Development and Evaluation of a Microbial Consortium for enhancing Fertility and Productivity in Saline Soil

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Abstract

Plant growth promoting rhizobacteria (PGPR) is a group of stress tolerant bacteria which possess unique properties that help in plant growth promotion under environmental stress conditions. These bacteria are promosing alternatives to chemical fertilizers and can be extensively used in organic farming. In this study, halotolerant microorganisms isolated from saline agricultural soil collected from Patharvala, Maharashtra have been investigated for their plant growth promoting ability. The isolates were qualitatively and quantitatively analzed for their plant growth promoting traits such as nitrogenase activity, IAA production potential, ability of phosphate solubilization, siderophore production using Ashby's nitrogen free medium, yeast malt dextrose medium, Pikovaskaya's medium and King's B medium respectively. Salkowski's method for quantification of the produced IAA yielded highest concentration of 6.10 mg/ml. Siderophore production potential of the isolates was analyzed using CAS reagent and quantified with the maxima of 12.86 PSU.

Initially, 76 halotolerant bacteria were isolated from saline soil, out of which three isolates belonging to the genus Azotobacter were selected based on their highest PGPR activity and used for the preparation of inoculum for seed germination assay. The germination rates of black eyed beans seeds with consortium, commercial biofertilizer and control were calculated as 87.5%, 90.9% and 69.6% respectively, confirming the effectiveness of the bacterial consortium and commercial biofertilizer for improving seed germination compared to control in salt stress conditions.

Keywords: Rhizobacteria, Indole acetic acid, PGPR.

Introduction

India has the population of 1.2109 billion (as on March 2011- https://knowindia.india.gov.in/) and agriculture is the largest occupation and contributor to India's GDP. Due to population explosion, the food demand is growing multifold every day. One of the possible way to combat food insecurity is increasing agricultural productivity[16].

Environmental stress such as water deficiency, temperature, heavy metal contamination, climate change, soil salinity and improper irrigation practices have caused great decrease in the crop productivity affecting overall food availability[31].

Soil salinization is a major challenge in agriculture which is globally affecting soil fertility and nutrient cycle[42]. Saline soil is attributed to the presence of excessive amount of soluble salts in the rhizosphere of plants. Accumulation of salt caused due to over irrigation and excessive use of chemical fertilizers is one of the major contributors of declining soil fertility[25]. Soil salinity causes formation of reactive oxygen species (ROS), reduced water absorption (water deficiency), osmotic stress, resulting in decreased photosynthetic activity and inhibiting seed germination[2].

One of the solutions to increase fertility and productivity of saline soil is the use of plant growth-promoting rhizobacteria (PGPR). It is a group of stress tolerant bacteria that colonize near plant roots, capable of inducing plant growth under stress conditions. These bacteria have ability to solubilize various compounds like phosphate, zinc, potash and can increase plant yield by producing plant growth-promoting hormones, siderophores, enzymes, cytokines, exopolsaccharides and enhancing nitrogen fixation[31]. Halotolerant microorganisms isolated from saline soil have been widely studied for their plant growth-promoting activity. Several studies have shown that plant seeds treated with salt tolerant bacteria *Pseudomonas putida* and *Pseudomonas fluorescens* have increased the germination rate by 51%.

Halotolerant *Bacillus amyloliquefaciens*, *Bacillus subtilis* have been studied for enhancing synthesis of antioxidants, nutrient absorption and ROS degradation in plants like *Oryza sativa* and *Cicer arietinum*[2]. A lot of research was done on *Kusshneria* sp. isolated from sea sediments which increase plant germination in chia, rice, alfalfa, lettuce and barley under alkaline-NaCl conditions[17]. *Enterococcus* sp. and *Enterobacter* sp. are known to promote growth and salt tolerance in plants like *Oryza sativa*, *Brassica napus* and *Zea mays. Micrococcus* sp. has been studied for production of indole acetic acid (IAA) and 1-Aminocyclopropane-1-carboxylate deaminase (ACC deaminase) enzyme which supports growth of *Arachis hypogaea* L. plant in saline conditions[1]. Halotolerant bacteria like *Serratia* and *Halomonas* have been exclusively studied for their biological control activities such as antifungal properties, production of HCN and NH_3[22].

The present study focuses on investigating the ability of halotolerant microorganisms to boost plant growth in saline soil and to promote their use in organic agriculture. The

objectives of this study include isolation and screening of halotolerant bacteria from saline soil for analyzing their plant growth promoting activity and to present PGPR as the possible alternative for chemical fertilizers.

Material and methods

Collection of samples: Soil samples were collected from 5 different salt affected areas which were irrigated using bore well or underground water. Total 3 types of agricultural soil were collected from Alkuti (Mhaskewadi, Alkuti: 19˚05'20.92"N, 74˚19'36.72"E), Patharvala (Patharvala, Newasa: 19˚49'69.68"N, 75˚11'16.64"E) and Shingnapur (Shani shingnaour: 19˚38'44.74"N, 74˚85'09.85"E) from Maharashtra State near Ahmednagar. All samples were collected in sterile plastic bags and preserved for further studies. Fig.1 explains the map of the locations from where soil samples were collected.

Sample analysis: The samples were analyzed using different parameters. Soil analysis included determination of pH, salt content, organic matter content, nutrient content and electrical conductivity. Three of these soil samples were found to differ in their salt content. The samples collected from Shingnapur were having highest salt content followed by the soil collected from Alkuti. The samples collected from Patharvala showed high production despite of having a considerable amount of salt in it. The soil sample collected from Patharvala was repeatedly autoclaved over a course of three days to be used for pot assay.

Primary screening for of plant growth promoting ability

Salt tolerance test: Sterile nutrient agar containing 5% salt was used to grow the organisms. The concentration of NaCl found in fertile soil ranges between 0.8 - 0.9%. Based on this percentage, 5% concentration of NaCl was selected for halotolerance test[5].

Nitrogen fixation test: Organisms isolated by salt tolerance test were screened for their nitrogen fixation ability. Isolates were grown on Ashby's mannitol nitrogen free agar medium and incubated at room temperature (30˚C) for 72 hours. Growth on this medium shows ability to fix atmospheric nitrogen[40].

Indole acetic acid (IAA) production test: Isolates were cultured in Yeast Malt Dextrose (YMD) broth and incubated at 28˚C for 5 days at 120 rpm in a shaking incubator. Centrifugation of the obtained broth was performed at 10,000 rpm for a duration of 10 minutes. Freshly prepared Salkowski's reagent (10 mM/L $FeCl_3$ in 35% *v/v* H_2SO_4) was added to the supernatant and incubated in dark for 30 minutes. Absorbance was checked at 530 nm using a colorimeter. Development of pink colour indicates production of indole acetic acid[38].

Phosphate solubilization test: Pikovaskaya's medium containing tricalcium phosphate as the sole source of phosphate was used to spot inoculate the isolates incubated at 37˚C for 2 days. Zone of clearance around the colony indicated the ability of the isolate to solubilize phosphate[11].

Potash solubilization test: The isolates were spot inoculated in Alexandrov's medium containing mica powder (insoluble potassium source) and incubated at room temperature (30˚C) for 2-3 days. Zone of clearance around the colony indicates ability of the isolate to solubilize potash[24].

Zinc solubilization test: Pikovaskaya's medium supplemented with 0.1g% zinc oxide was used to spot inoculate the isolates. The plates were incubated for 2-3 days. Zone of clearance surrounding the colony indicates positive result[35].

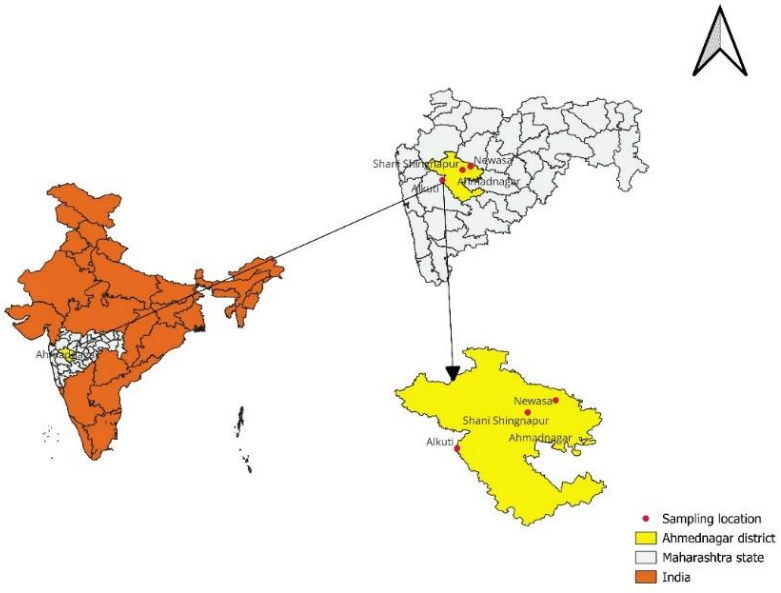

Fig. 1: The map of locations of sample collection
(This map was created using QGIS version 3.34.14 https://www.qgis.org)

Siderophore production test: The ability of the isolates to produce siderophore was determined using Chrome Azurol Sulfonate (CAS) reagent. 24 hours old cultures were inoculated separately on King's B agar medium containing CAS reagent and incubated at room temperature (30˚C) for 48 hours. Development of orange halo around the bacterial colony indicates production of siderophores[32].

Quantification of siderophores was done using 96 well plate assay. CAS reagent was added to the colony inoculated in the well of 96 welled plate along with media. Colour changed as a result of siderophore production quantified using ELISA plate reader.

Biochemical characterization: Biochemical characterization of the three isolates showing highest plant growth promoting activity was performed. The isolates were studied for production of catalase and oxidase enzyme. Methyl red test, Voges- Proskauer test, nitrate reduction test were performed according to the Bergey's Manual of Determinative Bacteriology[21].

Preparation of seeds: Healthy seeds of the plant black eyed bean were used as it is the major crop grown in the area from where soil sample was collected. 3 of the total isolates showing maximum traits of PGPR activity were selected and used for preparation of inoculum. 0.5 Macfarland standard of the 3 isolates was made. The slurry resulted by mixing of jaggery, activated charcoal and the inoculum was then used for coating all the seeds to be used for pot assay. Macfarland standard same as the consortium above, was prepared using organisms isolated from commercial organic fertilizer. The seeds were coated with this slurry prepared by adding inoculum made from commercial biofertilizer, jaggery and activated charcoal and planted in the soil. For preparation of the slurry, activated charcoal served as the substrate to which the organisms can adsorb and jaggery allowed proper adherence of the bacteria to the seeds.

Pot assay: Trays having 70-80 pots each were used for pot assay. The experimental pots were filled with sterilized soil collected from Patharvala. The tray was divided in such a way that it has equal number of pots for the seeds coated with consortium, commercial biofertilizer and control. All the seeds were planted into their respective pots. After rooting, the number of germinated and non- germinated seeds was recorded to study the effect of consortium as well as that of the commercially available biofertilizer on growth of plants in saline soil.

Results

In this study, 76 halotolerant bacteria were isolated from saline soil. Three of these isolates showing maximum traits of PGPR activity were selected and named as PS1, PS2 and PS3. (PS: Soil sample collected from Patharvala).

The bacteria were further studied for their PGPR activity such as ability to fix nitrogen, IAA production, phosphate solubilization, potash solubilization, zinc solubilization and siderophore production.

All the isolates grown on Ashby's medium showed ability to fix atmospheric nitrogen. Among various PGPR species, *Azotobacter* spp. is most studied for transforming infertile land into fertile one. It has been studied for nitrogen fixation, indole acetic acid production and stress regulation improving plant growth and development[36]. Nitrogen deficiency is a major reason for decreased fruit yield in plants like apple. In such cases, halotolerant nitrogen fixing bacterial strains *Bacillus subtilis* HG-15, *Bacillus velezensis* JC-K3 have been known to increase crop productivity and to prevent oxidative stress[13].

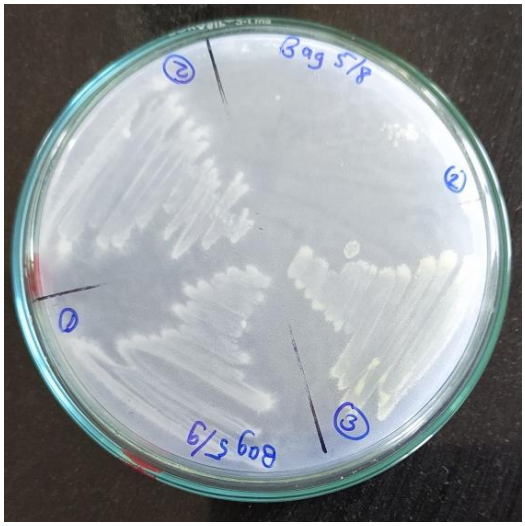

Fig. 2: Growth of nitrogen fixing bacterial isolates on Ashby's medium isolated from soil collected from Patharvala, Newasa

Fig. 2 shows the bacterial isolates streaked on Ashby's media having ability to fix nitrogen. Growth of the bacteria on this media indicates their ability to fix atmospheric nitrogen. The ability of isolates to produce indole acetic acid (IAA) was determined using Salkowski's reagent. Colorimetric analysis by checking absorbance at 540nm for quantification of the produced IAA allowed the selection of three bacterial isolates PS1, PS2 and PS3 giving highest IAA production of 6.19mg/ml, 4.24 mg/ml and 6.19 mg/ml respectively (fig. 3).

The production of indole-3- acetic acid (IAA) has positive influence on plant germination rate[14]. Symbiotic and non-symbiotic nitrogen fixing bacteria like Agrobacterium, Rhizobium, Klebsiella, Azotobacter have been exclusively studied for IAA production and increased crop productivity. Experiments were carried out to quantify IAA production by these organisms showing that the production of IAA increased synergistically with nitrogen fixation by symbiotic bacteria[29]. Studies on various Azotobacter species like *Azotobacter vinelandii*, *Azotobacter salineris*, *Azotobacter tropicalis* have revealed that these species exhibit pesticide tolerances and can promote plant growth by producing

considerable amount of indole acetic acid under stress conditions[7].

Fig. 3 illustrates the quantification of IAA produced by the bacterial isolates PS1, PS2 and PS3. Quantification was performed by Salkowski's method using colorimetric analysis at 540nm. The three isolates (PS1, PS2 and PS3) were studied for their ability to solubilize phosphate on Pikovaskaya's medium. The isolates were further analyzed based on the zone of solubilization within Pikovaskaya's medium as shown in fig. 4.

Many researchers studying PGPRs have paid attention to the characteristics of Azotobacter as a biological fertilizer. As reported, Azotobacter species has ability to solubilize organic as well as inorganic phosphate compounds[20]. *Azotobacter* spp. isolated from rhizoshperic soil can tolerate upto 0.8% NaCl and can exhibit plant growth promoting properties at high temperature regions. Fig. 4 displays the zone of clearance around bacterial colony indicating solubilization of phosphate in the culture media. The ability of the isolates to solubilize zinc was studied on Pikovaskaya's medium supplemented with 0.1g% zinc oxide. The isolates able to solubilize zinc have shown a zone of clearance around their colony (fig. 5).

Zinc is an important growth factor for plants and inoculation of zinc solubilizing bacteria instead of chemical zinc fertilizers has always been an eco-friendly option. Bacteria like *Bacillus glycinifermentants* and *Azotobacter* spp. are known to improve seed germination rate and grain yield in rice and wheat[41]. Fig. 5 shows the zone of clearance observed around the bacterial colony exhibiting zinc solubilization property.

The isolates were further tested for their ability to solubilize potassium. Zone of clearance around the colony on Alexandrov's medium indicates the ability of the isolates to solubilize potash. *Azotobacter nigricans* is one of the well-known and promising bacteria having plant growth promoting characteristics like ACC deaminase activity, IAA production, nitrogenase activity and potassium solubilization.

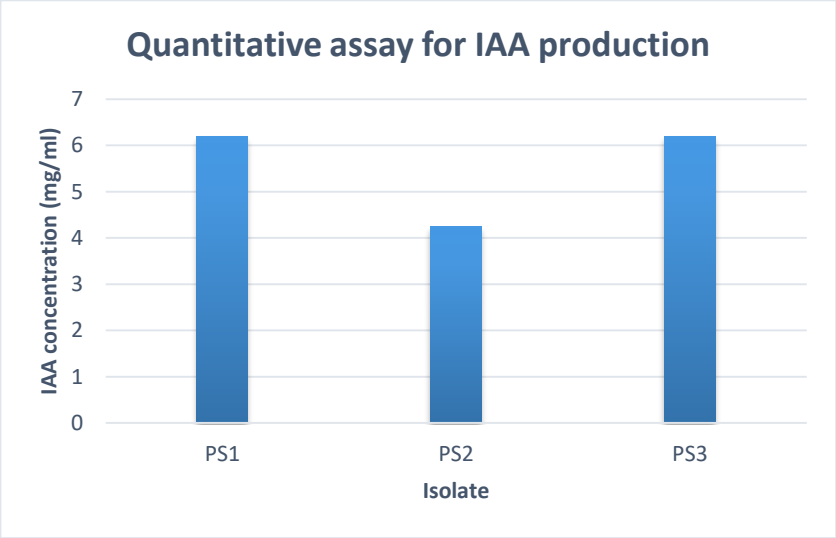

Fig. 3: Graphical representation of quantitative estimation of indole acetic acid produced by selected 3 bacterial isolates from soil sample collected from Patharvala, Newasa

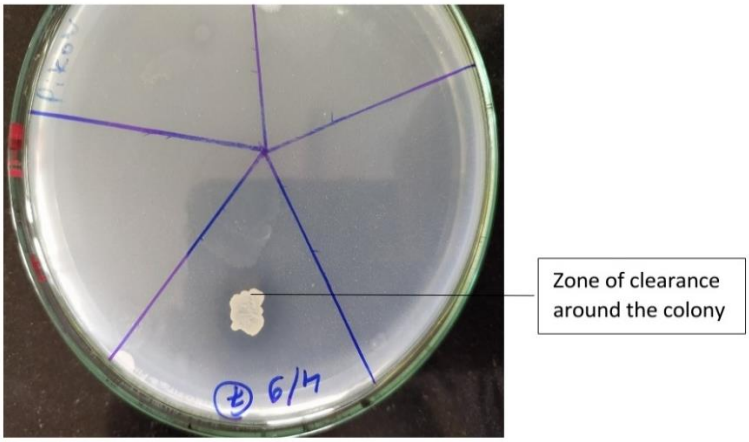

Fig. 4: Phosphate solubilizing bacteria on Pikovaskaya's medium isolated from the soil collected from Patharvala, Newasa

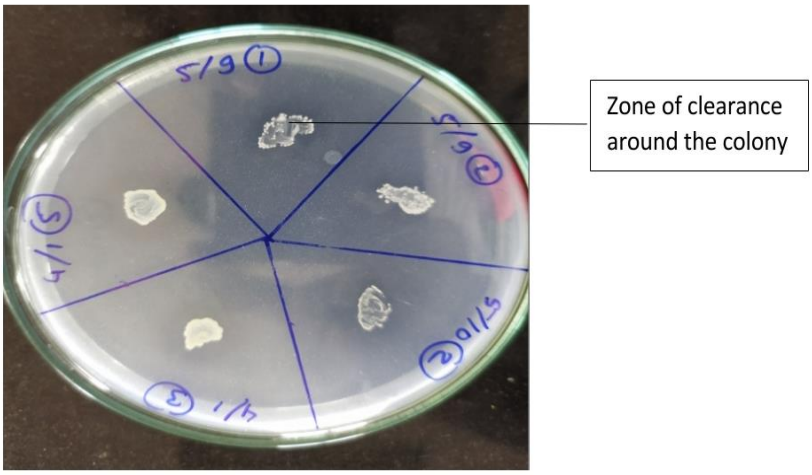

Fig. 5: Zinc solubilizing bacteria isolated from Patharvala soil sample grown on Pikovaskaya's medium (0.1g% zinc oxide) indicating positive result for zinc solubilization

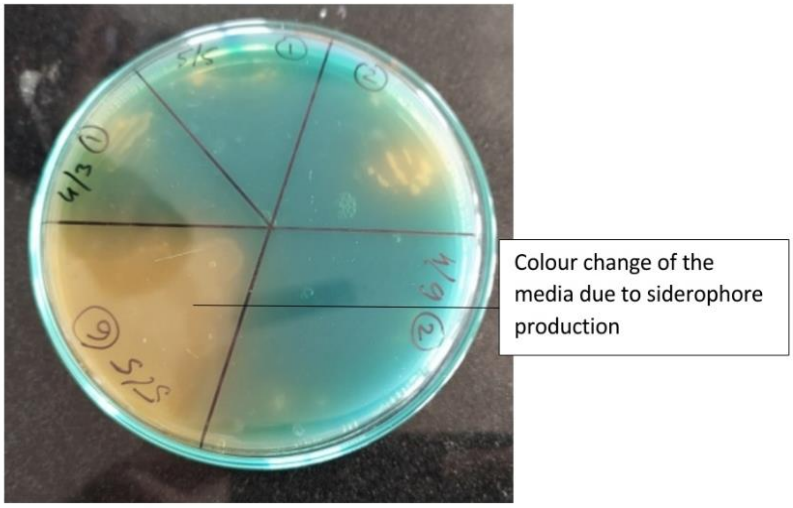

Fig. 6: Siderophore producing bacterial isolates on King's B media isolated from Patharvala soil sample

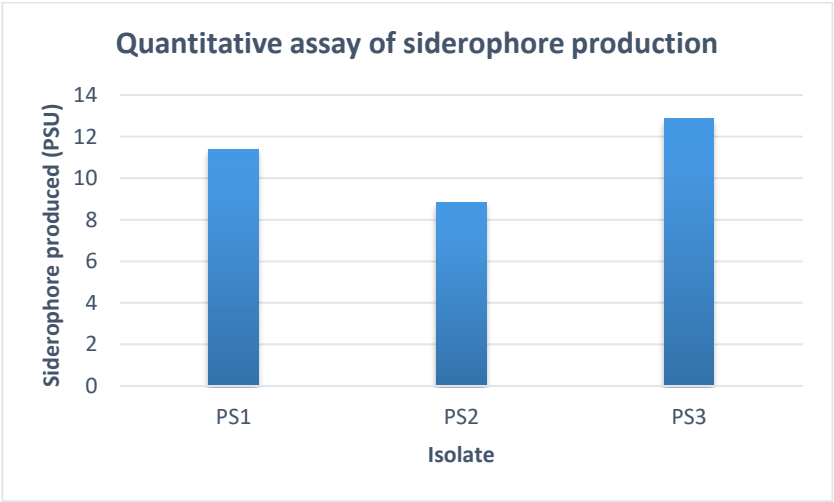

Fig. 7: Graphical representation of quantitative estimation of siderophore produced by selected 3 bacterial isolates

Inoculation of *Azotobacter nigricans* PR19 along with NPK treatments has revealed enhanced root elongation and increased seed germination in maize[30]. The bacteria isolates (PS1, PS2 and PS3) were studied for their ability to produce siderophore: an important plant growth promoting factor. King's B medium with CAS reagent was used to study the siderophore production while the siderophores produced by bacterial isolates were quantified using ELISA plate reader-

Thermo scientific Multiskan Go (Fig. 6 and fig. 7). Under stress conditions with iron deficiency, strains of *Pseudomonas* sp. have been studied for effective increase in apple yield[9]. *Azotobacter vinelandii* has been considered as a model nitrogen fixer as well as siderophore producer PGPR[15].

Fig. 6 illustrates siderophore producing bacteria on King's B media. The colour of the media changes due to CAS reagent which indicates siderophore production. Fig. 7 explains quantitative assay of siderophore production by bacterial isolates. Quantification was performed in 96 well-plate using ELISA plate reader.

The above tests allowed selection of three isolates PS1, PS2 and PS3 having highest PGPR potential which were then subjected to biochemical characterization. The tests were performed according to the Bergey's Manual of Determinative Bacteriology (eighth edition) as catalase test, oxidase test, methyl red test, Voges- Proskauer test, nitrate reduction test. Biochemical characterization allowed the identification of the bacterial isolates being used for the preparation of consortium. The isolates were identified as the genus Azotobacter (table 1).

Table 2 shows the results of biochemical tests performed for identification of the bacterial isolates PS1, PS2 and PS3. According to the biochemical characterization, the isolates belong to *Azotobacter chroococcum.*

The 3 isolates (PS1, PS2 and PS3) selected by screening their plant growth promoting activity were further used for the preparation of consortium for pot assay. The chemical analysis of the soil sample collected from Patharvala was performed. Table 2 shows the results for soil chemical analysis using different parameters such as pH, electrical conductivity, amount of copper, iron and zinc present in it. The plant growth promoting ability was studied by inoculating the seeds of black eyed bean seeds in the consortium (slurry) along with the slurry prepared by using commercially available organic fertilizer and a non-inoculated control. Fig. 9 compares the number of seeds germinated and not germinated when coated with consortium, biofertilizer with non-coated seeds in soil sample collected from Patharvala, Newasa.

The germination rates of black eyed beans seeds using consortium, commercial biofertilizer and control were calculated as 87.5%, 90.9% and 69.6% respectively. The chart illustrates that the seeds coated with bacterial consortium have better germination rate than the control. Both treatments (consortium and commercial biofertilizer) improved germination compared to the untreated seeds (control).

Table 1
Biochemical characterization of the bacterial isolates

Isolate	Catalase test	Oxidase test	Methyl red test	Voges-Proskauer test	Nitrate reduction test	Motility
PS1	+	-	NA	NA	+	+
PS2	+	-	NA	NA	+	+
PS3	+	-	NA	NA	+	+

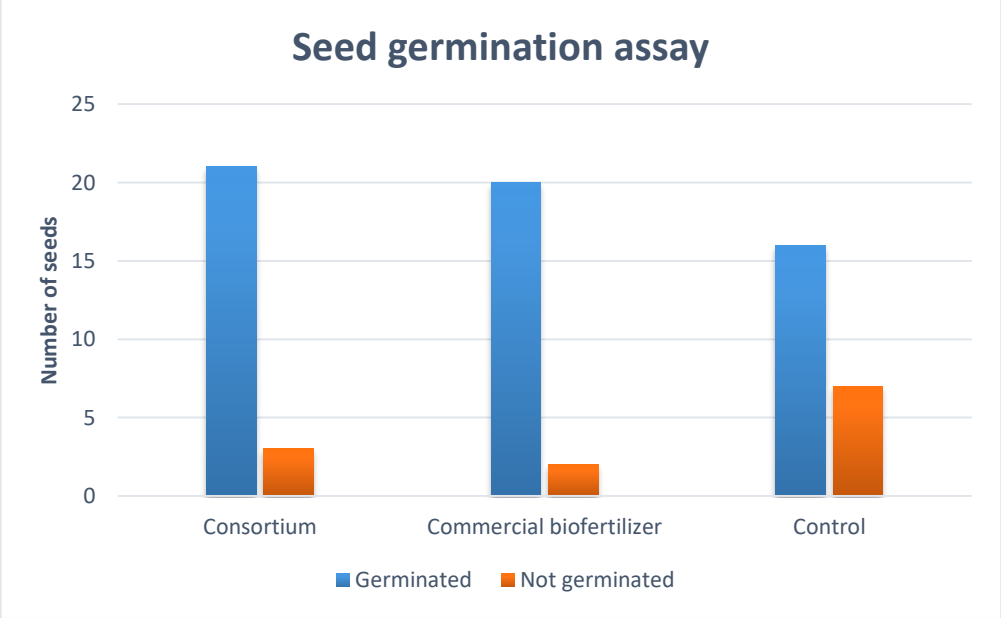

Fig. 8: Graphical representation of number of seeds germinated and not germinated with the inoculation of consortium, commercial biofertilizer and non- inoculated seeds

Table 2
Chemical analysis of Patharvala soil sample

Parameter	Amount
pH	7.5
Electrical conductivity	0.72
Iron (Fe)	4.538 ppm
Zinc (Zn)	0.718 ppm
Copper (Cu)	2.764 ppm

Discussion

PGPRs have been studied for their potential in boosting plant growth and productivity in saline soils. Several studies on salt-tolerant PGPRs have demonstrated that they can improve wheat crop yield in saline conditions by enhancing soil health, plant biomass, nutrient uptake and reducing sodium content in plant leaves[39]. Apart from plant growth promotion, PGPRs have been used in phytoremediation of salt-impacted sites.

Phytoremediation systems enhanced with PGPRs (PEPS) have shown positive effects on plant health in greenhouse as well as in the field experiments[10]. One of the effective way of improving plant development and production under saline conditions is the combination of salt-tolerant plants with beneficial soil microorganisms such as PGPR and arbuscular mycorrhizal fungi (AMF)[8,23].

PGPRs play a vital role in reducing salinity stress in plants through various mechanisms like exopolysaccharide synthesis, antioxidant machinery activation and increased nutrient uptake[3]. The use of region-specific PGPR consortia has potential in promoting plant growth in saline soils. The innovation of novel biofertilizers based on PGPR could provide a sustainable approach to improving productivity in salt-affected agricultural fields[3].

The use of PGPRs for plant growth improvement in saline soils has several future prospects: The use of salt-tolerant PGPRs and their metabolites could be a sustainable solution for increasing crop productivity and mitigating harmful effects saline soils in an eco-friendly manner[18]. Research on salt-tolerant PGPRs and their metabolites is still nascent and requires greater global attention. Positive effects of PEPS in greenhouse and field experiments suggest that phytoremediation of saline soil is possible within stipulated time using this approach[10]. Interestingly PGPR inoculation does not change the rates of salt uptake by plants.

PGPR enhanced plant biomass leads to greater salt uptake on a per area basis compared to untreated plants which contradicts with the above research[10]. In such cases, the combination of salt-tolerant PGPR with AMF could improve the saline soil recovery[8]. In conclusion, development of PGPR based biofertilizers can be an important strategy to increase fertility and productivity of saline soil[37]. Field studies could provide further research into this sustainable approach[3].

Conclusion

This study investigated the ability of halotolerant microorganisms isolated from saline soil to promote plant growth in salt affected agricultural fields. Tests carried out for determining nitrogen fixing ability, IAA production, phosphate solubilization, potassium solubilization, zinc solubilization and siderophore production allowed the selection of three isolates showing highest PGPR activity.

Pot assay of the inoculum prepared by mixing these three isolates has provided valuable insights into the plant growth promoting activity of halotolerant PGPR. Comparison of the seed germination assay with commercial biofertilizer suggested that the use of PGPR as organic fertilizers can potentially increase plant growth rate and yield in saline soil.

Acknowledgement

We are thankful to the Maharashtra Education Society, Principal of Abasaheb Garware College, Pune and Head, Department of Microbiology, Abasaheb Garware College, Pune, India.

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(Received 24th September 2025, accepted 08th October 2025)

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