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Studies on characterization and antifungal activity of plant growth promoting rhizobacteria isolated from semiarid soil against plant fungal pathogens.

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Abstract

Aim: Changes in global temperature leads to severe drought conditions that may hamper food production in some countries in arid and semiarid regions of the world. The chemical fungicides are toxic for soil health. This study deals with characterization and antifungal activity of plant growth promoting rhizobacteria (PGPR) from rhizosphere of wheat grown under semiarid soil (15.80% moisture).

Methodology: Out of 45 isolates, most potent PGPR were isolated, identified and studied for their different plant growth promoting traits. Isolates evaluated for antifungal activity against plant pathogenic fungi and zone of inhibition (ZOI) was taken as an indicator of antifungal property. PGPR were studied for the effect on seed germination of wheat.

Results: Among the five PGPR isolates *Pseudomonas fluorescens* was most potent antagonist with 35 mm of ZOI against *Aspergillus niger*, demonstrated maximum phosphate solubilization with phosphate solublization index 4.09, as well as showed highest rate of seed germination (80 %) compared to other PGPR. *Pseudomonas fluorescens* was found to be strong cyanide producing strain.

Keywords: Antifungal activity, Characterization, PGPR, Plant pathogenic fungi.

Introduction:

One of the foremost agricultural issues reducing crop yield is drought stress in arid and dry regions of the globe Changes in world air temperature are resulting in longer drought periods and a lot of severe drought conditions can hinder food production in some countries (Lau *et al*., 2012). Current methods to increase the ability of plants to tolerate drought stress involve the use of water-saving irrigation and genetic engineering of drought-tolerant transgenic plants. Sadly, the ways are effortful and highly technical and thus troublesome to apply in practice. Several plant growth promoting rhizobacteria (PGPR) are shown to boost drought stress effects in plants by reducing plant ethylene levels that are sometimes raised by unfavorable conditions (Arshad *et al*., 2008). Thus one alternative for growing plants beneath dry conditions is the use of PGPR. The rhizosphere is the slime zone of soil specifically influenced by the root system which provides an expensive supply of energy and nutrients for bacterium (Dobbelaere *et al*., 2003; Gray *et al*., 2005). The rhizosphere is inhabited by a diverse range of microorganisms and the bacteria colonizing this habitat are called rhizobacteria (Schroth *et al*., 1982).PGPR are a part of rhizobacterial community and occupy as much as 2 to 5% of total rhizobacterial population constituting a cardinal part of rhizosphere biota (Kevin-Vessey *et al*., 2003). Microorganisms are vital for agriculture in order to promote the circulation of plant nutrients and reduce the necessity of chemical fertilizers. The use of fertilizers, including chemical fertilizers and manures, to reinforce soil fertility and crop productivity has usually negatively affected the complex system of the biogeochemical cycles (Perrott *et al*., 1992; Steinshamn *et al*., 2004). PGPR affect plant growth in two different ways, indirectly or directly. The direct promotion of plant growth by PGPR entails either providing the plant with a compound that is synthesized by the bacterium, for example phytohormones, or facilitating the uptake of certain nutrients from the environment (Glick *et al*., 1995). The indirect promotion of plant growth occurs once PGPR reduce or forestall the harmful effects of one or more phytopathogenic organisms. This can

happen by manufacturing antagonistic substances or by inducing resistance to pathogens. Wheat *(Tritium aestivum L.)* is one in all the foremost cereal crop in India and worldwide. The quality of the nutritional components of this major cereal crop is of particular concern to producers and consumers throughout the globe (Walia *et al*.,2013). One of the main causative fungal diseases of wheat is *Fusarium graminearum* (Murray *et al*.,2009). The fungus, in addition to seedling blight and *Fusarium* head blight, causes different seed and soil-borne diseases of seedlings. Moreover, the pathogen also produces mycotoxins including trichothecenes such as deoxynivalenol (DON) which is potent inhibitor of eukaryotic protein biosynthesis and has involved in a number of human and animal mycotoxicoses (Obanora *et al*., 2013). Therefore, even low levels of these toxins in raw grain can make them unsuitable for human or animal consumption. Aspergillus spp. is storage mold responsible for production of aflatoxin (Hassan *et al*., 2009), Aspergillus flavus is a saprophytic soil fungus that infect and contaminates pre-harvest and post-harvest seed crops with the carcinogenic secondary metabolites aflatoxin (Amaike*et al*., 2011). In this work, we provide information on the isolation and identification of bacteria from the rhizospheric semiarid soil of wheat with potential to produce PGPR traits and antagonistic activity against *Aspergillus niger, Aspergillus flavus, Aspergillus glaucus, Aspergillus terreus, Fusarium oxysporum* and *Cladosporium*. The PGPR have the potential to be used in the biological control of these fungi and hence can be used as biofertilizers as well as biocontrol agent.

Materials and methods:

Collection of sample

Root samples of wheat (*Triticum aestivum*) plant from the semiarid soil (15.80% soil moisture) of Jamni region of Wardha district, Maharashtra during month of December, collected and placed in sterile plastic bag, stored at 4ºC in Laboratory of PG Department of Microbiology, Bajaj College of Science, Wardha for further analysis.

Isolation and Identification of Rhizobacteria:

The rhizosphere soil samples were processed within 24 hr for isolation of PGPR**.** Samples were serially diluted with sterile distilled water (10^{-1} to 10^{-7}) and each dilution was plated on peptone yeast extract dextrose agar, Pseudomonas isolation agar, King's B agar, Serratia differential medium, Jensen's medium and Nutrient agar respectively. After incubation for 24 h at 37°C, colonies were picked and maintained as pure cultures in nutrient agar slants with periodic transfer to fresh media (Aneja *et al*., 2001). The identification of isolates were done by cultural, morphological and biochemical characterization as described in Bergey's Manual of Systematic Bacteriology (Tein *et al*., 1979). Isolates were also evaluated for production of various enzymes like catalase, gelatinase, oxidase and amylase by using standard procedures. (Hsu *et al*., 1975; Berlemont *et al*., 1975).

Characterization of isolates for PGPR traits

Siderophore production

Siderophore production was determined on Chrome azurol S agar (CAS) by the method given by Schwyn and Neilands (1987). CAS agar plate divided into equal sectors and spot inoculated with PGPR isolates and incubated at 28^0C to 30^0C. Development of orange halos around the colonies indicates positive siderophore production (Schwyn *et al*., 1987).

Hydrogen Cyanide production (HCN)

Isolates were cultured on Nutrient agar medium supplemented with glycine (4 g l^{-1}). The production of HCN was detected after 48 hr by using whatman filter paper no. 1 soaked in 2 % sodium carbonate and 0.5% picric acid, fixed to the underside of the petri-dish lid which was sealed with parafilm before incubation at 28^0C to 30^0C. A change from yellow to orange, red, brown, or reddish brown was recorded as an indication of weak, moderate, or strongly cyanogenic potential, respectively (Lorck *et al*., 1948).

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Vol-10 Issue-10 No.03 October 2020

Assay for Indole acetic acid (IAA) Production

The isolates were freshly grown on their respective growth medium amended with tryptophan (500 µg/ ml) at 30^0C for 48 h. fully grown cultures were centrifuged at 8000 rpm for 10 min and were assayed for qualitative detection of IAA. 2 ml of supernatant of each isolate was mixed with two drops of orthophosphoric acid and 4 ml of salkowski reagent (1 ml of 0.5M $FeCl_3$ in 50 ml of 35 % $HClO_4$). Formation of pink colour indicates IAA production (Brick *et al*., 1991).

Production of Ammonia

The isolates were tested for the production of ammonia by inoculating in 10 ml peptone broth and incubated for 24 h at 30^0C. The test was performed by adding 0.5 ml of Nessler's reagent (Himedia) in tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino *et al*., 1992).

Phosphate solubilization activity and Phosphate solubilizing index

For the study of phosphate solubilizing ability, the isolates were first screened on Pikovaskaya's agar for solubilization of insoluble inorganic phosphate. Cultures were inoculated on center of agar plate under aseptic condition. Plates were incubated for 3 days at 30°C. Presence of clear zone (halo zone) around the colony indicates presence of phosphate solubilization ability. Phosphate solubilization index was calculated by measuring the colony diameter and the halo zone diameter by using following formula.

Phosphate Solubilization Index (SI) = (colony diameter + clearing zone) / colony diameter (Karpagam*et al*., 2014).

Heavy metal tolerance

The isolates were studied for its heavy metal tolerance potential on Muller-Hinton agar supplemented with varying concentrations of four heavy metals Cu, Hg, Zn, Pb. Plates were incubated at 37°C for 24 hr to allow diffusion of the metal into the agar, after incubation the plates were observed for the growth of isolate (Hassen *et al*.1998).

Effect on Seed Germination

To evaluate the plant growth promoting efficiency of PGPR isolates, their *invitro* effect was observed on wheat seeds. Pot culture experiment was conducted to study seed germination efficiency for a period of 10 days. Twenty wheat seeds were surface-sterilized with $HgCl_2$ (0.1%) and successively washed several times with sterilized water. Then seeds were soaked overnight with culture of rhizobacterial isolates. Wheat seeds not soaked in PGPR isolates were used as control. Then all the seeds were sown in six separate plastic pots and filled with autoclaved soil. Pots sowed with inoculated seeds were labeled as experimental and pots sowed with un inoculated seeds were labeled as control. Pots were incubated for 10 days at 37º C. Then these six pots were observed for germination of seeds after regular interval of time (Noshin *et al*., 2010).

Antifungal activity of PGPR isolates against plant pathogenic fungi

All the PGPR isolates were screened for antagonistic activity against six plant pathogenic fungi viz; *Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Aspergillus glaucus, Cladosporium* and *Fusarium oxysporum* by using agar diffusion assay. These fungi cultures were obtained from the P. G. Department of Botany, J.B. College of Science, Wardha and stored for the further experimental use at 4ºC. For the evaluation of antifungal activity, broth cultures of PGPR isolates were made and culture broth was centrifuged at 8000 rpm for 15 min. Czapek Dox agar medium was used to study the antifungal assay. After solidification of the medium, 0.01ml of each fungal broth culture was spread on the respective plates in uniform manner. Then by using the sterile borer, wells were prepared in each plate and 20 µl of supernatant of each PGPR isolates were added in each well and plates were incubated for 4-5 d at 30ºC. Amphotericin- B (AMB) was used as positive control. Inhibition of fungal mycelium around the well was noted as positive test and zone of inhibition was measured.

ISSN: 2278-4632
Vol-10 Issue-10 No.03 October 2020

Cell wall degrading enzyme production:

Chitinase activity:

A minimal salt medium containing colloidal chitin as sole carbon and energy source was used. The medium consisted of $Na2HPO_4$ - 6 g, $KH2PO_4$ - 3.0 g, NH4Cl -1 g, NaCl- 0.5 g, yeast extract - 0.0 5 g, colloidal chitin -1.0 % (w/v), agar-15 g and distilled water-1000 ml and incubated at 30ºC. Colonies showing zones of clearance against the creamy background were recorded as chitinase producing PGPR (Hsu *et al*., 1975).

Cellulase activity:

A preliminary qualitative analysis for cellulolytic activity was conducted by using Congo red dye. The PGPR isolates were grown on Carboxy Methyl Cellulose agar (CMC) containing (g l-1) $KH2PO_4$ -1.0, $MgSO_4$.7H2O -0.5, NaCl -0.5, $FeSO_4$.7H2O - 0.01, $MnSO_4$.H2O - 0.01, NH_4NO_3 -0.3, CMC-10.0, Agar-12.0. The pH was adjusted to 7.0 with 1 M NaOH. The CMC agar plates were incubated at 37°C for 5 d to allow the secretion of cellulase. At the end of the incubation, to visualize the hydrolysis zone, the agar medium was flooded with an aqueous solution of congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1 M NaCl for 15 min. Clear zone around the colony was indicative of cellulase enzyme production (Berlemont *et al*., 1975).

Results and Discussion

The rhizosphere of wheat plants fully grown underneath semi-arid soil (15.80% soil moisture) was tested to explore the diversity of plant growth promoting rhizobacteria. A total of 45 PGPR bacteria were isolated from the rhizosphere soil of the wheat plant. Five best potential bacterial isolates showing PGPR traits and demonstrating antifungal activities were selected for characterization. The isolates were identified as *Bacillus* spp.1, *Bacillus* spp. 2, *Azotobacter* spp., *Serratia marcescens* and *Pseudomonas fluorescens*. Among five isolates three were gram negative and two were gram positive and all were motile. *Bacillus* spp., *Azotobacter* spp. and *Serratia marcescens* were found to ferment glucose and sucrose with production of acid and gas. Only *Azotobacter* spp. could found to ferment lactose. Both *Azotobacter* spp. and *Serratia marcescens* were found to ferment mannitol also. *Bacillus* spp. 2 and *Pseudomonas fluorescens* did not found to ferment any sugar. The isolates were also found to produce catalase, oxidase, amylase, gelatinase, cellulase and chitinase enzymes. The results of morphological and biochemical characterization are depicted in Table 1. The isolates showed varied levels of PGPR traits such as siderophore production, phosphate solubilization, IAA, ammonia and HCN production. All PGPR bacterial isolates were found to produce indole-3-acetic acid (IAA), exerted ability for phosphate solubilization on the Pikovskaya medium with different efficacy. The maximum phosphate solubilization was found in *Pseudomonas fluorescens* with phosphate solubilization index 4.09. Less phosphate solubilizing activity was found in *Serratia marcescens* with phosphate solubilization index 3.1 (table 4). The production of ammonia and hydrogen cyanide observed in all the five isolates. Moderate cyanide production was observed in *Bacillus* spp., *Azotobacter* spp. And *Serratia marcescens* while *Pseudomonas fluorescens* were recorded as strong cyanide producing strain with brown colour of filter paper. In the present study all the bacterial isolates exhibited siderophore production on CAS medium. CAS assay is the universal assay for detection of siderophores. The principle of this assay is based on a color change of CAS from blue to orange resulting from siderophoral removal of Fe from the chrome azurol dye. Isolates were tested for their heavy metal tolerance ability. All the isolates were found to tolerate Cu, Zn and Pb at varying concentrations. *Bacillus spp.2* and *Azotobacter were* the only isolates that could tolerate Hg while rest of the isolates found to be very sensitive against Hg (Table 5). The important aspect of this investigation was to study the effect of PGPR isolates on seed germination of wheat plant *in vitro*. Our experiments showed that PGPR inoculation significantly enhanced seed germination of wheat over control. However, the speed of enhancement varied with bacterial strains. The results also indicate that the PGPR

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Vol-10 Issue-10 No.03 October 2020

strains initiate the germination of wheat seeds at the 3rd day of treatment connecting to active participation in germination system. The highest seedling height (18 ± 1.4 cm) was observed in seeds treated with *Pseudomonas fluorescens* then *S. marcescens* (14 ± 1.3) followed by *Azotobacter* spp. (13 ± 1.0), *Bacillus* spp.2 (10 ± 1.4) and *Bacillus* spp.1 (10 ± 1.5) after 7 d treatment as compared to 9 cm in control. These results were analyzed statistically by using Microsoft Excel tool and are depicted in Table 2. Similar experiment was done by Fatima *et al.*, (2009) supports our investigation. They reported the isolation of 3 PGPR strains ie. WPR-51, WPR-42 and WM-30 belonging to *Azotobacter* and *Azospirillum* from rhizosphere of wheat. These strains positively affected the germination of wheat as well as increased biomass and root shoot length when tested in pot experiments. Maximum root length was observed due to WPR-51 treatment (26.6 cm/plant) and mixture of three isolates (27.8 cm/plant). While shoot length was vice versa. A length of 39.4 cm per plant was observed due to treatment of WPR-51 while due to mixture shoot length was 36.1 cm plant. The WPR-42 and WM-3 treatments were not found so effective to increase root and shoot length as compared to control. The Rhizobacteria were screened for antifungal activity against *Aspergillus flavus, Aspergillus niger, Aspergillust erreus, Aspergillus glaucus, Cladosporium* and *Fusarium oxysporum* and zone of inhibition was taken as an indicator of antifungal property. On Czapek Dox agar medium all the five isolates were found to inhibit the growth of all plant pathogenic fungi. Among the six pathogens, *Aspergillus niger* was found to be most sensitive against PGPR isolates. *Pseudomonas fluorescens* was found to be most efficient antagonist against plant pathogenic fungi and showed zone of inhibition of 35 mm, 27 mm, 25 mm, 23 mm, 22 mm and 19 mm against *Aspergillus niger, Cladosporium, Fusarium, Aspergillus flavus, Aspergillus glaucus* and *Aspergillus terreus* respectively. Among the tested plant pathogenic fungi, highest antifungal activity of all the PGPR isolates was found to be against *Aspergillus niger* while *Aspergillus terreus* was found to be less sensitive plant pathogen against all the PGPR isolates. From this study it was found that *Pseudomonas fluorescens* was the most efficient plant growth promoting rhizobacteria and also the potent antagonist for plant pathogenic fungi. The detailed results of antifungal activity are depicted in table 3.

Conclusion

Present investigation elucidates the multifarious role of PGPR isolate *Pseudomonas fluorescens* obtained from semiarid soil of wheat rhizosphere, was found to exhibit different plant growth promoting traits which may be useful to promote the plant growth. The isolate demonstrated antifungal activity that may be useful in formulating new inoculants with combinations of different mechanisms of action, leading to a more efficient use for biocontrol of plant pathogenic fungi. This is efficient approach to replace chemical fertilizers and pesticides for sustainable agriculture. In view of these, PGPR as a biofertilizer and biocontrol agent could provide a means for reducing the plant disease incidences in addition to avoiding the use of chemical fertilizers and fungicides.

Table 1: Biochemical characterization and PGPR traits of isolates (+ means positive, - means negative)

Tests	***Bacillus* spp.1**	***Bacillus* spp.2**	***Azotobacter***	***S. marcescens***	***P. fluorescens***
Grams Reaction	+	+	-	-	-
Motility	Motile	Motile	Motile	Motile	Motile
Glucose fermentation	+	-	+	+	-
Sucrose fermentation	+	-	+	+	-
Lactose fermentation	-	-	+	-	-

Mannitol fermentation	-	-	+	+	-
H_2S Production	+	+	+	+	+
Indole test	-	-	+	-	-
Methyl red	+	+	+	+	+
Voges Proskaur test	-	-	+	-	+
Citrate test	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	-	+	+	+	+
Amylase	-	+	+	+	-
Gelatinase	+	-	+	-	+
Cellulase	+	+	-	+	+
Chitinase	+	+	+	+	+
Ammonia	+	+	+	+	+
Siderophore	+	+	+	+	+
HCN	+	+	+	+	+
Phosphate solublization	+	+	+	+	+

Table 2: Effect of PGPR isolates on Seed Germination (Mean and Std. Error)

Pots no.	No. of Wheat seed Germinated out of 20	Wheat seeds soaked into	Length of the shoots measured in (cm)		
			3rd day	5th day	7th day
1	8	Control	1.2 ± 0.24	4 ± 0.26	9.3 ± 0.25
2	11	*Bacillus* spp.1	1.7 ± 0.22	5 ± 0.15	10 ± 1.5
3	9	*Bacillus* spp.2	1 ± 0.10	5.3 ± 0.29	10 ± 1.4
4	10	*Azotobacter* spp.	1.6 ± 0.21	5.6 ± 0.18	13 ± 1.0
5	11	Serratia marcescens.	2 ± 0.09	4.4 ± 0.20	14 ± 1.3
6	16	*Pseudomonas fluorescens*	3.6 ± 0.23	12 ± 1.3	18 ± 1.4

Table 3: Antifungal activity of PGPR isolates.

Fungi used	Zone of Inhibition (in mm)					
	AMB	*Bacillus* spp.1	*Bacillus* Spp.2	*Azotobacter*	*Serratia marcescens*	*Pseudomonas fluorescens*
Aspergillus flavus	11	12	11	14	16	23
Aspergillus niger	13	18	21	14	15	35
Aspergillus terreus	10	11	11	12	12	19
Aspergillus glaucus	12	12	11	12	11	22
Cladosporium	14	12	17	16	14	27
Fusarium oxysporum	18	13	11	12	13	25

ISSN: 2278-4632
Vol-10 Issue-10 No.03 October 2020

Table 4: Phosphate solubilizing index of PGPR isolates.

Isolates	Days					
	1	2	3	4	5	6
	Phosphate solubilizing index					
Bacillus spp. 1	2.22	2.2	2.4	2.6	3	3.2
Bacillus spp. 2	2.5	2.5	3	3.3	3.7	3.8
Azotobacter	2.11	2.6	3	3.2	3.7	3.8
S. marcescens	2.25	2.33	2.4	2.6	2.69	3.1
P. fluorescens	2.5	3.0	3.8	3.9	4	4.09

Table 5: Heavy metal tolerance of PGPR isolates

Isolates	Heavy metals (mg/ml)			
	Cu	Hg	Zn	Pb
Bacillus spp.1	200	-	200	200
Bacillus spp.2	400	200	600	600
Azotobacter spp.	1000	200	1000	1000
S. marcescens	1000	-	1000	1000
P. fluorescens	600	-	400	400

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