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PGPR Isolated from rhizospheric soil of *Zanthoxylum armatum* DC. in Garhwal Himalaya

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ABSTRACT
The goal of this report was the identification of PGPR from *Zanthoxylum armatum* DC. A total of 11 genera were identified from rhizosphere of *Z. armatum*. A total of 32 isolates produced Indole acetic acid ranging from 0.69 µg/ml to 28.21 µg/ml, highest IAA being produced by *Pseudomonas* sp. A total of 28 (63.63%) bacterial isolates produced ammonia. Qualitatively, 21 (47.72%) isolates belonging to the *Pseudomonas* sp., *Bacillus* sp., *Flavobacterium* sp., *Klebsiella* sp., *Enterobacter* sp., *Serratia* sp. and *Azotobacter* sp genera displayed positive results towards phosphate solubilization and only 20 (45.45%) isolates exhibited positive results of siderophore production on CAS agar medium. The present study hence, provides a potential biofertilizer however, field studies of this isolate as soil inoculants in *Z. armatum* DC. are required in order to establish its actual performance.

Keywords: Rhizobacteria, PGPR, Biofertilizers, Plant Growth Promoting Activities.

1. Introduction
Modern agriculture relies on high input of agrochemicals which cause major environmental problems [1]. Feeding an increasing human population and reducing the impacts on the environment urges for low input agricultural practices. The rhizosphere [2] of plants is a zone of intense microbial activity, and some bacteria from this zone, termed rhizobacteria exhibit active root colonization in the presence of the existing native micro flora. Rhizobacteria that exert beneficial effects on plant development are referred to as PGPR [3]. Microorganisms are important in agriculture in order to promote the circulation of plant nutrients and reduce the need of chemical fertilizers as much as possible [4, 5, 6]. Exudations of the photosynthate from the plant roots provide a preferred ecological niche for the growth of microorganisms. The bacteria which are associated with plant roots and help in plant growth promotion are called Plant growth-promoting rhizobacteria (PGPR) [7]. Plant growth-promoting bacteria [8] were defined as free-living soil [9]. The mechanisms by which PGPR promote plant growth are not fully understood [10] But several mechanisms have been suggested by which PGPR can promote plant growth, including phytohormone production like indole acetic acid, gibberellic acid, cytokinins and ethylene [11] enhancing stress resistance, nitrogen fixation, stimulation of nutrient uptake and biocontrol of pathogenic microorganisms [12, 13, 14, 15] increasing the supply or availability of primary nutrients to the host plant [16] the synthesis of antibiotics, enzymes and fungicidal compounds [17, 18], solubilization of mineral phosphates and other nutrients [19] nitrogen fixation [20], antagonism against phytopathogenic microorganisms by production of siderophores [21], antibiotics and cyanide [22]. The strains mainly from genera such as *Pseudomonas, Azospirillum, Burkholderia, Bacillus, Brevibacillus, Enterobacter, Rhizobium, Erwinia, Serratia, Alcaligenes, Arthrobacter, Acinetobacter* and *Flavobacterium* have been reported so far. PGPRs have been shown to enhance plant growth significantly in different families of plant of commercial and agricultural importance [4].Till now no major studies regarding the effect of PGPR on medicinal plants has been undertaken.
Demand for medicinal plant is increasing in both developing and developed countries due to growing recognition of natural products such as herbal medicines and herbal supplements, being non-toxic and having no side effects and affordable prices. Herbal medicines are used to treat many conditions such as asthma, chough, cholera, toothache, eczema, premenstrual syndrome, rheumatoid arthritis, migraine, menopausal symptoms, chronic fatigue, irritable bowel syndrome and cancer, etc. Habitat loss and deforestation coupled with over harvesting has resulted in dwindling population of important medicinal plants around the world. So, direct extraction of

natural products from wild medicinal plants to satisfy the current requirement is fast becoming an unrealistic goal.

One of the most important medicinal plants is *Zanthoxylum armatum* DC. with a lot of benefits. The bark, fruits and seeds of *Z. armatum* are extensively used in indigenous system of medicine as carminative, stomachic and anthelmintic. The fruits and seeds are employed as an aromatic tonic in fever and dyspepsia. An extract of the fruits is reported to be effective in expelling round worms. Because of their deodorant, disinfectant and antiseptic properties, the fruits are used in dental troubles, and their lotion for scabies. They are also used to ward - off houseflies [23] Recently, there is a growing interest in PGPR due to their efficacy as biological control and growth promoting agents in many medicinal crops [24] Hence, the present work was aimed to find the alternative approaches like investigating indigenous PGPR for the presence of plant growth promoting traits and to select those PGPR which can be used for increasing the growth and yield of medicinal plants.

2. Material and Methods

2.1 Site description of soil sampling

Soil samples from the rhizosphere of *Zanthoxylum armatum* DC were collected from different altitudinal sites in different localities of Garhwal Himalaya between elevational ranges of 500 to 2000 meters. First site was Srinagar valley (30°12' 52. 92 N, 078° 48' 30.37 E) at 500-1000 meters (Devalgarh, Bugani, Badiyaargarh), second was Pauri adjoining areas (30°12' 37.25 N, 078°50'33.38 E) at 1000-1500 meters (Khanda, Dobhsrikot, Premnagar) and third site was Kedarnath wild life sanctuary (KWLS) (30° 37' 06. 35 N, 079°00'40.13 E) at 1500-2000 meters (Guptkashi, Kalimath, Ukhimath) in month of Nov to Feb (winter season).

2.2 Isolation and screening of rhizobacteria

1 gm rhizospheric soil was dissolved in 10 ml of sterile distilled water, making 10^{-1} dilution. 1 ml of this dilution was used to prepare further dilutions upto 10^{-7}. 1 ml of each dilution 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} were placed on Nutrient agar, in triplicate. Each such plate was incubated at 28±2 ^{0}C for 72 hrs [25].

2.3 Morphological and Biochemical Characterization

Well isolated colonies were picked up and different characteristics of colonies such as shape, size, elevation, surface margin, colour, colony form, pigmentation etc. were recorded. Selected isolates were biochemically characterized by Gram's reaction, carbohydrate fermentation, oxidase test, IMVIC tests, NO_2 reduction, Urease, Catalase, starch and gelatin hydrolysis as per the standard methods [26].

2.4 Plant Growth Promoting Mechanisms

2.4.1 Indole acetic acid (IAA) production test

All isolates were screened for IAA production. In brief, test bacterial culture was inoculated in the nutrient broth containing L- tryptophan (5 µg/mL), incubated at 28±2 °C for 5 days. Cultures were centrifuged at 3,000 rpm for 30 min and two mililiter of the supernatant was mixed with two drops of ortho-phosphoric acid and 4 ml of Salkowaskis reagent (50 ml, 35% perchloric acid; 1 ml 0.5 M $FeCl_3$). Appearance of red color indicates IAA production. OD (optimum density) was measured at 535 nm using spectrophotometer and shown as µg/ml [27, 28].

2.4.2 Production of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48-72 hrs at 28±2 ºC. Nessler's reagent (0.5 ml) was added in each tube. Development of brown yellow colour was a positive test for ammonia production [26].

2.4.3 Phosphate solubilization

Phosphate solubilization was detected by formation of transparent halos surroundings bacterial colonies on the Pikovskaya agar after 72 hrs incubated 28 °C [29].

2.4.4 Siderophore detection

Siderophore was detected by the formation of orange halos surrounding bacterial colonies on CAS agar plates after 48 hrs at 28±2 °C [30].

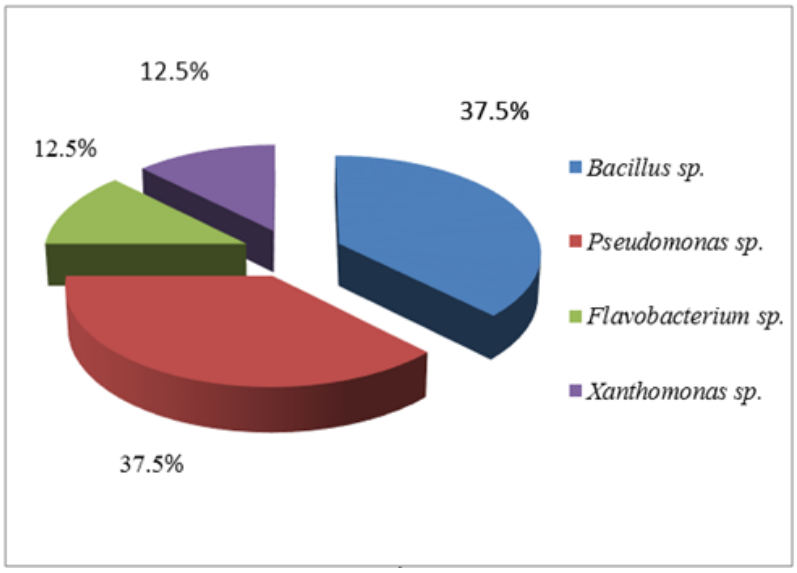

Fig 1: Percentage distribution of isolated microbial population of various bacterial genera from Srinagar

3. Results and Discussion

3.1 Isolation and identification of PGPR

All distinct bacterial morphotypes were identified on the basis of their colony characteristics (margin, elevation, form, color), morphology and biochemical characteristics [31]. A total 44 rhizobacteria were isolated from the rhizospheric soil of *Z. armatum* at different altitudinal sites. Maximum number of bacteria was to be found gram negative and rod shaped. In all 11 different genera were identified, 22 bacteria were isolated from site-3, followed by 16 isolates from site-2 and 8 from site-1. Maximum number of rhizobacteria was isolated from site-3. The organisms were identified as *Bacillus* sp. (29.5%) the most dominant genera followed by *Pseudomonas* sp. (25%), *Flavobacterium* sp. (11.36%), *Xanthomonas* sp, *Klebsiella* sp. (6.81% each), *Staphylococcus* sp, *Azotobacter* sp, (4.5% each), *Serratia* sp., *Enterobacter* sp., *Alcaligenes* sp., *Micrococcus* sp. and *unidentified* sp. were frequently present in rhizospheric soil.

In the Srinagar valley, total 8 rhizobacteria were isolated from rhizospheric soil of *Zanthoxylum armatum* DC. Four different genera were found in rhizospheric soil of *Z. armatum* (Fig.1). Among them 37.5% was *Bacillus* sp. followed by *Pseudomonas* sp. (37.5%), *Flavobacterium* sp. and *Xanthomonas* sp. (12.5%).

In the Pauri adjoining area, total 16 rhizobacteria were isolated from rhizospheric soil of *Zanthoxylum armatum* DC. Eight different genera were found in rhizospheric soil of *Z. armatum* (Fig.2). Among them 31.25% was *Bacillus* sp. followed by *Pseudomonas* sp. (25%), *Flavobacterium* sp. (12.5%), *Staphylococcus* sp., *Klebsiella* sp., *Xanthomonas* sp., *Serratia* sp. and *Enterobacter* sp. (6.25%).

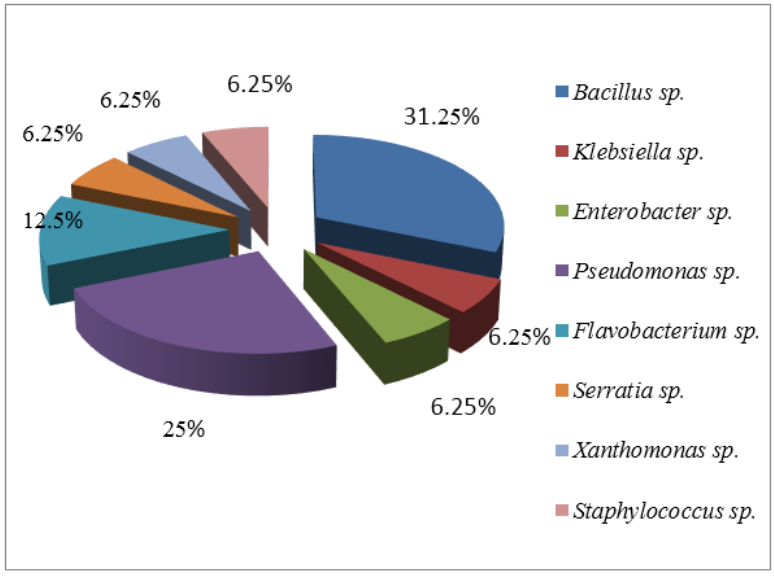

Fig 2: Percentage distribution of isolated microbial population belongs to the various bacterial genera from Pauri

In the KWLS, total 20 rhizobacteria were isolated from rhizospheric soil of *Zanthoxylum armatum* DC. Nine different genera were found in rhizospheric soil of *Z. armatum*. Among them 25% was *Bacillus* sp. followed by *Pseudomonas* sp. (20%), *Klebsiells* sp., *Flavobacterium* sp., *Azotobacter* sp. (10%), *Micrococcus* sp., *Staphylococcus* sp., *Alcaligenes* sp. and Xanthomonas sp. (5%).

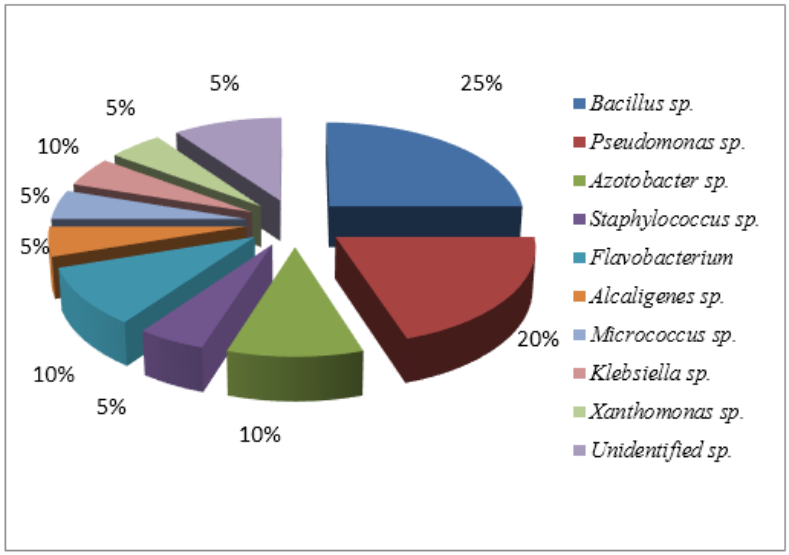

Fig 3: Percentage distribution of isolated microbial population belongs to the various bacterial genera from KWLS

The diversity indices were calculated and compared the bacterial diversity between different altitude *i.e.* Srinagar, Pauri and KWLS (Table: 1) the lowest Shannon-Wiener index was 0.81 from Guptkashi and highest was 2.81 from Ukhimath

Table1: Bacterial diversity index in *Zanthoxylum armatum* rhizosphere in Garhwal Himalaya

Indices	Srinagar			Pauri			KWLS		
	DG	BG	BD	KD	D S	P N	GK	KM	UM
Shannon index	1.5	1.0	1.0	1.37	1.0	2.24	0.81	2.28	2.81
Simpson index	0.38	0.5	0.5	0.44	0.5	0.22	0.63	0.26	0.143
Margalef SR Index	1.44	1.44	1.44	1.24	0.72	2.1	0.72	2.28	3.08

Devalgarh=DG, Bugani=BG, Badiyargarh=BD, Khandha=KD, Dobh Srikot=D S, Prem Nagar= P N, Gupkashi=GK, Kalimath=KM, Ukhimath=UM

3.2 Characterization of rhizobacteria of PGP traits

Each isolate was screened for plant promoting traits such as Indole acetic acid, ammonia production, phosphate solubilization and siderophore production. Plant growth promoting activities are compared in Fig 4.

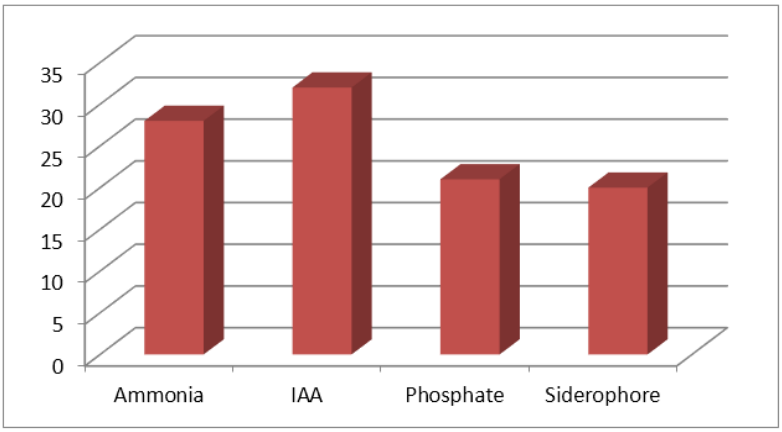

Fig 4: Plant growth promoting properties of isolates.

3.3 Ammonia Production

Bacterial isolates were tested for the production of ammonia in peptone water. In all 28 (63.63%) bacteria were observed to produced ammonia (Plate: 1). A total 6 isolates produced large amount of ammonia followed by13 moderate and 9 produced small amount of ammonia. Among them only three strains did not produce ammonia, these being identified as isolates belonging to the *Serratia* sp., *Staphylococuus* sp. and *Alcaligenes* sp.

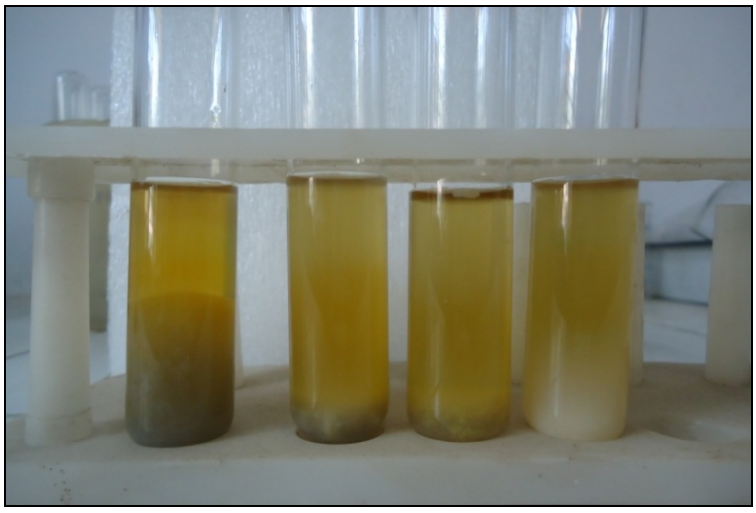

Plate1: Production of ammonia in peptone water

3.4 Indole-3- acetic acid production

IAA is one of the most important phytohormone and function as important signal molecule in the regulation of plant development. A Total 32 (72.72 %) produced IAA (Fig: 5, 6 and 7) that ranged from 0.69 µg/ml to 28.21 µg/ml. Highest IAA was produced by *Pseudomonas* sp. from site-1 followed by *Klebsiella* sp. (S_2KW_1) from site-2 and *Bacillus* sp. (S_3KW_9) from site-3. Lowest IAA was produced by *Micrococcus* sp. (S_3UW_3) from site-3. IAA production was illustrated in Plate 2.

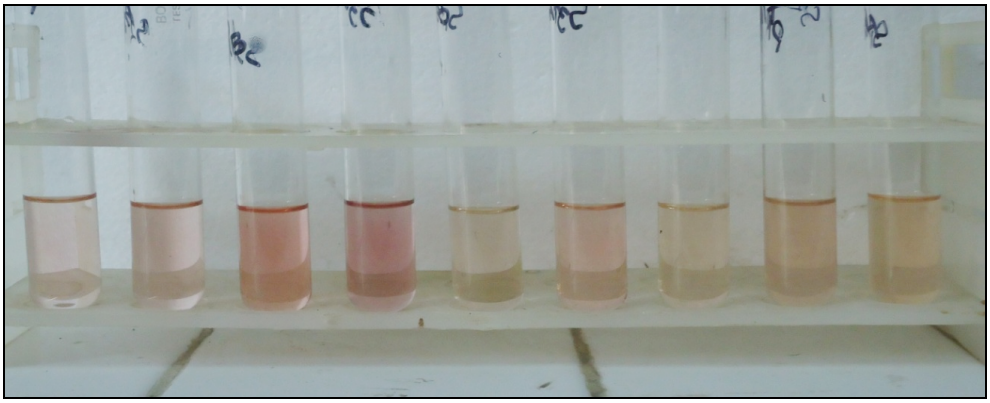

Plate 2: Indole-3- acetic acid production

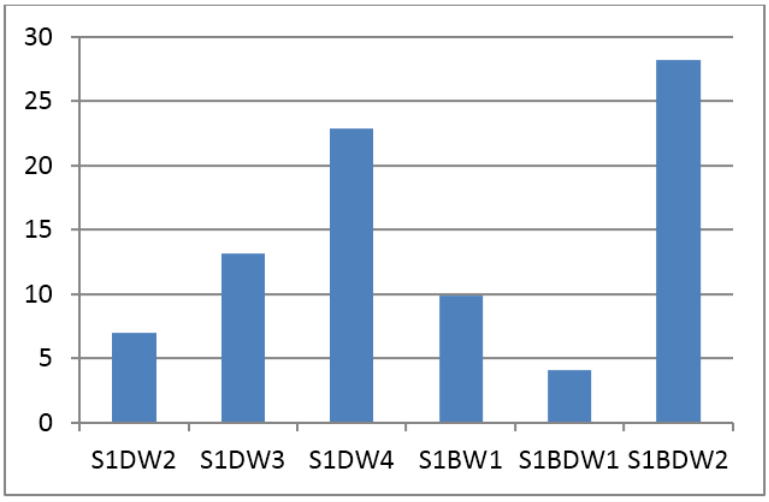

Fig 5: Indole-3-acetic acid production by bacterial isolates isolated from Site-I

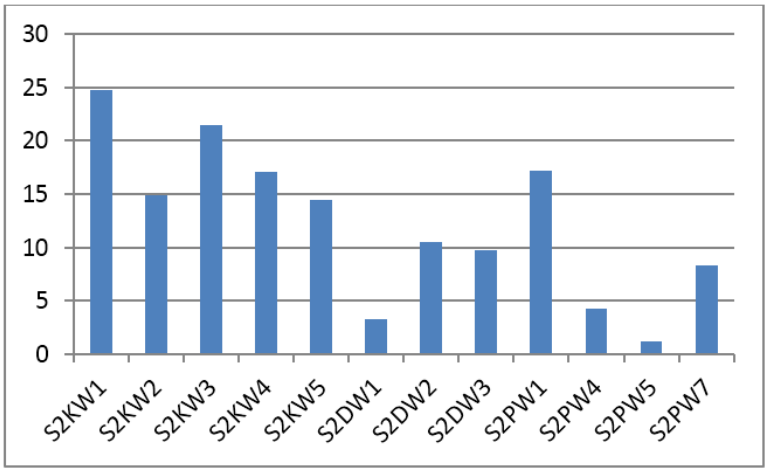

Fig 6: Indole-3-acetic acid production by bacterial isolates isolated from Site-II

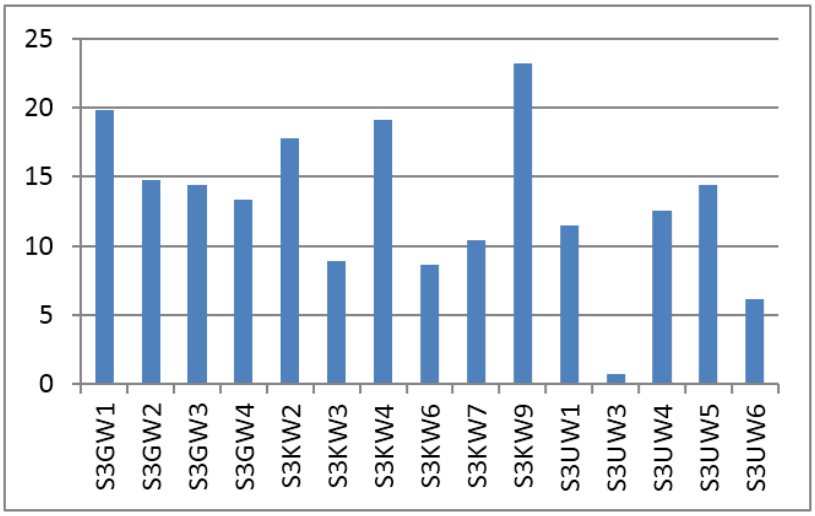

Fig 7: Indole-3-acetic acid production by bacterial isolates isolated from Site-III

3.5 Phosphate Solubilization

All the bacterial isolates in the present study were able to produce catalase. Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. Qualitatively phosphate solubilization was detected on Pikovskaya agar plate (Plate. 3), evident by halo around the inoculated spot. Total of 21 (47.7%) of isolates showed positive results, belonging to the *Pseudomonas* sp., *Bacillus* sp., *Flavobacterium* sp., *Klebsiella* sp., *Enterobacter* sp., *Serratia* sp. and *Azotobacter* sp. Phosphate solubilization from *Z. armatum* rhizosphere was observed in 3 isolates from Srinagar, 9 isolates from Pauri and 9 Isolates from KWLS.

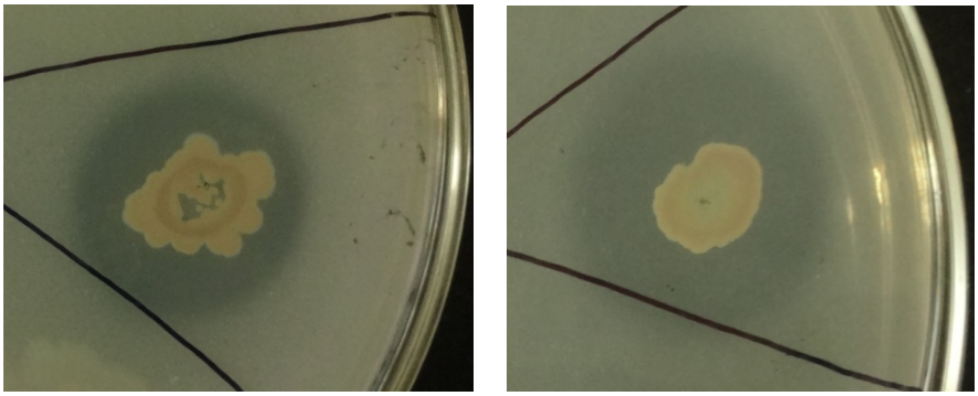

Plate 3: Phosphate Solubilization on Pikovskaya agar

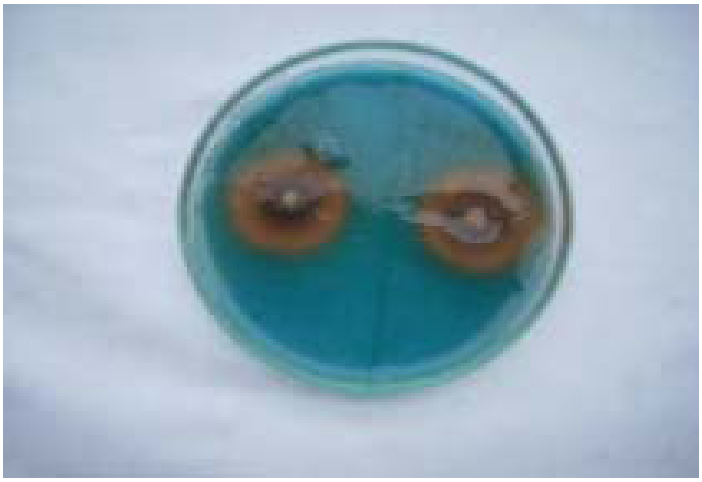

Plate 4: Siderophore Production on CAS agar medium

3.6 Siderophore Production

Siderophores provide a competitive advantage to producer organism over fungal pathogens for the absorption of available iron. The role of siderophore in the control of diseases has been reported [32]. A total of 20 (45.45%) isolates displayed positive results of siderophore production (Plate. 4) on CAS agar medium from Srinagar, Pauri and KWLS. Bacteria identified as belonging to the *Pseudomonas* sp. *Flavobacterium* sp., *Bacillus* sp., *Xanthomonas* sp., *Klebsiella* sp., *Alcaligenes* sp. and *Serratia* sp. were among the siderophore producing strains.

Microbial diversity studies are important in order to understand the microbial ecology in soil and other ecosystems [33]. Micro-organisms are environment specific; therefore, for understanding the microbial diversity and their applications, investigation on occurrence of various groups of microorganisms from different environment is essential [34].

PGPR colonize roots of plant and promote plant growth and development through a variety of mechanisms. The exact mechanism by which PGPR stimulate plant growth is not clearly known, although several mechanisms such as production of phytohormones, suppression of deleterious organisms, activation of phosphate solubilization and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth promotion [9]. Microbial diversity studies are important in order to understand the microbial ecology in soil and other ecosystems [33]. In this study it was observed that eight gram negative genera were present in rhizosphere. The rhizosphere is colonized by a predominantly gram-negative microbial community has been reported [35].

In our study *Bacillus* (27.27%) was dominant group [36]. *Bacillus* species are also a major component of the microbial flora, which live in close association with various types of agricultural crops. Earlier, studies also reported *Bacillus* as the dominant genera in the rhizosphere of *Oryza sativa,* Wheat and *Elaeagnus angustifolia* L., [36, 37, 38].

In the present investigation, predominance of *Bacillus* is due to its ability to efficiently use the nutrients provided by the plant through exudates. In additions, *Bacillus* has the ability to inhibit the growth of other strains. Many strains of *Bacillus* have been reported to produce substances that act as growth inhibitors for other microorganisms [39].

The variability in the performance of PGPR may be due to various environmental factors that may affect growth and exert their effects on plant. To achieve the maximum growth promoting interaction between PGPR and nursery seedlings it is important to discover how the rhizobacteria exert their effects on plant and whether the effects are altered by various environmental factors, including the presence of other microorganisms [40].

Maximum rhizobacteria produced ammonia documented that the observation of Kumar [41]. It has been reported that IAA production by PGPR can vary among different species and strains, and also influenced by culture conditions, growth stage and substrate availability [42]. The higher concentrations of phosphate-solubilizing bacteria are commonly found in the rhizosphere soil as compared to non rhizospheric soil [43]. The quantity and activity of microorganisms are determining factor of the productivity of any kind of soil [44]. Multiple PGP activities among PGPR have been reported by some other workers while such findings on indigenous isolates of India are less commonly explored [45]. In the present study isolates S_1BDW_2, S_2PW_5, S_2PW_7 and S_3GW_1 (*Pseudomonas* sp.), S_3KW_3 (*Klebsiella* sp.) and S_3KW_9 *Bacillus* were found to be most efficient PGPR which solubilized insoluble phosphorus, produced IAA, produced ammonia, produced siderophore and catalase. Such type of study is necessary as it advocates that use of PGPR as inoculants or biofertilizers is an efficient approach to replace chemical fertilizers. A number of studies suggest that PGPR enhances the growth, seed emergence, crop yield, and contribute to the protection of plants against certain pathogens and pests [46, 47, 48, 49, 50].

4. Conclusion

Genera *Bacillus* was dominant in rhizospheric soil of *Z. armatum*. The actual composition of the microbial community in the root zone is dependent on root type and plant species. Bacterial population was increased with increasing altitude. These studies concluded that the isolates which were positive for PGPR parameters could be further tested under field conditions. These are the preliminary studies for the selection of effective PGPR strains for consequent use as biofertilizer. The chemical fertilizers which are used for the increase of production are very expensive and harmful for human health besides being hazardous to environment. Hence, the isolates of PGPR obtained in the present study can be further tested for toxicological aspects and mass production, which might be useful for development of ecologically sustainable biocontrol strategy and biofertilizers of several plant in a sustainable manner, The findings of the present investigation highlighted that plant growth promoting rhizobacteria from rhizospheric soil may be exploited after strain improvement for local use.

5. Acknowledgement

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6. References

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