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Isolation of Plant Growth-Promoting Bacteria from Hilly Areas of Sheikh Badin, Khyber-Pakhtunkhwa Province, Pakistan

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Abstract

Plant growth-promoting bacteria are playing pivotal role in agriculture field and such organisms are the main focus of the researchers. This study was designed to explore the diversity of bacteria from the Sheikh Badin hilly areas of Khyber-Pakhtunkhwa, Pakistan. Total twelve (12) bacterial strains IOM-1 to IOM-12 were isolated using culture dependent method. The isolated bacterial strains were able to grow on T5 medium, R2A agar, TSA, and nutrient agar media. Optimum growth was recorded at 30-45 °C and at pH 7.0-7.2. All the isolated organisms were Gram-stain-

negative, aerobic and small rod-shaped. Strains were positive for oxidase and catalase activities. Sequence similarities of 16S rRNA gene showed, that isolated strains were belonging to the genus Microvirga (97.93-100 %) and the closest neighbors were M. jeongseonensis, M. terrestrus, M. calopogonii, M. splendida, M. guangxiensis, M. vignae, M. tunisiensis and M. zambiensis. According to phylogenetic analysis result, isolates were forming eight clades with their closest reference type strains. Organisms IOM-6, IOM-8, IOM-10, IOM-11, and IOM-12 were found to have nitrogen fixation capabilities, which revealed that these isolated bacterial strains will play an important role in plant growth promotion.

Keywords: Plant-Growth-Promoting Bacteria (PGPB), Sheikh Badin hilly areas of KPK, Pakistan, Microvirga, 16S rRNA gene, Phylogenetic analysis

INTRODUCTION

Plant-growth-promoting bacteria (PGPB) are integral components of the soil ecosystem and are important for maintaining plant variety, soil fertility, and nutrient cycling (Ramakrishna *et al.*, 2019). Plants benefit from a variety of interactions between microbes, and themselves. Microorganisms live and feed on plants, and in return, these microbes release chemicals that improve plant development, stress tolerance, and defense against hazardous infections (Schirawski *et al.*, 2018). They enhance development of plants by solubilizing nutrients, stabilizing nitrogen and generating growth hormones, suppressing diseases, improving soil structure and aiding nutrients uptake (Hayat *et al.*, 2010). Plant-Growth-Promoting Bacteria have several benefits for plant growth, such as improved yield, germination, root formation, and drought resistance (Karadayi *et al.*, 2016). *Bacillus, Pseudomonas, and Streptomyces* species also produce antibiotics and used to control crop diseases, while *Azospirillum, Bacillus and Pseudomonas* are commonly used in bio-fertilizers (Gómez-Godínez *et al.*, 2023).

Nitrogen-fixing bacteria reside within plant tissues, such as tubers and roots, as well as the interfere face between rhizosphere and soil, they play a crucial role in providing substantial quantities of mineral nitrogen essential for plant growth (Lazali *et al.*, 2017; Timmusk *et al.*, 2017). The process of nitrogen fixation has been observed both in symbiotic bacteria and non-symbiotic bacteria. Nitrifying bacteria, which enhance cereal crop productivity, have been identified belonging to the following genera: *Azotobacter,* Azospirillum, *Burkholderia, Beijerinckia, Clostridium, Gluconacetobacter, Herbaspirillum, Paenibacillus*, and *Methanosarcina* (Ladha *et al.*, 2016)

Zinc solubilizing bacteria such as Bacillus, Acinetobacter and Thiobacillus have the potential to solubilize insoluble source of zinc into soluble form like Zinc oxide (Zno), and enhance plant growth through improving zinc availability (Hussain et al., 2015; Sukhwal et al., 2022). Phytohormones; Abscisic acid, salicylic acid, gibberellic acid, Indole acetic acid (IAA), auxins, cytokinins and ethylene increases resistance against unfavorable conditions (Khan et al., 2020; Mohite, 2013).

Phosphate solubilizing bacteria also playing important role in crop production (Teng et al., 2019; Zhu et al., 2011). Actinomycetes and beneficial fungi also playing their role in the availability of potassium for the plants (Baba et al., 2021). Similarly, the role of Phosphate is also worth mentioning in plant metabolic activities in arid and semi-arid environmrnts (Billah et al., 2019). Zinc solubilizing bacteria play their important role in plant growth promotion (Sunithakumari et al., 2016).

The secondary metabolite, hydrogen cyanide (HCN) produced by bacteria protect the host plants against infections. The genera Rhizobium, Pseudomonas, Alcaligenes, Aeromonas and Bacillus species producing hydrogen cyanide (Sagar et al., 2018). Siderophores are produced by microorganisms, which play pivotal role to improve plants growth through availability of iron ions (Ahmed & Holmstrom 2014). Polysaccharides are also important for the plant development and help in resistance against stress conditions especially in drought (Fadiji et al., 2022). EPS-producing bacterium, Pantoea alhagi NX-11 helps the rice plants growth in drought condition (Sun et al., 2020).

Keeping in view the pivotal role of beneficial bacteria (Figure 1) in agriculture sector, the following study was designed to achieve the following aims and objectives.

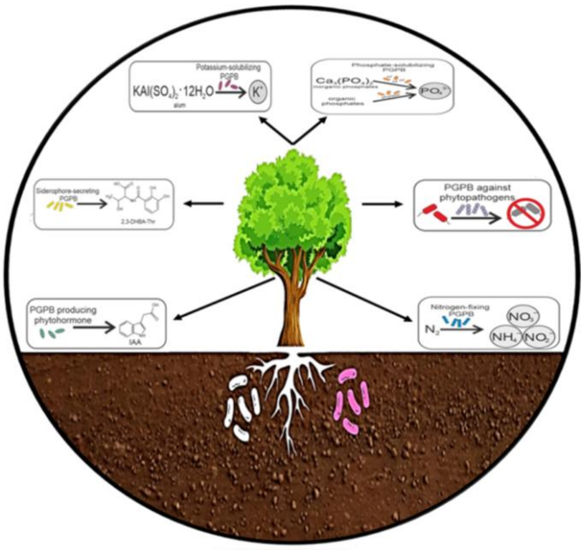

Figure 1: Numerous soil plant growth-promoting bacteria have a positive effect on plant growth and development through their ability to fix nitrogen from the atmosphere, make phosphates and potassium soluble, produce phytohormones and siderophores, and show resistance to plant diseases.

The current aimed to Isolation and Identification of bacterial strains from the soil of hilly areas in Sheikh Badin, Khyber Pakhtunkhwa, Pakistan.

MATERIAL AND METHODS

STUDY AREA AND SAMPLE

To know the diversity of PGPB, which help in promotion of plant growth, soil samples were collected from the Sheikh Badin hilly areas (24° 39' 20.5920'' N 68° 50' 14.0712'' E), Khyber Pakhtunkhwa Province of Pakistan (Figure 2).

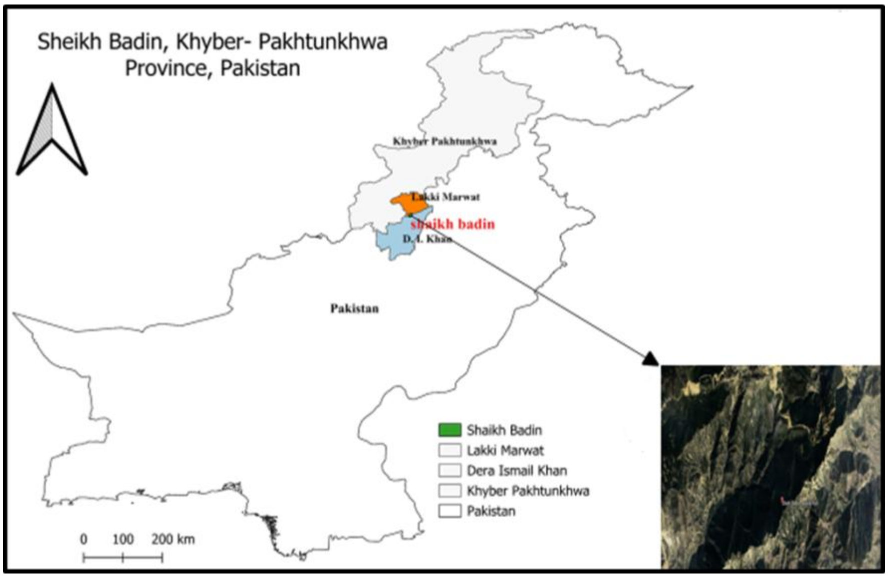

Figure 2: The map showing the location of bacterial isolates in the present study

The five soil samples collected from different sited were brought to Institute of Microbiology, Faculty of Veterinary and Animal Sciences (FVAS), Gomal University Dera Ismail Khan in sterilized bags and stored at 4 °C until further process. Bacteria were isolated by culture dependent method and identification by morphological, physiological and molecular techniques (Figure 2).

SAMPLE COLLECTION AND PROCESSING

Samples were collected from the soil of hilly areas of Sheikh Badin. Weight 5 grams of each soil sample. The soil samples, each weighing 5g, were subjected to agitation in a flask filled with sterilized water (50 mL) and multiple glass beads. This agitation occurred at a temperature of 37 °C for duration of 1 hour, with the flask being rotated at 200 revolutions per minute (r.p.m.). Bacterial strains were isolated on the following culture media after spreading 100 uL each sample at 25-45 °C and pH 6.0-9.0. To prepare T5 medium, first,

dissolve 1 g glucose; 2 g yeast extract; l g lotus extract; 0.5 g peptone and 1 g of $CaCO_3$ in 1000 mL of sterilized water. Once all components are dissolved, adjust the pH to 7.0-7.2 using an appropriate acid or base solution. After pH adjustment, add 20 grams of agar to solidify the medium. Then, autoclaved the culture medium at 121 °C for 25 minute before use. For preparing 1000 mL of nutrient agar we added beef extract; 3 g, peptone; 10g, agar: 18 g into1 L water and pH 7.0-7.2.

R2A agar medium with compositions (g L^{-1}): yeast extract, 0.6; glucose, 0.6; peptone; Casamino acid, 0.6; K_2HPO_4, 0.3; MgSO4. $7H_2O$; sodium pyruvate, 0.3; 0.05; agar, 18; pH, 7.2. The Tryptic soy agar medium consist of casein 17.0 g, soybean meal 3.0 g, Sodium chloride 5.0 g, dextrose 2.5g, dipotassium hydrogen phosphate 2.5 g, agar 15.0 g, pH 7.0-7.2.

All the isolated bacterial taxa were identified based on their morphological, physiological, and genomic characteristics. Oxidase activity was checked through the oxidation of Tetra methyl-p-Phenylene diamine, following the method used by (Kovacs 1956). Catalase activity was determined by addition of a drop of 3 % (v/v) H_2O_2.

RESULTS AND DISCUSSION

ISOLATED BACTERIAL STRAINS

The twelve (12) bacterial strains with ids; IOM-1, IOM-2, IOM-3, IOM-4, IOM-5, IOM-6, IOM-7, IOM, 8, IOM-9, IOM-10, IOM-11, and IOM-12) were isolated from soil collected from Sheikh Badin hilly areas Khyber Pakhtunkhwa Province of Pakistan. These isolated strains were routinely cultivated on T5 medium, R2A agar, TSA, and Nutrient agar. T5 agar medium was the best one for isolation of most of the bacterial strains (Figure 4.1). Growths were observed at temperature 25-45 °C (Figure 4.2), and at pH ranges of 6.0-9.0 (Figure 4.3). All the isolated organisms were short-rod shaped and Gram-stain-negative. They were positive for catalase and oxidase activities. The detail growth characteristics are provided in Table 4.1.

Organisms IOM-6, IOM-8, IOM-10, IOM-11, and IOM-12 were found to have nitrogen fixation capabilities, and were forming blue zone on malate medium without nitrogen after incubation at 37°C up to 24 h. Result revealed that isolated strains will play pivotal role in plant growth promotion.

Table 4.1: Isolated strains growth recorded at different temperatures and pH ranges

Isolated Strains	Temperature Range (25-45°C)	pH Range (6.0-9.0)
IOM-1	30-40°C (optimum 37°C)	6.5-8.5 (optimum 7.2)
IOM-2	35-40°C (optimum 37°C)	6.5-7.5 (optimum 7.0)
IOM-3	25-37°C (optimum 33°C)	6.0-9.0 (optimum 7.2)

IOM-4	30-40°C (optimum 37°C)	6.5-8.5 (optimum 7.2)
IOM-5	25-37°C (optimum 33°C)	6.0-9.0 (optimum 7.2)
IOM-6	30-45°C (optimum 37°C)	6.0-7.5 (optimum 7.0)
IOM-7	25-37°C (optimum 33°C)	6.0-9.0 (optimum 7.2)
IOM-8	30-42°C (optimum 37°C)	6.0-9.0 (optimum 7.2)
IOM-9	25-37°C (optimum 33°C)	6.0-9.0 (optimum 7.2)
IOM-10	30-37°C (optimum 37°C)	6.8-8.0 (optimum 7.0)
IOM-11	30-40°C (optimum 37°C)	6.5-8.0 (optimum 7.0)
IOM-12	30-40°C (optimum 37°C)	7.0-8.5 (optimum 7.2)

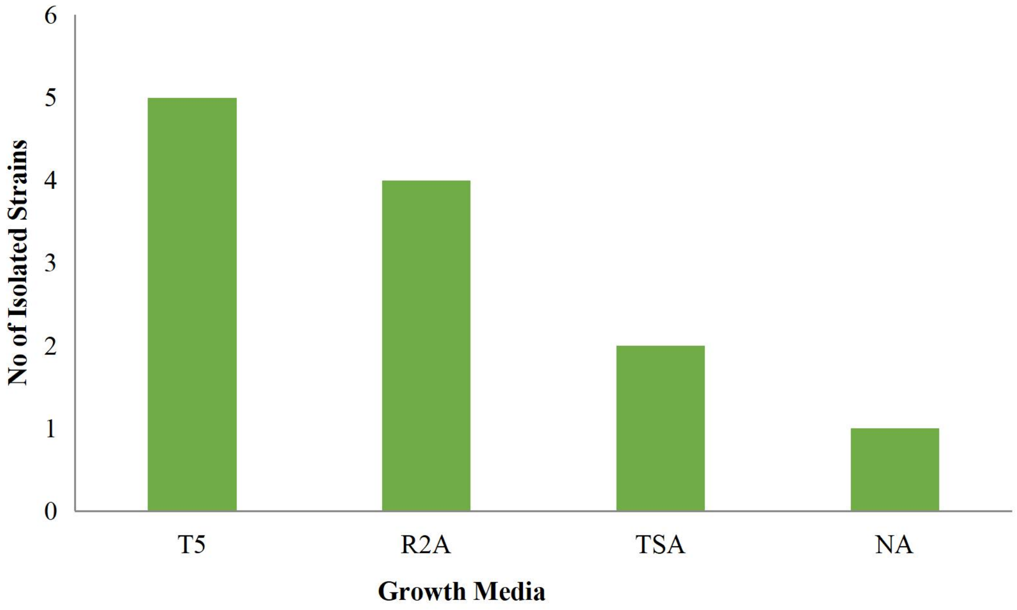

Figure 3 Number of isolates grown on different culture media

Table 4.2: 16S rRNA gene based sequence similarities of isolated strains (IOM-1, IOM-2, IOM-3, IOM-4, IOM-5, IOM-6, IOM-7, IOM-8, IOM-9, IOM-10, IOM-11, and IOM-12) with the closest strains in the genus Microvirga. Source of isolation and NCBI accession numbers are also provided for the isolated strains.

No	Isolated Strains	Closest Strains	Source of Isolation	Similarity 16S RNA gene (%)	NCBI Accession Numbers
1	IOM-1	M. terrestris BT290^{T}	Sheikh Badin	99.73%	PP724371
2	IOM-2	M. splendida BT325^{T}	Sheikh Badin	99.72%	PP724375
3	IOM-3	M. jeongseonensis BT688^{T}	Sheikh Badin	98.93%	PP724382
4	IOM-4	M.terrestris BT290^{T}	Sheikh Badin	100%	PP724386
5	IOM-5	M.jeongseonensis BT688^{T}	Sheikh Badin	99.68%	PP724387
6	IOM-6	M. calopogonii CCBAU 65841^{T}	Sheikh Badin	99.16%	PP724391
7	IOM-7	M.jeongseonensis BT688^{T}	Sheikh Badin	99.69%	PP724396
8	IOM-8	M.guangxiensis CGMCC 1.7666^{T}	Sheikh Badin	98.61%	PP724399
9	IOM-9	M.jeongseonensis BT688^{T}	Sheikh Badin	99.71%	PP724402
10	IOM-10	M.vignae BR 3299^{T}	Sheikh Badin	97.93%	PP724403
11	IOM-11	M. tunisiensis LmiM8^{T}	Sheikh Badin	99.74%	PP724407
12	IOM-12	M. zambiensis WSM3693^{T}	Sheikh Badin	99.17%	PP724410

DISCUSSION

In recent studies, the ever-growing global population has put significant strain on agricultural resources, leading to increase dependence on synthetic composts, pesticides, and development controllers to satisfy heightening harvest need (Hossain et al., 2023).

Over use of chemicals and pesticides are common practices to boost crop yields but have negative effects on soil health and biodiversity. The misuse of agro-chemicals impact on soil fertility, alter soil pH, deplete essential nutrients, and disrupt microbial communities impact on soil and reduced biodiversity, pesticides and fungicides can accumulate in soils and harming beneficial insects, birds and mammals (Adedibu, 2023).

Plant-Growth-Promoting Bacteria (PGPB) play a significant role in global bio-geochemical cycles, including regulating the dynamic of organic matter decomposing, and as well as they enhance development of plants by solubilizing nutrients, stabilizing nitrogen and generating growth hormones, suppressing diseases, improving soil structure, aiding nutrients uptake and increase productivity (Hayat et al., 2010). Diazotrophic, Azospirillum, Herbasspirillum, Gluconacetobacter, Azotobacter, Bacillus, Actinomycetes, Pseudomonas, Klebsiella, Enterobacter, Alcaligenes, Burkholderia, Pennisetum and Serratia species have been isolated from crops playing pivotal role in plant growth promotion (Sajjad et al., 2001; Zhang et al., 2022) Therefore, use of microbial inoculants of PGPB for the development of sustainable agricultural production is becoming a more extensively accepted practice in intensive agriculture in many parts of the world (Sezen et al., 2016). Microvirga species have been isolated from different environments playing their role in plant growth promotion. Microvirga species have the ability to fix nitrogen. M. lupini, M. zambiensis, M. lotononidis, M. guangxiensis, M. vignae, M. tunisiensis, and M. calopogonii are considered as symbiotic nitrifying bacteria (Li et al., 2020).

Nitrogen is essential nutrients for the production of amino acids, protein, nucleic acids etc and stone fruits trees, vegetables, and grains require an adequate annual supply for proper plant growth and productivity. For example tuber crops contribute 3.9 % to the global population's energy requirements. Specifically, sweets, potatoes account for 1.5%, Cassava for 1.9%, and Yams, and other tuber crops collectively for 0.3 %. This process convert N2 into inorganic nitrogen compound like ammonia (NH_3) enhancing the growth and yield of sugar cane, sorghum, maize, wheat, and cucumber (Zhang et al., 2022). Total 12 bacterial strains were isolated (IOM-1-IOM-12) forming clades with Microvirga strains. Some of our isolated strains have positive nitrogenase activity and form clades with nitrogen-fixing species in the phylogenetic tree.

CONCLUSION AND FUTURE PERSPECTIVES

This study report diversity of bacterial strains belonging to the genus Microvirga from hilly soil area of KPK, Pakistan. Morphological and phenotypic characteristics were used to identify bacteria. In order to probe in to their ancestry phylogenetic inference based on 16S rRNA gene sequences was performed to classify Microvirga strains up to species level. All isolates were belonging to 8 species of Microvirga. Isolated strains were short-rod shaped and catalase positive and oxidase positive. Optimum growth was recorded at 30–45 °C and at pH 7.0–7.2.

Furthermore, isolated organisms IOM-6, IOM-8, IOM-10, IOM-11, and IOM-12 were found to have nitrogen fixation capabilities. Such isolates can be used as bio-inoculant to enhance the crops productivity can be used as bio-fertilizers. These bacteria can substitute chemical fertilizers use, and can indirectly reduce the environmental pollution caused by the extensive use of

chemical fertilizers. Through the whole genome sequencing we can probe to the genes responsible for nitrogen fixation and production of secondary metabolites (plant growth hormones) responsible for plant growth promotion. So, we can explore the hidden capabilities of isolated strains from such extreme environments.

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