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Isolation of Rhizobacteria spp. and its effect on the Growth of *Glycine max* (L.) Merr.

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A B S T R A C T

Plant growth-promoting rhizobacteria (PGPR) are shown to have the natural capacity of environmental clean-up by replacing the use of chemicals in agriculture. The soil-born PGPR provides plant growth promotion through biostimulation and biocontrol activity. In the present study, rhizobacteria were isolated from various crop rhizosphere and screened for their biostimulation abilities like phosphate, potassium, and zinc solubilization and, ammonia, indole-3-acetic acid (IAA) and hydrogen cyanide (HCN) production. Total 60 morphologically distinct rhizobacteria were isolated from various crop fields. Among 60 isolates, 49 isolates showed phosphate and potassium solubilization ability, while 31 isolates showed zinc solubilizing activity. HCN production was detected in 19 isolates which indicates the biocontrol ability of these isolates. IAA production was detected in 12 isolates. Finally, 4 isolates, *Microbacterium indicum, Bacillus altitudinis, Staphylococcus sciuri, Pseudomonas fluorescens* that were showing maximum traits positive were selected for seed germination assay and these isolates were used as plant probiotics for in vitro plant growth promotion activities for *Glycine max* (L.) crop. The effect of isolates on the growth of crop plants was assayed and found that treated plants were superior over the control plants concerning percent germination, shoot-root length, and vigour index. It indicates that these isolates may be used as biofertilizers and bio inoculants for various crops as they enhanced plant growth via diverse mechanisms and offered an attractive strategy to replace chemical fertilizers and pesticides.

***Key words*:** PGPR, Rhizobacteria, IAA production, Mineral solubilization, HCN production, Plant probiotic

The uncontrolled use of chemical fertilizers in modern agriculture, particularly nitrogenous and phosphorus fertilizers, has resulted in significant soil, air, and water pollution. The use of these substances in excess seems to harm soil microorganisms and harms soil fertility and also pollute the environment [1]. To overcome this problem and to enhance soil fertility, the capacity of PGPR to directly boost the growth of many crops has proved its potential as biofertilizers [2]. Soil is an important natural environment for plants and is necessary for their growth because it contains key nutrients and beneficial bacteria. Soil is made up of important minerals and nutrients that are present in the form of organic matter, soil, and water making them available to plants [3]. "Rhizobacteria" are the bacteria that colonize the root [4]. These helpful microbes, known as plant probiotics [5-6], enhance plant growth through various direct and indirect methods, including nitrogen fixation and phosphate solubilization, and phytohormone as well as siderophore production [7]. Inoculants containing beneficial microorganisms applied to crops could allow sustain and increase agricultural productivity while using fewer resources and aid in the cultivation of marginal lands [8-9]. *Glycine max* (L) Merr. (soybean) is known as the "golden bean" of the twenty-first century and is one of India's most significant legume crops, contributing to soil fertility and offering a rich source of protein. Soybean seed inoculation with the suitable Rhizobium sp. at sowing is advised for maximum effectiveness [10]. Different studies have discovered that a portion of the nitrogen fixed by legumes is transferred to the crop, resulting in significant nitrogen production and an increased cropping system [11]. The major goal of this study was to isolate, screened and characterize the rhizobacteria from soybean rhizosphere and to see how these PGPR strains inoculation improves growth of soybean.

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MATERIALS AND METHODS

Sampling and Isolation of rhizobacteria
Collection of soil samples

Soybean roots and rhizosphere soil samples randomly collected from various locations of Nashik District, Maharashtra, India. Soil samples were taken from 3 to 20 cm

layers below the soil surface. Root adherent soil samples and uprooted plant samples were transferred to the sterile culture bottles and refrigerated at 4°C temperature in laboratory for further use.

Isolation of rhizospheric microorganisms

Firstly, 0.1 gm of soil collected from the rhizosphere of soybean was added to 10 ml of saline and shaken for 2 minutes. Sample solutions were kept stable for 10-15 minutes at room temperature for the settling of suspended particles at bottom of the flask. Primary isolation of rhizospheric bacteria was done on Nutrient Agar media. Serial dilutions of supernatant were prepared up to 10^{-9} and inoculated on the nutrient agar plate by spread plate technique and incubated overnight at 37°C. Specialized media used for isolation of PGPR was Yeast Extract Mannitol Agar, Ashbey's Mannitol Agar, Kings B Medium, Pikovskaya medium. Rhizobacteria isolated in primary isolation on Nutrient Agar medium were reisolated on the above-mentioned media. The PGPR isolates were preserved on nutrient agar slants for further characterization and identification.

Screening of PGPR traits

Nitrogen fixation

For detection of nitrogen fixation Jensen's media was used [12]. The medium contains (g/L), 20 g sucrose, 1 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g NaCl, 0.1 g Fe_2S0_4, 0.005 g Na_2MoO_4. $2H_2O$, 2 g $CaCO_3$, and 15 g agar. The PGPR isolates were inoculated on the Jensen's medium and were incubated for 4 days at 37°C temperature and observed for the presence of growth.

Mineral solubilization

a. Phosphate solubilization

All the isolates were tested for phosphate solubilizing activity by spot inoculation on Pikovskaya's medium containing tri-calcium phosphate (TCP). The medium contains (g/L), 10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_47H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4H_2O$, and 0.002 g $FeSO_47H_2O$. This method was described by Pikovsakaya in 1948. Plates were incubated at 37°C for 2-3 days. Zone of solubilization around colony indicates phosphate solubilization ability of isolate. The diameter of colony and halo zone around colony were recorded. The solubilization index (SI), which is obtained by the ratio of clearance zone diameter to colony diameter, can be used to calculate bacteria's mineral solubilizing ability. Mineral solubilization index (SI) can be calculated using the formula below [13-15]:

$$\text{Solubilization Index (SI)} = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}} \times 100$$

b. Potassium solubilization

Potassium-solubilizing bacteria are bacteria that are engaged in the solubilization of potassium from potassium-bearing minerals (KSB). They can transform insoluble mineral potassium into available potassium in the soil [16]. All the isolates were screened for potassium solubilizing activity by spot inoculation on modified Aleksandrov medium containing, 0.5 g glucose; 0.5 g $MgSO_4$. $7H_2O$; 0.1 g $CaCO_3$; 0.006 g $FeCl_3$; 2.0 g Ca $(PO_4)_2$; 3.0 g potassium aluminum silicate; and 20.0 g agar in 1 litre of distilled water. pH of the medium is adjusted to 7.2 by adding 1 N NaOH. The plates were incubated at 28±2°C for 3 days. The colonies exhibiting zone of solubilization were selected and the diameter of the colony and total zone of solubilization was recorded.

c. Zinc solubilization

All the isolates were tested for solubilizing efficiency by spot inoculation on modified Pikovskaya's agar medium containing 0.1% of $ZnCO_3$, as an insoluble zinc source [17]. The plates were incubated at 30°C for 48 hours. The colonies exhibiting zone of solubilization were selected and the diameter of the colony and total zone of solubilization was recorded to calculate the zinc solubilizing efficiency of the isolate.

Indole acetic acid (IAA) production

The rhizobacteria isolated were analyzed for Indole Acetic Acid (IAA) production ability described by Ehmann [18]. The isolated strains were tested for IAA production. 10 ml of nutrient broth supplemented with 1mg/ml Tryptophan was inoculated with 24 h old bacterial cultures and incubated for 72 hours on a rotary shaking incubator at 30±2°C. After incubation, the broth was centrifuged at 6000 rpm for 10 minutes. The supernatant (1 ml) was mixed with 2 ml of Salkowaski's reagent (0.5M $FeCl_3$ in 35% of perchloric acid) and kept in dark and incubated for 25 minutes. Colour change was recorded at 530nm absorbance. Non inoculated broth was used as control. Standard curve was prepared by plotting known IAA concentration 10, 20, 30, 40, 60,70, 80 and 90 µg/ml against 530 nm absorbance. IAA produced by isolates was calculated using standard curve.

Ammonia production

The Nessler's reagent method described by Cappuccino and Sherman [19] was used to detect ammonia production. All the 24 hr old bacterial isolates were inoculated in 10ml of 1% peptone broth. Tubes were incubated at 30±2°C for 48 hours on a rotary shaking incubator. After incubation 1ml of Nessler's reagent was added. The development of faint yellow to the dark brown colour indicated the production of ammonia [19].

HCN production

Hydrogen cyanide gas production of all the isolates was checked by the method described by Castric [20]. The isolates were inoculated onto a nutrient agar slant containing 4.4 g/L of glycine [21]. A Whatman filter paper no.1 soaked in 0.5% picric acid in 2% Na_2CO_3 was placed inside the tube. Tubes were sealed and kept for incubation at 30±2°Cfor 4-5 days. Filter paper turns brown indicates HCN production

Identification of selected PGPR strains by sequencing of 16S rRNA gene

Among four selected bacterial isolates, 12,17, 34 were identified by 16S rRNA gene sequencing at NCIM Pune, and identification of isolate 59 was done based on morphological and biochemical analysis.

Glycine max (L.) seed germination assay

Seed germination assay was performed by using 50 *Glycine max* seeds and 5 experimental sets for each isolate. Seeds were surface sterilized with a 2% (v/v) sodium hypochlorite solution for 10 min and with 70% ethanol for 30 sec, after that rinsed with sterile distilled water 3-4 times. For 30 minutes, the surface-sterilized seeds were immersed in individual bacterial solutions while being shaken at 120 rpm. Fifty seeds of soybean were placed in sterile petri dishes with filter paper and irrigated with sterile distilled water. The seeds were incubated in a dark condition at 30±2°C for 10 days, and the germinated seeds were counted every 48 hr. Seeds were

considered germinated when its radicle emerged by about 2 mm. The final percent seed germination rate was calculated [22]. The seedling vigour index was determined by measuring the root and shoot lengths of plant after 8 days [23] using the formula below:

Vigour index = (Mean root length + Mean shoot length) × Seed germination percentage

Statistical analysis

One way analysis of variance (ANOVA) was conducted at a significant difference level and a 0.05 level to test the effect of treatments on *Glycine max* (L.) plant root and shoot growth using Tukey HSD method [24]. The root and shoot lengths were measured and the data was statistically analyzed. Each treatment was tested with at least five replicates, with a standard deviation (SD) calculated and data expressed as mean ±SD. When P was ≤ 0.05, the observations were considered significant [25].

RESULTS AND DISCUSSION

Isolation of rhizospheric microorganisms

Seven distinct crop fields from Nashik region were selected for isolation of PGPR. In an isolation 60, morphologically distinct rhizobacteria were isolated on various culture media, such as, Nutrient agar, Pikovaskayas agar, Ashby's agar, Yeast extract mannitol agar, King's B medium. These isolates are used for further studies.

Screening of PGPR traits

Nitrogen fixation

All rhizobacteria isolated were cultured on Jensen's media, 50 isolates demonstrated nitrogen fixing potential among all rhizobacteria isolated from soil samples. Rhizobacteria growth promoting effects include nitrogen fixation [26]. Biswas [27] reported increased nitrogen uptake in rhizobia inoculated rice plants. It shows plant growth promotion ability of nitrogen fixing rhizobacteria which were isolated in the present study.

Mineral solubilization

a. Phosphate solubilization

Calcium phosphate present in the soil as a non-utilizable form is dissolved to convert in utilizable form is termed as mineral phosphate solubilization [28]. Among all 60 isolates, 49 isolates were able to produce a zone of solubilization around the colony on Pikovskaya's agar plates. Around 13 isolates showed considerable solubilization efficiency. Isolate number 59 showed a maximum solubilization index at 6.25. While isolate number 12 showed a 3.71 solubilization index. Isolate number 17 and 34 both have showen solubilization index 2.71 (Fig 1). The ability of the isolates to solubilize tri-calcium phosphate (TCP) showed greater capacity as compared to the reported results of Pathak *et al.* [29]. They reported the solubilization index of isolate PB-1 was 2.08, isolates PB-4 and VC-01 showed SI of 1.24 and 1.31 respectively, which was found to be less as compared to the present study. A mechanism for variation in solubilizing abilities of isolates may be due to the release of organic acids and their chelating abilities [30].

b. Potassium solubilization

Among 60 isolates 49 isolates showed potassium solubilization ability of which 22 isolates showed a solubilization index of more than 3. Isolate number 12 and 59 showed 3.14 and 3.75 solubilization index, while isolates number 17 and 34 showed 2.42 and 2.80 solubilization index respectively (Fig 1). In comparison to non-rhizosphere soil, the rhizosphere has a significantly higher concentration of potassium solubilizing bacteria [31]. Meena *et al*. [32] has reported the ability to solubilize the silicate rocks by *Bacillus spp*. has been found effective among other potassium solubilizers.

c. Zinc solubilization

Among 60 isolates, 31 isolates had shown a zone of solubilization on modified Pikovskaya's medium, confirming their zinc solubilizing activity. Around 8 isolates showed solubilization index of more than 3. Isolate number 59 showed a 4.75 solubilization index, while isolates number 12, 17, and 34 showed a solubilization index of 2.85, 2.71 and 2.71 respectively (Fig 1). Sharma *et al.* [33] has isolated *Bacillus* from soybean rhizosphere soils and screened for Zn a solubilization ability with 0.1% zinc in the form of ZnO, $ZnCO_3$, and Zn $(PO_3)_4$ as insoluble zinc sources and reported similar results with the present study, in which *Bacillus* species showed the greater diameter of solubilization on all the three zinc compounds.

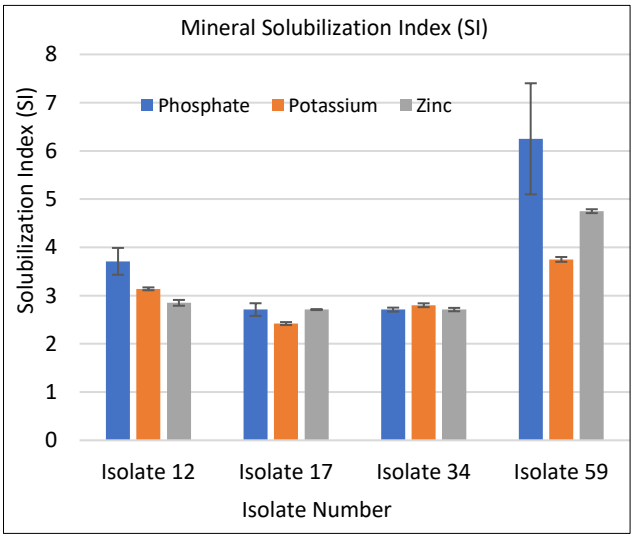

Fig 1 Phosphate, potassium and zinc solubilization index of 4 selected efficient rhizobacteria

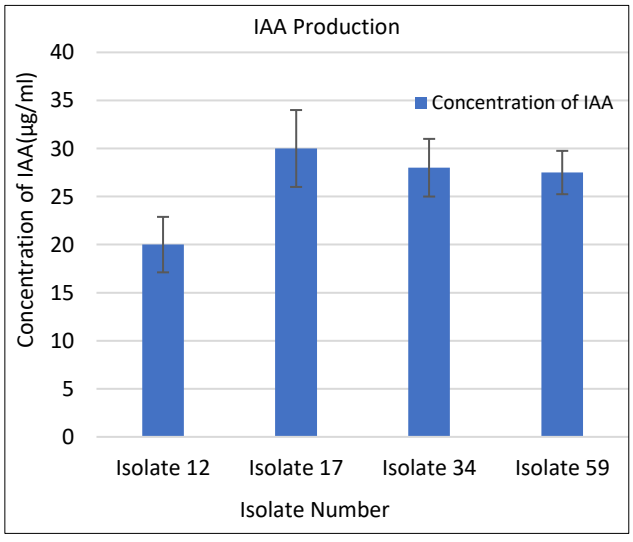

Fig 2 IAA production

IAA production

The PGPR enhances crop growth and development by producing phytohormones [34-35]. The growth-promoting effects of rhizobacteria may be due to the phytohormone production [36]. Auxins are important phytohormones. IAA is major auxin that promotes plant growth through cell division, elongation, and differentiation [37]. Qualitative and quantitative measurements of IAA production showed that out of all 60 isolates 12 isolates produced IAA ranging from 15.5 µg/ml to 30 µg/ml. The highest amount of IAA was produced by isolate number 17 at 30 µg/ml. Isolate number 12, showed 20 µg/ml while both, isolate number 34 and 59 showed 27.5 µg/ml IAA production (Fig 2). Isolate number12, 17,34 and 59 had shown a considerable amount of IAA production which indicates the plant promotion potential of these isolates. *Pseudomonas spp* has been reported for producing 23.006 µg/ml amounts of indole-3-acetic acid [38]. Hidayati *et al*. [39] has reported 114.83 mg/ml IAA production by *Providencia sp.* which is high as compared to present study. IAA production by PGPR has been reported to vary between species and strains, as well as being impacted by culture conditions, growth stage, and substrate availability [40].

Ammonia production

The ammonia production was tested by Nesler's reagent in which, all the isolates showed positive test while isolate numbers 3,6,11, 24, 30 showed negative test. Ammonia generation is a major characteristic of PGPR, and it is the feature that allows plants to develop in an indirect manner [41]. In the present study, most of the isolates were showing ammonia-producing ability which can enhance plant growth.

HCN production

Rhizobacteria are associated with biocontrol activity of plant against plant pathogen by producing low molecular weight metabolites, such as hydrogen cyanide, which involves in antifungal activity [42]. In present study 30 isolates were showed HCN production which indicates biocontrol ability of isolate. Isolate number 2, 6, 11, 12, 17, 19, 22, 31, 34, 53, 59 were showed maximum HCN production. Ahmad *et al.* [43] has described that antifungal effect can be linked with HCN producing ability of rhizobacteria isolates.

Identification of selected PGPR strains by sequencing of 16S rRNA gene

Four selected rhizobacteria showing biostimulation and biocontrol potential were identified based on morphological, biochemical, and 16S rRNA sequencing. As shown in table three potential isolates were identified by sequencing analysis (600bp) and Phylogenetic analysis. Isolate 59 was identified as *Pseudomonas fluorescence* based on their morphological and biochemical characteristics (Table 1).

Table 1 Identification of selected PGPR strains by 16S rRNA analysis

Selected Rhizosphere isolates	Sequence analysis	
	Closest NCBI database match	Percentage of identities
12	*Microbacterium indicum*	97.39 %
17	*Bacillus altitudinis*	100 %
34	*Staphylococcus sciuri*	100 %

Multiple PGPR traits of isolates

Among all isolates, four isolates were showed phosphate, potassium, and zinc mineral solubilization, IAA, Ammonia, and HCN Production ability which indicates direct as well as indirect PGPR plant growth stimulation potential of isolate. Based on their mechanism, four potential isolates showing all six PGPR trait positive were selected for *glycine max* (L.) plant seed germination and growth studies (Table 2).

Table 2 Multiple PGPR traits of isolates

Isolate	Mineral Solubilization			IAA production	Ammonia production	HCN production
	P	K	Zn			
Microbacterium indicum	+	+	+	+	+	+
Bacillus altitudinis	+	+	+	+	+	+
Staphylococcus sciuri	+	+	+	+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+

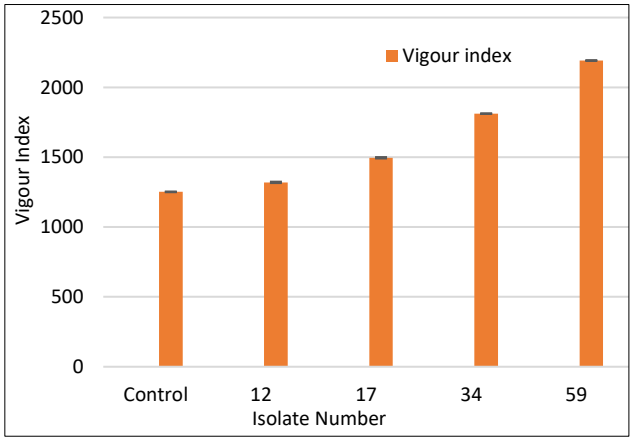

Fig 3 Vigour index of *Glycine max* (L.)

Seed germination assay of glycine max (L.)

The seed germination assay provides the visual in vitro confirmation of whether the germination rate and efficiency are getting influenced or not when seeds are treated with rhizospheric, bacterial suspension. The germination percentage value, length of root, and shoot on day 8 were some criteria for evaluation of whether a particular isolate is influencing seed germination and growth or not. Joshi *et al*. [44] has reported 81.9% seed germination and 1820 vigour index in *Pseudomonas* treated soyabean plant which is less as compared to present study. In present study among the four isolates *Pseudomonas fluorescens* (59 number isolate) showed highest percent seed germination, 92% and vigour index, 2192 in soybean plant which was followed by *Staphylococcus sciuri* (isolate number 34) 90% seed germination percent and 1812 vigour index while *Bacillus altitudinis* (isolate number 17) and *Microbacterium indicum* (isolate number 12) was showed seed germination percent 88% and 85% respectively and vigour index of 1496 and 1320 respectively. All isolates showed greater results as compared to control, untreated plant showed 83% and 1252 germination percent and vigour index respectively (Table 3, Fig 3). *Brassica juncea* and *B. oxyrrhina* inoculated with *Bacillus spp.* SN9 showed plant tissue enhanced with biomass and accumulation of nitrogen [45]. Buensanteai *et al*. [46] has reported IAA producing *Bacillus amyloliquefaciens* KPS46 enhanced growth of *Glycine max* (L.) similar results showed by *Bacillus altitudinis* treated soybean

plant in present study, were it has showed increase in seed germination % and vigour index as compare to control plant (Table 3).

Table 3 Seed germination assay of *Glycine max* (L.)

Name of isolate	Seed germination % (*Glycine max*)
Control	83
Microbacterium indicum	85
Bacillus altitudinis	88
Staphylococcus sciuri	90
Pseudomonas fluorescens	92

Statistical analysis

One-way ANOVA was conducted at a significant difference level and a 0.05 level was detected. Rhizobacteria isolates showed a significant effect on seed germination, shoot and root growth of soybean plants because the p-value is <0.05 (Table 4-6).

The *f*-ratio value is 56.57353. The *p*-value is <0 .00001 (Table 5). The result is significant at $p < 0.05$. Tukey's HSD procedure facilitates pairwise comparisons within ANOVA data. The F statistic indicates an overall difference between sample means (Table 5). Tukey's HSD test allows determining a significant difference between the various pairs of means (Table 6).

Table 4 Summary of data for seed germination (%)

	Treatments					
	Control	12	17	34	59	Total
N	5	5	5	5	5	25
∑X	410	426	430	453	457	2176
Mean	82	85.2	86	90.6	91.4	87.04
$\sum X^2$	33630	36302	36986	41043	41773	189734
Std. Dev.	1.5811	1.3038	1.2247	0.5477	0.8944	3.7359

Table 5 Result details of statistical analysis

Source	SS	df	MS	
Between-treatments	307.76	4	76.94	$F = 56.57353$
Within-treatments	27.2	20	1.36	
Total	334.96	24		

Table 6 Pairwise comparisons of seed treatment

Pairwise comparisons			HSD.05 = 2.2071 HSD.01 = 2.7606	Q.05 = 4.2319 Q.01 = 5.2933
T_1:T_2	M_1 = M_2 =	82.00 85.20	3.20	Q = 6.14 (p = 0.00263)
T_1:T_3	M_1 = M_3 =	82.00 86.00	4.00	Q = 7.67 (p = 0.00023)
T_1:T_4	M_1 = M_4 =	82.00 90.60	8.60	Q = 16.49 (p = 0.00000)
T_1:T_5	M_1 = M_5 =	82.00 91.40	9.40	Q = 18.02 (p = .00000)
T_2:T_3	M_2 = M_3 =	85.20 86.00	0.80	Q = 1.53 (p = 0.81221)
T_2:T_4	M_2 = M_4 =	85.20 90.60	5.40	Q = 10.35 (p = 0.00000)
T_2:T_5	M_2 = M_5 =	85.20 91.40	6.20	Q = 11.89 (p = 0.00000)
T_3:T_4	M_3 = M_4 =	86.00 90.60	4.60	Q = 8.82 (p = 0.00004)
T_3:T_5	M_3 = M_5 =	86.00 91.40	5.40	Q = 10.35 (p = 0.00000)
T_4:T_5	M_4 = M_5 =	90.60 91.40	0.80	Q = 1.53 (p = 0.81221)

Where, T_1 = control, T_2= 12 , T_3=17, T_4=34, T_5= 59 ($T_1$to T_5 is Treatment 1 to 5)

CONCLUSION

The present study emphasizes the ability of Plant Growth-Promoting Rhizobacteria (PGPR) extracted from the rhizosphere of Nashik District to improve the growth of *Glycine max* (L.) and investigates the biostimulation potential of this novel rhizobacterium. Total 60 morphologically distinct colonies of rhizobacteria were obtained. Among them, 4 isolates, *Microbacterium Indicum, Bacillus altitudinis, Staphylococcus sciuri, Pseudomonas fluorescens* were selected that showed maximum phosphate, potassium, and zinc solubilization. Biocontrol activity of isolates was checked by HCN production test. Quantitative Indole-3-acetic acid (IAA) production showed the efficiency of isolates to produce phytohormone, it indicates the biostimulation capability of isolates. The 4 isolates which were showing maximum traits positive were selected for the study of seed germination & vigour index in *Glycine max* (L.). Inoculation of plants with PGPR plant probiotic isolates promotes soybean growth potential through a biostimulation mechanism. It shows a promise in the future of rhizobacteria which would help to improve the yield of *Glycine max* (L.).

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LITERATURE CITED

1. Youssef MMA, Eissa MFM. 2014. Biofertilizers and their role in management of plant parasitic nematodes. *E. Jr. Biotechnol. Pharm. Research* 5: 1-6.
2. Babalola OO. 2010. Beneficial bacteria of agricultural importance. *Biotechnol. Letters* 32(11): 1559-1570. doi:10.1007/s10529-010- 0347-0. PMID:20635120.
3. Patel K, Goswami D, Dhandhukia P, Thakker J. 2015. Techniques to study microbial phytohormones. In: Bacterial metabolites in sustainable agroecosystem. Ahmedabad: Springer International Publishing. pp 1-27.
4. Kloepper JW, Lifshiftz R, Zablotowicz RM. 1989. Free living bacterial inocula for enhancing crop productivity. *Trend Biotechnology* 6: 39-44.
5. Spence C, Alff E, Shantharaj D, Bais H. 2012. Probiotics for plants: Importance of rhizobacteria on above ground fitness in plants. *In*: (Eds) Maheshwari DK. Bacteria in Agrobiology: Plant Probiotics. Germany: Springer. pp 1-14.
6. Berlec A. 2012. Novel techniques and findings in the study of plant microbiota: Search for plant probiotics. *Plant Science* 193/194: 96-102. doi: 10.1016/j.plantsci.2012.05.013 PMID: 22794927
7. Lugtenberg B, Kamilova F. 2009. Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiology* 63: 541-556. doi: 10.1146/annurev.micro.62.081307.162918 PMID: 19575558
8. Bhardwaj D, Ansari M, Sahoo R, Tuteja N. 2014. Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. *Microbial Cell Factories* 13(1): 66. doi: 10. 1186/1475-2859-13-66.
9. Ahmad M, Pataczek L, Hilger T, Zahir Z, Hussain A, Rasche F, Schafleitner R, Solberg V. 2018. Perspectives of microbial inoculation for sustainable development and environmental management. *Frontiers in Microbiology* 9: 2992.
10. Jaga PK, Sharma SK, Sharma KB. 2021. Effect of inoculations in soybean [*Glycine max* (L) Merr.] on productivity and soil properties in soybean-wheat system on vertisols in central India. *Int. Jr. Curr. Microbiology App. Science* 10(2): 3520-3529. doi: https://doi.org/10.20546/ijcmas.2021.1002.387
11. Mayer J, Buegger F, Jensen ES, Schloter M, Heb J. 2003. Residual nitrogen contribution from grain legumes to succeeding wheat and rape and related microbial process. *Plant and Soil* 255: 541-554.
12. Jensen BB, Cox RP. 1988. Measurement of hydrogen exchange and nitrogen uptake by mass spectrometry. *Methods in Enzymology* 167: 467-474.
13. Afzal A, Bano A. 2008. Rhizobium and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum*). *Int. Jr. Agric. Biol.* 10(1): 85-88.
14. Sane SA, Mehta SK. 2015. Isolation and evaluation of rock phosphate solubilizing fungi as potential bio-fertilizer. *Jr. Fertil. Pesticides* 6(2): 156-160.
15. Nyugen C, Yan W, Tacon FL, Lapyire F. 1992. Genetic variability of phosphate solubilizing activity by monocaryotic and dicaryotic mycelia of the ectomycorrhizal fungus *Laccaria bicolor* (Maire). *Plant Soil* 143: 193-199.
16. Nguyen C, Yan W, Le TF. 1992. Genetic variability phosphate-solubilizing activity of the ectomycorrhizal fungus *Laccaria bicolor* (Maire) P.D. Orton. *Plant Soil* 143: 193-199.
17. Zeng WC, Zhang Z, Gao H, Jia LR, Chen WY. 2012. Characterization of antioxidant polysaccharides from Auricularia auricular using microwave-assisted extraction. *Carbohydrate Polymers* 89(2): 694-700.
18. Ehmann A. 1977. The Van Urk-Salkowski reagent-a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. *Journal of Chromatography* 132: 267-276.
19. Cappucino JC, Sherman N. 1992. *Microbiology: A Laboratory Manual*. 3rd Edition, Benjamin/Cumming Pub. Co., New York.
20. Castric PA. 1975. Hydrogen cyanide, a secondary metabolite of Pseudomonas aeruginosa. *Canadian Journal of Microbiology* 21(5): 613-618.
21. Miller RL, Higgins VJ. 1970. Association of cyanide with infection of birdsfoot trefoil by *Stemphylium loti*. *Phytopathology* 60: 104110.
22. Khan MA, Ungar IA. 1984. The effect of salinity and temperature on the germination of polymorphic seeds and growth of *Atriplex triangularis* Willd. *Am. Jr. Botany* 71: 481-489.
23. Abdul-Baki AA, Anderson JP. 1973. Vigour determination in soybean seed by multiple criteria. *Crop Science* 13: 630-633.
24. Garcia J, Schmidt JE, Gidekel M, Amélie, Gaudin CM. 2021. Impact of an antarctic rhizobacterium on root traits and productivity of soybean (*Glycine max* L.). *Journal of Plant Nutrition* 44(12): 1818-1825.
25. Baykov AA, Evtushenko OA, Avaeva SM. 1988. A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. *Anal. Biochemistry* 171: 266-270.
26. Urquiaga S, Cruz KHS, Boddey RM. 1992. Contribution of nitrogen fixation to sugar cane: nitrogen-15 and nitrogen-balance estimates. *Soil Sci. Soc. America Proc*. 56: 105-114.
27. Biswas JC. 1998. Effect of nitrogen fixing bacteria on growth promotion of lowland rice (*Oryza sativa* L.). *Ph. D. Thesis*, Department of Soil Science University of Phillipines, Los Banos.
28. Goldstein AH. 1995. Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. *Biol. Agric. Horticulture* 12(2): 185-193.
29. Pathak R, Paudel V, Shrestha A, Lamichhane J, Gauchan DP. 2017. Isolation of phosphate solubilizing bacteria and their use for plant growth promotion in tomato seedling and plant. *Journal of Science Engg. and Technology*13(2): 61-70.
30. Vyas P, Gulati A. 2009. Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing *fluorescent Pseudomonas*. *BMC Microbiology* 9(174): https://doi.org/10.1186/1471-2180-9-174
31. Padma SD, Sukumar J. 2015. Response of mulberry to inoculation of potash mobilizing bacterial isolate and other bio-inoculants. *Global Jr. Bio. Sci. Biotechnology* 4: 50-53.

32. Meena VS, Maurya BR, Verma JP, Meena RS. 2016. Potassium solubilizing microorganisms for sustainable agriculture. Springer.
33. Sharma SK, Sharma MP, Ramesh A, Joshi OP. 2012. Characterization of Zinc-solubilizing bacillus isolates and their potential to influence zinc assimilation in soybean seeds. *Jr. Microbiol. Biotechnology* 22(3): 352-359.
34. Glick BR, Bashan Y. 1997. Genetic manipulation of plant growth promoting bacteria to enhance biocontrol of phytopathogens. *Biotechnol. Adv*., 15: 353-378.
35. Volpin H, Philips DA. 1998. Respiratory elicitors from *Rhizobium meliloti* effect intact alfalfa roots. *Plant Physiology* 116: 777-783.
36. Chabot R, Antoun H, Cescas MP. 1996. Growth promotion of maize and lettuce by phosphate-solubilizing Rhizobium leguminosarum biovar phaseoli. *Plant Soil* 184: 311-321.
37. Asgher M, Khan MIR, Anjum NA, Khan NA. 2015. Minimizing toxicity of cadmium in plants-role of plant growth regulators. *Protoplasma* 252: 399-413.
38. Mohite B. 2013. Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *Jr. Soil Sci. Plant Nutrition* 13: 638-649. doi: 10.4067/S0718-951620130050 00051
39. Hidayati U, Chaniago IA, Munif A, Siswanto, Santosa DA. 2014. Potency of plant growth promoting endophytic bacteria from rubber plants (*Hevea brasiliensis* Mull. Arg.). *Journal of Agronomy* 13: 147-152.
40. Mirza SM, Ahmad W, Latif F, Haurat J, Bally R, Normand P, Malik KA. 2001. Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane in vitro. *Plant and Soil* 237: 47-54.
41. Yadav J, Verma JP, Tiwari KN. 2010. Effect of plant growth promoting rhizobacteria on seed germination and plant growth chickpea (*Cicer arietinum* L.) under in vitro conditions. *Biological Forum* 2(2): 15-18.
42. Dowling DN, O'Gara F. 1994. Metabolites of Pseudomonas involved in the biocontrol of plant disease. *Trends in Biotechnology* 12: 133-141
43. Ahmad F, Ahmad I, Khan MS. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research* 163(2): 173-181.
44. Jaya J, Tomar DS, Titov. 2018. Seed quality parameters of peanut and soybean as influenced by seed treatment with different microbial inoculants. *Int. Jr. Curr. Microbiol. App. Science* 7(1): 2660-2668.
45. Buensanteai N, Yuen GY, Prathuangwong S. 2008. The biocontrol Bacterium Bacillus amyloliquefaciens KPS46 produces auxin, surfactin and extracellular proteins for enhanced growth of soybean plant. *Thai Journal of Agricultural Science* 41(3/4): 101-116.
46. Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E. 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* 324(5930): 1064-1068.