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Assessment of *Pseudomonas* spp. for Growth Promotion, Biocontrol and Stress Tolerance Applicability towards Organic and Inorganic Pollutants

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ABSTRACT

Organic and inorganic pollutants have deleterious effect in the environment, crops productivity, microbiome and soil fertility. The persistence uses of toxic compound like pesticides not only affect soil health but also agitated ecosystem. Use of Plant Growth Promoting rhizobacteria can play an important role towards achieving the objectives of sustainable ecosystem. *Pseudomonas* group of bacteria are known for their remediation properties but are lesser explored for their growth promotion attributes and to alleviate other abiotic and biotic stress in the agriculture system. The paper presents multifarious potential of *Pseudomonas* isolates obtained from agriculture fields in the vicinity of Ghaziabad, U.P (PGPM2, 3, 4, 5). Biochemical tests for identification suggested that these isolates belong to *Pseudomonads* group of organisms. Further, molecular characterization through 16S RNA gene sequence analysis confirmed their identity as PGPM2- *Pseudomonas* species, PGPM3- *Pseudomonas plecoglosida,* PGPM4- *Pseudomonas* and PGPM5- *Pseudomonas* respectively. Production of essential hormones such as indole acetic acid, gibberellic acid and phosphate solubilisation activity have endorsed the plant growth promotion attribute by these isolates. Production of ammonia and secretion of lytic enzymes like lipase by PGPM2 and PGPM3 indicate stronger bio-control abilities in these isolates as compared to other isolates, all isolates showed positive for the bio-film formation, a positive attribute that helps in colonising and surviving harsh environments. Bangle method established the strong antagonistic activity that these isolates possess against 3 important plant root pathogens namely *Macrophomina phaseolina* (↓41%) followed by *Sclerotium rolfsii* (↓36-15%) and *Fusarium oxysporum* (↓21%) respectively. Isolates PGPM2 & PGPM3 showed maximum tolerance against salt (500MM) while isolates PGPM2 and PGPM4 showed better tolerance to Pesticides monocrotophos and dimethoate. (PGPM2- MIC and LD50 and PGPM3 –MIC AND LD50). Host plant bioassay with pearl millets (bajra) revealed maximum growth promoting efficiency (root length and shoot length) by PGPM2. The manuscript provides a biochemical and physiological evidence that native *Pseudomonas* isolates not only promote plant growth but also possess unique ability to tolerate adverse abiotic stress conditions such as tolerance to salt, organic (pesticides) and inorganic pollutants thus offering multiple growth supporting advantages to the plant host.

Key words : *Abiotic, Biotic, Bioremediation, Pathogen, Pesticides, Salt.*

Introduction

Microorganisms play a vital role in maintaining soil dynamics and soil fertility, they are crucial to enhance plant productivity while maintaining the soil health. Agriculture soils are most complex habitat

varying residual pesticides different condition of pH, temperature and salinity directly or indirectly affect the indigenous micro flora and the productivity of crops. Area around the plant roots harbours enormous number of microorganisms that directly or indirectly stimulate plant growth and these microorganisms are known as plant growth promoting rhizobacteria (Kloepper *et al.*, 1980; Ryu *et al.*, 2004). They show diverse biotic activities like providing nutrients, eliminating deleterious species and specific breakdown of organic pollutant (Gusain and Bhandari, 2019; Kang *et al.*, 2013). Fluorescent *pseudomonads* have been repeatedly studied for their bioremediation abilities but were lesser explored for their plant growth traits (Ahemad *et al.*, 2008).

Increasing crop plant productivity and enhancing resistance or tolerance against various stress factors has become major aim for modern agriculture (Kang *et al.*, 2013). Several strategies could be employed to manage the deleterious effects of both abiotic and biotic stress factors on plants. However, in agriculture both plant growth and yield are repeatedly impacted by factors such as temperature, pH, salinity, drought and other pollutants. Therefore, if the PGPR are expected to support plant growth, and play a meaningful role in perking up the plant growth, these rhizobacteria will have to survive under different stress conditions (Dal Bello *et al*., 2002 and Mehmood *et al.*, 2018).

This manuscript is an effort to identify such candidate microbes with complementary plant growth supporting abilities along with biocontrol and stress tolerance properties. The choice of *Pseudomonas* isolates for the study is due to earlier reports where individual *Pseudomonas* spp. were identified with either plant growth promoting or organic pollutant degrading abilities. None the less carefully verified experimental studies were lacking where the potential of *Pseudomonas* isolates across the entire spectrum of plant growth promotion including plant growth, resistance to plant pathogen and tolerance to abiotic stress route can be established. There is a requirement to specifically identify such *Pseudomonas* isolates that can tolerate and counter abiotic stress conditions including varying conditions of salinity, pH residual pesticides and heavy metal toxicity, which happens to be the mandate of this paper. In the present study we have isolated native bacterial spp from agricultural fields of Western UP, India and experimentally verified their plant growth promotion and biocontrol properties along with abiotic and biotic stress tolerance against salt, temperature, pH, heavy metals and pesticides. The manuscript also presents the characterization of these native *Pseudomonas* isolates that offer holistic growth support to host plants.

Materials and Methods

Native PGPR isolation from agriculture field

Soil sampling was done by the protocol of Kumar *et al.*, 2012. Collection of rhizospheric soil samples was carried out from agricultural plots of Millets (*Pennisetum glaucum*), Sorghum (*Sorghum bicolor*), and non-cultivated grass collection in Western UP, India was. For sample collection, 5 different locations within a specific plot area were picked randomly and from each location rhizospheric soil sample along with roots was collected from a minimum of 3 position. All the sample from one location were pooled and airdried samples were passed through a 2mm sieve to obtain homogenous soil sample. In a 250 ml of conical flask, 100ml of sterile distilled water was taken and ten grams of homogenized soil was added. The flask was shaken for 10 min on a rotary shaker. One milliliter of suspension was added to 10 mL sterile water vial and shaken for 2 min. Serial dilution technique was performed. An aliquot (0.1 mL) of this suspension was spread on Kings B agar medium to observe bacterial colonies. Typical bacterial colonies observed over the streak were maintained under the names PGPM2, 3, 4 and 5 respectively. Morphological characteristics of the colony of each isolate were examined on KB agar plates and pure culture of each isolate was maintained on KB agar. *P. fluorescens* MTCC 2421 was used as a reference culture which was procured from MTCC IMTECH, Chandigarh in all the experiment and it was maintained on KB/KB agar. Each of the following experiments was conducted with every native isolate (i.e); PGPM2-5 and all the experiments were conducted in triplicate.

Biochemical analysis of native isolates

All the isolates were studied for their microscopic morphology by performing gram staining. Biochemical tests for identification and characterization of bacterial isolates were carried out based on Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). For each test respective media were prepared and 100µl freshly grown culture was in-

oculated in the media and incubated for 24 to 48 hr at 30°C. Triple sugar agar test, citrate test, urease test, starch hydrolysis, methyl red test, gelatin hydrolysis test and amino acid test were carried out as per the protocol explained by Egamberdiyeva, 2005.

Sugar utilization tests: Utilization of amino acids (Lysine and Ornithine) and utilization of sugars; (Adonitol, Lactose, Arabinose and Sorbitol) was performed using biochemical test kit KB002 TM HiAssorted (for Gram-negative rods) from Himedia. 50µl of overnight grown culture from each PGPM isolates inoculated and incubated for 18hrs at 30°C. Observations were done after incubation based on color reaction chart provided in the kit.

Phenotypic characterization: For the phenotypic characterization bacterial isolates were tested for their sensitivity towards a variety of antibiotics such as Ampicillin, Chloramphenicol, Carbenicillin, Colistin, Gentamicin, Kanamycin, Polymyxin, Penicillin-G, Spectinomycin and Tetracycline. These antibiotics were named as A, B, C, D, E, F, G, H and I respectively. 100µl freshly grown bacterial culture was inoculated aseptically on luria agar plate as a spread plate to obtain a lawn growth. Discs of above-mentioned antibiotics were taken with a sterilized forceps and placed over the inoculated agar plate. Plates were incubated for 18 to 24h at 37°C. Observations were recorded by measuring the zone of inhibition around the antibiotic discs, in case of every isolates i.e., PGPM1-5 respectively.

Pigment production test unique to identify members of *Pseudomonas* group: 100µl overnight grown cultures of PGPM1-5 were inoculated in fluorescein and phycocyanin agar respectively. The plates were incubated in incubator at 30ºC for 24 hr. After incubation time the plates were checked for the appearance of yellow pigmentation.

16S rRNA Gene sequencing and phylogenetic analysis of PGPR isolates

Characterization of all native isolates was carried out at molecular level using 16sRNA gene amplification followed by partial sequencing method. Total genomic DNA of overnight grown culture at 30±2°C, log phase cultures of bacterial isolates PGPM2, 3, 4 and PGPM5 was obtained using CTAB method (Doyle 1991). The 16s rRNA region of the ribosomal DNA gene cluster of bacterial isolates was amplified with the standard set of forward and reverse primers, 27F (5'AGAGTTTGATCCTGGC TCA) and 1492R (5'GGTTACCTTGTTAC GACT), respectively. The PCR run for the bacterial isolates constituted: 5 minutes of denaturation at 95°C; 35 cycles (30 sec at 94°C), annealing (60 sec at 55°C), and synthesis (90 sec at 72°C), followed by a final extension for 5 minutes at 72°C.

Tests: for biochemical markers that designate plant growth promoting properties

Harmone production

IAA production

To 20 ml Luria broth 1% filter sterilized L-tryptophan solution was added. The medium was individually inoculated with 0.1 ml freshly grown bacterial cultures PGPM1-5 respectively and kept for 48 h at 28° ± 2°C. After incubation, centrifugation was done at 5000 rpm for 15 mins and supernatant collected. For IAA estimation to 2ml of supernatant, two drops of ortho-phosphoric acid and 4 ml of Salkowaski's reagent (50 ml, 35% perchloric acid, 1ml 0.5 N Ferric chloride solution) were added. IAA production was indicated by pink color development (Gordon and Weber, 1950).

Gibberellic Acid: One ml of overnight grown bacterial culture was re-inoculated in 45ml nutrient broth and inocubated for 72 h at 28°C ± 2°C at 140 rpm. After incubation the medium was centrifuged at 10,000 rpm for 15 min. The culture supernatant was acidified (2.5 pH) using 1N HCl and extracted by adding equal volume of ethyl acetate in three stages to obtain 5ml extract. Extract was evaporated and used for TLC analysis as described by Bhalla *et al*., (2010).

Nutrient mobilization

Phosphate solubilization: Pradhan *et al*., 2006 described method to detect the ability of organisms to solubilize phosphate. Each isolate was spot inoculated using sterile tooth picks onto Pikovskaya's Agar medium containing (per liter) tricalcium phosphate (2.5 g), glucose (13 g), $(NH_4)SO_4$ (0.5 g), NaCl (0.2 g), $MgSO_4.7H_2O$ (0.1 g), KCl (0.2 g), yeast extract (0.5 g), $MnSO_4$ trace, $FeSO_4$.7H2O trace, agar (15 g), and pH 7.2. Plates were incubated for three to five days at 28°C ± 2°C. The results statement assay was performed using this method.

Nitrate reduction: Nitrate Reduction biochemical test kit KB002 TM HiAssorted (for Gram-negative rods) from Himedia. 50µl of overnight grown cul-

ture were inoculated and incubated for 18hrs at 28±2°C. After incubation observations were done based on color change reaction in the kit.

Siderophore production: Schwyn and Neilands (1987) described the method to check siderophore production on Chrome-azurol S (CAS) medium. The bacterial strains were grown on LB media. After overnight incubation, the cultures were centrifuged at 10000 rpm for 10mins. 0.5 ml of supernatant was taken and 0.5ml of CAS reagent was added and checked for change in colour (from blue to yellow) was measured at 630nm using spectrophotometer. Siderophore production was quantities using the formula given below:

Percentage of siderophore production = control (abs) - sample (abs) / control (abs)*100

Assessment of biocontrol properties

Assay for ammonia production: All the isolates PGPM1-5 were tested for ammonia production in peptone water medium containing peptone 10g, NaCl 5g, yeast extract 5g, (per liter) and pH adjusted to 7.6. In 10 ml medium, 100µl freshly grown cultures were inoculated individually in each tube and incubated for 48h at 28±2°C. After incubation the broth was centrifuged, and Nessler's reagent (0.5 ml) was added to supernatant (Kumar *et al.,* 2014).

HCN production: All the isolates were screened to produce hydrogen cyanide as per the protocol given by Ahemad *et al*., 2008. In nutrient agar, glycine (4.4 g/L) was added, and isolates were grown as culture. Whatman filter paper (No. 1), soaked in 2% sodium carbonate in 0.5% picric acid solution was placed on the innerside of the petri plate is upper lid. Plates were sealed with parafilm and incubated at 28±2°C for 4 days (Kumar *et al.,* 2014).

Enzyme assay: Enzyme production in isolates was checked for a variety of enzymes such as: protease, lipase and amylase on respective substrates namely: Protease casein agar, lipase tributyrin agar and amylase starch agar. All the above-mentioned media plates were spot inoculated with freshly grown cultures of PGPM1-PGPM5. Protease production was determined using skim milk agar medium, that contained (per liter) pancreatic digest of casein 5g, yeast extract 2.5g, glucose 1g, skim milk solution 7%, and 15g of agar (Montville 1983). Microorganisms producing lipase were screened using tributyrin agar plates (TBA) containing (per liter) peptone 5 g, beef extract 3 g, tributyrin 10 ml (autoclaved separately and added before pouring plates), agar-agar powder 30 g and pH adjusted to 7.2. Post inoculation the bacterial cultures were incubated at 28 ± 2°C for 48 h. Zone of clearance around the colony indicated them as lipase producer (Veerapagu *et al.,* 2013).

Other biochemical tests

Biofilm formation: All the bacterial isolates PGPM1- PGPM5 were grown in King's B broth medium in individual polypropylene tubes and incubated for 36-48 hrs at 28±2°C. After incubation, the medium was decanted and the tubes were washed with phosphate buffered saline (pH 7.3) and dried. Then 0.1% crystal violet stain was added, allowed to stay for 1 min and decanted. The tubes were for the formation of a violet ring at the top (Ansari and Ahemad, 2018).

Emulsification: 20 ml of sterilized distilled water with 10 µl of crude oil was added to a Petri dish (90mm diameter) followed by the addition of 10 ìl of culture broth to the oil surface. Observations recorded after 15 minutes for appearance of clearing zones on the oil surface (Cipinyte *et al*., 2011).

Assessment of biotic stress tolerance

Tolerance against plant pathogen: Screening for biocontrol activity was carried out using the bangle method as described by (Lim *et al.,* 1991) to identify antagonistic potential of isolates against common root pathogen *Macrophomina phaseolina, fusarium oxysporium* and *Sclerotium rolfsii* pathogens. These pathogens were obtained from Central Research Institute for Dryland Agriculture (CRIDA), Hyderabad, India. All the fungal isolates were maintained on MDA (*M. phaseolina* and *S. rolfsii*) and on PDA (*F. oxysporum*) at 30±2^{0}C. In this method we have placed the fungal disk at the centre of the media and the bacterial cultures were introduced using heat sterilized bangle which form a circle around the central fungal inoculum. Percentage of growth inhibition in the bacteria challenged pathogen over unchallenged pathogen (control) was measured by the formula.

% Inhibition of radial mycelial growth =C-T/Cx100
Where C= Control, T= Treatment
C = Radial colony diameter (in cm) of control (i.e., growth of pathogen unchallenged with native isolates)
T = Radial colony diameter (in cm) of treatment (i.e., growth of pathogen challenged with respective native isolate)

Assessment of abiotic stress tolerance

Tolerance against temperature: Isolates PGPM1-PGPM5 were checked for their tolerance towards a range of different temperature i.e., 25, 30, 35, 40 and 45ºC respectively. 100µl overnight of grown cultures were inoculated into 10ml king's B broth and incubated at 150 rpm 28±2ºC for 24hr. After incubation an aliquot of the culture was taken, serially diluted in fresh broth and CFU (colony forming unit) calculation was done for each isolate at every temperature treatment (Paulucci *et al.,* 2015).

Tolerance against salts: test abiotic stress tolerance of the isolate's salinity was taken as one of the parameters, NaCl incorporated into 10ml LB medium at different concentrations (0, 50, 100, 150, 250, 500, 750 and 1000mM respectively). 100 µl of overnight freshly grown culture was inoculated in NaCl amended media and incubated for 18hrs at 30ºC, 150 rpm. After incubation OD was measured taken at 595nm of isolates (Paulucci *et al.,* 2015).

Tolerance against pesticides stress: For pesticide stress tolerance, monocrotophos and dimethoate were taken. They belong to the class of organophosphate pesticide. 10-150 ppm concentration ranges were tested for the tolerance study by native isolates. Overnight grown culture 100µl was inoculated into 10ml medium supplemented with the respective pesticide concentration. After incubation the growth of the isolates were recorded at 610nm (Shaheen and Sundari, 2013)

Plant bioassay Assessment of growth promotion: To check the plant growth promotion ability of bacterial isolates, pearl millets (bajra) was chosen as the host. Bajra seeds were soaked overnight and after 18hrs seeds surface was sterilized for 1 min in 1% solution of sodium hypochlorite, followed by a wash in 95% ethanol and 8 successive rinses with sterilized distilled water. Sterilised seeds were sown in 135 mm sterilized glass petri dishes (25 seeds per dish and three triplicates treatment) containing thin layer of absorbent cotton wetted with 50 ml of sterilized distilled water. Surface sterilized seeds individually coated with overnight grown PGPM1, 2, 3, 4 and 5 served as treatment T1, T2, T3, T4, and T5. Petri dishes were incubated at 30°C in darkness (Amalraj *et al.,* 2015).

Results and Discussion

The 4 distinct pure cultures of bacterial species were found to be gram negative and rod-shaped. Biochemical characterisation of these negative rods tabulated in Table 1 was studied based on Bergey's Manual of determinative Bacteriology (1957). Based on biochemical identification test results the isolated bacterial spp. show nearest relation to the bacterial *Pseudomonas* genera MTCC 2421. *Pseudomonas* reference culture from MTCC is hence referred to as PGPM1. The 4 bacterial isolates were named as PGPM2, PGPM3, PGPM4 and PGPM5 respectively. All the native isolates PGPM 1-5 were appeared as circular, convex, slimy colonies with entire smooth margins on KBA and they all are odourless. Slime production of isolates is reported due to production of laven from sucrose by fluorescence *Pseudomonads* (Nishimori *et al.,* 2000). Surface growth of colonies with no embedded expansion represented aerobic

Table 1. Biochemical characterization of PGPM isolates

	Pseudomonas	PGPM1	PGPM2	PGPM3	PGPM4	PGPM5
Gram staining	Negative	N	N	N	N	N
TSI	Negative	N	N	N	N	P
Citrate	Positive	P	P	P	P	N
Urease	Positive/Negative	N	N	N	N	N
Catalase	Positive	P	P	P	P	P
Starch	Positive/Negative	P	P	P	P	P
Methyl Red	Negative	N	N	N	N	N
Indole	Negative	N	N	N	N	N
Phenylalanine deamination (TDA)		N	N	N	N	N
H_2S Production	Negative	N	N	N	N	N
Fluorescin	Positive/Negative	P	N	P	P	N
Pycocynin	Positive/Negative	P	P	P	P	N
Score card		10	9	10	9	7

nature of isolates. For citrate utilization test all strains are positive for except PGPM5 similarly for TSI test all cultures showed positive utilization of sugar except PGPM5 but no gas production was seen in the medium. Triple Sugar Iron (TSI) test medium determine the ability of a microbe to produce gases/hydrogen sulfide (H_2S) on utilization of specific carbohydrates *i.e.,* glucose, lactose and sucrose or peptone A no. of tests Methyl red, Indole test, Phenylalanine deamination (TDA), H_2S production and urease were negative for all the strain (Figure 1). For catalase production all the strains were positive. As it determines the bacterial ability to breakdown the hydrogen peroxide in oxygen/water and thus producing bubbles indicate catalase enzyme production. As evidenced from Table 1 show characteristic features of *Pseudomonas* genera. All the PGPM isolates were positive for the starch hydrolysis and citrate Starch hydrolysis test was performed to test the hydrolysis of starch by bacteria due to production of the extra cellular enzyme alpha-amylase. All these tests are based on the principle metabolic changes results in media culture change either by pH change and substrate utilization or addition of the reagent (Cappuccino and Sherman, 2007).

These bacterial cultures were also tested for sugar utilization. All bacterial isolates were positive for glucose utilization except for PGPM5. PGPM5 was the only culture to use adonitol as carbon source and rest all PGPM was negative for lactose, arabinose and sorbitol utilization (Table 2). Bacterial cells normally use sugars as the energy source whereas some bacterial species can use arabinose or xylose (Beisel and Afroz., 2015). Shruti *et al*., (2013) have reported *pseudomonas* culture with ability to utilize glucose, maltose, xylose and fructose.

Bacterial phenotypic identification was done through antibiotic sensitivity assay and results were tabulated in Table 2. All the isolates were resistant to colistin antibiotic. PGPM2, 3 and 4 was found to resistant against carbenicillin and penicillin-G antibiotic whereas PGPM5 was found moderate towards these antibiotics. For PGPM2 showed sensitivity towards tetracycline antibiotic whereas remaining PGPMs was resistant to it. PGPM3 was sensitive against chloramphenicol antibiotic and PGPM4 and 5 were resistant to it. For gentamicin antibiotic PGPM 4 was the only one which showed sensitivity towards it and rest all the PGPM were found as resistance spp. In the sensitivity assay all the PGPMs differ in their sensitivity and resistant pattern towards particular antibiotic which make them uniquely identical from each other isolates Figure 2. Secretion or production of the antibiotic substances is studied extensively for various applications in biotechnology. Resistance or sensitivity towards a particular antibiotic or a group of antibiotics can also serve as an important phenotypic character that can

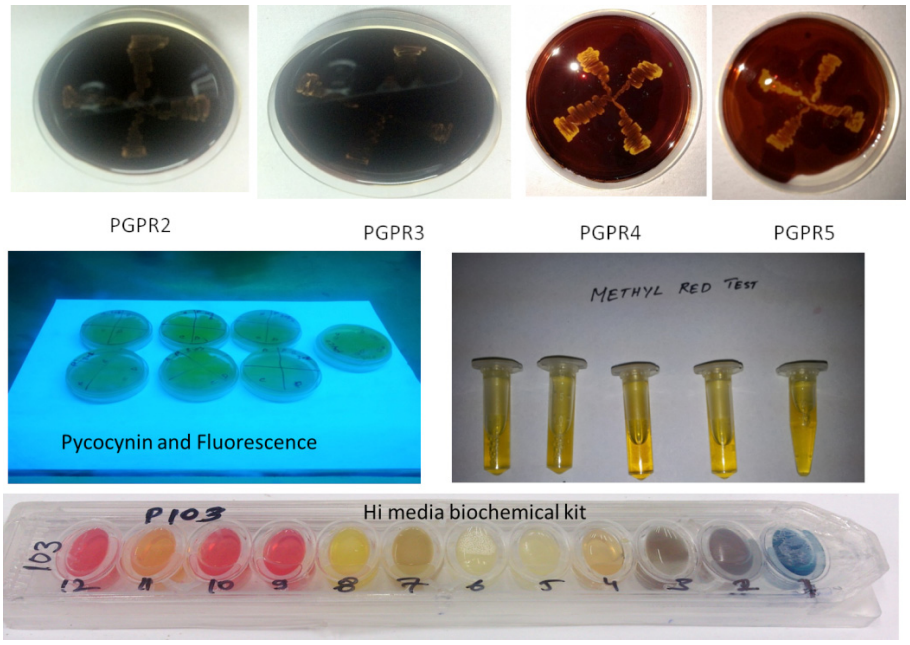

Fig. 1. Biochmecal tests with PGPMs

Table 2. Sugar utilization in PGPM strains

Tests	PGPM1	PGPM2	PGPM3	PGPM4	PGPM5
Glucose	P	P	P	P	N
Adonitol	N	N	N	N	P
Lactose	N	N	N	N	N
Arabinose	N	N	N	N	N
Sorbitol	N	N	N	N	N

be used to characterize the bacterial species (Compant *et al*., 2005).

For pigment production tests among all the isolates PGPM 2, 3 and PGPM1 showed pigmentation when grown on King's B media this is due to media carbon source, energy source, pH change or aeration (Meyer and Abdallah, 1978; Kumar *et al.,* 2014). Further they were also tested to produce other pigment like fluorescein and phycocyanin, PGPM2 and PGPM3 again showed positive pigmentation on agar plate and thus they were positive for the production of fluorescin and pycocynin pigment (Figure 1). In *Pseudomonas* genera exhibit distinguishing yellow- green pigment production under different condition (drought, salinity and water logging) and nutrient supply (Meyer *et al.,* 1978).

Initial characterization through morphological and biochemical assays revealed that these strains belong to the *Pseudomonas* genera. Further confirmation was obtained by the 16S rRNA sequence analysis. PGPM2 was found as *Pseudomonas* spp. and PGPM3 was found as *Pseudomonas plecoglocida* (Figure 3).

Different studies reported plant growth promoting abilities with the help of microorganism present in the soil system (Kumar *et al.,* 2014). Researchers have observed that these growth promoting effects are influenced by biotic and abiotic factors including presence of other bacterial species and the soil types (Kang *et al.,* 2013, Ahemad *et al.,* 2008). Growth hormone is essential for the development of plant and hence the microbes that are able to secrete plant hormones are more likely preferred in establishing beneficial (Apine and Jadhav, 2011). In the present investigation 5 isolates were screened *in vitro* for IAA production. Indoles acetic acid production was checked in all the PGPM isolates and showed that all isolates were positive for indole acid production,

Table 3. Phenotypic characterization of PGPM

	A	B	C	D	E	F	G	H	I	J
PGPR2	S	R	M	R	M	S	S	R	R	S
PGPR3	S	R	S	R	M	M	S	R	R	R
PGPR4	S	R	R	R	S	S	S	R	S	R
PGPR5	M	M	R	R	R	S	R	M	R	R

S = sensitive, R = resistant, M = moderate

*A-Ampicillin, C-Chloramphenicol, B-Carbenicillin,D- Colistin, E-Gentamicin, F-Kanamycin, G-Polymyxin, H-Penicillin-G, I-Spectinomycin and J-Tetracycline

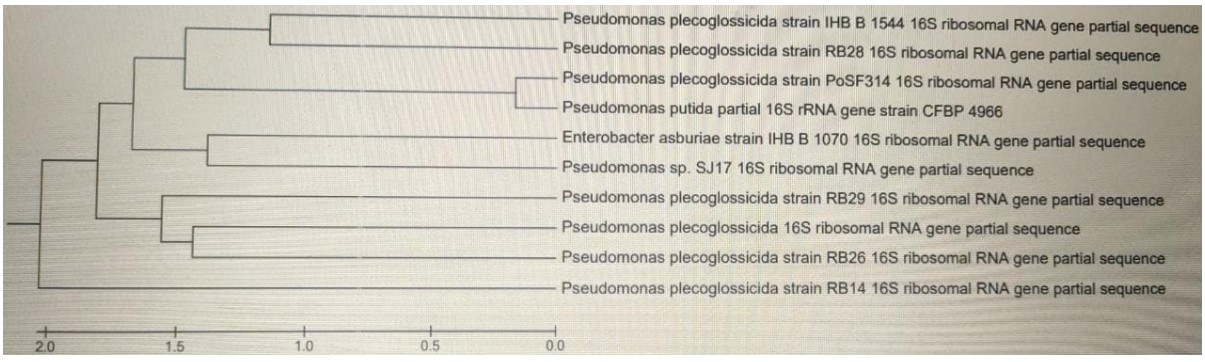

Fig. 3. Phylogenetic tree of bacterial spp.

as they were able to utilize L-tryptophan as source of nitrogen. The ability to use tryptophan and release of IAA found in the present study is in accordance with literature reported (Ali *et al.*, 2010; Mohite *et al.*, 2013). IAA is important in induction in cell elongation and cell division all subsequent steps for plant growth and development. Various studies reported for IAA production in *Pseudomonas* and other plant friendly microbes such as *Azospirilum* and *Azotobacter* spp (Apine and Jadhav, 2011). Lower concentrations of auxins (pytoharmone) reportedly stimulate root elongation, whereas higher concentrations inhibit the root elongation (Madhaiyan *et al.*, 2007). Another group of phytoharmone is gibberellic acid which involved in the process of Gibberellins (GAs) are plant hormones that regulate growth and influence various developmental processes, including stem elongation, germination, dormancy, flowering, sex expression, enzyme induction, and leaf and fruit senescence. In this study all the strains were found positive to produce gibberellic acids based on TLC and the results were in the conformity with the study by Bhalla *et al*., 2009 (Table 5). Rf values for gibberellic acid was observed in the study are 0.85 for PGPM 2 and 3, 0.86 for PGPM3 and for PGPM5 0.87, which is like GA3 hormone reported by Bhalla *et al.*, 2009. PGPR can enhance plant growth directly by providing plants with nutrients such as nitrogen via nitrogen fixation or by supplying phosphorus from soil bound phosphate (Kasim *et al.*, 2016; Berg, 2009). PGPR are known for their ability to synthesize several plant growth hormones such as auxins and cytokinins (Berg, 2009; Kumar *et al.*, 2014; Yang *et al.*, 2009).

On the phosphate solubilization test, it was observed that except PGPM 5 all other isolates showed ability to use the phosphate media for their growth. However, there is no clear zone of solubilization hence we can say that PGPM1-4 has weak phosphate solubilizing activity while PGPM 5 is negative for phosphate activity (Kumar *et al.*, 2014). Test to produce siderophore revealed maximum siderophore production in PGPM3 (33% SU) followed by PGPM1 and PGPM5 that is (25% and 21%) respectively moderate production in PGPM 4 showed. PGPM are generally known to produce secretes low-molecular mass iron chelating agent either homologous siderophores or heterologous siderophores (Khan *et al*., 2018). All the strain was positive for siderophore production. Means these microbes were able to use natural environment iron primarily occurs as Fe^{3+}, which is likely to form insoluble hydroxides and oxyhydroxides thus becoming in accessible to plants (Rajkumar *et al*., 2010). Siderophores ligands synthesised by microbes, not only help in iron nutrition of the host with which these microbes are associated fungal pathogen but also help in outcompeting other soil. Role of siderophores in control of diseases has been well documented by Baker *et al.*, 1986. Pathogenic mycelial growth suppression and inhibition of spore germination were the primary effects of antagonistic bacterial strains. Reported in literature in the bacte-

Table 4. Production of enzyme and other biochemical properties of all strains

	Tests	PGPM1	PGPM2	PGPM3	PGPM4	PGPM5
Growth hormone	IAA	+	+++	++	++	+
Growth hormone	IAA	+	+++	++	++	+
	Gibberellic acid	+	++	++	+	+
Markers indicating nutrient mobilization	Phosphate Solubilization	Weak reaction	Weak reaction	Weak reaction	Weak reaction	Weak reaction
	Nitrate Reduction	P	P	P	P	P
	siderophore production	+++	++	++++	++	++
Markers indicating biocontrol properties	Cellulase	-	-	-	-	-
	Lipase	+	+	+	+	+
	Protease	-	-	-	-	-
	Ammonia production	++	++++	+++	++	++
	HCN production	-	-	-	-	-
Amino acid	Lysine	N	N	P	P	P
	Ornithine	N	N	P	P	N
Other biochemical	Biofilm	++	+++	+++	++	++
	Emulsification	+	+	+	+	+

rial cell membrane iron form iron-siderophore complex which reduced to Fe^{3+} to Fe^{2+} which is further released by them through the siderophore via the inner and outer membranes. Thus, siderophores act as solubilizing agents for iron from unavailable compounds under iron limiting conditions (Indiragandhi *et al*., 2008). Numerous studies highlighted enhanced siderophore-mediated Fe-uptake by siderophore producing rhizobacterial inoculation (Rajkumar *et al.,* 2010). Sharma and Johri (2003) reported the role of the siderophore-producing *Pseudomonas* strain GRP3 on iron nutrition in vigna radiate. They have observed a decline in chlorotic symptoms and an increase in iron, chlorophyll a and chlorophyll b contents in strain GRP3 inoculated plants as compared to control, plants after 45 days of inoculation.

All isolates were found positive for lipase production. Ammonia production showed by PGPM2 maximum followed by PGPM3. PGPM1, PGPM4 and PGPM5 showed same colour change activity in the form of ammonia production. Starch hydrolysis was only showed by PGPM1, PGPM2, PGPM3 Cellulose, protease and HCN production were negative for all the PGPM's. These enzymes have a role to play in host growth promotion by assisting in breakdown of complex soil and elimination natural predator. It is evident that a variety of microorganisms also exhibit hyperparasitic activity, attacking pathogens by excreting cell wall hydrolases (Chernin and Chet, 2002).

PGPM seem to maintain plant growth over biotic stress in the agriculture field through antagonist activity against plant pathogen as there was contention for space, nutrients in ecological niches with the positive effects for farmers on the user, consumer or the environment (Ahemad *et al.,* 2008). Antagonistic activity of all the isolates were also tested against three plant pathogens *Macrophomina phesolina*, *Fusarium oxysporium* and *Sclerotium rolsfi* and the diameter were measured in cm. For *Macrophomina* spp. PGPM 4 and 2 and showed maximum inhibition by 53%for *Fusarium* spp. PGPR 3, 4 and 2 showed maximum inhibition by 31%, and for *Sclerotium spp.* PGPR 3 and 2 showed inhibition of 26%. *Pseudomonas* bacteria were recorded as highly aggressive colonizers in rhizosphere region of various crop plants and there are reports broad spectrum antagonistic activity against plant pathogens like nematodes (Almaghrabi *et al.,* 2013; Ramadan *et al.,* 2016; Yadav *et al.,* 2017; Kejela *et al.,* 2017). In biocontrol studies, isolates also showed better growth inhibition of plant pathogen in terms of percentage inhibition. PGPM 2 and PGPM4 showed maximum inhibition of plant pathogen *Macrophomina phaseolina* (!41%). PGPM3 and PGPM4 showed inhibition *for Sclerotium rolfsii* (↓31%). PGPM 2 and PGPM3 again showed maximum inhibition for *Fusarium oxysporum* (↓26%). This is further supported by Dal Bello *et al.* 2002, that PGPR have been employed to control several plant pathogens, including *Fusarium* spp. biological control could be achieved either by using the ability of several PGPR strains to antagonise the disease-causing agents or inducing plant resistance. In addition to some species of *Pseudomonas* and *Bacillus* was also reported to induce systemic resistance in plants against invading pathogens and antagonists to root-knot nematodes of *Meloidogyne* spp. (Ryu *et al.,* 2004; Prasad *et al.,* 2015; Farahat *et al.,* 2017).

Table 5. Antagonistic properties against root pathogen by PGPM

ROOT PATHOGEN	PGPR1	PGPR2	PGPR3	PGPR4	PGPR5
Macrophominaphaseolina	26%	41%	41%	53%	41%
fusarium oxysporium	23%	15%	31%	31%	16%
Sclerotium rolfsii	22%	26%	26%	21%	19%

Table 6. Pesticides tolerance by PGPM isolates

Pesticides	Parameters	PGPM1	PGPM2	PGPM3	PGPM4	PGPM5
Monocrotophos	LD50	35PPM	55PPM	45PPM	45PPM	40PPM
	MIC	100PPM	130PPM	110PPM	110PPM	110PPM
Dimethoate	LD50	25PPM	60PPM	25PPM	40PPM	35PPM
	MIC	100PPM	120PPM	100PPM	110PPM	110PPM

Another stress is temperature in the agriculture fields. We have tested the tolerance range of our isolates over a wide range of temperature under which they are able to grow well or not. All PGPM showed maximum growth at 30-35°C. Among *Pseudomonads*, PGPM2 showed maximum growth followed by PGPM3 and PGPM1.

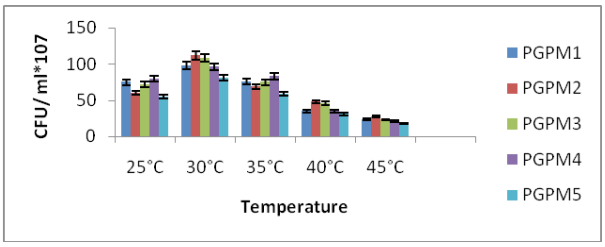

Fig. 4. Temperature tolerance of PGPM isolates

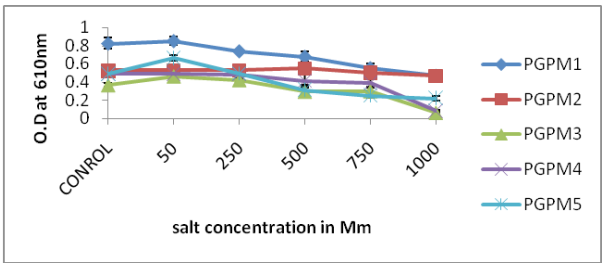

Fig. 5. Salt tolerance by PGPM isolates

Several reports published to account saline condition in agriculture land, and it is reported that year by year the saline portions are increasing. (Deshwal and Kumar, 2013; Paul and Nair, 2008; Costa *et al.*, 2020). Increased salinity in the soil results in decreased plant productivity and mineral uptake compared to soil without salinity reported by Han and Lee (2005). The isolates were also tested towards the tolerance of salinity in the range of 50, 100, 150, 250, 500, 750 and 1000MM solution of NaCl. All the isolates were able to grow up to 1000M solution though isolates growth decrease as the concentration of the salt increases. Under salt stress conditions all the isolates were able to grow and showed maximum tolerance level against it. PGPM1 was found to be most tolerant species even at higher concentration i.e 1000 mM the growth was slow as compared to control. The application of 100 mM of NaCl to peanut plants inhibited the nodule formation by *Bradyrhizobium* strains (Dardanelli *et al.*, 2009). Similarly, Ahmad *et al.* (2011) observed that salinity stress significantly reduced plant growth but inoculation with PGPR containing ACC deaminase and rhizobia enhanced plant growth, thus reducing the inhibitory effect of salinity. Kohler *et al.* (2010) conducted experiments and observed that plants inoculated with *P. mendocina* had significantly greater shoot biomass than the control plants at various salinity levels. Abbaspoor *et al.* (2009) mentioned that PGPR *Pseudomonas fluorescens* 153, 169, *P. putida* 108 effectively multiply in saline soil. Few plants growth promoting Pseudomonas had ability to survive under saline condition and enhanced the plant growth in maize (Kausar and Shahzad, 2006), cotton seedling (Yao *et al.*, 2010) and *Cicer arietinum* L. (Mishra *et al.*, 2010).

Along with pesticides all the PGPM showed sensitivity in term of their growth. Among *Pseudomonads*, with monocrotophos pesticides PGPM2 showed highest LD50 at 55ppm and MIC at 130ppm followed by PGPM3, PGPM4 and PGPM5. With dimethoate PGPM2 again showed LD50 at 60ppm and MIC at 120ppm, followed by PGPM4, PGPM5 and PGPM3. PGPM1 showed least tolerance towards these pesticides in terms of LD50 and MIC. Pesticides in the agriculture fields have a vast impact on microbial community and plant productivity. After implication pesticide residue has become major concern nowdays. PGPM can help plants to grow on contaminated soils with inherent toxicity. With this reference the isolates were studied having multiple PGPM characteristics were also checked for their ability to grow in presence of pesticides in the present study. All PGPM were found to tolerate the pesticide concentration ranging from 10-100ppm and their LD50 and MIC were tabulated. The evolving organic farming research is coming up with has developed many techniques to replace chemical fertilizers either completely or partially (Divya and Kumar, 2011). To ensure sustained productivity in farming system, it become necessary to replenish the reserves of nutrients which was removed from the soil due to excessive chemical inputs in agriculture. Application of PGPR to induce abiotic stress tolerance in plants is extensively investigated as an attractive strategy to control plant stress (Dimkpa *et al.*, 2009; Kasim *et al.*, 2013; Gupta *et al.*, 2019). PGPR utilize several mechanisms to induce abiotic stress tolerance in plants (Dimkpa *et al.* 2009; Yang *et al.*, 2009; Gong *et al.*, 2020). Biological control could be utilized to manage both biotic and abiotic stress factors. Plant growth promoting rhizobacteria (PGPR) are known for their abilities to induce plant defence/tolerance, promote plant

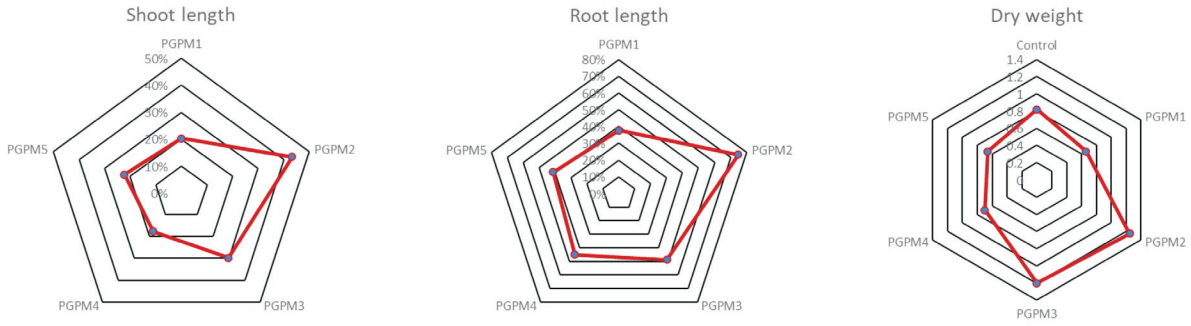

Fig. 6. Host plant treated with PGPMs

growth as well as antagonise several plant pathogens and have been considered as potential biocontrol agents (Vives *et al.*, 2018; Errickson and Huang, 2019).

Pearl millets were chosen for the plant bioassay study. PGPM treatment with host plant has shown a positive effect on the plant growth in terms of root length, shoot length, and plant dry weight. Among all PGPM, PGPM2 showed highest root length (77.6%), shoot length (76.3) and dry weight (125mg) followed by PGPM3 (RL69.8, SL65.3% and DW 120mg) as compared to control growth. After that PGPM1 showed RL increment of 47.3% and SL 43.6% followed by PGPM4 (RL42.9%, SL43%) and PGPM5 (RL42.8%, SL42%) showed better growth in comparison to control (Figure). PGPM2 and PGPM3 showed maximum growth on pearl millets in term of root length shoot length and dry weight. As compared to uninoculated control of all PGPM's showed higher growth as they are having inherent PGP properties. *Burkholderia gladioli* reported to colonize and proliferate in the root area of wheat and bajra thus increasing its shoot and root length as compared to control (Gunjal and Kapadnis, 2013). Egamberdieva (2010) showed that bacterial strains *Pseudomonas* sp. and *P. fluorescens* were able to colonize the rhizosphere of both wheat cultivars. The bacterial strains *Pseudomonas* sp. and *P. fluorescens* significantly stimulated the shoot and root length and dry weight of wheat. Various study intends that PGPM increased the plant growth, seed emergence and crop yield, and protect plant against plant pathogen (Kejela *et al.*, 2017; Khan *et al.*, 2020; Kumar *et al.*, 2018).

Conclusion

The accomplishment of *Pseudomonas spp* as a potential plant growth promoter and biocontrol agent encouraged research into a new level. It would be very advantageous to identify and established specific microbes with cumulative abilities of plant growth promotion, biocontrol and alleviate stress in the agricultural fields. In our study all the PGPM's have the ability to promote plant growth by secreting growth hormone, nutrient mobilization and other enzyme production but highest activity was recorded with PGPM2 and PGPM3. Apart from the growth promotion these spp. was also able to withstand various biotic and abiotic stress level which was extensively studied in this paper. Again, PGPM2 and PGPM3 in plant bioassay showed maximum growth on peral milltes in term of root length shoot length and dry weight. As compared to uninoculated control all the PGPM's showed higher growth as they are having inherent PGP properties. These strains are promising for agriculture application since they were able to grow quickly in broth condition, having different growth promoting attribute, protection against plant pathogen and are able to tolerate salinity, pesticides stress condition.

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