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Mini Review

Plant Growth Promoting and Biocontrol Potential of *Pseudomonas* sp. Strains on Sorghum (*Sorghum bicolor*) Plant

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Abstract

Extensive studies on the use of biocontrol agents (BCA's) to control diseases caused by plant pathogenic microorganism, to reduce the over usage of chemical inputs and to minimize broad use of fungicides, which leads to resistance in plant pathogens. In sustainable agriculture, plant growth promoting (PGP), and BCA's have emerged as eco-friendly alternatives to most of the chemical pesticides. In the present study, six *Pseudomonas* spp. strains were screened for various PGP traits *viz*., Indole-3-Acetic Acid (IAA), Phosphate solubilization, siderophore activity, hydrogen cyanide (HCN) production and furthermore the strains were characterized for *in vitro* antifungal activity against various plant pathogenic fungi, drought, and temperature tolerance. The strains P21-ABF and P22-DSK has shown effective PGP traits and antagonistic activity against *Rhizoctonia solani* and *Fusarium oxysporum*. The most prospective strains P21-ABF and P22-DSK were selected to perform *in planta* biocontrol studies on sorghum seeds. The most prospective strain P21-ABF upon molecular characterization was identified as *Pseudomonas aeruginosa*.

Keywords: Plant Growth Promotion; Biocontrol; Agriculture and Plant Pathogens

Introduction

Plant growth promoting bacteria (PGPB) have an important role in agriculture especially eco-friendly sustainable agriculture practices. These bacteria not only minimize the usage of chemicals, but they also help in accumulation plant minerals. PGPB perhaps, develops a useful (beneficial) interactions with plants especially by colonizing roots and they enhance plant strength and also increase soil [1]. These beneficial effects can be direct or indirect results on plants. Growth promotion as direct mechanism by PGPR includes production of secondary metabolites that enhances plant growth such as growth hormones like Auxins [2], cytokinins, gibberellins [3]. Indirect mechanism occurs *via* the inhibition of pathogens by the production volatile compounds such as hydrogen cyanide and Iron (Fe) chelating siderophores [4]. PGPR also antagonize plant pathogens and/or induce systemic resistance in the plant to various fungal and bacterial diseases [5,6]. Because plants lack effector-triggered immunity to many soilborne pathogens, they rely on PGPR for defence against pathogen attack [5]. Rhizosphere has wide microbiome populations in the form of epiphytes (on surface) and endophytes (inside plant tissues) [7]. For the last 30 years usage of microbial inoculants as commercial formulations has increased extensively which is an important alternative to minimize application of synthetic nutrients which helps towards healthy environment [2]. In bacteria genera *Pseudomonas* sp. is most studied because of having unique characteristics of processing multiple PGP traits. Their PGP activities include production of HCN, siderophores, pectinolytic enzymes, antimicrobial compounds, phosphate solubilizing enzymes [8,9]. Therefore, in the current research

we hypothesized that rhizobacteria isolated from arid rhizosphere soils may induce and support plant growth and biocontrol activity under biotic or abiotic stress condition. To report this hypothesis, we isolated six *Pseudomonas* sp. strains and were investigated to produce HCN, siderophores, phosphate solubilization and biocontrol activity.

Materials and Methods

Screening for PGP traits indole-3-acetic acid

Luria Bertani (LB) broth with added 5mmol tryptophan media was inoculated with 1% overnight culture (0.1 OD at 600nm) grown in LB broth and incubated at 28 °C for 48-72h on incubator shaker. Cell pellet was obtained by centrifugation at 5000rpm for 5min and the resulted supernatant was mixed with freshly prepared Salkowsky reagent and incubated for 1h at room temperature under dark conditions which develops pink pigmentation and absorbance was measured at 530 nm spectrophotometrically [10]. By Bradford method the auxin (IAA) was estimated in terms of protein using the cell pellet and concentration was expressed as mg/mg cell protein.

Siderophore production

To determine siderophore production 10*µl* of bacterial culture was spot inoculated on Chrome Azurol S (CAS) agar plates and incubated at 28°C for five days. Development of orange halo around the colony was considered as positive for siderophore production [11].

Production of HCN

The production of HCN was detected by streaking of test bacterial culture on King's B agar plates prepared by adding 0.4% glycine and plates were incubated with Whatmann No. 1 filter paper flooded with picric acid solution (0.5% picric acid in 2% sodium carbonate) located in the upper lid of petri dish. To prevent the escape of volatile compounds plates were sealed with parafilm. After 24-48 h, color change from yellow to orange of filter paper was observed [12].

Phosphate solubilization activity

For qualitative estimation of phosphate solubilization activity, 10µl test bacterial culture was spot inoculated on Pikovskya's agar plate and incubated for 3-5 days at 30°C. Halozone around the colony indicates the phosphate solubilization positive and is measure using the below formula [13]:

Phosphate solubilization index (PSI) = A/BX100 (A-Colony + Halozone diameter and B-Colony diameter).

Screening for drought and temperature stress

Trypticase soya broth (TSB) with different water potentials (-0.05, -0.15, -0.30, -0.49, -0.73 and -1.03 MPa) was prepared by adding appropriate concentrations of polyethylene glycol (PEG6000) [14] and was inoculated with 1% of overnight raised cultures of the bacterial isolates in TSB and incubated at 28°C for 24h in shaking incubator at 120 rpm. Three replicates of each isolate and each concentration were prepared. Bacterial growth was measured spectrophotometrically at 600 nm. Same method was followed to screen the test bacteria for temperature stress tolerance ranged 28°C to 65°C [15].

Antifungal activity

Determination of the antifungal activity of test bacteria was performed according to the cross streak method by Fernando [16]. In short, the PDA plate was inoculated with 24h old pure test bacteria as single streak at both the ends of petri plate and incubated at room temperature for 48h. After incubation, the test plates and control plates were spot inoculated with 5 mm fungal pathogen disc in the centre and without disturbing plates were incubated at 28 ± 1°C for another 5 - 7 days depending on the fungi tested. By comparing the growth of fungi in both test and control plates were measured and statistical data was prepared to observe the inhibition of fungi by test bacteria [17].

In planta plant growth and biocontrol activity on Sorghum

Agricultural field soil collected at Agriculture research farm, PJTSAU campus, Hyderabad, India, was used to study the efficacy of two drought tolerant PGP *Pseudomonas* sp. strains (P_{21}-DSk and P_{22}-DSK) in alleviating plant growth and biocontrol effects in host plant sorghum (*Sorghum bicolor*) as described earlier [15]. Sorghum seeds were surface sterilized with 0.1% $HgCl_2$ followed by 70% ethanol and washed multiples times with sterile distilled water. After drying seeds were inoculated with test bacteria either with liquid form or carrier based solid form having ~10^8 cfu/mL or/g and dried under cool place and planted into plastic pots filled with sterile soil. Different treatments of inoculated and uninoculated samples were replicated three times, maintaining three plants per pot. The pots were kept under greenhouse conditions and daily sprinkling with sterile distilled water. After 30 days of germination the plant lets were assed for root & shoot length and dry biomass.

Molecular characterization

The selected bacterial isolates were screen for microscopic, morphological and biochemical characterization according to Bergey's manual of determinative bacteriology. For molecular analysis, genomic DNA was extracted [18], and the 16S rRNA gene

was amplified by polymerase chain reaction (PCR) using universal forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse (5′-AAGGAGGTGATCCAGCCGCA-3′) primers using standard conditions (initial denaturation 94 °C for 5min; 30 cycles of denaturation at 94 °C for 30s, annealing at 50 °C for 40s, extension at 72 °C for 90s; final extension at 72 °C for 7min. The PCR product (~1500bp) was purified and sequenced (Xcelris Lab, Ahmadabad, India). The sequence (16S rRNA gene) obtained was compared with the existing data base of 16S rRNA gene sequence present at NCBI GenBank.

Results

PGP and antifungal activity

Based on cultural and morphological characteristics a total of six strains isolated from different rhizospheric soil samples were selected tentatively as *Pseudomonas* spp. based on the initial morphology and fluorescence nature under UV light on King's B agar media. Isolates screened for drought and temperature tolerance in TSB at varying water potential and different temperature. Out of five potential *Pseudomonas* spp. strains, only two could be able to grow at minimum water potential of –1.03 MPa and at higher temperature of 45°C (Figure 2 and 3), and all strains were screened *in vitro* for PGP traits. All strains were able to produce IAA and strain P22-DSK showed highest production (50.4 ± 1.1 µg/mg) and strain P21-ABF (37.6 ± 1.2 µg/mg) is next among other strains (Table 1). P-solubilization activity was confined only to 70% isolates and the highest solubilization index was confined to strain P21-ABF (189 ± 2.2) and strain P22-DSK showed second highest (158.30 ± 1.9) among other strains, but all the stains produced ammonia (99%) and interestingly siderophores and HCN were observed only in one strain P21-ABF (Table 1 and figure 1). Interestingly only two strains P21-ABF and P22-DSK were effectively inhibited the pathogenic fungal growth *Rhizoctonia solani* and *Fusarium oxysporum* strains. Strain P21-ABF showed 84% and 74% inhibition and strain P22-DSK showed 79% and 68% growth inhibition, respectively.

Strain	IAA (µg/mg protein)	Phosphate Solubilization Index	HCN Production	Siderophore Activity	*In vitro* % of radial growth inhibition of pathogenic fungi	
					Rhizoctonia	*Fusarium*
P21-ABF	37.6 ± 1.2	189.33 ± 2.2	+	+	84.48 ± 4.20	79.34 ± 3.90
P22-DSK	50.4 ± 1.1	158.30 ± 1.9	-	-	70.23 ± 2.88	68.71 ± 3.21
P4-DSK	31.8 ± 1.0	150.40 ± 1.8	-	-	-	-
P1	33.0 ± 1.2	-	-	-	-	-
PF-R5	27.6 ± 0.9	-	-	-	-	-
PF-SA4	19.2 ± 1.0	-	-	-	-	-

Table 1: PGP and antifungal activity of test isolates. Numerical values are mean ± SD (n = 3).

IAA: Indole Acetic Acid; HCN: Hydrogen Cyanide; +: Positive; -: Negative.

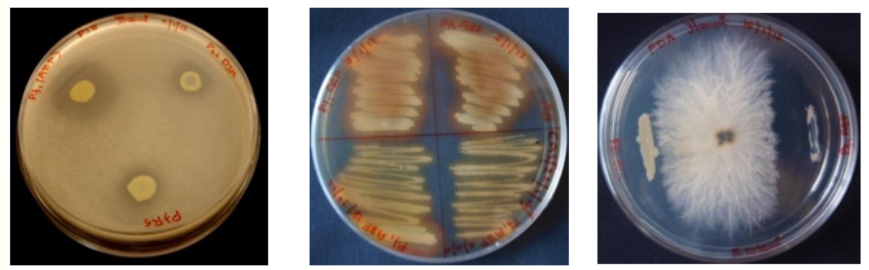

Figure 1: Showing *in vitro* PGP and antifungal activity: A; P-solubilization, B; Siderophore activity, C; Antifungal activity of P21-ABF against *Rhizoctonia solani*.

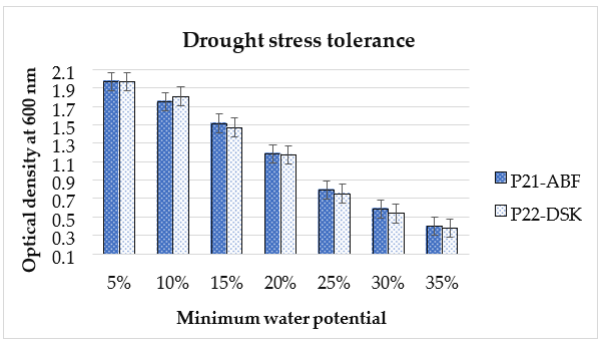

Figure 2: *In vitro* drought stress tolerance of test isolates, mean ± SD (n = 3).

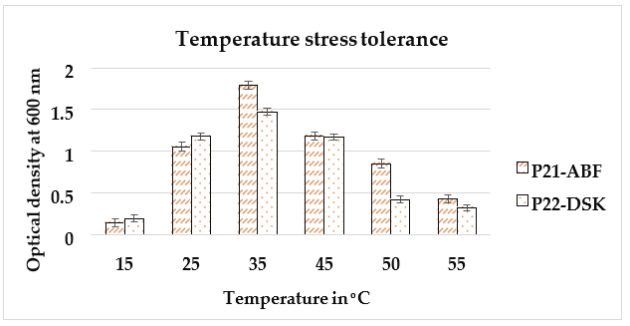

Figure 3: *In vitro* temperature stress tolerance of test isolates, mean ± SD (n = 3).

In planta assay

Plant growth promoting and biocontrol ability of microbial inoculation studies of two *Pseudomonas* spp. strains P21-ABF and P22-DSK were evaluated in Sorghum as model plant. Different test treatments such as seeds without any treatment as control, seeds treated with antagonistic bacteria, seeds with fungal pathogen and seeds with both antagonist and fungal pathogen. In all treatments, seeds were surface sterilized with 0.5% NaOCl (Sodium hypochlorite) and followed by washing with sterile distilled water for several times. After 25 days the physiological parameters like shoot, root lengths and dry mass of the seedlings were evaluated (Table 2). Results highlighted the positive effects of strain P21-ABF as a very effective growth promoter, thus significantly increasing the shoot (~ +17%), root (~ +70%) and dry mass (~ +84%) than control plants.

Molecular characterization of *Pseudomonas* sp. strain P21-ABF

The effective isolate selected based on PGP characters, abiotic & biotic stress tolerance was characterized based on microscopic, morphological, and biochemical studies. Gram staining method revealed that the isolate P21-ABF was gram -ve, motile, rod-shaped bacteria. On King's B agar medium colonies appeared as creamy, smooth, shiny, circular, convex with greenish pigmentation observed under UV light. From molecular analysis, 16s rRNA gene sequence blast showed 99% homology with existing gene data and the strain P21-ABF was identified as *Pseudomonas aeruginosa*.

Treatment	Inoculum	Shoot length (cm)	Root length (cm)	Dry mass (gm)
T1	Only seed	38.5	31.5	0.125
T2	Seed + *Rhizoctonia*	18.5	21.4	0.130
T3	Seed + *Fusarium*	22.4	23.9	0.121
T4	Seed + P21-ABF	43.6	65.2	0.345
T5	Seed + P22-DSK	41.5	61.7	0.302
T6	Seed + *Rhizoctonia* + P21-ABF	40.0	47.1	0.204
T7	Seed + *Fusarium* + P21-ABF	38.1	56.4	0.261
T8	Seed + *Rhizoctonia* + P22-DSK	35.1	39.3	0.279
T9	Seed + *Fusarium*+ P22-DSK	37.7	45.4	0.285
T10	Seed + *Rhizoctonia* + *Fusarium* + P21-ABF	45.3	53.8	0.231
T11	Seed + *Rhizoctonia* + *Fusarium* + P22-DSK	42.0	49.1	0.222

Table 2: Physiological parameters of *Sorghum* plants after treated with different test controls.

Cm: Centimeter; gm: Gram.

Discussion

The PGPR can competitively and effectively colonize plant roots and promote plant growth by reducing the population of deleterious bacterial/fungal phytopathogens. Plants were continuously exposed to various stress conditions (biotic and abiotic) and is considered as serious problem to agriculture affecting crop yield. The outline of stress (biotic and abiotic) tolerant microbial bioinoculants in agricultural soils can alleviate various stress conditions in crop plants by lowering stress-induced ethylene production. Stress tolerant bacteria/fungi can survive in these conditions and attach to the seed surface or colonize roots, results in the deamination of ACC, which is the precursor of ethylene, in plant cells through the production of ACC deaminase enzyme. This leads to lowering of the plant ethylene level and facilitates the growth and development of plants [19].

In our results, strains P21-ABF, P22-DSK and P4-DSK showed phosphate solubilization, except P1, PF-R5 and PF-SA4. These phosphate solubilizing microbes (PSM) have an important role in plant growth by making available insoluble phosphorus to soluble phosphates to plants. Therefore, the widespread and commercial application of PSM in plant growth promotion helps to reduce both escalating cost of phosphate fertilizers and to maintain the fertility of soil. Considering abovementioned points, the PSM presented with multiple PGP traits have more prospective to be used to enhance the plant productivity and growth [20]. Among many plant growth promoting rhizobacteria *Pseudomonas* and *Bacillus* spp. were extensively studied genera of phosphate solubilization activity [21]. The group of bacteria belongs to *Bacillus, Pseudomonas, Acinetobacter* spp. were evidenced to be as effective p-solubilizers, as well as phytohormone producers [22]. IAA production by microbes has positive effect on root system elongation and development which helps in the uptake of water and essential nutrients, this may lead to the increased root growth and develop a healthy plant as compared to control. All the six strains showed significant IAA production with the difference in their values. Out of six, two isolates produce higher IAA concentration with the value of 50.4 µg/mg for P22-DSK, 37.6 µg/mg for P21-ABF compared to previous reports of Rana., *et al*. [23]; Zhang., *et al*. [24]. Among the isolates only P21-ABF can be able to produce HCN and siderophore. Hydrogen cyanide production is a control mechanism by bacterial antagonists against broad range of phytopathogens [25]. Beneficial PGPR produce such secondary metabolites which were considered as BCAs against various plant pathogens. Also, HCN have indirect ability to increase the availability of soluble phosphate and iron to plants, resulting in enhanced plant growth [26]. Siderophores of PGP bacteria can promote plant growth through improved direct iron availability to plants under iron deficient conditions or by eliminating the availability of iron to pathogens [27]. Biocontrol ability was considered as important characteristics for the isolates whether they produce PGP traits or not. Amongst all, only two isolates were able to inhibit *F. oxysporum* and *R. solani* were therefore identified as PGPR. Isolates showing biocontrol ability were prospective PGPR because of their indirect effect on plant growth by inhibiting various pathogens [28]. Earlier reports suggest plant root exudates plays an important role in the alteration of rhizobacterial populations and can affect the incidence of antagonistic bacteria [29].

Two out of six isolates P21-ABF and P22-DSk were able to grow at a minimum water potential (-1.33 MPa). Low osmotic levels (-0.30 MPa) and EPS-production by stress tolerant bacteria were probably because of the naturalization in the semiarid habitats. EPS production is possible reason to a response to matric stress [30]. Microbial EPS has exclusive water retention 45and cementing characteristics, which protect bacteria against desiccation and various stress conditions through improved soil fertility and formation of soil aggregates at root level [31,32]. Indeed, an increase in EPS production in *A. brasilense* Sp245 was regarded as responsible protection under extreme drought situations [33]. The drought tolerance of the two rhizobacteria could be explained by production of EPS. Similarly, these two strains were also able sustain at a maximum temperature of 55°C and the optimum growth of these two strains was at 35°C where the growth of bacteria was high compared to all the temperature tested. Though there was a significant reduction in the bacterial growth as the temperature increases. Our present results were likely to the study conducted by Ali., *et al*. [34] he demonstrated increased growth of wheat plants by temperature tolerant *P. putida* strain AKMP-7. Based on preliminary studies on PGP traits like biocontrol and abiotic stress tolerance, bacterial isolates P21-ABF and P22-DSK were further screened for pot studies to evaluate growth promotion of Sorghum (*Sorghum bicolor* L). The initial bacterial count of P21-ABF and P22-DSK per seed was 1×10^7 colony forming units, respectively. We performed different treatments with different combinations of bacteria with and without pathogen. These two bacterial isolates significantly enhanced the growth sorghum plants (Table 2). However, of the

two isolates, P21-ABF showed significantly higher growth promotion in terms of increase root, shoot and dry biomass weight than P22-DSK in all combinations performed. Previously, many PGPB species viz., *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Rahnella* were reported for their ability enhance growth promotion studies on various plants [35]. A *B. pumilus* strain isolated from maize root surface has shown growth promoting effects on maize plants under greenhouse conditions [36]. Similarly, *B. subtilis* strain able to produce IAA and enhanced plant growth by increasing the number of root hairs [37].

To confirm the identity of the most prospective bacterial isolate P21-ABF, 16S rDNA gene sequencing was carried out since the 16S rDNA gene is the most used marker for reasoning the phylogenetic relationship between bacteria due to its universal distribution, highly conserved nature and its rate of evolution [38]. 16S rDNA gene analysis of 1500 bp sequence of P21ABF showed 99% sequence similarity with *P. aeruginosa* upon blast on NCBI GenBank. The bacterial isolate P21-ABF identification was much likely to the previous studies by [39] and his group isolated plant growth promoting, and antagonistic *Bacillus* spp. have earlier been reported for their stress tolerance against salinity, temperature and desiccation.

Conclusion

In PGPR world there were number of examples of effective biocontrol agents, the future challenge is not to prove that biocontrol is possible, but to improve efficacy and robustness of biocontrol activity, most importantly in field level studies. This will be achieved through a better understanding of the antagonism mechanisms, plant–microbe interactions and processes as well as microbial ecology in the soil and rhizosphere. In the present study, the *Pseudomonas* sp. strain P21-ABF was selected as a prospective strain to be used as a microbial inoculant in a stressful environment as it showed stress tolerance against all tested stress levels, exhibited antagonisms and multiple PGP activities. These types of prospective strains need in the process of sustainable agriculture strategies in various biotic and abiotic stress conditions. Since the strain P21-ABF was identified as *Pseudomonas aeruginosa* which is regarded as the human pathogen and identified some clinical relevant due to which these type of strains cannot be used as microbial inoculants for further field level studies, but the research carried out in the present study will be helpful as a model work to carry out the experiments on other PGP strains activity of P21-ABF against *Rhizoctonia solani*.

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