance. According to the FLUENT data, the riser/combustor maximum temperature is around 1400 K or 1130 $^{o}$C at the primary burning sector in the bed center. According to velocity contours, the greatest velocity in the center-oriented riser/combustor peaks at 3.3 m/s. The CO and $CO_2$ both mass fraction counters show maximum concentration in the center geometry, whereas lower CO concentration is found in parallel geometry. The lowest level of $NO_x$ is established in the parallel geometry at around 15 ppm, whereas the counter contours establish the maximum level of $NO_x$ at about 31 ppm. Circulating Fluidized Bed Combustor is found to be the most advantageous and effective technology for producing power from Thar lignite coal and reducing emissions.

**Keywords:** Two-phase gas-solid flow model, Thar coal, Circulating fluidized bed, Hydrodynamic, Computational fluid dynamics, Riser, Emissions reduction

## 1. INTRODUCTION

Energy is widely acknowledged as one of the most crucial components of societal well-being and a prerequisite for long-term development. A consistent energy resource and demand are essential components for every government when it comes to offering consumers energy that is economical, ecological, and clean [1]. Over a third of the world's electricity is still produced by coal, the most widely produced mineral on earth [2]. About 37 % of the electricity produced globally was from thermal coal. Coal has continued to be a significant energy source for many years, especially while emission reduction technologies are gradually implemented [3]. According to International Energy Agency (IEA) in the coal forecast for 2021, all-time-high production of 8,111 Mt occurs in 2022.

Pakistan has enormous energy needs, and because of this, the constituency is most concerned about power outages lasting a long period [4]. For many years, Pakistan's main source of energy has been imported fossil fuels. Over 185 billion tons of coal are in Pakistan's reserves, although only 175.5 billion tons are from Thar coal [5, 6]. In the industrialized region, a significant number of oil and gas plants have been replaced by coal-fired power plants. Exploiting natural resources, such as

Received: September 2022; Accepted: December 2022
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the Thar coalfield, which can produce 100,000 MW, could be a practical solution to fulfill the predicted demand [7]. Coal still serves as a source of energy generation in Pakistan, where its share of the country's energy mix is 12.8 % [5]. Pakistan needs to understand the most recent coal technologies because of its vast coal deposits [8]. Coal's adverse environmental consequences are caused by the emissions of several pollutants such as $SO_X$, $NO_X$, CO, and $CO_2$ [9]. The current coal consumption makes it difficult to reduce air pollution from coal power plants. It is essential to create a system or tools to control these harmful coal emissions [10].

Combustion is a multifaceted procedure containing sequential heterogeneous and homogeneous reactions of a fuel with an oxidizer. The important combustion stages contain, heating and drying, devolatilization and volatile combustion, and char burning. In recent years, CFBCs are extensively used for power generation, because of their superior burning efficiency and relatively better control of emission gases [11, 12]. Fluidized bed combustion technology is being used more often around the world, which has prompted researchers to improve the design and lower emissions through additional tests and modeling [13, 14]. Surface reaction kinetics and diffusional mass transference work together in the complex coal-burning process. Due to the variety of configurations and their multiple effects on the solid-gas two-phase flow, there has been minimal research on the intake and outlet effects on the hydrodynamics in risers or combustors, but no precise classification or understanding has been reached.

Therefore, it is crucial to comprehend the riser inlet sections' hydrodynamic properties [15]. It has been demonstrated that a CFB's riser output geometry significantly affects the hydrodynamics of the component. Knowing the effects of various variables and factors on emission actions is crucial for effective emission management, particularly to reduce emissions of inadequate burning products [16-18]. With a production capacity of 175 billion tons, Thar lignite coal requires special consideration when integrating with modern technologies. Generally speaking, conventional boilers devalue coal and produce high $NO_x$ levels. Therefore, CFBC technology has been developed and is used by modern enterprises and power locations due to reduced environmental risks and higher energy production efficiency [19]. A reduction in combustion emissions increased fuel investment, and higher combustion efficacy is all necessary for improving a combustion structure. Coal is an abundant fuel resource that can be used to produce and transform it into affordable energy. However, producing and using coal has an impact on the environment [20].

ANSYS FLUENT software comprises an extensive variety of physical modeling abilities that are used for turbulence, model flow, heat transfer, and reaction for engineering applications. Modeling with ANSYS code will reduce investment and working costs also simulation tool can solve multiple models in a single file which ultimately reduces the simulation time by three phases, pre-processing, solver, and post-processing [21]. Numerous studies have been published about CFD modeling of coal combustion in the CFB riser. Peters *et al.* [22] reported that the CFD codes deliver a shortcut to contract the comprehensive info in CFB risers for gas-solid flow. Hussain *et al.* [23] investigated that voidage beside the riser elevation is disturbed by the geometry of the CFB riser. The burning performance of low-ranking coal from Duki and Chamalung Baluchistan was used in a CFB Combustor. They found that increase in temperature has produced a rise in the volume of discharge gasses.

Considering all the above facts and challenges, current research is focused on mechanisms or modeling that especially reduce emissions. A simple CFB Combustion Riser was modeled using ANSYS Fluent software for the computational fluid dynamics (CFD) combustion analysis of Thar coal reserves. To reduce investment and operational costs, as well as emissions gases, this study analyzed the burning performance of low-grade Pakistani coals from Thar in a CFB riser. Before we obtain actual performance data from the future power plant in Pakistan useful to reduce power or electricity shortage, local coal is carefully analyzed.

## 2. MATERIALS AND METHODS

Thar coal used in this investigation was obtained from Thar Block-II. Eighty (80) samples n duplicates were chosen for evaluation in the current investigation. The results are a mean value of eighty

representative samples taken from Pakistan's Thar Block-II coalfield. Physicochemical characteristics are analyzed for all coal samples as per ANSYS software requirements. Data collection was done using ASTM standard procedures such as Proximate Analysis (D-3172-5) and Ultimate Analysis ASTM (D-3176, D-5373). For physicochemical analysis, a Memmert oven and a Vulcan muffle furnace were used to perform a Proximate Analysis to determine the amounts of moisture, volatile, fixed carbon, and ash. Ultimate Analysis was performed with an Elementar CHNS analyzer to examine the H, C, S, and N components in the samples of coal.

Circulating Fluidized Bed Combustion (CFB) principle is the foundation of the combustion system, which uses coal as a fuel. A wide range of material modeling capabilities is included in ANSYS software 19.0, which is used in industry to model turbulence, response, heat transfer, and flow.

### 2.1 CFB Simulation

The CFB simulation diagrams are shown in (Figure 1). It consists of a primary and secondary cyclone, an airstream blower, a solid intake system, a stainless-steel supply, and a quick Plexiglas column. On a riser with quadrilateral dimensions of 2649 mm in height, 72 mm in depth, and 265 mm in width, the 2D simulation work was performed. The 2D design was chosen because it required the least amount of processing work and was sympathetic to the flowing outlines in risers with different exit geometry [17]. The operative limitations were chosen since they had been proven effective in large CFBCs. Fluent Inc. developed and owns the CFD reference tool FLUENT, which created a simulation. Sand components, air, and solid-gas components were used in that order. Table 1 summarizes the constraints used in the simulation model.

### 2.2 Boundary Conditions

All volume fractions and speeds of related segments are fixed at the intake. As a result of the uncondensed gas segment potential, the pressure at the intake is uncertain. The primary gas velocity and solid segment are being measured, as seen in

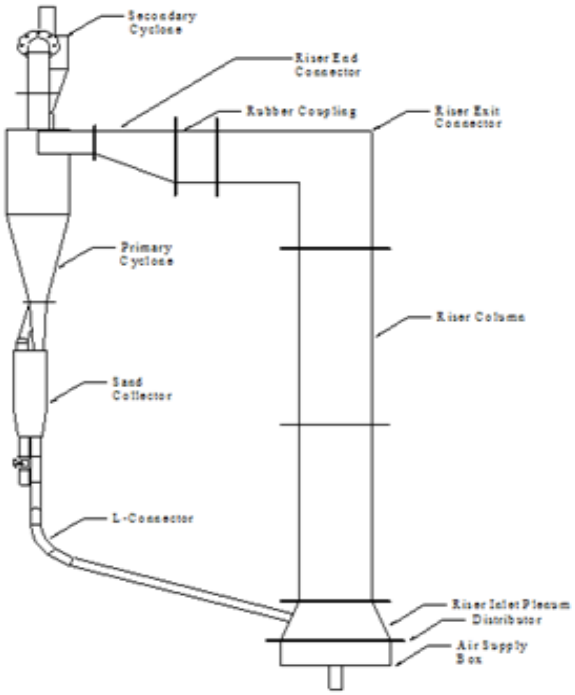



**Fig. 1.** Typical CFB riser geometry [17]

**Table 1**. The variables used in simulation work

| Constraint | Values Range |
|---|---|
| Dimensions of Riser (LXWXD) mm | 2649 X 265 X 72 |
| Velocity (Particle) | 2 m/s |
| Velocity (Gas) | 3~5 m/s |
| Particle (sand) properties | Density = 2500 kg $m^{-3}$<br>Diameter= 100X $10^{-6}$ m |
| Air Properties | Density= 1.225 kg $m^{-3}$<br>Viscosity= 1.79 x $10^{-5}$ kg/m.s |
| Height of the distributor's sand intake | 200 mm |
| Granular properties | 0.95 is the coefficient of particle-particle restitution.<br>Restitution coefficient for particle walls of 0.9 |
| The fraction of sand by volume | Within 10% of the volume percentage of sand, 0.03 utilized as the Algebraic Slip Mixture Model provides reliable predictions. |
| Multiphase model | Eulerian granular multiphase model |

(Table 2). The meshing process was completed using ANSYS FLUENT. Fine meshing was carried out in the direction of the riser in and out segments to analyze them in an improved fashion. Aspects were changed to achieve confluence below relaxation. The tolerance for confluence was set at 0.001.

The models used in ANSYS FLUENT to simulate the combustion of solid fuel are displayed in (Table 2).

**Table 2**. ANSYS FLUENT simulated models used

| Model | Settings |
|---|---|
| Space | 2D |
| Viscous | k-epsilon standard turbulence model |
| Time | Steady |
| Wall Treatment | Standard Wall Functions |
| Radiation model | P1 Model |
| Heat Transfer | Enabled |
| Species Transport | Generalized Finite Rate |
| Reactions Model | Eddy Dissipation |
| $NO_X$ Model | Thermal & Prompt |

### 2.3 Geometries of the Riser/Combustors

For the geometries of the risers/combustors, we have to consider the three different types according to their respective geometries. First, there is the center flow, counter flow, and parallel flow as shown in (Figure 2).

The grids are created using tri elements with an interval count of 10. An unstructured non-uniform triangle grid/mesh was used, due to the non-premixed combustion model, gas-solid being randomly spaced and do not follow any single pattern and having a strongly swirling flow inside the combustor/Riser shown in Figure 3. The iterations were calculated to an approximated time of 1 hour and 36 minutes. All the boundaries are also defined.

## 3. RESULTS AND DISCUSSION

The combustion characteristics of coal form the basis of the technology used for the power plant. The Thar Block-II coal assessment means data shows (Table 3) that moisture is high, volatile matter content is at low to medium levels, while sulfur and carbon are found low to moderate levels. The composition of the Thar Block-II coal is inputted as follows;

**Table 3**. Composition of Thar Block-II Coal

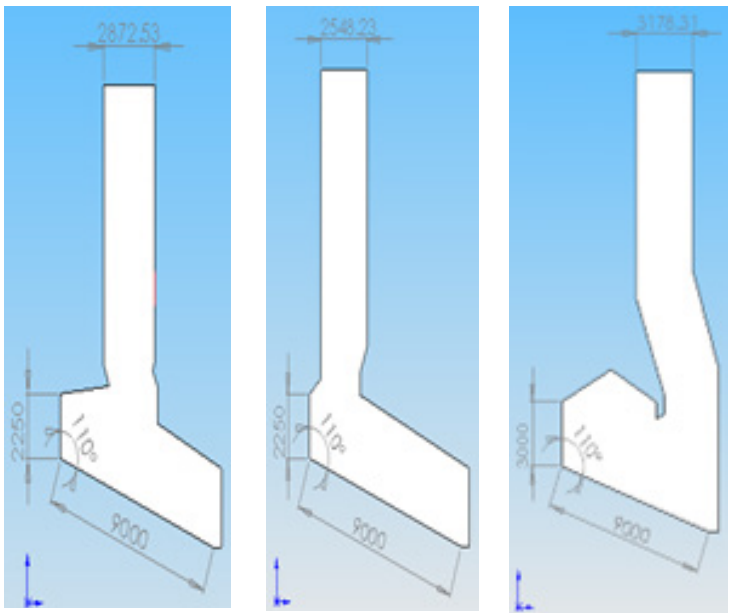
| Proximate Analysis | Weight (%) |
|---|---|
| Moisture | 46.26 |
| Volatile Matter | 28.85 |
| Fixed Carbon | 18.47 |
| Ash | 6.42 |
| **Ultimate analysis (DAF)** | |
| Carbon | 67.29 |
| Hydrogen | 4.30 |
| Nitrogen | 0.52 |
| Oxygen | 26.01 |
| Sulfur | 1.88 |

DAF= Dry Ash Free

Coal is burned using a variety of riser/combustors and combustion techniques. The main combustor is divided into three sections, primarily the burnout area, the combusting area, and the drying area. To start modeling gas flow, the properties of gaseous emittance and bed temperature are imported into the FLUENT program. The results demonstrate all of the combustion processes, including drying, devolatilization, char burning, and ash generation. The convergence of each simulation requires between 4,000 and 8,000 iterations.

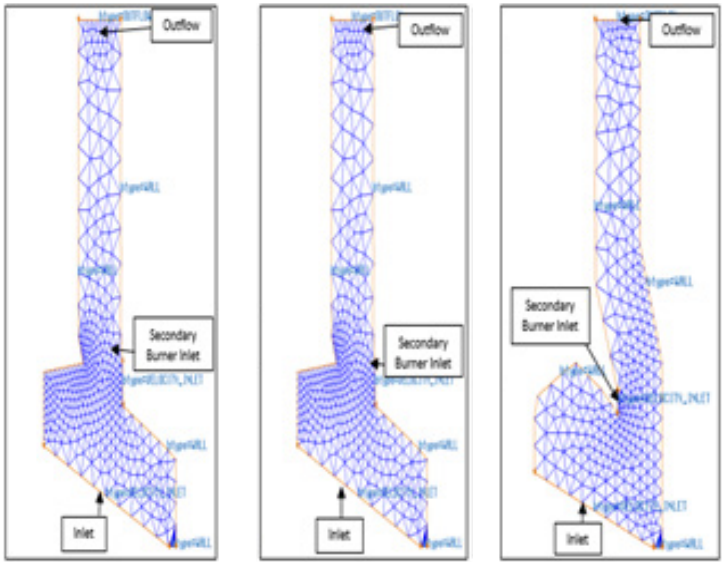
In (Figure 4) the static pressure contours in all three geometries calculate a maximum static pressure of 0.36 to 0.11 pascal and a minimum static pressure of -1.9 to -1.24 pascal. These statistics are small in comparison to atmospheric pressure; hence we can determine that the term strategy pressure in the compartment won't be caused any trouble.

The velocity contours (Figure 5 & Figure 6) show that the maximum velocity of the center-oriented riser/combustor is 3.3 m/s. We can see that as flue gases exit the primary chamber, all three velocities are at their maximum near the neck region. According to the velocity stream contours, the left-side wall at the neck area on the counter scenario may have issues due to fouling, slagging, and corrosion. In the third scenario, the right side of the neck wall may present us with a similar challenge. This is because the gas discharge stream




(a) Center Flow (b) Counter Flow (c) Parallel Flow

**Fig. 2.** Geometries of the Riser/Combustors (mm)




(a) Center Flow (b) Counter Flow (c) Parallel Flow

**Fig. 3.** Meshing of Coal Solid Fuel Combustor

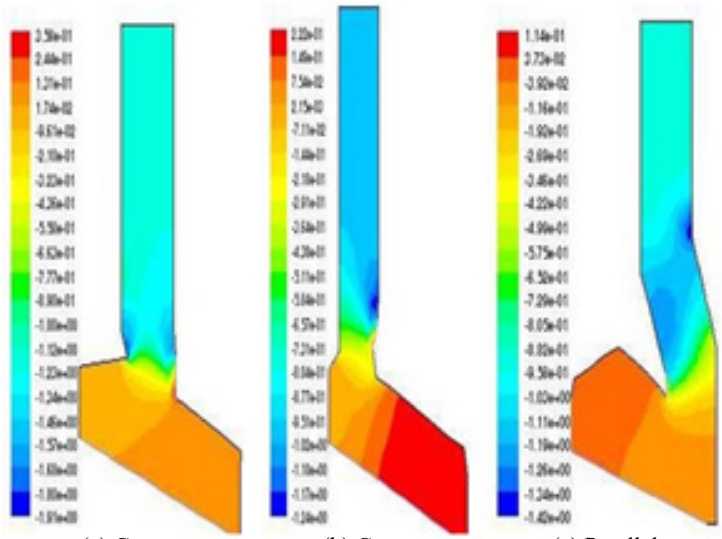
is concentrated close to the wall.

In (Figure 7) the central portion of the grate experiences the highest heat in all three scenarios, with the extreme temperature falling between 1400 K and 1440 K. Due to the high temperature in this area, volatiles is burned.

The burning of the volatiles is defined by two reactions:

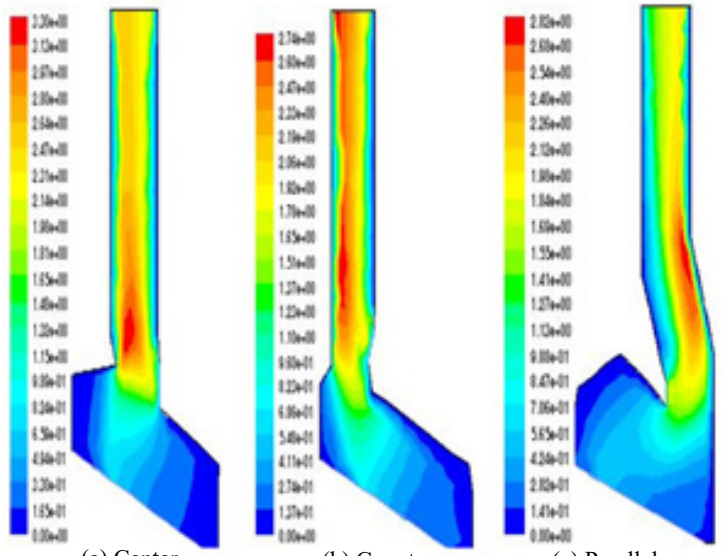
$2CO + O_2 \rightarrow 2CO_2$
$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$

The CO mass fraction contours (Figure 8) show that the central region of the grate, where devolatilization from the waste bed occurs, is where it is concentrated. Then, it is burned with oxygen to make $CO_2$. The center region contains both the lowest $O_2$ and greatest $CO_2$ concentrations (Fig. 9). We can observe (Figure 10) the lowering of $O_2$ at the secondary combustor area at the neck after $CH_4$ produced $CO_2$ and $H_2O$ in response. The contours (Figure 11) indicating the amount of $NO_x$ in ppm highlight the area of the geometry where it is frequently focused in the upper region. The lowest level is established by the parallel geometry



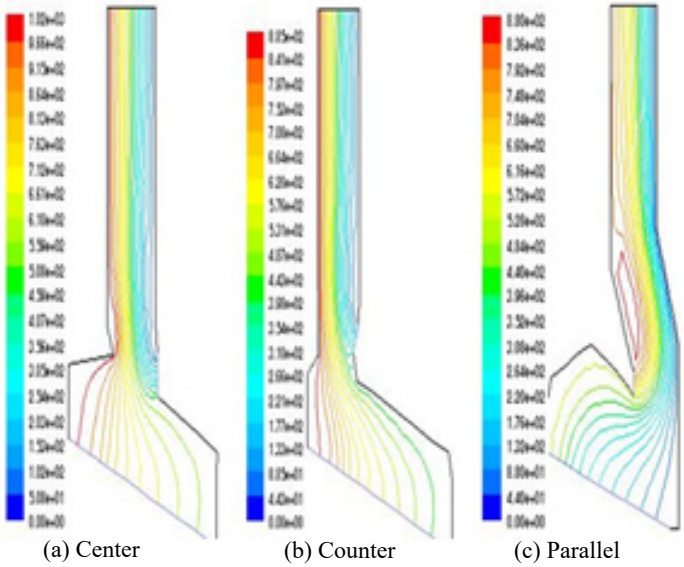
(a) Center (b) Counter (c) Parallel

**Fig. 4.** Contours of Static Pressure



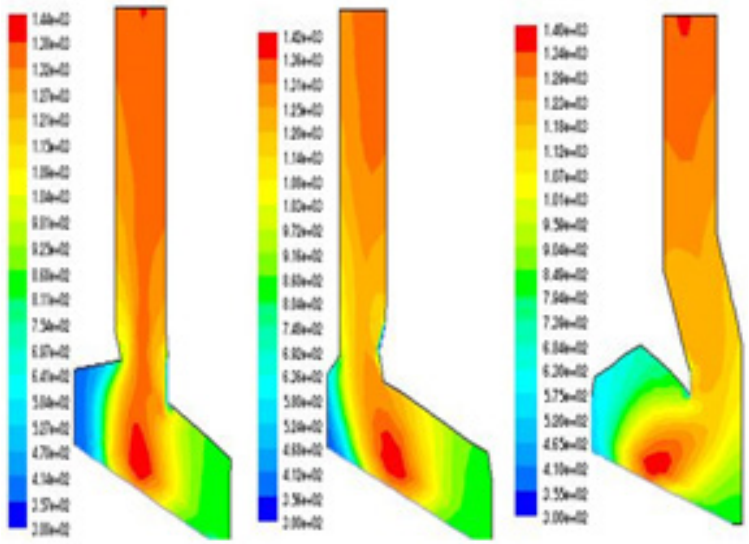
(a) Center (b) Counter (c) Parallel

**Fig. 5.** Contours of Velocity



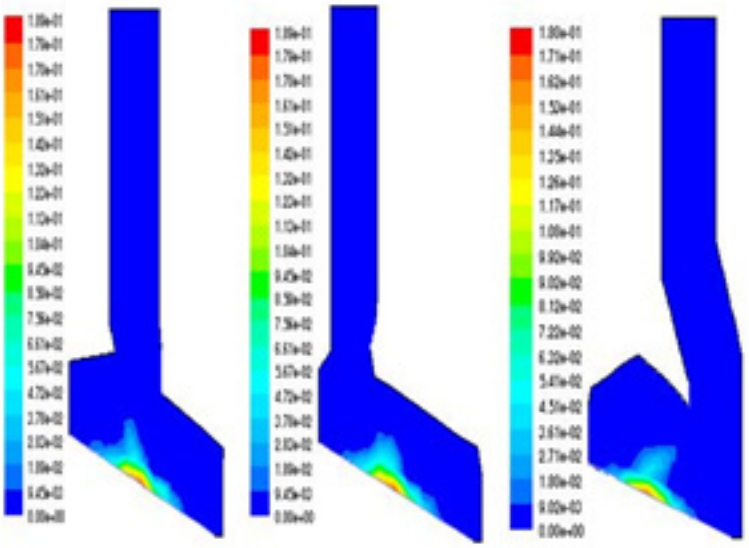
(a) Center (b) Counter (c) Parallel

**Fig. 6.** Contours of Velocity Stream



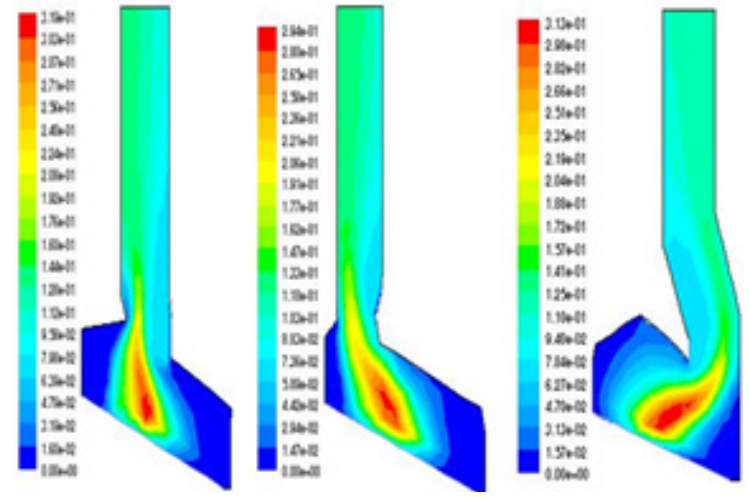
(a) Center (b) Counter (c) Parallel

**Fig. 7.** Contours of Static Temperature



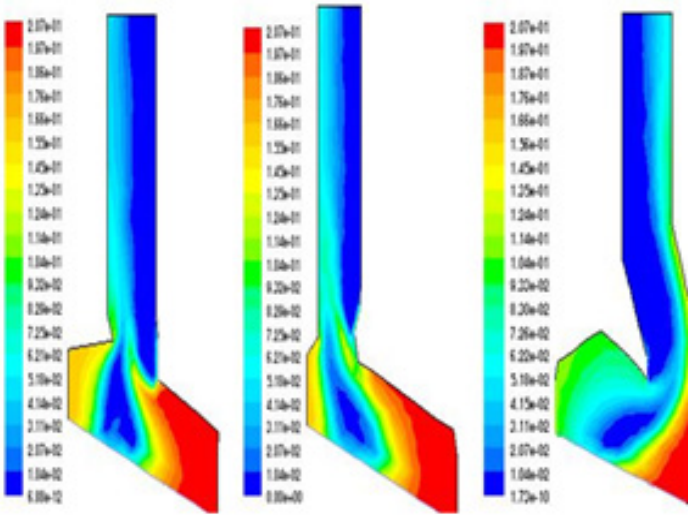
(a) Center (b) Counter (c) Parallel

**Fig. 8.** Contours of Mass Fraction of CO



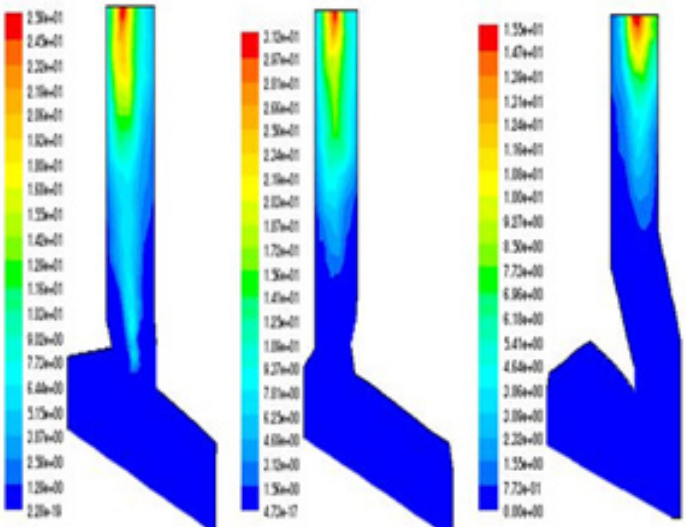
(a) Center (b) Counter (c) Parallel

**Fig. 9.** Contours of Mass Fraction of $CO_2$



(a) Center (b) Counter (c) Parallel

**Fig. 10.** Contours of Mass Fraction of $O_2$



(a) Center (b) Counter (c) Parallel

**Fig. 11.** Contours of $NO_x$ (ppm)

at around 15 ppm, while the greatest level is established by the counter geometry at about 31 ppm.

## 4. CONCLUSION

A CFD model was developed to examine the hydrodynamics of gas-solid flow in a circulating fluidized bed riser using the commercial ANSYS FLUENT software. The parametric examination of the two-phase gas-solid stream hydrodynamics of a CFB riser was used to model the impact of various riser exit forms. For analyzing mixing performance, the CFD model for the gas segment and the viscosity of static particles in the solids segment with a k-ϵ turbulence model were used. The FLUENT data indicate that the highest temperature within the compartment is around 1400 K, or nearly 1130 °C, at the primary burning sector in the bed center. The highest velocity in the center-oriented riser/combustor peaks at 3.3 m/s, according to velocity contours. The CO and $CO_2$ mass fraction contours show that it is concentrated in the center geometry and lower CO concentration is found in parallel geometry. The lowest level of $NO_x$ is established by the parallel geometry at around 15 ppm, while the greatest level is indicated by the counter contours at about 31 ppm. To reduce emissions, it is determined that the Circulating Fluidized Bed Combustor is the most advantageous and efficient technique for producing power from Thar lignite coal. However, to validate the results of

CFD modeling, further studies may be conducted and actual data from a current fluidized combustor may be used.

## 5. ACKNOWLEDGMENTS

The authors would like to give special recognition to Environmental Research Center, Bahria University Karachi Campus Pakistan, and DHA Suffa University Karachi-Pakistan for providing the facilities for this research work.

## 6. CONFLICT OF INTEREST

There is no conflict of interest among the researchers of this study.

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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 71-79 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)740

Pakistan Academy of Sciences

Research Article

# Surveillance of *Berberis* Species across Poonch Division of Azad Jammu and Kashmir

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**Abstract:** *Berberis* is one of the most important medicinal plant and it has a great medicinal value. *Berberis* has such pronounced medicinal values that it is used to cure many diseases and has exhibited great therapeutic effects among the local communities throughout the world. Diversity of *Berberis* is uncertain to great extent in Poonch division of Azad Jammu and Kashmir. *Berberis* specimens were collected from four districts comprising fifteen tehsils of Poonch division of Azad Kashmir. About 40 prominent locations were visited during flowering and fruiting stages during 2016-17. A total of seven species and a sub species were identified on the basis of morphological studies. Studies showed that identified *Berberis* species were present in all the four districts. Shanon and Simpson indices were used for calculating diversity of *Berberis* species in the study area along with calculating species evenness and equitability. Diversity indices indicated that there was moderate diversity of *Berberis* species within different districts and different tehsils. Simpson diversity index indicated that there is an 87 % chance that two individuals selected randomly from the study area would be different. Species evenness indicated that each specie identified from each tehsil had maximum chance of occurrence and each identified species is present in every tehsil. Species equability also indicated similar kinds of results which indicated that different species were evenly distributed in each tehsil. It was concluded that there is moderate diversity of *Berberis* species in Poonch division of Azad Jammu and kashmir. All the identified species were present in all the districts. The present study will advance our knowledge regarding identification and distribution of *Berberis* species in Poonch division.

**Keywords:** Abundance, *Berberis,* Diversity Studies, Shannon Index, Simpson Index

## 1. INTRODUCTION

Barberry (*Berberis* spp.) is a well-known medicinal plant, which has long been used in the world in many old civilizations [1]. This plant is deciduous, evergreen and semi-evergreen small tree or shrub, with regular spines, with inflorescence as racemes, umbels, or solitary red-orange, orange to yellow which grows up to 4 m high and under different ecological conditions [2, 3].

The prominent member in dicotyledonous genus in the Berberidaceae family is the *Berberis*; they are evergreen shrubs or small bushes, woody and spiny in nature. The recent reports showed that [4], the Berberidaceae family had 15 genera and 650 species around the world. *Berberis* is distributed in many parts of the world like Africa, America, Europe and Asia. In Pakistan *Berberis* is distributed across the mountainous regions (1400-3500 m above sea level). These areas include Kashmir, Khyber Pakhtunkhwa, and nothern areas of Baluchistan. *Berberis* is enriched with important chemical compounds. Due to presence of these compounds *Berberis* has a vital role in many systems of medicine [5].

Simple simulation tests and data regarding abundance of species can be used to measure species diversity, equitability, and richness. Most recommended indices or measures of species diversity are Shanon's index, Simpson's index and

Received: September 2022; Accepted: December 2022
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Fager's index [6]. These indices are commonly used indices for measuring species diversity as they face less criticism as compaired to others. Measures of species richness depends upon size of the sample. Variability in sample size can be misleading and richness and equitability esitimating methods for species can not be applied [6].

Diversity is one of the key component of an ecosystem which can be measured or estimated at different levels and in many ways. Diversity can be present within genes, within individuals of a population, within different species of the same genera, within communities and ecosystems [7]. The major problem in estimating biological diversity is that it is not properly defined and is ambiguous [8]. The survival of an area depends upon it's biodiversity. If an area donot have variation in it's flora and fauna then it has no value. As Reet said , "diversity, in essence, has always been defined by the indices to measure it" [9]. At species level in a community alpha diversity is opposed to β-diversity and γ-diversity: [10] which is also known as species number or species richness, eveness or equitability is the relative abundance of species. The two factors are also used for estimating the diversity of species within an area [9, 11].

Due to human over exploitation and environmental fluctuation in the Himalayan region, floral diversity has greatly reduced. Forest vegetation fragmentation into small patches is mainly due to anthropogenic disturbance. There is a great need of research regarding floral biodiversity identification and conservation in Poonch valley of Azad Jammu and Kashmir. The studies revealed that there were 30 endangered, 145 vulnerable and 68 species near to extinction. The valley determined deterioration in plant diversity because of maximum exploitation of vegetation. For sustainable use *in-situ* and *ex-situ* conservation, afforestation and harvesting by controlled methods can be a solution [12].

Taxonomic studies and identification of *Berberis* species is difficult due to high phytochemical and morpho-pathological variations. These variations may be due to Hybridization and environmental effects [12-14]. Field identification is often made challenging by overlapping characters especially in stem, leaves flower and berry size. In some of the plant species, serrations and leaf texture vary from environment to environment and with plant's age [13-16]. The present study was the first attempt to explore the diversity of *Berberis* species in each district of Poonch division as it was not well documented; it will serve as a baseline to study diversity and evolutionary relationships of *Berberis.*

## 2. MATERIALS AND METHODS

### 2.1 Study area

The state of Azad Jammu and Kashmir has a total area of 13,297 square kilometres. The total study area which comprises four districts and fifteen tehsils is about 2792 square kilometres, in which district Poonch is about 855 square kilometres, Bagh 770 square kilometres, Sudhnoti 569 square kilometres and Haveli 598 square kilometres. The study area is mostly hilly and mountainous [17].

Azad Jammu and Kashmir has a broad range of climatic conditions depending upon the altitude range (360 m South to 6325 m North). It has dry sub-tropical (South) to moist temperate (North) climate. The average rainfall is ranges from 1000 mm to 2000 mm. Northern districts have 30 to 60 percent precipitation in the form of snow. The snow line is about 1200 m in winters but reduces to 3300 m in summers [17].

### 2.2 Localities selection for specimen collection

For sampling, species identification and to cover the maximum part of study area depending upon the road links sites were selected and those sites were at least 10-15 kilometres apart from each other. The slected sites were grassy fields, orchards, field crops, mountainous regions, forests with bushes and high trees. The coordinate data and altitude of each locality was noted by using Altimeter.

### 2.3 Field visits and collection of specimen

Field visits were performed in the study area of Poonch division of Azad Jammu and Kashmir during flowering and fruiting stages in 2016 and 2017 for the collection of specimen and recording morphological parameters. Collected specimens were submitted to Pakistan Museum of Natural History, Islamabad for the morphological

identification and confirmation of *Berberis* species.

### 2.4 Processing of collected specimen

All the specimen were dried, numbered and preserved by applying standard herbarium techniques [18-20].

### 2.5 Storing of specimen in field

The specimen soaked in 10 percent formalin solution were placed in paper folders and stored in plastic bags.

### 2.6 Pressing and drying

The specimen were brought to the Lab., placed on a paper sheet and pressed with wooden plant presser. The papers between the specimen were changed every day for about 3 to 4 days and the sheets containing specimen were placed in well ventilated and warm place.

### 2.7 Mounting

Glued strips were used to mount the dried specimens on herbarium sheets. Sheets containing specimen were provided with all the standard information like local name, botanical name, locality, family, altitude, date of collection, specimen number and collector's name. All the specimen were deposited in the department of Plant Breeding and Molecular Genetics and Pakistan Museum of Natural History, Islamabad.

### 2.8 Identification of specimen

Identification was done by comparing;

- With already identified specimen at Pakistan Museum of Natural History, Islamabad.
- With taxonomic keys of Flora of Pakistan [15].
- With specimen images and other diagrams.

### 2.9 Statistical analysis

Diversity indices were calculated on the basis of tehsils as well as districts. Shanon weiner and Simpson's indices were used for diversity estimation. Beside diversity estimation eveness and and equitability of the species was also estimated using computer based software PAST 4.03.

## 3. RESULTS AND DISCUSSION

The central theme of ecology is diversity and it has a useful impact on an ecosystem. Measures of diversity most of the time regarded as gauges of security and protection of biological system. The collection and processing of diversity data is time consuming so it is hard to define and difficult to calculate. However, diversity in plant species is considered as fundamentals for plant species recognition. The presence and numerical conformation of total species in an ecosystem is known as its biodiversity. Establishment of diversity depends upon the stability of time and environment [22]. Generally, low diversity is yielded by homogeneous conditions whereas high diversity is yielded by heterogeneous conditions [23].

The richness of individuals within different species and indicating the variety of species present in a habitat are reliable methods of representing co-efficient of diversity in a sample or a habitat [24]. The estimates of floral diversity represent the number and functions of an ecosystem. If heterogeneity of the species is high, it will result in more floral diversity and thus results in higher index or co-efficient of diversity [25].

Two diversity indices evenness and equability were used for the calculation of short-term and long-term changes and continuous monitoring of species in the area. The measures of diversity are considered reliable when more than one indices are used, only one index for measuring diversity in an ecology can be misleading. The single index that measures the observed changes in the diversity can give a wrong impression as the changes occurred in an ecosystem cannot be covered by a single index [26].

### 3.1 Identified *Berberis* species and their taxonomic characters

#### *3.1.1 Berberis lycium*

Table 1 showed that *Berberis lycium* is diploid having 2n = 28, a shrub 2-3 m tall erect or suberect, semideciduous. Stem and branches

were pale, whitish to greyish. Spines were trifid, 1-2 cm long yellowish to straw coloured. Leaves 2.5-6 × 5.5-12 cm oblanceolate to ovate or elliptic grey or white below, openly veined 2-4 spines were present at margins. Racemes 10-25 flowered and 3-6 cm long rarely shorter. Flower 0.5-0.8 mm across usually pale yellow in colour. Pedicels were 0.6-1.2 cm long. Outer sepals much smaller than the middle and inner sepals; inner sepals were 0.45-0.5 cm long 0.3 cm broad, obovate. Ovules usually 4, shortly stipitate. Berries were 0.7-0.8 cm long and 0.5 cm broad blackish, ovoid with heavy grey-white bloom. Seeds 0.3-0.4 cm long.

### *3.1.2 Berberis parkeriana*

Data presented in Table 1 showed that *Berberis parkeriana* is diploid having 2n = 28, is a shrub very similar to *Berberis lycium* but leaves usually greenish below, epapillose and berries slightly larger. Stem was pale to whitish. Leaves were 3-6.5 × 6-12.5 cm, spines were absent at the margin of leaves. Thorns were tri-fid and 1.5-2.5 cm long. Flowers were yellow in colour. Racemes were 8-25 flowered. Berries were 0.8-0.9 cm long and 0.6-0.7 cm broad usually obviate blackish in colour. Seeds were usually 2-4 in number.

### *3.1.3 Berberis ulcinia*

Table 1 depicted that *Berberis ulcinia* is small glabrous shrub, diploid in nature having 2n = 28, 1-2 m tall much branched and densely spiny with 0.5-1 cm long internodes and reddish-brown stem. Thorns were tri-fid 1-1.5 cm long. Leaves were 0.5-1.5 cm long and 0.2-0.4 cm broad and linear-lanceolate or very oblanceolate, often entire to 1-2 spinulose at the margins, veined and minutely spined tip. Inflorescence 3-6 flowers were orange yellow in colour and were 0.5 cm in diameter, pedicellate, pedicel 0.3 to 0.4 cm. Sepals 0.64 cm, petals 0.41 to 0.46 cm. Stamens 6 and 0.33 to 0.39 cm long. Ovules 3 to 5. Berries black, globose, 0.5 × 0.25 cm. Seeds usually 3-5 in number.

### *3.1.4 Berberis royleana*

*Berberis royleana* diploid in nature having 2n = 28, shrub 1.5-3 m tall stem and branches were red brown. Spines were tri-fid usually red in colour and 1-1.5 cm long. Internodal distance was 1-2.5 cm. Leaves were oblong-obovate 0.7-1.6 × 0.6-1.3 cm. Raceme 3-8 fold fascicled or subumbellate 1-1.6 cm long. Pedicels 0.5-1 cm long. Berries were blackish, pruinose grey, oblong, 0.75 cm long 0.4 cm broad (Table 1).

### *3.1.5 Berberis orthobotrys*

Shrub 1-1.5 m tall diploid in nature having 2n = 28, stem dark red sometimes orange yellow or pale brownish (Table 1). Moreover, internode distance was 1-2.5 cm, 1-2.5 cm long reddish or brownish spines usually 3-fid were present. Leaves were 1-3×5.5-1.7 cm, spinulose at the margins grey beneath, subsessile shortly petiolate. Raceme usually 5-25 fold and 1.6-3.2 cm long. Flowers 0.7-1.2 cm across, yellow to pale yellow in colour. Sepals 0.4-0.7 cm long. Stamens were shorter than the petals. Ovules were 3-5. Berries oblong, sub-ovoid 0.7-1 × 0.5-0.6 very variable in colour sometimes red or dark coloured when dried, often 3 seeded, seeds 0.3 cm long.

### *3.1.6 Berberis brevissima*

The species *B. brevissima* is a shrub diploid in nature having 2n = 28 with whitish or pale, short, sub spreading almost glabrous stem and branches. Spines of tri-fid and usually 0.5-1 cm long. Leaves were 1-2 × 0.3-0.6 cm, 1-2 spinulose at the margin, pale sub-pruinose below, acute-sinulose at apex. Inflorescence 5-10 flowers yellow in colour, 2-5 fruited fascicled or sub-fascicled long up to 1 cm. Berries sub-globose, 0.35-0.5 cm long somewhat blackish in colour. Style 0.1 cm long berries were 2-3 seeded and 0.2-0.25 cm long (Table 1).

### *3.1.7 Berberis kashmiriana*

Table 1 showed that *B. kashmiriana* is a shrub 1-2 m tall diploid in nature having 2n = 28. Stem is glabrous pale yellow in colour. Internode distance was almost 2-3.5 cm, spines were usually 1-2 cm long and were of tri-fid. Leaves were 3-6 × 1-1.8 cm. Leaves were narrow obovate-oblong they were very short petioled, green epruinose below, spinulose at the margins, rediculately veined. Racemes were 3-4 cm long, 8-13 fold. Flowers were 0.95-1.35 cm across. Pedicels 0.8-1.7 cm long. Stamens slightly shorter than petals. Ovules 3-4 stipitate. Berries oblong 0.9-1 × 0.5 cm dark red

and very shortly stylose.

### *3.1.8 Berberis orthobotrys* sub spp. *capitata*

Erect glabrous shrub diploid in nature having 2n = 28, stem red-brown to pale-whitish in colour was present. Leaves were 1-2 × 0.5-1.5 cm, elliptic-obovate, serrated at the margins, petiolated, greenish on both sides and veined. Racemes were short usually 9-20 flowers. Fruits were 0.7-0.8 × 0.5-0.6 cm, oblong, sub-obvoid pale reddish when dried. Pedicels 0.5-10 cm long, 4 ovules, sessile, 2-3 seeded. Seeds were brown in colour (Table 1).

In the present study two indices the Shannon-Wiener's diversity index and Simpson's index were used for the calculation of diversity. Figure 1 is constructed on the basis of coordinate data shows the prominent collection sites of Poonch division of Azad Jammu and Kashmir. Grays reported the validity of indices application on biological data [25]. The Shannon-Wiener's diversity index is dependent upon distribution and suffers least criticism of validity. The calculated values of Shannon's index at various tehsils ranged from 2.05 to 2.08 (Table 2). Diversity indices greater than 2 indicated that there is moderate diversity of *Berberis* species within different districts and different tehsils. Simpson diversity index indicated value of 0.87, which indicated that there is 87 % chance that two individuals selected randomly from the study area would be different. It indicated huge variability among the individual plants that could result in stable population in the area. This variation might be the result of natural crossing resulting in interspecific and intraspecific genetic exchange among individuals or could be the environmental differences. These results could be confirmed via molecular analysis which is least affected by environment. On the other hand, it indicated that there is 13 % chance of the species identified from the area would be same.

Species evenness is the relative abundance of each specie in an area. Table 2 depicted the values ranging from 0.98 to 1 indicating that each specie identified from each tehsil had maximum chance of occurrence and each identified specie is present in every tehsil. Species equability also indicated similar kind of results. Equability values ranged from 0.99 to 1 which indicated that different species were evenly distributed in each tehsil.

Diversity indices from four districts of Poonch Division of Azad Kashmir were presented in the Table 3. The Table showed that from district Poonch 916 individuals were studied from these individuals eight species were identified. From district Bagh 688 individuals were studied and eight species were identified. From district Sudhnoti 1206 individuals were studied and eight species were identified. From district Haveli 737 individuals were studied and eight species were identified. Simpson diversity index indicated the value of 0.87 which indicated that there is 87 % chance that two individuals selected randomly from each district would be different. It also indicated that there is 13 % chance that the species identified from the area would be same. The values calculated by Shannon's index at different districts were 2.07 indicating that there is moderate diversity within different districts. Species evenness is the relative abundance of each specie in an area. Table 3 depicted the values ranging from 0.98 to 1 which indicated that each specie identified from each district has maximum chance of occurrence and each identified specie is present in every district. Species equability also indicated similar kind of results. Equability values ranged from 0.99 to 1 which indicated that different species were evenly distributed in the study area.

It was observed that with increase in altitude percentage distribution of the species decreases while in low altitudes it was high. The decrease in species distribution is due to human interaction, deforestation, soil erosion, infrastructure development, encroachment pressure, collections of medicinal plants, low number of species, overpopulation, global warming and harsh environmental conditions [27]. In the middle part of the altitudinal gradient, species diversity was high in the tree layer. Due to above mentioned factors it decreased in both upper and lower altitudes. For effective conservation plan which can be implemented by knowing the indigenous flora, species identification and classification, habitat ecology, affecting the population of plants and anthropogenic factors, particularly those of threatened and vulnerable either locally or internationally [28]. Due to over exploitation and high rate of utilization species like *Geranium*

**Table 1**. Calculated values of studied characters for identification of *Berberis* species from Poonch division of Azad Jammu and Kashmir

| | Ploidy | P.H | Spines | Spine colour | Leaf area | Leaf type | Racemes | F. colour | Pedicels | Ovules | Berry size | Berry colour | Seeds |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***B. lycium*** | 2n = 28 | 2-3 m | Trifid, 1-2 cm | Yellowish | 2.5-6 × 5.5-12 cm | Oblanceolate to ovate | 10-25 | Yellow | 0.6-1.2cm | 4 | 0.7-1×0.5-0.6 cm | Heavy grey | 2-4 |
| ***B. parkeriana*** | 2n = 28 | 2-3 m | Trifid,1.5-2.5 cm | Yellowish | 3-6.5 × 6-12.5 cm | Epapillose | 8-25 | Yellow | — | — | 0.7-0.8 × 0.5-0.6 cm | Obviate blackish | 2-4 |
| ***B. ulcinia*** | 2n = 28 | 1-2 m | Trifid 1-1.5 cm | Brownish | 0.5-1.5 × 0.2-0.4 | Linear-lanceolate | 3-6 | Orange yellow | — | 3-5 | 0.9-1 × 0.5 cm | Black | 3-5 |
| ***B. royleana*** | 2n = 28 | 1.5-3 m | Trifid 1-1.5 cm | Redish | 0.7-1.6 × 0.6-1.3 cm | Oblong-obovate | 3-8 | Yellow | 0.5-1 cm | 3-5 | 0.75×0.4 cm | Blackish | 2-4 |
| ***B. orthobotrys*** | 2n = 28 | 1-1.5 m | Trifid 1-2.5 cm | Reddish or Brownish | 1-3×5.5-1.7 cm | Subsessile | 5-25 | Pale yellow | 3-5 cm | 3-5 | 0.7-1×0.5-0.6 cm | Red or dark coloured | 3 |
| ***B. brevissima*** | 2n = 28 | 1.5-3 m | Trifid 0.5-1 | Yellowish | 1-2 × 0.3-0.6 cm | Spinulose | 5-10 | Yellow | — | 3-5 | 0.8-1×0.5 cm | Blackish | 2-3 |
| ***B. kashmiriana*** | 2n = 28 | 1-2 m | Trifid 1-2 cm | Brownish | 3-6×1-1.8 cm | Obovate oblong | 8-13 | Yellow | 0.8-1.7 cm | 3-4 | 0.9-1 × 0.5 cm | Dark red | 3 |
| ***B. orthobotrys* sub spp. *capitata*** | 2n = 28 | 1-3 m | Trifid 1-2 cm | Brownish | 1-2 × 0.5-1.5 cm | Elliptic-obovate | 9-20 | Yellow | 0.5-10 cm | 3-4 | 0.7-0.8 × 0.5-0.6 cm | Pale reddish | 2-3 |

**Table 2**. Calculated values of diversity indices from tehsils of Poonch division of Azad Jammu and Kashmir

| | Rawala kot | Thorar | Hajira | Abbaspur | Bagh | Dheerkot | Hari Gehal | Baloch | Dolian Jattan | Mang | Pallandri | Trarkahal | Khurshid Abad | Mumtaz Abad | Haveli |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 | 8.00 |
| Individuals | 234.00 | 220.00 | 206.00 | 256.00 | 267.00 | 205.00 | 257.00 | 249.00 | 261.00 | 218.00 | 248.00 | 230.00 | 276.00 | 244.00 | 217.00 |
| Shannon | 2.06 | 2.07 | 2.05 | 2.07 | 2.08 | 2.07 | 2.06 | 2.07 | 2.08 | 2.06 | 2.07 | 2.06 | 2.06 | 2.06 | 2.07 |
| Simpson | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 | 0.87 |
| Evenness | 0.98 | 0.99 | 0.97 | 0.99 | 1.00 | 0.99 | 0.98 | 0.99 | 1.00 | 0.98 | 0.99 | 0.98 | 0.99 | 0.98 | 0.99 |
| Equitability | 0.99 | 0.99 | 0.99 | 1.00 | 1.00 | 0.99 | 0.99 | 1.00 | 1.00 | 0.99 | 0.99 | 0.99 | 0.99 | 0.99 | 1.00 |

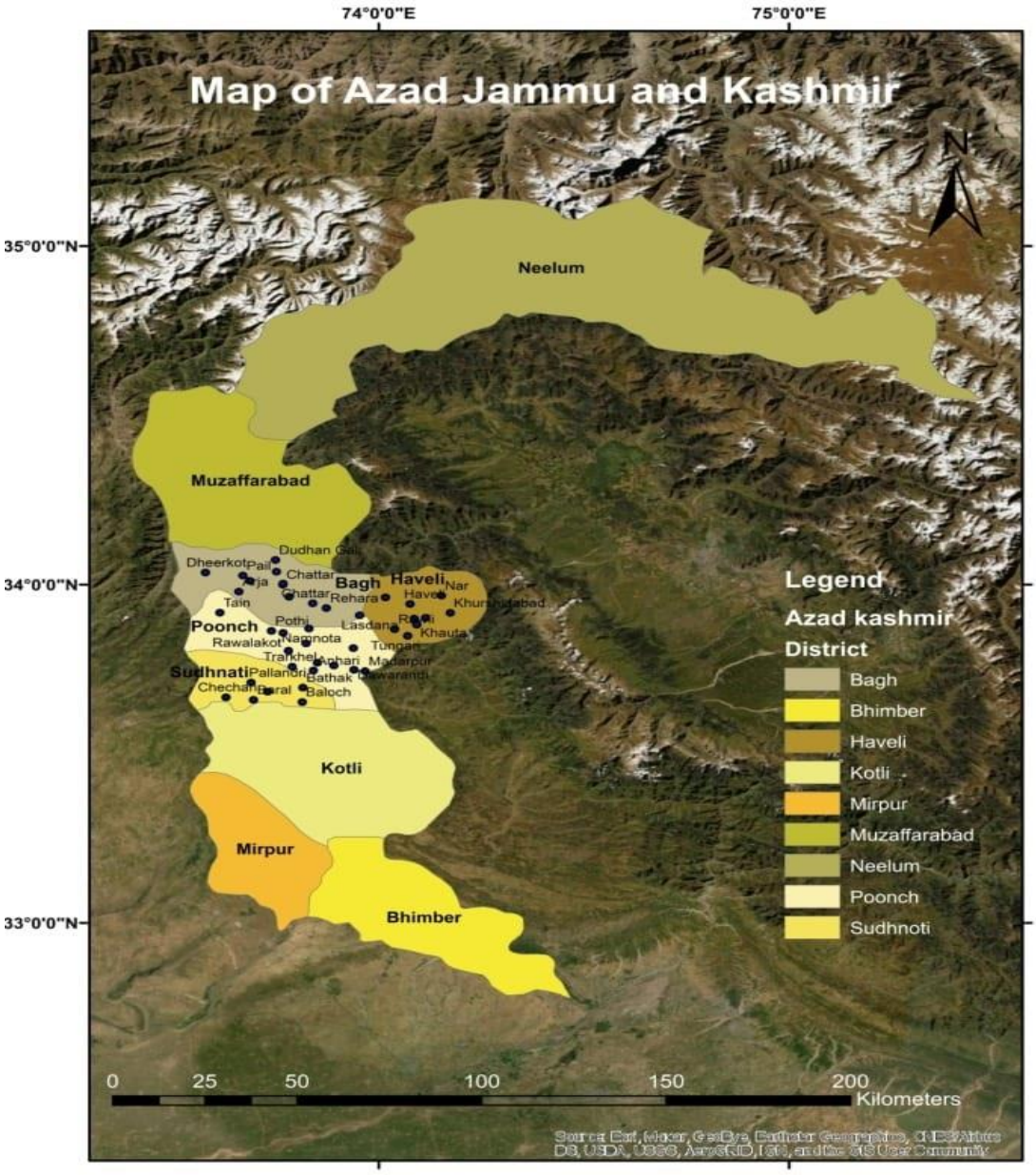


**Fig. 1.** Map of Azad Jammu and Kashmir showing data collection sites based on coordinate data

**Table 3.** Calculated values of diversity indices from districts of Poonch division of Azad Jammu and Kashmir

| | Poonch | Bagh | Sudhnoti | Haveli |
|---|---|---|---|---|
| Species | 8 | 8 | 8 | 8 |
| Individuals | 916 | 688 | 1206 | 737 |
| Simpson | 0.87 | 0.87 | 0.87 | 0.87 |
| Shannon | 2.07 | 2.07 | 2.07 | 2.07 |
| Evenness | 0.99 | 0.99 | 0.99 | 0.99 |
| Equitability | 0.99 | 1.00 | 1.00 | 1.00 |

*wallichianum, Bistorta amplexicaule, Saussurea lappa, Aconitum heterophyllum, Ajuga bracteosa, Jurinea dolomiaea* and *Berberis lycium* are on the verge of extinction [29]. There is always an increasing demand of these species while their production and conservation declined in the recent past few years. The relationship between functioning of ecosystem and species diversity is always directly proportional. The diversity loss now evolved as a worldwide issue that gained substantial consideration and attention. Floral degradation and disturbance of ecosystem has greatly affected the funna of that ecology. In most of the areas, over grazing of grass lands, over exploitation and destruction of biodiversity resulted in less production and of plant populations which have impacts on human society. To run the ecosystem properly, productivity and population balance have sublime importance which also affects ecosystem functions [30].

## 4. CONCLUSION

On the basis of present study it was concluded that there is moderate diversity of *Berberis* species in Poonch division of Azad Jammu and kashmir. All the identified species were present in all the districts. Identified *Berberis* species were evenly and equally distributed within the study area.

## 5. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 81-92 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)741

Pakistan Academy of Sciences

Research Article

# Distribution Pattern of Tree Species and Richness along an Altitude Gradient in the Sub-Alpine Temperate Zone of Hindu Kush Mountainous Forests, Pakistan

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**Abstract:** Lal-Koo Mountains Forest (LMF) is the most extensive vegetation type in the largest Hindukush Mountainous ranges of Pakistan, however highly overlooked compared to the Himalayan and Karakorum ranges. Here, we studied the conifer tree species regeneration, diversity, basal area, density, and species richness of the Lal-Koo Mountains Forest (LMF) along the altitude gradient. We used the quadrate (10 m × 10 m) sampling method for vegetation analysis at 54 different locations between 1970-3120 m elevations. We found a total of 115 species belonging to 58 families. We find the maximum value of Shannon's -Winner index 3.603 at 2115 m and Simpson's Diversity Index at 0.91 at 2290 m along an altitude gradient in lower elevation ranges. The current finding revealed that observed tree species richness shows a unimodal pattern with a peak at 2400 m in the mid–elevational range followed by a basal area peaked at 2300 m across the elevation gradient. We concluded that the high growth ratio of regenerates is due to open areas (free canopy space) likely available due to severe deforestation at low altitudes. In Lalkoo, tree density did not follow a regular trend, although the highest values were obtained around 2400 m and 2600 m along altitude. Our results also indicate that there is a narrow elevational range at high altitudes (near the timberline) measured from 2750 m – 3120 m, of the gradient. Furthermore, we discovered broader altitude ranges in the midst (relating well with the theory behind the mid-domain effect) in the range of 2345 m – 2750 m, but the lower altitude range assessed from 1970 - 2345 m does not reveal precise data for the reported species richness, which is a deviation from Stevens elevational Rapaport rule.

**Keywords:** Lalkoo Forest, Hindukush Mountains, Pakistan, Regeneration, Elevation gradient, Species richness, Diversity, domain effect.

## 1. INTRODUCTION

Altitude is a combination of multi-environmental variables that interact with other factors like topography, aspect, soil texture, nutrients, and inclination of slope that couple forest composition [1-2]. The idea about Altitudinal variation that exists among tree species was also assessed multiple times in forest ecosystems in relation to topographic and soil variables [3], observed from floristic composition, species richness [4] and evenness, resilience, and total plant production [1]. Moreover, habitat variability is an essential component in determining biodiversity along the altitudinal gradient [5]. Mutually dependent interactions, such as the latitudinal inclination of

Received: September 2022; Accepted: December 2022
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diversification, latitudinal variations of species abundance, and links across diversity and productivity, are interrelated across spatial patterns due to geographic restrictions and the impacts of territory. Recently latitudinal patterns of species richness have also been the focus of biological research that affects the distribution of individual species [6-7].

However, little research sought to evaluate the function of identification of the mid-domain effect (MDE) in determining species abundance and uniformity peaks at mid-level heights in a range region [8]. According to this research, MDE is affected by the species including the prevalence of relative range widths and median, the availability, placement, and constraining the ability of border restrictions, and the sampling place [9].

The mid-domain effect (MDE) has been proposed as a null explanation for diversification contours and motif perception [8]. Colwell and Lees [8], greatly launched the concept, stating that "given a domain with geometric limitations, the overlaps of species ranges grow towards the center of the domain if they are put haphazardly, giving a uni-model sequence of species abundance along the slope." In typically, the MDE is better for species with a large range compared for those with a small range. The notion of MDE piqued the interest of scholars who wanted to apply it to other analyses.

Using various measurements of vegetation analysis like tree's Diameter at Breast Height (DBH), Forest Denseness, soil analysis, aspects, and temperature are considered as important variables, significantly affecting vegetation patterns. However, factors like subtropical precipitation patterns are observed, showing a tendency in rise along an elevation gradient of 3500- 4000 meters and then decrease at various elevation ranges in lower altitudes influenced by topography in the mountainous forests [7]. Similarly, high altitude changes in the composition of soil and drastic decrease in soil temperature greatly affect tree's Diameter at Breast Height (DBH) as well. The percentage of sand documented [10] decreased by 1 % for 152.47 feet within altitudes of 6400 and 9600 feet, whereas the percentage of silt declined by 1 % per 188.23 feet within the identical levels. Magnesium (Mg) amd Potassium (K) concentration in soil rise with elevation by almost 1 mg/kg every 63.82 feet and 1 mg/kg every 30.92 feet, respectively. These features imply that numerous aspects associated with the altitudinal gradient, such as soil type and ambient temperature, can be investigated [11], availability of soil nutrients and depth of soil concerning both B and A/O horizons influences flora in a geographical area [12]. Aside from these considerations, it has been proposed that species abundance is directly influenced by elevations as well as climatic conditions, therefore species variability of various life forms generally diminishes with rising height, and extremely tiny life forms persist at extreme elevations [13].

Lalkoo Mountains forest (LMF) is the most extensive vegetation type in the largest Hindu Kush Mountainous (HKM) ranges of Pakistan. Ecologically these mountains (HKM) are moist temperate forest zones, that are con-jointly located with great mountainous ranges of the Himalayas and Karakoram [3]. Both the Himalayan and Karakorum Mountain ranges are studied enormously, under research focusing on ecology, however, the Hindu Kush Mountains (HKM) ranges are highly ignored and overlooked [14-16]. The Lalkoo mountainous forest is floristically diverse and one of the world's biodiversity hotspots [17]. In addition, these mountains are distributed within a very small geographical extent making, between an elevation gradient of 1800 and 4400 m with great economic importance for timber usage and crown coverage [14, 18]. Like other moist temperate forests, Lalkoo presents a high richness of tree species like *Abies* (Fir), *Picea* (Spruce), *Quercus* (Oak), and *Pinus* (Blue pine). All these characteristics make this study, ideal for an altitudinal pattern of tree species. We studied species richness patterns along the altitudinal gradient which is an advanced tool for analysis of biodiversity for both floral [18] as well faunal communities [19-21].

The main objective of the current work is to analyze the effects of environmental indicators on plant species and diversity patterns along an altitudinal gradient in Lalkoo mountainous forests, Pakistan. Besides, our primary objective, we were also interested to focus on conservation and devise management guidelines to conserve the biodiversity of these mountainous forests. Lastly, we discussed some baselines for future management [22-23].

## 2. MATERIALS AND METHODS

The study was conducted in the Lalkoo mountainous forest (LMF) Swat District of Hindukush, Pakistan which stretch across the Himalayas and Karakorum Mountain ranges. Geographically Lalkoo area lies within the coordinates of 35°08′ 27.62″ N and 72°23′09.12″E with an altitudinal range from about 1581 m a.s.l. and reaches the highest peak of alpine pastures of 3849 m a.s.l. However, the present observations were assessed between 1970 and 3120 m.a.s.l. The famous visiting spot Gabin Jaba (locally means honey marshes) with an elevation of 2581m a.s.l., is also located in the research area. The annual temperature of the investigated area was recorded at 13.96 °C to 22.25 °C. The amount of relative humidity is maximum in July, August, and September having 73.55 %, 80 %, and 69 % respectively. The mean annual rainfall recorded in Lalkoo is 1777 mm. Maximum rainfall observed in July and August with a mean total rainfall of 228.9 mm and 220.9 mm while minimum rainfall occurred in November and December which is recorded at 42.4 mm and 78.4 mm (data source PMD; http://www.pmd.gov.pk).

We studied tree various species along an elevation gradient. The research region is categorized as moist temperate woodland and contains alpine, sub-alpine, and grassland zones. The basal area was calculated as (GBH)2/4pi, where pi 14 = 3.14. We also estimated the species' dominance. The basal area was measured as DBH ≥10 cm and 1.3 m above the ground, while regeneration (Seeding and Sapling) was measured at DBH <10 cm, and height >30 cm. All the trees inside the plot were counted, and their girths were determined using a measuring tap-adopting the method [24]. Slope, elevation, and aspect for each plot are recorded as well. Slope and aspect were measured by compass; elevation was measured by the altimeter.

### 2.1 Field survey

From June 2016 to December 2019, fieldwork was carried out. We picked six separate locations, referred to as Site-A, Site-B, Site-C, Site-D, Site-E, and Site-F (Figure 1), along the height on various hill-slopes from 54 sampled plots (quadrats) ranging in altitude from 1970 m to 3095 m (Table 1). Each hill-slopes was sorted into nine elevation zones/sections, and plots SIQ-I, SIQ-II, SIQ-III, SIQ-IV, SIQ-V, SIQ-VI, SIQ-VII, SIQ-VIII, and SIQ-IX were used to analyze them. The distance between two neighboring plots was preserved at least 115 m, and sampling plots were considered spatially isolated. The following are the predominant biological aspects of these Lalkoo mountainous forests (LMF) Swat District locations.

#### *2.1.1 Site-A*

This site starts near Shaheed Bela Khawar (SBK) located at 35° 08′ 45.95″ N and: 72° 23′ 17.47″ E (SIQ-I) and reaches to the peak of Barjo Sar (BS) located at 35° 09′ 25.30″ N and: 72° 25′ 19.59″ E(SIQ-IX) with 270° (W), 225° (W-S), 315° (W-N). The elevation range from bottom to top is 2125 m to 3045 m.

#### *2.1.2 Site-B*

This site starts near the entrance to Koz Lolkoo (KLK) is dominated by *Pinus wallichiana*. It is located on 35° 09′ 23.65″ N and 72° 23′ 21.37″ E, with elevation from 2200 m a.s.l., to 3120 m a.s.l., Famous small peak's local names are Bar Sange Sar, Tango Sar, and Oonani Sar. This site ends with Splo Sar (SPS) with a slope from 180° to 270° (S-W) along nine sampled plots.

#### *2.1.3 Site-C*

This site starts in the lower elevation range an area called Dunkacha (DK) and ends with the highest range Julba Sar (JS), which is affected by deforestation. The altitude of the area varies from 2000 m a.s.l., situated (35° 08′ 11.846″ N, 72° 23′ 28.34″ E) near to Bar Kale Lalkoo (BKL, DK) and reaches to forest Peaks of Julba sar (35° 09′ 04.34″ N, 72° 24′ 45.48″ E) with altitude 2920 m a.s.l., This site has slope ranging from 270 (W), 225 (W-S), and 45 (N-E) from SIQ-I to SIQ-IX. Famous site peak's local names are Gadro Dowl, Bata, and Char.

#### *2.1.4 Site-D*

This site has an extensive area with different undulating slopes and diverse vegetation types of trees like *Taxus wallichina* and *Aesculus indica* that are scattered in the thick vegetation of *Abies pindrow* and *Picea smithiana*. This site starts

from Kar Khawar (KK) in the lower altitude of 2175 m (35° 09′ 16.75″ N, 72° 20′ 59.96″ E) and continuous with the peaks of high altitude at 3095 m of Kapar Sar (KS). This situated between 35° 09′ 16.75″ N and 35° 09′ 39.83″ E). This site is almost East facing with aspect 90, while the only plots SIQ-II, SIQ-III, and SIQ-VI are 135 E-S facing.

### *2.1.5 Site-E*

This area has some cultivated land at lower altitudes, starting with the lower altitude of 1970 m, Dunkacha (DK: N 35° 08.489, E 72° 22.989), and reaches to sub-alpine zone dominated by *Quercus* spp. in the highest peaks called Landai Sar (LS: 35° 09′ 16.81″ N, 72° 21′ 06 .09″ E) with an altitude of 2890 m. All the aspects recorded at this site are East facing while SIQ-II and SIQ-IV are East-West with all the surveyed area.

### *2.1.6 Site-F*

This site starts from elevation with an elevation of 2000 m in the area of Lower Lalkoo (LL), located at 35° 08′ 44.44″ N and 72° 23′ 28.38″ E, and reaches high altitudes of 2920 m in peaks of Shalkho Sar (SS) located at 35° 09′ 12.32″ N and 72° 24′ 45.50″ E. The aspects W (270), W-N (315), and W-S (225) were unique to this site.

## 2.2 Data Analysis

We used general linear models (Using SPSS) and Microsoft Excel 2010 to examine the relationships of regeneration, species tree density basal area, and richness with elevation. To determine inter-correlations among predictor variables, we used Pearson correlations, which can cause collinearity effects, and fitted values were compared with standardized residuals for each significant predictor. Using Shannon's and Simpson's diversity indexes, we calculated plant biodiversity. The Shannon's index (H') was calculated as (H' = ΣPi*ln Pi), whereby, Pi is the significance value of a species as a proportion of all individuals. Simpson's Diversity Index was calculated using the formula C = ΣPi2, where C is Simpson's diversity and Pi is described previously [25].

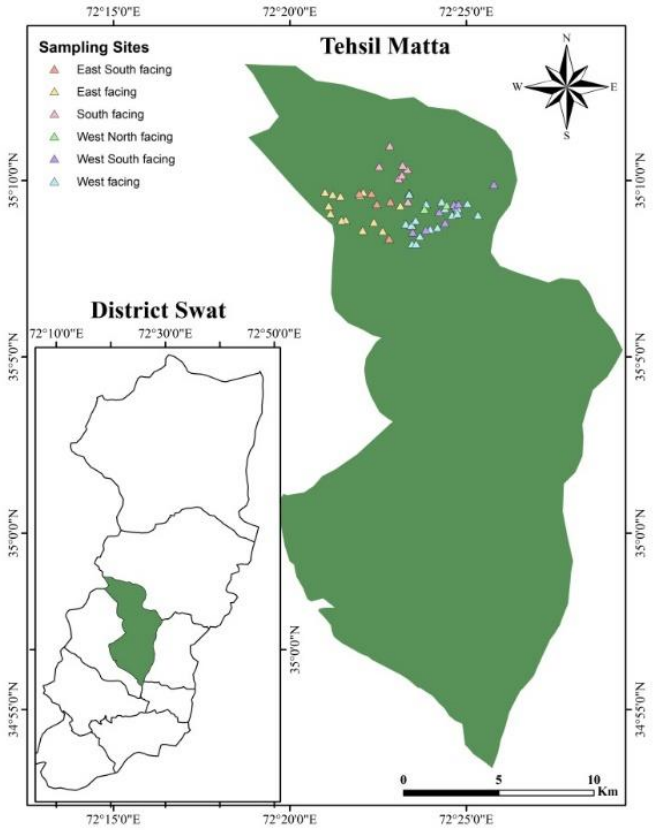


**Fig. 1.** Geographical locations of study sites

**Table 1.** Geographical details of study sites

| Sites Name | Latitude | Longitude | Elevation range | Slope range |
|---|---|---|---|---|
| Site-A | 35° 08'45.95" | 72° 23' 17.47" | 2125-3045 m | 225°-315° |
| Site-B | 35 09 23.65 | 72 23 21.37 | 2200-3120 m | 180°-270° |
| Site-C | 35 08 11.84 | 72 23 28.34 | 2000-2920 m | 45°-270° |
| Site-D | 35 09 16.75 | 72 20 59.96 | 2175-3095 m | 90°-135° |
| Site-E | 35 08.489 | 72 22.989 | 1970-2890 m | 176°-265° |
| Site-F | 35 08 44.44 | 72 24 45.50 | 2000-2920 m | 225°-315° |

We selected Quadratic regression using GLM models (better fit) for comparison of selected variables. We compared real species richness to null model predictions to determine the role of the mid-domain effect [26], in which domain bounds can also be characterized by soft and hard borders [27]. The mid-domain effect (MDE) concept of Colwell and Lees, [27] provides a straightforward and supplementary insight on species abundance gradients, that is effectively presented in a review of the literature. According to the Mid-domain effect (MDE) theory, the arbitrary allocation of species geographic ranges of varied sizes inside an area (or domain) constrained by hard bounds results in a peak in species diversity in the center of the domain [27]. We evaluated each species' range and reported it as the disparity between the lowest elevation of 1970 m and the greatest elevation of 3120 m along the species diversity gradient [27]. Throughout sampling, range size is frequently overlooked. Range sizes were also utilized to assess the robustness of the null model to ranging midpoints [28] with 95 % prediction curves relying on 1000 simulations at every 115 m elevational domain without substitution by empirical range sizes. Model fitness (GLM) was determined by regressing empirical species abundance against the average of modeled richness. We assumed that the sampling effort was equivalent at all locations (9 quadrats/site) and that as the number of people grows, so does the richness of the studied species. When contrasted to altitude, most of our data-set variations exhibit a hump-shaped association (see scattered plots) [28].

## 3. RESULTS

According to the current study, the largest peak of Shannon's-Wiener Index of diversity (H') was recorded between 2.043 (at 2315 m) to 4.334 (at 2575 m), indicating more species in an ecosystem, hence H' values of more than 2 have been recognized as a medium to high diverse (Table 2). Thus, the Lalkoo mountainous forest (LMF) has a high species variety, particularly at mid-height.

While the lowest value of the Shannon index was recorded at 0 .923 (2920 m) at Site-F(SIQ-IX). We recorded the highest value 1.74 of the Simpson index (D') at the elevation range of 2345 m, Site-C (SIQ-VI). However, the second maximum value across the total elevation gradient was recorded as D'=1.63(2690 m) and D'=1.61(2460 m) while the lowest value D'=0.2 was recorded consecutively in two sampling plots at Site-F (SIQ-VIII and IX) near the timberline at an altitude of 2805 m, and 2920 m, respectively. A such similar pattern of tree species in timberline area was reported by Rawal *et al*. [29]. We found characteristics decline in species density, basal area, and regeneration towards the end of high elevation across the altitudinal gradient. Regression between basal area and elevation reveals a strong quadratic association ($r^2=0.012$, $P<0.05$) as well as a hump-shaped relation with a peak around 2400 m. Nevertheless, regeneration has a very low $r^2$ value ($r^2=0.004$, $F=0.281$, $df=52$, $p=0.053$; slope=-0.000, intercept = 6.237) and a uniformly distributed motif (Fig. 2) close to the ecotone layer, but due to excess deforestation at lower altitudes, the capacity of regeneration is greatest due to more available space and light availability.

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We found characteristics decline in species density, basal area, and regeneration towards the end of high elevation across the altitudinal gradient. Regression drawn between basal area and the elevation shows a significant quadratic relation (r2=0.012, P<0.05) and shows a hump-shaped relationship with a peak at around 2400 m. However, regeneration shows a very low r2 value (r2=0.004, F=0.281, df=52, p=0.053; slope=- 0.000, intercept=6.237) and has with uniform distribution pattern (Figure 2) near ecotone layer however due to excess deforestation at the lower altitudes the capacity of regeneration is maximum due to more available space and light availability.

The tree's density shows a significant correlation with elevation (r2=9.00, p < 0.05, t=1.99: two-sample t-test). Maximum variation of trees density was observed at mid-elevation (2300-2600 m) in the mixed dominant zone of *Pinus wallichiana, Abies pindrow*, and *Picea smithana*) between 2300-2600 m. The response of species richness to elevation was modeled employing general linear modeling [31]. Species richness was negatively correlated with elevation showing quadratic model (r2=0.227, p>0.05, t=1.99, Intercept = 15.0938, Slope= -0.0039, F=19.11, t=6.55) that followed a hump-shaped maximum richness at 2400 m elevation.

Our analysis revealed that out of total species richness (710) the maximum species richness 18 % was analyzed for *Pinus wallichiana* (121) followed by 17 % by *Abies pindrow* (121), 11 % Picea smithiana (81) and 7 % for *Quercus dilatata*. The tree's density shows a significant correlation with elevation (r2=9.00, p<0.05, t=1.99: two-sample t-test). Maximum variation of trees density was

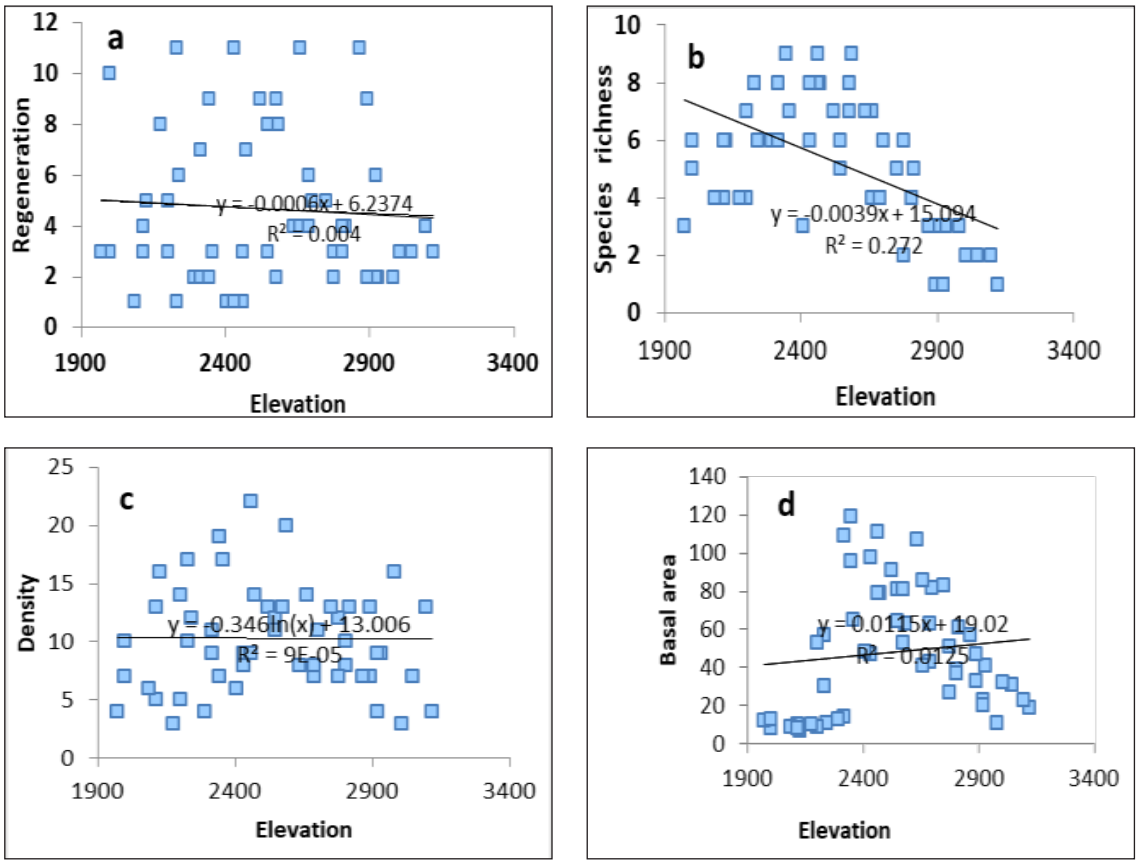


**Fig. 2.** Observed species richness and diversity parameters (a), Regeneration (b), Species richness (c), Density (d), Basal area of trees along an elevation gradient of Lalkoo, Hindukush Mountainous Range.

observed at mid-elevation (2300-2600 m) in the mixed dominant zone of *Pinus wallichiana, Abies pindrow* and *Picea smithana*) between 2300-2600 m. The response of species richness to elevation was modeled employing general linear modeling [31]. Species richness were negatively correlated with elevation showing a quadratic model (r2=0.227, p>0.05, t=1.99, Intercept= 15.0938, Slope= -0.0039, F=19.11, t=6.55) that followed a hump-shaped maximum richness at 2400 m elevation. Our analysis revealed that out of total species richness (710) the maximum species richness of 18 % was analyzed for *Pinus wallichiana* (121) followed 17 % by *Abies pindrow* (121), 11 % *Picea smithiana* (81), and 7 % for *Quercus dilatata* as shown in Figure 3.

Our results revealed that the maximum species richness in the range of 2200 to 2700 m, (having overlapping boundaries) shows mid-elevation peaks especially in species richness which is due to the increasing overlap of species ranges towards the center of the domain as the extent of elevation is bounded by highest and lowest elevations conforming mid-domain effect (MDE).

## 4. DISCUSSION

When GLM models for altitude and topography were compared independently, it was discovered that height is a stronger determinant of species richness then surface topography for all species and virtually all species groupings. Additionally, our findings reveal that species diversification, as measured by Shannon's index (H') and Simpson's index (D'), varies significantly in response to fluctuations in the altitudinal inclination in abundance (number of species) and constancy in the sampling sites.

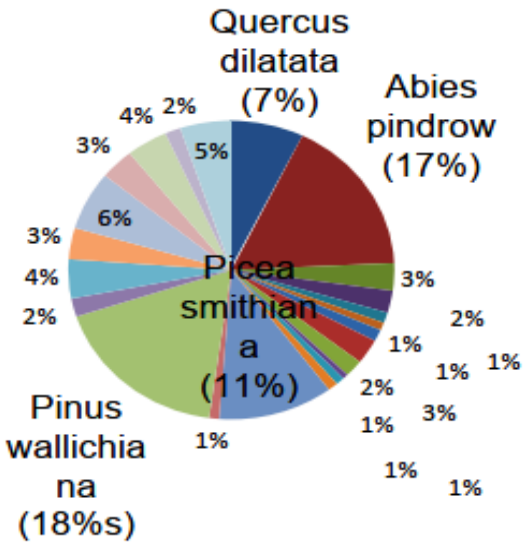


**Fig. 3.** Range size distribution of dominant trees observed along an elevation gradient in Lalkoo Swat.

Table 2. Results of simple linear regression of basal area, tree density, species richness, and regeneration with elevation gradient in Lalkoo forest.

| | Intercept | Slope | D.f | *F* | *P* | $R^2$ | *t* statistics |
|---|---|---|---|---|---|---|---|
| Trees basal area | 19.02 | 0.0110 | 52 | 0.35 | 0.001 | 0.012 | 0.81 |
| Tree's density | 11.45 | -0.0004 | 52 | 1.1E-05 | 0.05 | 9.001 | 1.99 |
| Species richness | 15.09 | -0.0039 | 52 | 19.11 | 2.71 | 0.722 | 6.55 |
| Regeneration | 6.24 | -0.0061 | 52 | 0.28 | 0.053 | 0.004 | 1.89 |

High values of Shannon's diversity were recorded H'=3.603 at Site-F (SIQ-II) with elevation 2115 m, H'= 3.591 at Site-F (SIQ-I) with elevation 2000 m, and H'= 3.860 at Site-F (SIQ-VII) with elevation 2690 m. The larger value of H' (>2) the greater will be species diversity of the ecosystem [32]. The high value of Simpson's index, D'=0.91 were analyzed in the elevation range of 2290 m, at Site-D (SIQ-II) and the lowest range of Simpson's index D'=0.3 were recorded at Site-F (SIQ-IX) in the lower elevation 2920m. Greater the value of Simpson's index (0 >1) represents no diversity. Our results obtained revealed the high biodiversity of trees in Lalkoo mountainous forest (LMF) Swat District of (the Hindukush range mountains). To ensure prolonged biodiversity, aspects of vegetation must be kept at many natural sizes, from genetic and species divisions to habitats and landforms [33].

We found significant correlation between saplings (DBH<10 cm, height>30 cm) and elevation (r2=0.005, p>0.05, t=1.948, Intercept=6.237, Slope=-0.0006, F= 0.2960). Across the elevation gradient higher sapling density (8-11) was observed in the lower elevation range of 2175 m a.s.l., to 2430 m a.s.l., which is due to factors like open area available to sapling due to severe deforestation and open canopy compared with a dense canopy that is probably due to light requirement of the seedlings. It is also revealed that seedlings are light-demanding require direct solar radiation and tolerate spring frost. Shrestha *et al*. [34] found that the seedlings grow best in a sunny position. However, Scott *et al*. [35] also reported that they best grow in shade. While frequently lower values of sapling density (<2 to >5) were recorded in the higher altitudinal gradient between 2660 to 3045 m. But frequent data available regarding sapling growth also revealed that besides temperature other environmental variables like slope, aspect, altitude, soil moisture are regarded as a significant predictor for sapling abundance. So, from our research analysis, it is obvious that sapling density decreased with altitude [35-37].

We also discovered that the area of altitudinal bands in Lalkoo increases steeply (from 2125 m) with increasing altitude and then decreases (to 2775 m), following a hump-shaped sequence of life-forms in the mid altitudes ranging with a peak about 2400 m. Species richness, as measured by the Shannon index, exhibited a hump-shaped dispersion to height [38-39]. Hump-shaped dispersion is typical in the Himalayas and other moist temperate forests [40-41].

The present investigation revealed that the highest species richness appears at intermediate altitudes and decreases monotonically with increasing elevation ranges [38]. On several occasions in the Himalayas and moist temperate forests like Lalkoo, a uni-modal dispersion sequence of vegetation types of abundance such as bryophytes, ferns, epiphytes, and species high prevalence of numerous animal communities were ascertained, indicating that this is the broad On Sense On elevational trend vigorously encountered in the research area [42].

At a broader level, the mid-elevation peak in plant diversity is influenced by a variety of factors such as soil factors, temperature, humidity, and the mid-domain effect (MDE), all of which are regarded as major indicators of the mid-elevation peak in plant species richness distribution patterns [43-45]. The mid-elevation peak in species richness was determined by randomly placing elevation slopes selected from a specified range size distribution in a geographic location with strict limits [43, 46]. It is further stated that while considering the function of the mid-domain effect, the organisms that occupy the same boundaries (hard and soft) must be examined jointly. Tree species richness diversity pattern in Lalkoo significantly deviated from MDE null model. Although the Lalkoo mountainous forest has ecological and floristic continuity with the greatest Hindukush mountainous ranges most of our study sites in the lower elevation ranges exhibit not climatologically or geographically hard boundaries so the deviation might be caused by the limitation to the applicability of MDE (modeled by mid-domain) within the observed area. It is also significantly highlighted that the degree of departure of the MDE peak from the empirical distribution implies that other factors such as climatic variables and evolutionary history factors play a significant role in interpreting the reported species abundance patterns. Despite the existence of numerous eco-physiological restrictions to vegetation types of growth beyond the timberline at higher elevations, the impact of these hurdles on tree species spreading is thought to be minor [47-49].

The present investigation revealed that MDE gives explanatory detail about tree species richness distribution patterns along elevation gradients [50-51]. According to the most recent MDE research, species borders that coincide along an altitudinal inclination promote increasing confluence of species ranges toward the center of a restricted geographical region [46, 52]. As a result, MDE forecasts a hump-shaped abundance of spatial variability in a mountainous environment like Lalkoo, with the highest diversity at mid-elevation ranges [26]. However, numerous studies have demonstrated that, in addition to MDE, the elevation trend of species richness can be influenced by area, size, climatic condition, and evolutionary history [51-53], which may provide better perspectives on the biodiversity distribution together across elevation gradients [54]. The total mean basal area 2538.33 (t-Test: Paired Two Sample for Means) was found between <7 ± >119 for trees with greater than 4 cm diameter at breath height. The tree basal area shows the highest peak at mid-elevation (at 2400 m) however maximum increase was observed from the 2315 m to 2775 m elevation range. The overall minimum basal area was found at 2125 m a.s.l., Site-A(SIQ-I) So this has been revealed that the lower value of basal area at lower altitudinal ranges is due to human interference like deforestation that affected most of the forest area in the lower altitudinal range near Lalkoo villages.

Human intervention is the primary source of an imbalanced environment; some estimations claim that between one-third and one-half of the earth's topography has been seriously altered by anthropogenic activities [54-55]. Due to the frigid climate patterns at higher altitudes, most tree species have a restricted elevation range size, resulting in a decrease in the basal area at higher elevations. Some tree species have a narrow elevation range along elevation slopes in mountainous forests [18].

## 5. CONCLUSION

The present study revealed that Lalkoo mountainous forest (LMF) Swat reserves has a reasonably good tree species pattern along an altitudinal gradient. Visual observations of the forest area in its lower altitudes towards higher altitudes show four distinct vegetation overlapping zones that is Blue–Pine zone dominated by Pinus wallichiana for which maximum tree species diversity was observed between 2125 to 2520 m a.s.l., . Fir – Spruce zone is dominated by Picea smithiana and Abies pindrow that shows maximum diversity between 2635 to 2750 meter, Spruce –Oak zone is dominated by Abies pindrow, Quercus semicarpifolia, Taxus wallichina and Picea smithiana in some sites that shows maximum diversity between 2865 to 2920 m a.s.l., and also Alpine–Sub-alpine flora that include mostly scrubby vegetation formed by Juniferous communis that is observed above 3300 m.

The regeneration potential of the sapling is found maximum of 10,11/Unit sample in the lower elevation range2000 m a.s.l., and 2230 m a.s.l., which is due to the high availability of free canopy space due to heavy deforestation near Lalkoo villages. The elevation pattern of species richness followed a uni-model (hump-shaped) pattern peak was at 2400 m a.s.l., Tree density was not uniform along the elevation gradient however high species density were found at around 2600 m a.s.l., Maximum basal area was observed in the mid-elevation range of 2500 m show a peak of quadratic pattern. At mid-elevation range across the elevation, gradient found maximum tree species diversity [56] which corresponds to theory of the mid-domain effect. The study suggests that the distribution pattern of tree species is greatly regulated by altitude and climatic factors [56], however factors like past historical factors that are not evaluated in the present investigation also play important role in Hindukush mountainous range forest Lalkoo Swat.

## 6. DECLARATION

I declare that: (i) the results are original; (ii) the same material is neither published nor under consideration elsewhere; (iii) approval of all authors has been obtained, and (iv) in case the article is accepted for publication, its copyright will be assigned to Pakistan Academy of Sciences. Authors must obtain permission to reproduce, where needed, copyrighted material from other sources and ensure that no copyrights are infringed upon.

## 7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 93-97 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)759

Pakistan Academy of Sciences

Research Article

# New Distributional Record of *Urentius hystricellus* (Richter, 1870) (Hemiptera: Tingidae) from Southernmost Region of Punjab, Pakistan

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**Abstract:** *Urentius hystricellus* is well known phytophagous and invasive true bug of family Tingidae. From Pakistan, this family is poorly studied despite having significant economic importance. Present species was identified with the help of most relevant and published literature. Specimens were mounted on triangular cards for morphological studies. New distributional data of *U. hystricellus* is included. Brief diagnosis, host plant and remarks on biology and current distribution in Pakistan are added. Line drawing of adult and fore wing along with digital photographs are also given.

**Keywords:** Lace bug; Invasive species, Southern Punjab, Distribution

## 1. INTRODUCTION

Family Tingidae is mainly distributed in tropical and temperate regions with approximately 2600 described species across the world [1, 2]. The tingid fauna of Pakistan is poorly investigated. So far, twelve species have been described based on both material and literature records from Pakistan [3, 4]. Members of this family also known as Lace bugs and have been reported as pests of various cultivated and ornamentals [5, 6]. Many species of lace bugs have been documented as oligophagous in nature but few species described as polyphagous pests associated with several plant families [2]. Association between plant and lace bugs results in the form of plant injuries such as gall formation and leaves staining which also leads to stunting plant growth and significant economic losses [1, 7]. Morphologically these bugs can be recognized by the following characters; small body size (5-6 mm in length), pronotum triangular, backwardly extending over scutellum; head with lateral expansions of the thorax; wings usually with the pattern of elevated ridges and sunken membranous throughout; antennae four segmented; tarsi one or two segmented; ocelli absent [8-12].

Approximately 300 genera belonging to the family Tingidae have been described worldwide; of which 8 genera were recorded from Pakistan [4,13]. Genus *Urentius* is one of the small and widely distributed all over the world [2]. Members of this genus can be separated from other genera by the combination of the following characters;

Received: September 2022; Accepted: December 2022
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whole body with spinules; head depressed laterally; pronotum with three rows of carinae, middle carina longest; wings with 1-2 series of spines on outer margin; tarsi two segmented [14]. Several studies based on exploration, description and distribution of Tingid fauna investigated that, a total of 7 species of genus *Urentius* were described from Old World, 5 from Africa and Southern Palaearctic region, one from Australia and 2 species from Oriental region [2]. However, little information is available on the tinged fauna of Pakistan with twelve described species in several genera [3, 4]. Moreover, all species belonging to genus *Urentius* have been observed in association with economically important plant and caused serious damage. In present study, we have recorded one species *Urentius hystricellus* (Richter, 1870) infesting brinjal plantation for the first time from Southern Punjab of Pakistan.

## 2. MATERIALS AND METHODS

Various insect collection tours were conducted during 2021-22 in district Rahim Yar Khan of Punjab province. During these tours specimens of lace bugs associated with brinjal plantations were collected through aspirator. All specimens were collected and preserved in 75 % ethanol. Collected materials were identified using most relevant and available literature likewise [1,10,15,16]. Diagnostic characters of these tinged were observed with the help of NOIF XSZ 107 BN stage microscope. Micrographs were prepared using Amscope 18-megapixel camera attached to the same microscope. Helicon focus software was used for stacking images. Stacked pictures were cleaned with the help of Adobe Photoshop. Hand drawing of adult, and fore wing was performed manually. Identified specimens were deposited in the Department of Entomology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan. Distribution Map of studied taxon has been drawn with the help of ArcGIS software.

## 3. RESULTS AND DISCUSSIONS

### 3.1 *Urentius hystricellus* (Richter, 1870)

**Host Plant:** *Solanum melongena* L.

**Global distribution:** Israel, Tanzania, Zimbabwe, Uganda, Yemen, Zambia, Botswana, Ghana, Kenya, Mozambique, Namibia, Nigeria, South Africa (Transvaal) and USA [2], Sudan [16], Thailand, Africa, Egypt, Senegal, Ethiopia, Niger, Uganda [17], and India [18].

**Distribution in Pakistan (Fig. 1):** Pothwar: Rawalpindi, Islamabad, Chakwal, Jhelum [4]; South Punjab: Liaqatpur, Khanpur, Rahim Yar Khan, Sadiqabad (Current study).

**Systematics account:** *Tingis hystricellus* Richter, 1870: 84; *Urentius echinus* Distant, 1903b:134; *Urentius olivaceus* Distant, 1909c:115; *Urentius aegyptiacus* Bergevin, 1930a:18.

**Material examined:** Liaqatpur (Agricultural land) (28.9394°N′70.94874°E), 23-vi-2021, 07♂ and

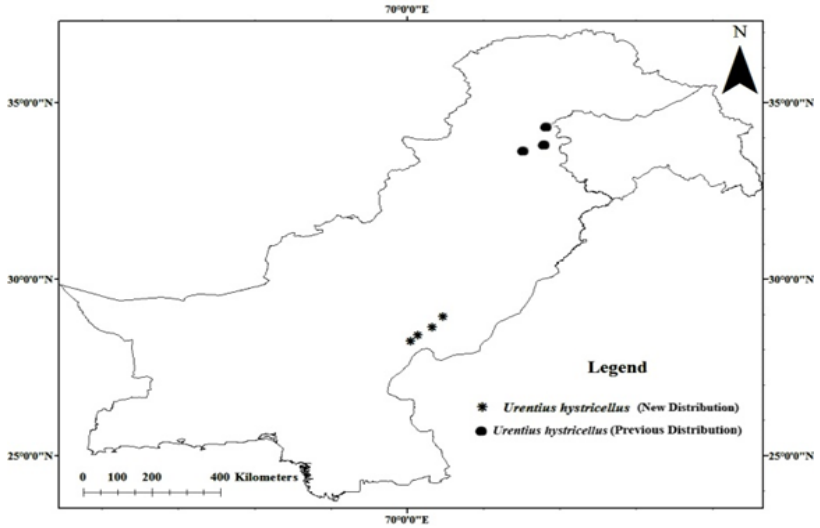


**Fig. 1.** Distribution map of *Urentius hystricellus* (Richter, 1870) in Pakistan

05♀; Khanpur (28.63318°N′ 70.65737°E), 05-vii-2021, 04♂ and 03♀; Rahim Yar Khan (Cropped area) (28.42116°N′70.29887°E), 12-vii-2022, 02♂ and 03♀; Sadiqabad (28.24696°N′70.09795°E), 15-viii-2022, 01♂ and 02♀.

**Diagnosis:** Body pale ochraceous dorsally; with distinctly long spines along lateral aspect (Fig 2A, Fig 3A). Head with three spines (Fig 2B, Fig 3A). Antennae dark brownish; small, having small row of 15-18 setae in overall view (Fig 2D); apical segment thicker comparatively; basal segment sub globose; second antennal segment elongated and triangular (Fig 2D); pronotal sheath covering head, posterior margin of sheath reaching toward ocular margin dorsally; pronotal disc carinate irregularly (Fig 2A, Fig 3A), inner margin with continuous single row of cell running toward costal margin; outer margin with series of 11-12 long spines conspicuously (Fig 3A). Wings hyaline (Fig 2C); costal margin broad. Double rows of cell in hemelytron costal area (Fig 2C, Fig 3B); slight brownish patches present on wing. Legs with a number of small, conspicuous setae; tibia and tarsi dark brownish comparatively in overall view.

## 4. CONCLUDING REMARKS

*Urentius hystricellus* was found in association with brinjal plants and caused chlorotic spotting appearance on leaves. High infestation of this pest leads in dryness and reduces the beauty of plant. According to the latest published records, *Urentius hystricellus* (Richter, 1870) has been considered

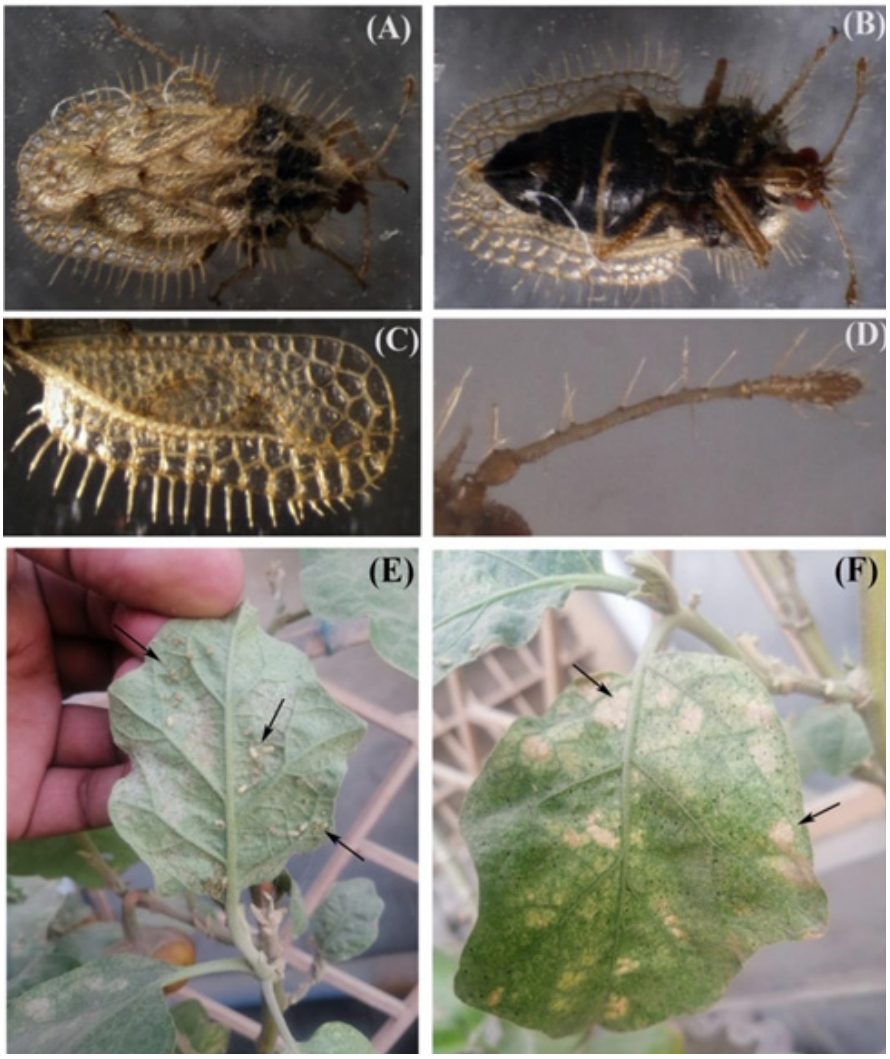


**Fig. 2.** *Urentius hystricellus* (Richter, 1870); (A) Habitus dorsal view (B) Habitus ventral view (C) Forewing (D) Antenna (E) Live specimen on brinjal leaf (F) Damaging symptoms

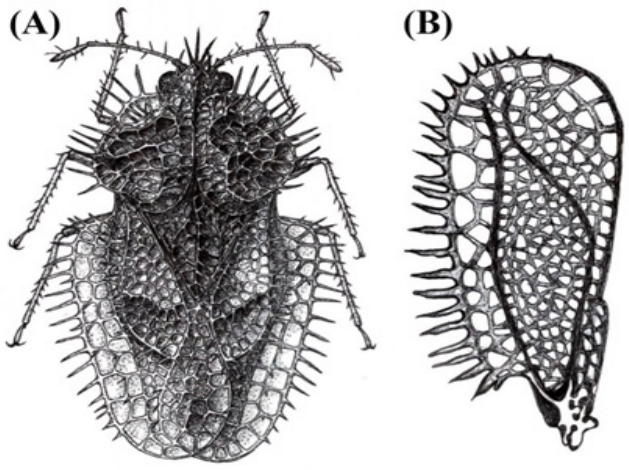


**Fig. 3.** Line Drawing of *Urentius hystricellus* (Richter, 1870)
(A) Habitus in dorsal view (B) Fore wing

as invasive species of family Tingidae with wide range of distribution across the world [2]. However, this species was only recorded from Pothwar region of Pakistan. In present study, we have also noticed the distribution of *U. hystricellus* in southernmost region of Punjab.

## 5. ACKNOWLEDGEMENTS

We are grateful to the Department of Entomology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan on provision of necessary facilities for prepration of digital images of studied materal. We are immensely thankful to anonymous reviewers for their constructive comments.

## 6. CONFLICT OF INTEREST

The authors declared no conflict of interest

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*Proceedings of the Pakistan Academy of Sciences: B Life and Environmental Sciences* 59(4): 99-113 (2022)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(59-4)811

Pakistan Academy of Sciences

Research Article

# Assessment of Soil Chemical Properties for Monitoring and Maintenance of Soil Fertility in Probolinggo, Indonesia

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**Abstract:** Soil is paramount to sustaining living in biomass production, water quality control, climatic mitigation, and biodiversity endurance. Closely associated with sustainable agriculture, it degrades soil in the long run, robbing the soil of its production capacity and food-generating ability. In Probolinggo, a regency in Indonesia, intensifying the use of chemical fertilizers and pesticides yet a declining trend in yield production was discovered. This research analyzed the acid, nitrogen, organic carbon, and nutrients focusing on phosphor, potassium, iron, and manganese contents. Organic carbon/nitrogen ratio, soil organic compound rate, and cation exchange capacity were also discussed in order to illustrate the correlations among chemical substances and their roles in soil and plant maintenance. While such a study has yet to be performed in Probolinggo, the results should show the degree of land deterioration and future attempts at damage control and correction open to facilitate. Employing a simple random method, soil and plant samples were collected from 18 villages in six districts and their chemical contents were compared to the standard set in Government Regulations No 150/2000. The results showed low N-total, P-Bray, P-Olsen, K, C-Organic, and C/N ratio availabilities at 0.18, 13.88, 14.41, 0.37, 1.36, and 7.38 respectively, contrasted to high rates on pH (5.94), Fe (153.46 mg $kg^{-1}$) and Mn (37.96 mg $kg^{-1}$). Biomass production is conclusively imperative to fix the land composition and meet the plant nutrient requirements through an organic approach; fertilizers from digester biogas are therefore recommended. This action requires field agricultural advisors to raise awareness of sustainable agriculture.

**Keywords:** Environmentally Friendly, Organic Approach, Soil Deterioration, Soil Fertility Evaluation, Sustainable Development Goals, Sustainable Farming

## 1. INTRODUCTION

A natural body as the result of complex biogeochemical and physical processes, soil is imperative in sustaining the living [1, 2]. Not only limited to plant substrate, but its role also expands as far as an exponential part of biodiversity by regulating the water cycle and nutrients [3, 4]. Vastly contributing to the ecosystem, soil is crucial to sustainable development goals (SDGs) concerning biomass production, water quality control, climatic mitigation, and biodiversity endurance [5, 2]. Quality soil holds the potential to solve hunger and poverty issues while retaining robust health [6, 7] key to welfare, making it the most valuable asset of a nation [4].

Received: November 2022; Accepted: December 2022
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While soil health is associated with sustainable agriculture [8, 9], it degrades soil in the long run due to supplying food for the ever-increasing human population [10, 11], robbing the soil of its production capacity and food-generating ability [11, 12]. Agricultural practices deprive soil properties [2, 13] have been reported in Uruguay after an agricultural production period between 2012 and 2014 [14], in Iran after forestland conversion to tea plantation [15], and in Italy where food self-sufficiency plummeted to less than 80 % [12].

Out of various agricultural practices and land exploitations, monocropping is one of the most detrimental methods toward soil quality [16–18]. Another cause is low irrigation water quality [19, 20]. Pesticides are also liable for affecting microorganisms [21, 22], particularly in enzymes entailing carbon, nitrogen, sulphur, and phosphor cycles [23, 24] While fertilizers may be helpful, excessive use of chemical fertilizers reduce soil biodiversity and function [25, 26] by triggering soil acidification and soil crusting that suppress the growth of organic matters [2, 27, 28].

Running soil fertility evaluation (SFE) should be feasible to assess the degree of soil deterioration and its result should help in determining the appropriate biomass treatment for each condition – some countries like Nigeria [29], the Philippines [30], and Indonesia [31, 32] have based their fertilizer application guides on it. Analyzing the chemical properties of soil – e.g. acid, organic carbon, nutrients – is one of the attainable approaches.

Soil deterioration is a serious issue in Indonesia; since the start of modern agricultural practices in the 1970s, biomass-producing areas have been exploited all year long. In addition to monocropping, excessive pesticides on rice production in Java has been reported [33] – a certain site applied up to 24.6 kg of pesticides $ha^{-1}$ $yr^{-1}$ [34], and farmers in another locale violated the recommended amount of chemical pesticides [35]. The preliminary survey in Probolinggo Regency, a regency in the province of East Java, Indonesia, discovered intensifying use of chemical fertilizers and pesticides yet a declining trend in yield production.

Soil quality studies had been performed in a number of areas in Indonesia, among them are of apple plantations [36], cassava fields [37, 38], paddy fields [39, 40] and post-paddy fields [41], oil palm plantations [2, 42–46], rubber [47], sugarcane [48–50], even volcanic soils [51] and drylands [52–54]. A few researchers had also compared different fields at once in Bengkulu [55] and West Java [56]. However, not one of the such studies had been run in Probolinggo Regency. The aforementioned researches mostly put N, P, and K contents as their parameters – only some of them discussed about the C-organic content, pH rate, and micronutrient availability. Further, studies on Mn, Fe, SOC, and CEC contents in Indonesia are scarce. The case in the surveyed area calls for a more thorough soil quality study for biomass production to detect any occurrence of soil deterioration. In addition to the prevalently analyzed factors, the infrequently examined ones are put into consideration. Further, in the soil, the chemicals N, P, and K contained in a number of local commodities are also scrutinized.

## 2. MATERIALS AND METHODS

### 2.1 Location

Conducted in Probolinggo Regency, East Java, Indonesia, in 2020, a number of six districts – with three rural villages in each to make 18 in total – were randomly appointed to represent the regency: Krucil District (Krucil Village, Bremi Village, and Betek Village), Tiris District (Ranu Agung Village, Tiris Village, and Andungsari Village), Gading District (Mojolegi Village, Wangkal Village, and Kaliacar Village), Pajarakan District (Karangpranti Village, Pajarakan Kulon Village, and Sukomulyo Village), Krejengan District (Kedung Caluk Village, Sumber Kalimoho Village, Seboro Village), and Kraksan District (Semampir Village, Sidopekso Village, and Kregenan Village) (Figure 1). The GPS details for the six districts are listed in Table 2.

Each district has its type of soil. While Pajarakan is of regosol and alluvial, Kraksaan soil is dominated by alluvial with a small part of andosol. Krejengan has equal portions of regosol and alluvial. Regosol takes most of Gading, leaving a little for andosol. Tiris is mostly regosol with a latosol touch, and Krucil is mostly regosol with bits

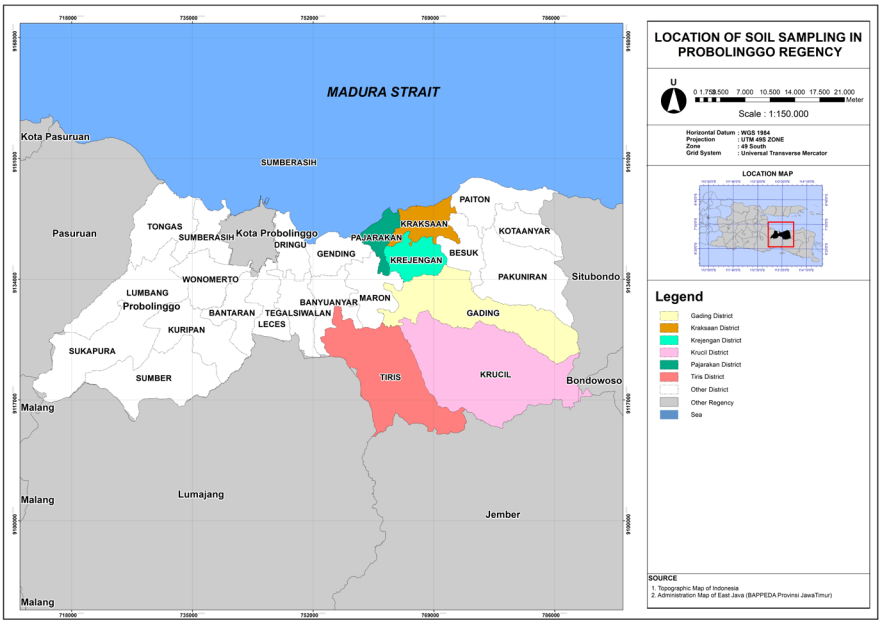


**Fig. 1.** Location soil sampling (Krucil District, Tiris District, Gading District, Pajarakan District, Krejengan District, and Kraksan District)

of latosol dan andosol.

Average rainfall from 2015 to 2020 at six soil sampling locations as follows: Pajarakan (119 mm), Kraksan (144 mm), Krejengan (155 mm), Gading (108 mm), Tiris (133 mm), and Krucil (78 mm). According to Schmidt-Ferguson, the climate types and Q are Pajarakan (140.00 % - E), Kraksan (120.00 % - E), Krejengan (120.00 % - E), Gading (83.33 % -D), Tiris (133.64 % - E), and Krucil (100.00 % - D). As a complement, cropping system data at six research locations are Pajarakan (1x paddy, corn), Kraksan (1x paddy, corn), Krejengan (1x paddy -100 %, 2x -30 % paddy, corn, soybean), Gading (paddy 1x), Tiris (paddy 1x), and Krucil (paddy 2x).

Referring to Government Regulation No. 150/2000 that the solum critical benchmark is ≤ 20 cm, only six locations are ≥ 50 cm. As no soil crust was encountered, all districts were considered secure at this point. A series of the preliminary survey was conducted to identify soil characteristics in each village. Then, soil and plant samples were gathered for chemical analyses.

## 2.2 Materials

Soil and plant sample selection were of simple random sampling, taken from three different sites in each village at any time during the research period. Once a plant was chosen, the area around it was leveled and cleaned from grass and rubbles, then dug as deep as 5 cm to 20 cm to take the soil sample. A lead tube was probed into the ground with the help of a wooden block until three-quarters part of it was buried. Another tube was pressed inside the first one at 1 cm deep to compact the dirt and separated it. The tube was then dug out of the ground along with some extra dirt to protect it from direct contact with the shovel. Excessive dirt on the upper end of the tube was carefully leveled and then covered with a plastic lid. The lower end received the same handling. The top lid was then labeled with information on gaining depth, date, and location.

## 2.3 Methods

Both soil and plant were tested for their acid (pH), organic carbon (C-organic), and nutrient (N, P, and K) contents. Acidity was measured with a pH meter. Organic carbon content was assessed on Walkey and Black method. As for nutrient content, N was calculated by employing the Kjeldahl method and P by Olsen and Bray, while K, Fe, and Mn with Atomic Absorption Spectrophotometry (AAS).

All results were then compared to the parameters set by the Indonesian government as declared in the

Government Regulations No 150/2000 on Land Deterioration Management for Biomass Production [57] – except for Fe which went with the standard set by the Soil Research Center of the Ministry of Agriculture [58] – to determine the land condition as listed in Table 1.

**Table 1.** Soil fertility scales based on chemical aspects

| | Scale | | |
|---|---|---|---|
| | **Low** | **Medium** | **High** |
| N-total | 0.2 | 0.2 to 0.5 | 0.5 |
| P-Bray2 (ug $g^{-1}$) | 20 | 20 to 40 | 40 |
| K (me 100 $g^{-1}$) | 0.3 | 0.3 to 0.6 | 0.6 |
| Mn (me100 $g^{-1}$) | 0.2 | 0.2 to 0.6 | 0.6 |
| C-organic (%) | 4 | 4 to10 | 10 |
| Fe (mg $kg^{-1}$) | 3 | 5 | 9 |

Source: [57, 58]

Organic carbon/nitrogen ratio (C/N ratio), soil organic compound (SOC) rate, and cation exchange capacity (CEC) were also discussed to illustrate the correlations among chemical substances and their roles in soil and plant maintenance.

## 3. RESULTS AND DISCUSSIONS

### 3.1 Chemical Properties in Soil

The chemical properties in soil represent its ability to provide balanced nutrients for plants. The test results are recorded in Table 2 below.

### 3.2 Acidity and CEC

Affecting a vast number of biochemical processes in soil, pH is the master variable [59]. With an average of 5.94 in the depth of 0 cm to 25 cm, the general acidity level is within the critical benchmark rates between 4.5 and 8.5. The soil of eight villages came out quite acidic at 5.91, while four villages were neutral to fairly neutral at 7.02. In four villages, the soil conditions were passable despite being acidic at 5.11 since their N, P, K, Mn, and Fe contents were enough to nourish plants.

The fertilizer administration in the study area is deduced to be the reason for low soil pH in some sampling sites. Ammonium fertilizers run through the nitrification process to form nitrate. In such process, the released $H^+$ ions turn the soil more acidic [59, 60]. Phosphorus and potassium can decrease soil pH after years of use [61]. Years of land use for agriculture typically increases $H^+$ ions which, consequently, accelerate soil acidification [62]. Plants grown in acidic land are prone to stresses, such as H and Mn poisoning and nutrient deficiency [59].

Acid also affects microbial diversity in soil [63, 64] and its presence indicates nutrient availability [66]. Neutral pH expands CEC, optimizing P and other nutrient contents in soil [6, 22, 66]. Out of all samples, the lowest rate was 10.49 me $gr^{-1}$ while the highest was 55.06 me $gr^{-1}$ – the ones with high CECs were taken after fertilizer application.

### 3.3 Organic Carbon Content, SOC, and C/N Ratio

While the C-organic should be at least 4 %, the average rate in samples was 1.35 % with the lowest quantity of 0.62 and the highest of 2.67 – these figures characterize badly-damaged soil. This finding corroborates an earlier report that more than 77 % of paddy fields in Java had low organic carbon [26, 67]. That there is inadequate vegetation due to monoculture in the research sites and that farmers barely recover soil biomass are deduced to be the reasons [2, 49, 50].

C-organic content reflects the amount of soil organic compound (SOC), another fertility factor [2, 68] and the most vital component resulting from interactions among producer, decomposer, and mineralogy [60, 70], attributable to its ability to improve soil chemical, physical, and biological characteristics [10, 28].

SOC is the key indicator of soil quality and agricultural sustainability [71]. SOC content relies heavily on nitrogen binding in soil [2, 67]. The SOC test results came out between 1.07 % and 4.62 % for all 18 samples, and seven samples reached the level of as low as 1.65 %. SOC is associated with vegetation type, hydrology, and organic matter.

Referring to the low SOC rate, it is proven that cultivation and land management can cause it, leading to land damage in the long run [71]. Disproportionate use of chemical pesticides and fertilizers suppresses the growth of nutrient-producing biota and, consequently, diminishes

**Table 2.** Soil chemical properties of 18 villages in six districts in Probolinggo Regency

| District | Village | pH $H_20$ | pH KCl. | C-organic (%) | N-total (%) | C/N (%) | SOC (%) | P-Bray (%) | P-Olsen (%) | Available K (%) | CEC me $gr^{-1}$ | Fe mg $kg^{-1}$ | Mn mg $kg^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pajarakan | Karang Pranti | 6.1 | 5.2 | 1.22 | 0.16 | 8 | 2.11 | 22.49 | - | 0.17 | 55.06 | 238.10 | 36.70 |
| 7° 46' 17.458" S 113° 22' 35.044" E | Pajarakan Kulon | 6.9 | 6.2 | 1.76 | 0.20 | 9 | 3.04 | - | 5.79 | 0.29 | 51.27 | 92.50 | 51.27 |
| | Sukomulyo | 6.6 | 5.7 | 1.14 | 0.15 | 8 | 1.97 | - | 7.96 | 0.17 | 53.75 | 265.7 | 53.75 |
| Kraksaan | Semampir | 6.7 | 6.0 | 1.39 | 0.17 | 8 | 2.41 | - | 15.12 | 0.48 | 50.18 | 142.90 | 28.72 |
| 7°45' 30.42" S113°23'46.46"E | Sido Pekso | 7.9 | 7.0 | 1.09 | 0.14 | 8 | 1.89 | - | 28.77 | 0.33 | 43.40 | 47.60 | 37.77 |
| | Kregenan | 6.4 | 5.7 | 1.13 | 0.14 | 8 | 1.95 | 16.93 | - | 0.47 | 48.12 | 190.50 | 47.95 |
| Krejengan | Kedung Caluk | 6.1 | 5.2 | 0.75 | 0.11 | 7 | 1.29 | 19.59 | - | 0.60 | 36.58 | 263.70 | 17.58 |
| 7° 48' 16.081" S 113° 25' 5.077" E | Sumberkatimoho | 6.5 | 5.7 | 0.62 | 0.12 | 5 | 1.07 | 37.69 | - | 0.49 | 39.61 | 190.50 | 11.03 |
| | Sebaroh | 5.6 | 4.9 | 1.31 | 0.22 | 6 | 2.26 | 16.22 | - | 0.31 | 38.44 | 142.90 | 18.64 |
| Gading 7°50'39.7" S 113°26'02.2" E | Mojolegi | 5.6 | 4.8 | 1.57 | 0.19 | 8 | 2.72 | 13.56 | - | 0.06 | 10.49 | 92.50 | 31.83 |
| | Kaliacar | 5.2 | 4.8 | 1.39 | 0.23 | 6 | 2.41 | 8.70 | - | 0.58 | 20.99 | 190.50 | 42.38 |
| | Wangkal | 6.0 | 5.2 | 1.47 | 0.19 | 8 | 2.54 | 3.84 | - | 0.46 | 34.37 | 47.60 | 17.47 |
| Tiris | Ranu | 5.0 | 4.4 | 0.94 | 0.15 | 6 | 1.63 | 9.25 | - | 0.40 | 24.19 | 238.10 | 58.25 |
| 7°94'75402" S 113° 39'4. 2622" E | Agung | 5.5 | 4.5 | 1.41 | 0.21 | 7 | 2.43 | 8.78 | - | 0.08 | 15.12 | 142.90 | 22.16 |
| | Andung Sari | 4.9 | 4.4 | 1.02 | 0.14 | 7 | 1.76 | 2.95 | - | 0.60 | 32.51 | 95.20 | 50.93 |
| | Tiris | | | | | | | | | | | | |
| Krucil 7°56'35,4"S 113°28'29,8"E | Betek | 5.2 | 4.5 | 1.26 | 0.15 | 8 | 2.18 | 2.16 | - | 0.57 | 26.91 | 47.60 | 60.56 |
| | Krucil | 5.6 | 5.0 | 2.26 | 0.24 | 9 | 3.91 | 10.97 | - | 0.28 | 26.38 | 190.50 | 43.39 |
| | Bremi | 5.2 | 4.9 | 2.67 | 0.38 | 7 | 4.62 | 21.18 | - | 0.37 | 27.10 | 142.90 | 52.85 |
| Average | | 5.94 | 5.23 | 1.36 | 0.18 | 7.38 | 2.34 | 13.88 | 14.41 | 0.37 | 35.25 | 153.46 | 37.96 |
| Compare Standards of Table 1 | | | | Very low | Low | | | Very low | | Medium | | Very high | Very high |

SOC [72]. Supporting the finding, applying both chemical and organic fertilizers for years in China is effective in enhancing SOC [63]. Sufficient fertilizer – combined with cattle manure – should encourage SOC content [71] and tackle the issue.

A well-balanced C-organic and nitrogen contents support microorganisms in decomposition [48, 72, 73] and the C/N ratio indicates the speed of the process [2, 74]. From the average ratio of 7.38 %, it is conclusive that the soil is not damaged. However, further treatments should be undergone to prevent it from declining – compost application is needed when the SOC drops to 11 %, and land relaxation is required if it reaches 15 %.

### 3.4 Nutrient Content

From Table 2, it is derived that N-total values are 0.11 % to 0.38 %, averaging 0.18 %, which is below the standard of 0.2 %. It is similar to the N-total in post-paddy fields in Labuhanbatu, Indonesia [41]. Samples from Sebaroh, Kaliacar, Andung Sari, Krucil, and Bremi Villages are medium, and none are high. The non-existent high N-total value corroborates another soil analysis on several agricultural lands in West Java [56] and adds urgency to the call for management to enhance nitrogen content in agricultural fields throughout Java recommended by a previous study [26, 75]. An essential nutrient for plants [24, 76], nitrogen determines plant growth [32, 77] and therefore significantly influential towards harvest. Nitrogen is assimilated by the plant into amino acid, the main component of protein [78] that is responsible for forming chloroplast, mitochondria, and other structures where biochemical processes in plants occur. Nitrogen is also the key element of nucleic acid, serving as genetic matter, that controls plant growth and sustainability [79]. Nitrogen deprivation leads to stunting in plants [80], so an optimal supply of nitrogen should maximize production [81].

A few features are viewed to be the causes of low N-total content in this study. The fact that tropical lands naturally contain less nitrogen has been proven by contrasting Java with Japan [75] since higher temperature accelerates organic matter decomposition [2, 28]. Improper use of pesticides in agricultural practice not only eradicates nitrogen-generating bacteria [21, 83] but also restrains the work of enzymes in the nitrogen cycle [23, 24].

As for phosphate, the contents of (2.16 to 37.69) ug $g^{-1}$ – averaged at 6.30 ug $g^{-1}$ – are far lower than even the low P-Bray standard of 20 ug $g^{-1}$. The low P contents in samples are linked to high acidity [32]. Samples from Karang Pranti, Sumber Katimoho, and Bremi are regarded as a medium, and none is high. This outcome validates the report on low P contents in West Java [56], although the same study also informed about the several sites containing high P. It contradicts the high P contents found in post-paddy fields in Labuhanbatu [41] apparently due to much P fertilizers added in every sowing period. Research held in Bogowonto Catchment, Central Java, noted higher P content in agricultural lands than in non-agricultural ones due to human intervention through fertilizers [84].

Another component in nucleic acid forming [79], along with nitrogen, phosphorus is valuable for plants [76] in ensuring sustainability for its roles in cell multiplication, respiration, and photosynthesis. Its importance spreads to energy storage and distribution via adenosine diphosphate (ADP) and adenosine triphosphate (ATP) formation [80, 85]. Energy gained from photosynthesis and carbohydrate metabolism is stored in the form of phosphate compound and later used in growth and reproduction [86], which makes this substance crucial in seed formation and root expansion [87].

The potassium (K) contents in samples span between 0.06 and 0.60, averaged at 0.17, which is below the benchmark of 0.3. Out of all tested villages, nine are medium and one is low and six are very low. A leachable material, K is easy to get washed away. It is often bound to clay and other organic substances, and it mostly gets absorbed when attached to fine soil particles. As such, K is prone to erode to rain or wind exposure [88]. Soil typically erodes more in harder rain [89] and harder wind [90]. The climate of the study area is indicated to be a cause of low K content in some sites. Another consideration is the shallow irrigation canals. Since the deeper a canal is, the more water there will be and the more K+ ions will be absorbed [91], adding depth to those canals should help to answer the problem. Furthermore, the low potassium content is observed to be the effect of high CEC, which fortifies soil in keeping the substance within and decelerating its release. Some areas with high K contents are due to agricultural manipulation. However, continuous and excessive

use of fertilizers can wane potassium in soil [92].

A macro monovalent compound in soil, K is necessary for plant physiological processes [76] despite being excluded from basic organic compound formation since most enzymes require it as an activator [93], including protein synthesis in producing ATP [80]. K also supports the energy translocating process to various parts of the plant [93], regulates the opening and closing of stomata [80], and even strengthens the plant to resist diseases and stress [94, 95].

Iron contents are recorded at between (47.6 and 265.7) mg $kg^{-1}$, averaging at 153.45 mg $kg^{-1}$, which is high above the standard. Three villages are high and 19 are very high. Similarly, manganese contents extend over (11.03 to 60.5) mg $kg^{-1}$, averaged at 37.95 mg $kg^{-1}$ – five villages are high and 13 are very high. In addition to redox potential and pH content [96], the intensive use of inorganic fertilizers for decades has left residual buildups of Fe and Mn in soil. Mn helps to transport Fe throughout the plant [97] in binding phosphate for growth [98], and only a small amount of < 1 000 mg $kg^{-1}$ is required to activate protein-forming enzymes. While too little Fe and Mn lead to micronutrient deficiency [99, 98], too much of them is malicious for plants [100, 101].

The farmer's behavior in using pesticides is also influential in its production [23, 25]. In regards to controlling pests, diseases, and weeds, pesticides (as well as herbicides) have been reported to add nutrients to the soil, including Fe and Mn [102, 103]. However, their use must be controlled so as not to cause damage to the soil and the environment [21–28, 33, 35]. Several researchers, among others [28, 104], suggest using biomass ash to increase micronutrients, i.e. potassium, calcium, and magnesium. The referring to local wisdom is also recommended by some researchers [24, 83, 105–107], while the application of biological fertilizers is suggested by other researchers [22, 54, 66].

### 3.5 Chemical Properties in Plants

Ensuring nutrient adequacy in the soil is key to satisfying crops. The contents of N, P, and K towards cassava, coffee, corn, paddy, tobacco, shallot, chili, and melon as local commodities were studied and then administered in Table 3.

The research reveals the N-total values of plants are ranged between 0.81 % and 3.86 %, while P is 0.03 % and 0.64 % and K is 0.4 % and 4.01 %. Conclusively, the dominant weight of the plant biomass is nitrogen, followed by phosphate and calcium respectively.

Comparing the results of the soil test (Table 2) with the plant test (Table 3), it is certain that the N and K contents in the soil are lower than the ones in the plant while the P content is higher. That plant requirement on P is lower than on N and K should be the reason. Furthermore, P is not easily soluble in water, causing it to progress slowly, since most of it is inorganic. Paddy, cassava, corn, tobacco, melon, and chili absorb higher percentages of N and K, draining them from the soil.

This study has revealed the low content of several important substances in soil. Further action should be carried out in Probolinggo Regency by decomposing all organic waste and kitchen waste into organic fertilizer, which result is then returned to agricultural lands. The decomposition of organic matter should be done anaerobically in a communal or household scale digester [108–110]. A biogas digester as such doubles the benefit for society as well as the environment by providing clean, renewable energy [111, 112] and producing two types of organic fertilizers, i.e., liquid and solid. Ideally, this biogas digester should be installed with inlet pipes from excrete disposal to septic tanks in each household [99–101]. Since there is a possibility of decomposition fluctuation due to various feedstocks, several researchers recommended a two-stage digester to overcome the problem [113–115].

Moreover, the farmers in Probolinggo should be educated to prevent excessive land management. Tilling aims to loosen the soil in preparation for seeding [116] so that the plants' roots will be able to penetrate the soil and respire more easily. However, too much handling wears soil organic matter away faster [117]. Land handling limitation in organic agriculture is the potential to improve the total stock of soil organic matter [118].

**Table 3.** N, P, and K contents in cassava, coffee, corn, paddy, tobacco, shallot, chili, and melon samples in Probolinggo Regency

| Location | Commodity | N – Total (%) | P (%) | K (%) |
|---|---|---|---|---|
| ***Pajarakan District*** | | | | |
| Karang Pranti | Corn | 1.62 | 0.29 | 3.85 |
| Pajarakan Kulon | Paddy | 2.18 | 0.22 | 1.89 |
| Sukomulyo | Melon | 3.86 | 0.64 | 3.53 |
| ***Kraksaan District*** | | | | |
| Semampir | Paddy | 0.81 | 0.11 | 2.72 |
| Sido Pekso | Tobacco | 2.47 | 0.12 | 3.19 |
| Kregenan | Tobacco | 1.92 | 0.16 | 3.20 |
| ***Krejengan District*** | | | | |
| Kedung Caluk | Tobacco | 2.56 | 0.16 | 4.01 |
| Sumberkatimoho | Shallot | 3.37 | 0.16 | 2.49 |
| Sebaroh | Paddy | 1.07 | 0.35 | 1.20 |
| ***Gading District*** | | | | |
| Mojolegi | Paddy | 1.24 | 0.20 | 0.93 |
| Kaliacar | Chili | 3.74 | 0.21 | 3.89 |
| Wangkal | Corn | 0.82 | 0.03 | 0.40 |
| ***Tiris District*** | | | | |
| Ranu Agung | Cassava | 3.61 | 0.25 | 0.65 |
| Andung Sari | Coffee | 2.34 | 0.16 | 1.31 |
| Tiris | Cassava | 2.09 | 0.16 | 1.32 |
| ***Krucil District*** | | | | |
| Betek | Cassava | 2.05 | 0.07 | 0.65 |
| Krucil | Coffee | 2.35 | 0.11 | 1.57 |
| Bremi | Cassava | 2.31 | 0.30 | 2.74 |

## 4. CONCLUSION

The low nitrogen and C-organic contents and medium-to-high acidity underline the general deficiency of agricultural lands in Probolinggo Regency. As for nutrients, the low contents of nitrogen, phosphor, and calcium and the high content of manganese depict the imbalance in their soil production and plant consumption. This calls for biomass production to fix the land composition as well as meet the plant nutrient requirements through an organic approach. Involving organic fertilizers is therefore recommended, especially sludge and slurry from anaerobic digestion. Further research to map out land deterioration for biomass production is expected to run in other areas so that proper treatments can be performed in damaged soil and preventive steps can be taken to avoid it from happening. This action requires the role of field agricultural advisors to make people aware of sustainable agriculture.

## 5. ACKNOWLEDGEMENTS

The author wishes to extend his gratitude towards the Regent, the Rural Development Planning Agency, the Rural Agriculture Office, and the Environmental Agency of Probolinggo Regency. The author would also like to thank the Institute of Research and Community Center of Brawijaya University.

## 6. CONFLICT OF INTEREST

The authors declared no conflict of interest

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3. R.K. Robert, and C.R.L.Thompson. Forming patterns in development without morphogen gradients: differentiation and sorting. *Cold Spring Harbor Perspectives in Biology* 1(6) (2009).
4. D. Fravel. Commercialization and implementation of biocontrol. *Annual Reviews of Phytopathology* 43: 337359 (2005).


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