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Characterization of plant growth promoting rhizobacteria

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Abstract
In this study, we evaluated 12 selected rhizobacterial isolates for various *in vitro* plant growth-promoting traits. The functional diversity of the isolates with respect to beneficial traits demonstrated that all the isolates scored positive for nitrogen fixation, P, K and Zn solubilization; production of substances that promote plant growth, such as IAA, siderophore and ammonia. Two isolates demonstrated the ability to produce HCN. Further, quick analysis of PGP traits of the isolates on wheat was carried through paper towel test. With respect to germination percentage, all the isolates were significantly superior over control. The seedling fresh weight and dry weight were also found maximum in the seedlings treated with *S. tanashiensis* strain AUDT573 (1.98 g and 0.33 g, respectively).

Keywords: IAA, siderophores, IAR, plant growth promotion, paper towel test, etc.

Introduction
Plant Growth Promoting Rhizobacteria (PGPR) are a complex blend of bacteria that colonize plant roots and aid in their growth. While referring to the microorganisms intimately linked with the rhizosphere area, Kloepper and Schroth (1978) [15] coined the term "PGPR." Only 1-2 percent of the microorganisms in the rhizosphere have been shown to support the growth and development of plants (Antoun and Kloepper 2001) [3]. Using plant growth-promoting rhizobacteria (PGPR) as plant growth and health-stimulating agents is a biological method (Welbaum *et al.*, 2004) [37]. Beneficial bacteria colonize the roots of plants and play a crucial part in the growth of plants in several ways. These organisms present at the interface with the plant improve plant health *via*a number of mechanisms which may be direct or indirect (Pieterse *et al.*, 2014) [24].

Due to the exudates generated by plant roots, which serve as the primary food supply for microorganisms and facilitate effective geochemical cycling of nutrients, the rhizosphere is a hotspot for microbial interactions. In order to increase the development and production of agricultural crops and preserve the sustainability of agro-ecosystems, it is crucial to screen and choose effective PGPRs and use them in integrated strategies.

Wheat (*Triticum aestivum* L.) globally important is one among the top ten most popular crops and is widely cultivated around the globe (El-Lethy *et al.*, 2013) [7]. It is the second most staple food in various South Asian nations, including India. Worldwide, wheat is cultivated across an area of 279.9 million hectares, yielding a harvest of 779.6 million metric tonnes, with productivity of 2.8 metric tonnes per hectare (USDA, 2022) [35]. In contrast, India's wheat cultivation spans around 31.12 million hectares, resulting in an output of 109.59 million metric tonnes, achieving a higher productivity level at 3.5 metric tonnes per hectare (USDA, 2022) [35]. In order to enhance crop plant productivity, more than 143.88 million tonnes of chemical fertilizers are required annually. Despite being effective in increasing agricultural yields, these fertilizers have been shown to be harmful to both the health of the soil and the populations of both people and animals. The use of inorganic fertilizers and PGPR inoculation therefore represent promising agricultural approaches that can play a crucial role in crop protection, growth promotion or biological disease control, and sustained soil fertility. Applying microbial inoculants helps to reduce the use of chemical pesticides (Dilantha *et al.*, 2006) [6].

Although the primary mode of action of many PGPRs is via improving the availability of nutrients for the plant in the rhizosphere region, PGPRs are known to use one or more direct and indirect modes of action to boost plant growth and health (Glick 1995) [10]. Hence, in this current study, our efforts were focused on evaluating various characteristics related to the PGP

capabilities of wheat under controlled *in vitro* conditions.

Materials and Methods

Rhizobacterial cultures and their maintenance

Twelve rhizobacterial isolates (*Streptomyces shandoggensis* strain AUDT217, *Streptomyces parvus* strain AUDT248, *Streptomyces rimosus* strain AUDT502, *Streptomyces tanashiensis* strain AUDT573, *Streptomyces lavendule*strain AUDT617, *Streptomyces racemochromogenes* strain AUDT626, *Streptomyces spectabilis* strain AUDT656, *Streptomyces sclerogranulatus* strain AUDT690, *Pseudomonas azotoformans* strain AUDP203, *Pseudomonas marginalis* strain AUDP242, *Pseudomonas marginalis* strain AUDP279 and *Bacillus cereus* strain ACVB49 cultures available at the Microbial Genetics Laboratory, Department of Microbiology, College of Agriculture, Dharwad were used in the study. The actinobacterial cultures were maintained on Starch Casein Agar (SCA) at 28 ±1 °C, whereas, *Pseudomonas* sp. and *Bacillus cereus* strain ACVB49 were maintained on King's B agar and Nutrient agar, respectively. Single colonies were obtained by four-way streaking. Once purified, the isolates were tested and stored as glycerol stock at -80 °C.

Assessing the beneficial traits

ACC deaminase activity

ACC deaminase activity was determined following the procedure detailed by Palaniyandi *et al*. (2014) [21]. The bacterial isolates were cultivated using three distinct conditions (Dworkin *et al*., 1958) [41]: a minimal medium devoid of the nitrogen source (serving as the negative control), a minimal medium supplemented with ammonium sulfate (serving as the positive control), and a minimal medium supplemented with ACC at a final concentration of 3 mmol L^{-1}. Incubation of all agar plates took place at a temperature of 28 ± 2 °C for a period of 7 days. Isolates that exhibited growth on ACC-supplemented plates similar to that observed in $(NH4)_2SO_4$-containing medium were classified as positive for ACC deaminase activity.

Ammonia production

The isolates were examined for ammonia production by inoculating freshly. Grown bacterial cultures (at concentrations of 10^6 spores/mL for actinomycetes and 10^7 cfu/mL for bacteria) into tubes filled with 10 mL of peptone water. These tubes were then incubated at 28 ± 2 °C. Subsequently, 0.5 mL of Nessler's reagent was added into each tube, and the appearance of a yellow to brown coloration was monitored as an indicator of a positive result in the ammonification test (Cappuccino and Sherman in 1992) [4].

HCN production

The hydrogen cyanide-producing capability of the isolates was evaluated using the paired plate method (Lorck, 1948) [16] using their respective medium changed with glycine @ 4.4 g l^{-1}.

The procedure involved spreading the isolates onto HCN medium. Sterile discs made from Whatman No. 1 filter paper were prepared to match the diameter of Petri plates. These discs were then saturated with a 0.5 percent alkaline picric acid solution (0.5% picric acid (W/V) in 1% solution of sodium carbonate) and placed on the lids of each Petri dish. To capture volatile substances, the Petri plates were sealed with parafilm, ensuring no gaps were left. Subsequently, the sealed plates were incubated at a temperature of 28 ± 2 °C for a duration of 4 days. As a control, plates without rhizobacterial inoculation were used. The change in color of the. filter paper from yellow to brown was considered indicative of hydrogen cyanide (HCN) production.

Indole-3-acetic acid (IAA)

IAA production was quantitatively evaluated by growing bacterial isolates in TSB (MacFaddin, 1985) [7] supplemented with 2 mg/mL tryptophan as a precursor of IAA and incubated for 4 days at 28 ± 2 °C. The culture was centrifuged at 3000 rpm for 30 minutes. Then, a 2 mL portion of the supernatant was added along with the 100 µL of orthophosphoric acid and 4 mL of Salkowski's reagent, consisting of 50 mL of 35% perchloric acid and 1 mL of a 0.5 M FeCl3 solution. This mixture was left to incubate in darkness for 1 hour. The pink color development was taken as an indication of IAA production (Slama *et al*. in 2019) [32].

In vitro N_2 fixation

N_2 fixation by the rhizobacterial. isolates were estimated using the procedure outlined by Xu and Zheng (1986) [39]. Initially, the capacity of the isolates to grow on Norris N-free medium (Ranganayaki and Mohan, 1981) [26] were recorded by spotting 10 µl of the culture suspension on the plates and incubating at 28 ± 2 °C for 4 days.

Siderophore production

The siderophore-producing capability of the rhizobacterial isolates was evaluated using the universal CAS (Chrome Azurol S) assay. CAS reagent was prepared following the methods given by Schwyn and Neilands (1987) [29]. CAS agar plates were created by mixing 20 ml of CAS reagent with 180 ml of the respective sterilized media. The isolates were spot-inoculated with 10 µl and then incubated at 28 ± 2 ºC. An uninoculated plate served as a control. The development of a. yellow-orange halo around the bacterial colonies was considered indicative of siderophore production.

Mineral phosphate solubilization

Tri calcium phosphate (TCP) was used as a model insoluble P source for measuring the solubilization of insoluble inorganic phosphate. The petri plates containing sterilized TCP agar medium (Sundara Rao, 1963) [33] were spot-inoculated with 10 µl of cultures and the plates were incubated at 28 ± 2 °C.

Potassium solubilization

The solubilization of potassium by rhizobacterial isolates was examined by using the Aleksandrow's agar medium (Aleksandrov *et al*., 1967) [2]. The supplementation with 0.1% potassium aluminium silicate was done to the medium. Actively growing rhizobacterial cultures were spot-inoculated with (10 µl) onto Petri plates containing this medium, then incubated at a temperature of 28 ± 2 °C. The presence of a clear z0ne around the bacterial colonies was interpreted as the positive indication of potassium ('K') solubilization.

Zinc solubilization

The test isolates were subjected to screening to assess their capacity to solubilize zinc in TRIS mineral agar medium (Sharma *et al*., 2012) [31]. This medium was altered with the addition of 0.1 percent insoluble ZnO. Actively growing

cultures (10 µl) were spot-inoculated onto the medium and then incubated at a temperature of 28 ± 0.2 °C. Colonies that displayed a solubilization zone were identified as positive for zinc solubilization.

Quick analysis of growth promotional activities of rhizobacterial isolates on wheat through paper towel test

Wheat seeds were subjected to germination test in triplicates using germination paper as substrate. Prior to seed coating, wheat seeds were surface sterilized by soaking in 75% ethanol for 5 min followed by thorough rinsing in sterile distilled water and drying in a laminar flow cabinet. The seeds were bioprimed with rhizobacterial suspension (1×10^8 cfu/mL or 1×10^6 spores/mL) for 1 h and dried (Hadj Brahim *et al.*, 2022). The coated seeds were placed on wet paper towel and covered with another wet paper towel. The two paper towels were rolled carefully in a wax paper and tied with a rubber band and placed in germination chamber maintained at 25 ± 1 °C in an upright position. The seedlings were observed after 10 days for appearance of symptoms *viz.*, seed rot and seedling blight. In addition, following observations were made (Zucconi *et al.*, 1981) [40].

Germination percentage (GP)

The germination percentage was calculated using the following formula: (Ruan *et al.*, 2002) [28]

$$GP\ (\%) = \frac{\text{Total number of germinated seeds}}{\text{Total number of seeds tested}} \times 100$$

Seedling fresh and dry weight

After 5 days of germination, 10 seedlings per treatment were randomly selected to measure their fresh weight. Subsequently, they were dried overnight in an oven at 60°C for 72 hours, and their dry weight was subsequently determined (Patel *et al.*, 2017) [22].

Results

Assessing the beneficial traits of rhizobacteria

Among 12 rhizobacterial isolates, 9 rhizobacterial isolates developed brown colour upon addition of Nessler's reagent indicating positive for the ammonia production but all the rhizobacterial isolates produced IAA as detected by the Salkowaski's reagent. In case of 6 among 12 isolates showed an orange-colored halo-zone around the colony on dark blue-colored agar plates signifies the production of siderophore (Table 1).

HCN Production

The Picric acid assay was used to assess the ability of the rhizobacterial isolates to produce HCN. Among the 12 rhizobacterial isolates characterized for HCN production, AUDT 502 and AUDT 573 were capable of changing the colour of filter paper from yellow to brown indicating the positive results.

***In vitro* nitrogen fixation**

The ability of rhizobacterial isolates to fix nitrogen was ascertained by spotting cultures on Norris N-free agar (Ranganayaki and Mohan, 1981) [26] and growth of rhizobacterial isolates was taken as positive. Among 12 rhizobacterial isolates, 11 rhizobacterial isolates (AUDP203, AUDP 242, AUDP 279, ACVB 49, AUDT 217, AUDT 248, AUDT 502, AUDT 573, AUDT 626, AUDT 656 and AUDT 690) were able to fix nitrogen.

Table 1: Elucidation of the beneficial traits of rhizobacteria

Isolate	N_2 fixation	Siderophore Production	Ammonia Production	HCN Production	IAA production
P. azotoformans AUDP203	+	+	+	-	+
P. marginalis AUDP 242	+	+	+	-	+
P. marginalis AUDP279	+	+	+	-	+
B. cereus ACVB49	+	+	+	-	+
S. shandoggensis AUDT217	+	-	+	-	+
S. parvus AUDT248	+	-	+	-	+
S. rimosus AUDT502	+	-	+	+	+
S. tanashiensis AUDT573	+	+	+	+	+
S lavendule AUDT617	-	-	-	-	+
S. racemochromogenes AUDT626	+	-	-	-	+
S. spectabilis AUDT656	+	-	-	-	+
S. sclerogranulatus AUDT690	+	-	+	-	+

Note: "+" indicates positive w.r.t. beneficial traits, "-" indicates negative w.r.t. beneficial traits

ACC deaminase activity

The ability of efficient rhizobacterial isolates to produce ACC deaminase enzyme was examined by spotting the cultures on the minimal agar medium. The results indicated that each isolate was able to grow by using ACC as nitrogen source thus, scored as positive for the ACC deaminase activity (Table 2).

Table 2: ACC deaminase activity of rhizobacterial isolates

Isolate	$(NH_4)_2SO_4$ as N source	-ve control	ACC as N source
P. azotoformans AUDP203	-	-	++
P. marginalis AUDP 242	+	+	+++
P. marginalis AUDP279	+	+	+++
B. cereus ACVB49	+	+	++
S. shandoggensis AUDT217	+	+	++
S. parvus AUDT248	-	-	++
S. rimosus AUDT502	+	+	+++
S. tanashiensis AUDT573	+	+	+++
S lavendule AUDT617	+	+	++
S. racemochromogenes AUDT626	+	+	++
S. spectabilis AUDT656	+	+	+++
S. sclerogranulatus AUDT690	+	+	+++

Solubilization of minerals

The isolates capacity to solubilize phosphate was ascertained by observing for formation of clear zone of around the colony on their respective media. The results indicated that every 12 rhizobacterial isolates formed clear halo-zone on TCP, Aleksandrow's and TRIS minimal medium indicating solubilization of phosphate, potassium and zinc, respectively (Table 3).

Table 3: Qualitative estimation of mineral solubilization by rhizobacterial isolates

Isolate	Phosphate	Zinc	Potassium
P. azotoformans AUDP203	+	+	+
P. marginalis AUDP 242	+	+	+
P. marginalis AUDP279	+	+	+
B. cereus ACVB49	+	+	+
S. shandoggensis AUDT217	+	+	+
S. parvus AUDT248	+	+	+
S. rimosus AUDT502	+	+	+
S. tanashiensis AUDT573	+	+	+
S lavendule AUDT617	+	+	+
S. racemochromogenes AUDT626	+	+	+
S. spectabilis AUDT656	+	+	+
S. sclerogranulatus AUDT690	+	+	+

Quick analysis of growth promotional activities of rhizobacterial isolates on wheat through paper towel test

With respect to germination percentage, all the treatments were significantly superior to untreated control. The highest percentage of germination was observed the seeds treated with, *S. tanashiensis* strain AUDT573 (96.07%) followed by *S. rimosus* strain AUDT502 (95.73%) when compared to control (56.73%). The seedling fresh weight and dry weight were found maximum in the seedlings treated with *S. Tanashiensis* strain AUDT573 (1.98 g and 0.33 g, respectively) followed by *S. rimosus* strain AUDT502 (1.95 g and 0.32 g, respectively) when compared to control (1.50 g and 0.29 g, respectively) (Table 4).

Table 4: Quick analysis of growth promotional activities of microbes on wheat (Paper Towel test)

Isolates	Germination percentage (%)	Seedling fresh weight (g)	Seedling dry weight (g)
P. azotoformans AUDP203	55	1.77	0.30
P. marginalis AUDP 242	58	1.78	0.31
P. marginalis AUDP279	56	1.72	0.29
B. cereus ACVB49	54	1.69	0.26
S. shandoggensis AUDT217	58	1.75	0.32
S. parvus AUDT248	60	1.58	0.26
S. rimosus AUDT502	95	1.95	0.32
S. tanashiensis AUDT573	96	1.98	0.33
S lavendule AUDT617	88	1.89	0.29
S. racemochromogenes AUDT626	96	1.90	0.31
S. spectabilis AUDT656	59	1.86	0.25
S. sclerogranulatus AUDT690	90	1.70	0.31
Control	56	1.50	0.29
S.Em. (±)	0.97	0.42	0.25
C.D. @ 1%	2.92	1.19	0.79

Discussion

Rhizobacteria are known to fix nitrogen thereby stimulate plant growth, maintain the nitrogen in soil and thereby improve soil quality (Franche *et al*., 2009) [8]. Numerous studies showed that few non-symbiotic species of *Streptomyces* (Sellstedt and Richau, 2013) [30], *Pseudomonas*

sp. (Mirza *et al*., 2006) [18] and members of some *Bacillus* spp. have nitrogen fixation capabilities. P-solubilizing microorganisms are involved in the conversion of insoluble P to bioavailable forms thereby making it accessible to plants (Rajput *et al*., 2013) [25]. The *Streptomyces* (Jog *et al.*, 2014) [14], *Bacillus* and *Pseudomonas* presence in the rhizospheric soil improves plant assimilation of P. The secretion 0f organic acids that can dissolve inorganic phosphorus or *via* the secretion of phosphatases are the major mechanisms involved in P solubilization. Similarly, K solubilization also result in acidification of Aleksandrow's broth or the chelation of cations that bind to K. The screening for solubilization of the inorganic zinc showed that each of the 12 rhizobacterial isolates possessed the capability to solubilize inorganic zinc. These findings are in accordance with Suriyachadkun *et al*. (2022) [24] who showed that zinc-solubilizing rhizobacteria (*Streptomyces*) had higher solubilizing ability in the medium containing ZnO.

Indole-3-Acetic Acid (IAA) is an auxin that is common and natural and is resulted from L-tryptophan metabolism in microorganisms (Davies, 2004) [5]. The biosynthesis of IAA by soil-dwelling *Streptomyces* spp. has been well-documented in various studies (Vurukonda *et al*., 2018) [36]. IAA production was seen across every tested rhizobacterial strains at varying concentrations, likely attributed to differences in their physiological characteristics.

In this study, the rhizobacterial isolates exhibited ammonia production in liquid culture tubes (peptone water), evident from the change in color from light yellow to brown. Narula and Gupta (1987) [19] demonstrated that inoculating wheat and barley with ammonia-releasing strains led to increased dry weight. In our study, with the exception of three rhizobacterial isolates, all others demonstrated positive ammonia production.

Among volatile compounds considered as antibiosis molecules, HCN plays a role in sequestering iron, biological control of phytopathogens (Gu *et al*., 2020) [11] and increasing free phosphates for plant assimilation (Rijavec and Lapanje, 2016). Out of the rhizobacterial isolates subjected to testing, only two isolates were positive for hydrogen cyanide (HCN) production. The capability of PGPR to produce HCN is not limited to specific genera, which has led to their application as biofertilizers 0r biocontrol agents to bolster crop production (Agbodjato *et al*., 2015) [1]. PGPR also exhibit the ability to synthesize a variety of siderophores, which possess a strong affinity for iron. Consequently, this leads to a reduction of iron availability to pathogenic agents, including plant pathogenic fungi (Whipps, 2001) [38].

The rhizobacteria can boost the availability of nutrients for plant through fixing nitrogen, solubilizing inorganic phosphorus, potassium, zinc and producing of siderophores (Patni *et al*., 2018) [23]. The rhizobacteria also protect plant from pest and pathogen by producing cellulose, pectinase, xylanase, amylase and gelatinase (Glick, 2012) [9]. The enhanced efficacy of these strains within plants can be attributed to their diverse beneficial characteristics, including their heightened capacity for indole-3-acetic acid (IAA) production. Similar findings were reported by Imperiali *et al*. (2017) [13], who observed that treatments involving different Pseudomonad strains and mycorrhizal fungi contributed to improved plant health and productivity. In conclusion, the bacterial isolates consisting of N-fixer, P-solubilizer and K solubilizer incremented the growth of the wheat compared t0 untreated control (Negi *et al*., 2022) [20].

Conclusion

The isolates were found possess multiple plant growth promotional traits which was evident through *in vitro* studies as well as paper towel test. We consider these traits for further research to determine their biocontrol activities and evaluation under greenhouse and field conditions for plant growth promotion and biological control of pathogens.

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