Effect of salt tolerant *Bacillus* sp. and *Pseudomonas* sp. on wheat (*Triticum aestivum* L.) growth under soil salinity: A comparative study

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Abstract

This study was conducted to examine the comparative effect on wheat plant health inoculated with the two different rhizobacterial strains *Bacillus* sp. (JG3) and *Pseudomonas* sp. (JG7) under soil salinity. Total seven potential salt tolerant strains were isolated from the saline soils of BBAU-Lucknow. The bacterial strains have been investigated for nitrogen fixation, phosphate solubilization, ammonia, indole acetic acid and hydrogen cyanide production activities. Based on morphological and biochemical activities the strains JG3 was designated as *Bacillus* sp. and the strain JG7 was designated as *Pseudomonas* sp. Both the strains witness positive for the different plant growth promoting traits. In comparison of strain JG7, strain JG3 inoculated wheat seeds enhance plant height by 32.32%, root length by 37.84%, fresh weight by 28.2% and dry weight by 15.51% in FYM amended soils. We observe in this study that seeds treated with *Bacillus* sp. found significantly effective in plant growth promotion compared to *Pseudomonas* sp. in saline soil. Based on the comparative experimental study reported herein, it is pointedly observed that the use of salt tolerant PGPRs are effective for facilitating plant health in salt stress environments.

Introduction

Salinity is one of the most common environmental stress factors that adversely affect plant growth and crop production in cultivated areas worldwide.[1,2] Primary saline conditions appear naturally in environment yet anthropogenic activities are responsible for secondary salinity. Increased urbanization and deforestation are two important human derived activities for salinity. A number of reports are on soil salinization and their influences on crop productivity, land degradation and ecological disturbances are reported worldwide. Excess amount of salts effected physical chemical as well as the biological properties of soils. Plant health in saline soils is considerably decline owing to poor nutrition, osmotic stress and reduced microbial diversity.[3]

Wheat is major cereal crops in India and mainly cultivated in rain-fed areas. Salinity is a major constraint, which hampers wheat production, causing a loss of about 65% in yield in moderately saline soils.[4] Salt stress inhibits photosynthesis, protein synthesis, and other metabolic processes in plants.[2] The development of salt stress tolerant varieties through genetic engineering and plant breeding technology is often innovative technology but a long drawn process taking months to years for successful development. Microbial technology in agriculture is one the needed technology at present and future for sustainable crop productions.[5]

Since the elaboration of rhizospheric concept by Hilter in 1904 various rhizospheric microorganisms from different groups have been reported for their plant health promoting activities. The plant growth promoting rhizobacteria not only encourage plant health but emerged as important component of salt stress management.[6,7] The different PGPRs *Bacillus, Pseudomonas, Azospirillum, Agrobacterium, Achromobacter, Serratia,* have been reported for their PGP activity under different ecological conditions. Among these genus Bacillus the Gram positive and Gram Negative Pseudomonas are most extensively studies rhizobacteria facilitating plant health. *B. amyloliquefaciens, B. licheniformis, B. megaterium, B. pumilus*, and *B. subtilis,* are some important member of genus Bacillus and reported for plant growth and stress managements.[8,9] An increased agricultural production in response to *Pseudomaonas* inoculation has been reported through different mechanism.[10] Application of salt tolerant PGPR can be beneficial technology for wheat cultivation in saline areas. PGPR colonise roots and enhances root health through proving nutrient to plants and in return plant provide exudates to PGPR. At present there is dire need of fruitful sustainable agricultural technology in climate change scenarios. The objectives of this study are: i) Isolation and purification of the rhizobacterial strains from saline environment; ii) Efficacy of salt tolerant potential of isolated rhizobacterial strains; iii) Assays biochemical and plant growth promoting traits of salt tolerant strains; iv) *In vitro* study on potential of selected isolates from *Triticum aestivum* under soil salinity.

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Contributions: SRV designed the experiment and compiled whole manuscript. JG performed experiment. JSS analyzed data, refined scientific languages and guided experiment.

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Materials and Methods

Collection of soil samples

Soil sample were collected from the rhizosphere of grasses growing on non-fertile saline
soils form the premises of Babasaheb Bhimrao Ambedkar University, Lucknow (Figure 1).

Isolation of rhizobacterial strains

1 gram of rhizospheric soil was diluted in 9 mL of MQ water and serially diluted up to 10-6 and spread on nutrient agar media. Plates were incubated for 24 hours at 28±2°C and development of colonies was observed. The colonies were purified and preserved in nutrient agar slants at 4°C.

Rhizobacterial salt tolerance efficacy

All the isolates were examined for NaCl tolerance capacity (up to 1000 mM) in nutrient broth as well as streaked on NA

plates. In broth condition growth was monitored with UV-Vis double beam spectrophotometer at 610 nm.

Phenotypic characterization

Phenotypic characterization of all bacterial isolates was done according to *Bergey's Manual of Systematic Bacteriology* 2010 (Table 1).

Gram's staining

Late log phase culture of isolated strains were smeared on glass slides and fixed under Bunsen burner. The staining of all isolates were done according to Coico (2005)[11] and observed under phase contrast microscope.

Biochemical tests

Citrate utilization test

Citrate utilization test were done according to Koser (1924).[12] Freshly grown bacterial culture were streaked on Simmon Citrate agar plate and incubated for 24 hours for 28±2°C.

Amylase production test

Amylase production test were done according to Palleroni and Holmes (1981).[13] Spot inoculation was done on starch agar medium and was incubated for 24 hours at 28±2°C. After 24 hours the plate was flooded by iodine solution. A transparent zone around colonies appears for positive result.

Catalase test

The catalase activity was done according to Graham and Parker (1964).[14] Smear of bacterial culture was made on clean slide with help of inoculating loop. On pouring few drops of 3% hydrogen peroxide, bubbles of oxygen were observed on the slide.

Casein hydrolysis

Casein agar hydrolysis was performed with the method of Seeley and Van Demark (1970).15 Skimmed milk agar media were prepared and bacterial strains were spot inoculated on the plates and incubated for 24-48 hour at 28±2°C.

Carbohydrate utilization assays

Twelve carbohydrate utilization tests were examine with carbohydrate utilization kit (Himedia)-Mumbai (KB-009A) India.

Plant growth promoting test of isolated rhizobacterial strains

Phosphate solubilization

Phosphate solubilization test were performed according to Pikovaskaya (1948).[16] Isolated strains were spot inoculated on Pikovaskaya agar medium and incubated for 5-7 days at 28±2°C. Phosphate solubilization index (PSI) was calculated by using the formula.

Nitrogen fixation

N2 fixation ability of isolates were analyse on Jenson N agar medium according to Jenson (1954).[17] Appearance of growth on Jenson agar indicates efficiency of N fixation by isolate.

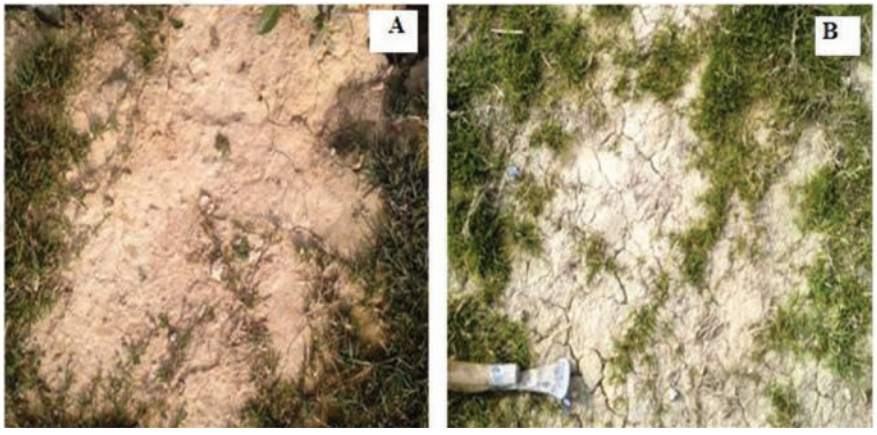
Siderophore production ability of isolates were determined according to Shwayn and Neilands (1987).[18] Appearance of orange colour zone on cash dye containing plate shows positive iron chelation.

Hydrogen cyanide production

The HCN production test was done according to Lorck (1948).[19] The isolates were subculture on Kings B agar plate supplemented with glycerol (15 mL L^{-1}). The filter paper was dip in Picrate/Na_2CO_3 solution and placed on upper lid of Petri plate and incubated at 28±2°C after sealing. Colour changes from yellow to orange, red, brown, or reddish brown was recorded as an indication of weak, moderate, or strongly cyanogenic potential, respectively.

Ammonia production

The NH3 production of isolated bacterial strains was done according to method of Cappucino and Sherman (1992).[20] Peptone water broth was prepared in test tubes and was inoculated by bacterial strains and incubated for 48-72 hours at 28°C. 1 mL of Nesseler's reagent was poured in test tubes. Change in colour from yellow to orange was observed.



Siderophore production | Indole acetic acid production

Figure 1. Photographs of sampling sites of BBAU-Lucknow. (A) Sampling site 1 (B) Sampling site 2.

Table 1. Phenotypic characterization of selected isolates.

Isolates morphology	JG1	JG2	JG3	JG4	JG5	JG6	JG7
Size (cm)	0.1	0.2	0.1	0.1	0.1	0.1	0.1
Form	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Edge	Entire	undulate	Entire	Curled	Entire	Undulate	Entire
Elevation	Crateriform	Flat	Flat	Convex	Flat	Convex	Convex
Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	EPS producing
Pigment	No	Yes	Yes	No	Yes	Yes	Yes
Colour	Off white	White	Off white	Light yellow	Orange	Yellow	Pale yellow

The IAA production test was done according to Gorden and Weber (1951).[21] The strains were inoculated on minimal broth containing different tryptophan concentrations of 50 and 100 µg L^{-1}. The test tubes were incubated for 48-72 hours at 28±2°C. The cultures were centrifuged at 6000 rpm for 10 minutes. About 4 mL freshly prepared Salkowaski reagent is added in 1 mL of supernatant. Appearance of Cherry Red colour is observed for IAA production. For quantification assay optical density was observed at 530 nm in double beam UV visible spectrophotometer.

Antibiotic sensitivity test

The antibiotic sensitivity test was done according to method of Bauer *et al.* (1996)[22,23] This test was performed on Mullar-Hilton agar media plates by placing antibiotic disc of Vancomycin, Kanamycin, Polymyxin-B and Erthromycin. After keeping it for 24-48 hours at 28±2°C zones were formed around the disc were observed.

Experimental design

In vitro complete randomized design pot experiment was performed to study inoculations effect on wheat plant growth. The plastic pots were filled with sterilized soils introduces with primary salinity according to Bharti *et al.* (2013).[24] FYM is used as a carrier in bioformulations yet FYM is also used as organic supplement to soils. The experiment conducted for 4 weeks and irrigation was done with non-saline MQ water. On 29th day the plants were pulled out from pots and analyse for growth parameters.

A. Control (Sterilized soil);
B. Sterilized soil + Farmyard manure;
C. Sterilized soil + Farmyard manure+ Strain JG3 (*Bacillus* sp.);
D. Sterilized soil + Farmyard manure + Strain JG7 (*Pseudomonas* sp.).

Statistical analysis

The collected data were subjected to statistical analysis for analysis of variance (ANOVA) performed with IBM SPSS 20.0. All values are in mean of triplicate ± standard error. The result was considered significant at $p<0.05$

Results

Rhizobacterial isolates

Total seven salt tolerant rhizobacterial

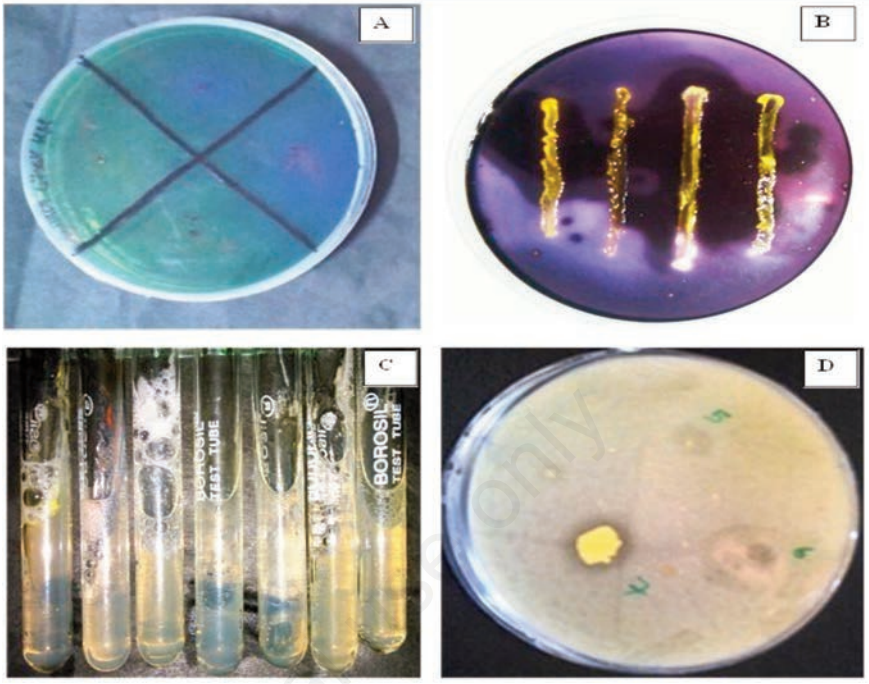


Figure 2. Biochemical activities of isolates. (A) Citrate utilization (B) Amylase production (C) Catalase test (D) Casein hydrolysis.

Figure 3. Development of farmyard based formulation with selected isolates in laboratory. Seed bio-priming.

Table 2. Biochemical activities of isolates rhizo-bacterial strains.

Biochemical activities of isolates	JG-1	JG-2	JG-3	JG-4	JG-5	JG-6	JG-7
Casein hydrolysis	-	-	+++	+	+	+	++
Starch hydrolysis	-	-	++	-	++	+	-
Catalase activity	++	+	++	-	-	++	++
Citrate utilization	-	+++	–	+++	-	-	++
Gram staining	-	+	+	-	-	+	-

strains with different morphology on nutrient agar medium were isolated and purified for the study (Table 1).

Biochemical test of rhizobacterial isolates

Different biochemical test of all seven isolates are described in (Table 2) and shown in (Figure 2). For citrate utilization capacity of isolates only two isolates JG2 and JG4 were found positive and remaining shows negative result for the test. Amylase production efficiency was shown positive by three strains JG3, JG5 and JG6 and other not found positive. For catalase test, out of 7 bacterial isolates, one isolates (JG3) was excellent positive (+++), two (JG1, JG6) were found moderately positive (++) and two strains (JG2, JG7) were slightly positive and two (JG4, JG5) were found negative (-). Strain JG3 was found excellently positive for casein hydrolysis while JG7 moderate positive, JG4 and JG5 slightly positive and JG1 and JG2 are negative for the test.

Carbohydrate utilization test

Different biochemical test of all seven isolates were given in Table 3.

Plant growth promoting traits of isolates

Plant growth promoting traits of isolates were described in (Table 4) and (Figure 4). IAA production was seen highest by strain JG7 16.3 µgml-1 followed by strain JG3 14.8 μgml^{-1} with amendment of 100 $\mu gL^{-1}L^{-1}$ of tryptophan.

Development of bio-formulation of selected strains

The bio-formulation of selected best PGPR strains (JG3 and JG7) were done according to procedures of Vidhyasekaran and Muthamilan (1995).23 FYM is used as carrier material for strains (Figure 3).

Wheat cultivars was procured form the local market and were surface sterilized with 70% ethanol followed by 2% Sodium hypochlorite solution for 10 minutes and rinsed in sterile MQ water. Seeds were dipped in sterilized MQ water for control and bacterial suspensions for 10 minutes and dried overnight in laminar.

Comparative study of plant growth parameter

The comparative experiment was shown in Figure 5. FYM amendment with soil enhances plant height by 10.06% while inoculation with strain JG3 enhances plant height by 84.56% and seeds inoculated with JG7 enhance plant height by 48.99% comparative untreated control (Figure 6A). Root length was increased by 15.62% in FYM amended soils, 101.56% in JG3+FYM amended soil, 57.81% in JG7+FYM amended soil compare to control (Figure 6B). Fresh plant weight was highest in JG3 inoculated plants 83.41% followed by 51.25% in JG7 inoculated plants and 14.07% in FYM amended soils (Figure 6C). While wheat plant dry weight was enhance by 94.04% in JG3+FYM amended soil 72.61% in JG7+FYM amended soil and 38.09% in

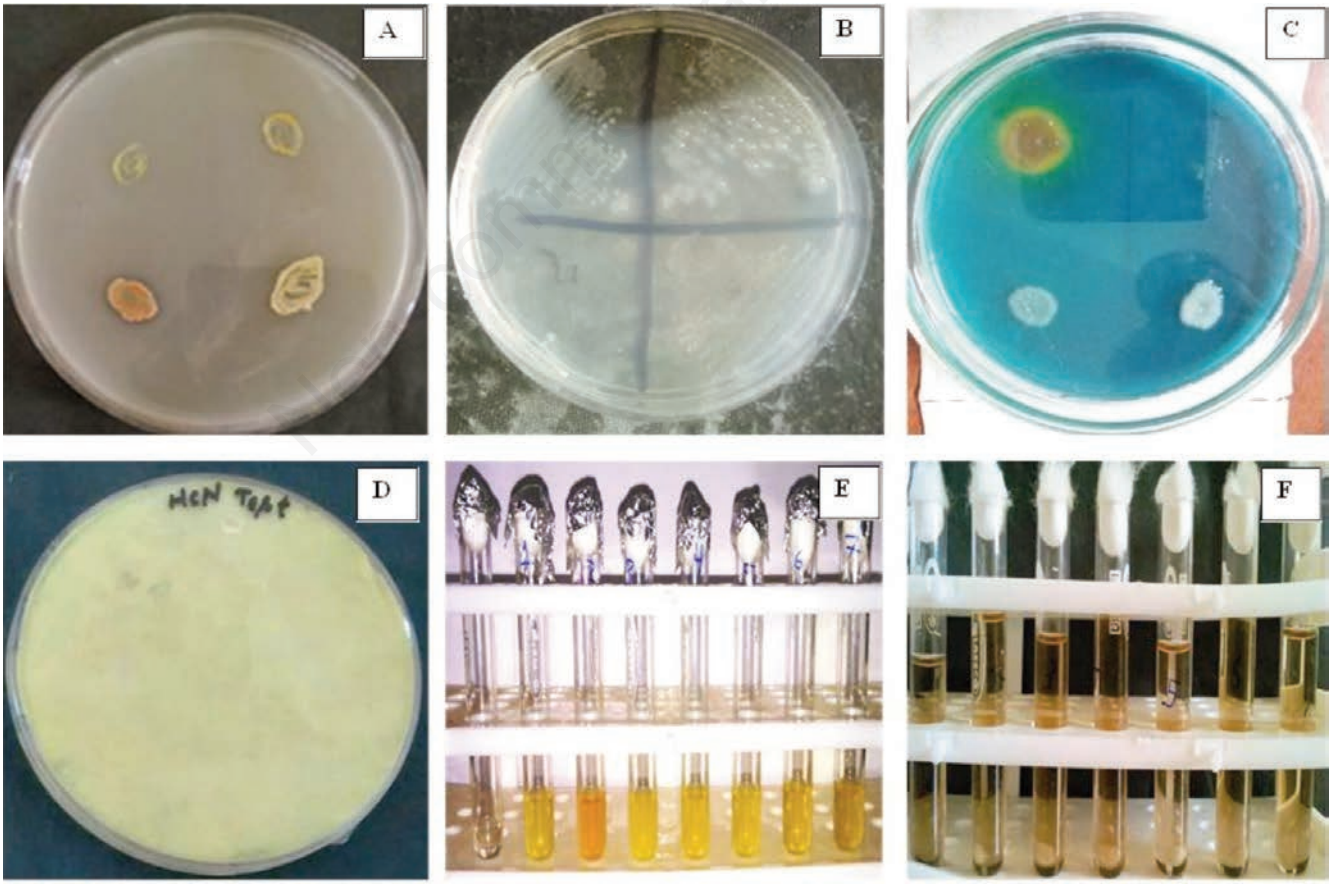


Figure 4. Photograph of plant growth promoting traits of salt tolerant isolates. (A) Phosphate solubilization (B) Nitrogen fixation (C) Siderophore production (D) HCN production (E) Ammonia production (F) IAA production.

FYM treated soil (Figure 6D). Thus in compare to strain JG7 we observe strain JG3 enhances plant height by 32.32%, root length by 37.84%, fresh weight by 28.2% and dry weight by 15.51% compared to FYM amended plants.

Discussion and Conclusions

In the present study seven salt tolerant rhizobacteria were isolated form naturally saline soils and assayed for their plant growth promoting potential and ability to mitigate saline stress of wheat plants. Two best potential isolates on the basis of their morphology and biochemical activities were screened and observed for wheat plant growth promotion under *in vitro* conditions. These isolates were designated as *Bacillus* sp. (Strain JG3) and *Pseudomonas* sp. (Strain JG7). Plant growth promoting rhizobacteria enhance plant health through various known and unknown plant growth promotion mechanisms.[24] Variety of rhizobacterial strains form both group *Bacillus* and *Pseudomonas* are also introduce for plant growth promotion under soil salinity.[25] In this study, *Bacillus* sp. (Strain JG3) and *Pseudomonas* sp. (Strain JG7) were found significant for different PGP traits. Seeds of wheat inoculated with strain JG3 exhibited significant plant growth enhancement than the strain JG7 under primary soil salinity. Wheat plant height, root length, fresh and dry weight was significantly reduced due to salinity stresses. The present study revealed that the variations in

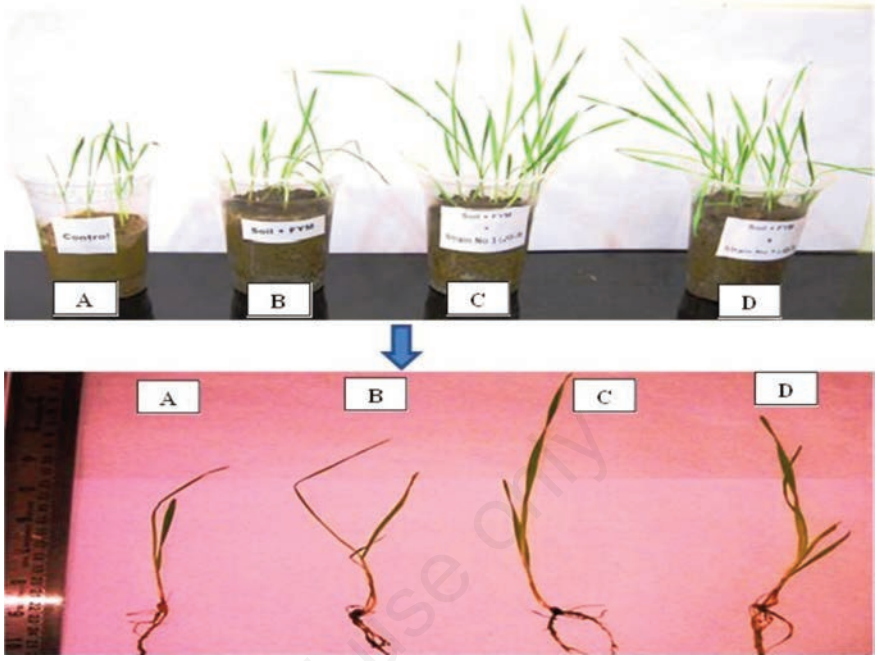


Figure 5. Comparative study of two different isolates on wheat plant growth promotion under secondary soil salinity.

Table 3. Carbohydrate utilization of rhizo-bacterial strains.

Carbohydrate utilization	JG-1	JG-2	JG-3	JG-4	JG-5	JG-6	JG-7
Lactose	+	+	+	-	+	-	+
Xylose	+	+	-	-	-	-	+
Maltose	+	-	-	+	-	+	+
Fructose	+	-	+	+	+	+	+
Dextrose	+	-	-	+	-	-	-
Galactose	-	+	-	-	-	+	-
Raffinose	-	-	-	-	-	-	-
Trehalose	-	+	+	+	+	-	-
Melibiose	-	+	+	-	-	+	-
Sucrose	-	-	+	+	+	-	+
L-Arabinose	-	-	-	-	-	-	-
Mannose	-	+	+	-	+	-	-

Table 4. Plant growth promoting activities of isolated rhizo-bacterial strains.

PGP traits of isolated strains	JG-1	JG-2	JG-3	JG-4	JG-5	JG-6	JG-7
IAA production	+	++	++	+	+	-	+++
Nitrogen fixation	-	-	+++	++	++	-	++
Siderophore production	-	-	++	-	-	-	-
Phosphate solubilization	-	+	-	-	-	-	-
Ammonia production	+	+	+	+	+	+	++
HCN production	-	+	+	-	-	-	+++

(+++ = excellent positive, ++ = moderate positive, + = slightly positive, - = negative).

plant growth parameters between non-inoculated and inoculated plants with JG3 and JG7 were statistically significant (Figure 6). Therefore, it may be concluded the the application of ST-PGPR is innovative and eco-friendly approach for reclamation of degraded agro- ecosystems. Decline demand of agrochemical in crop fields protects soil health and ease detrimental effects on human health.[26]

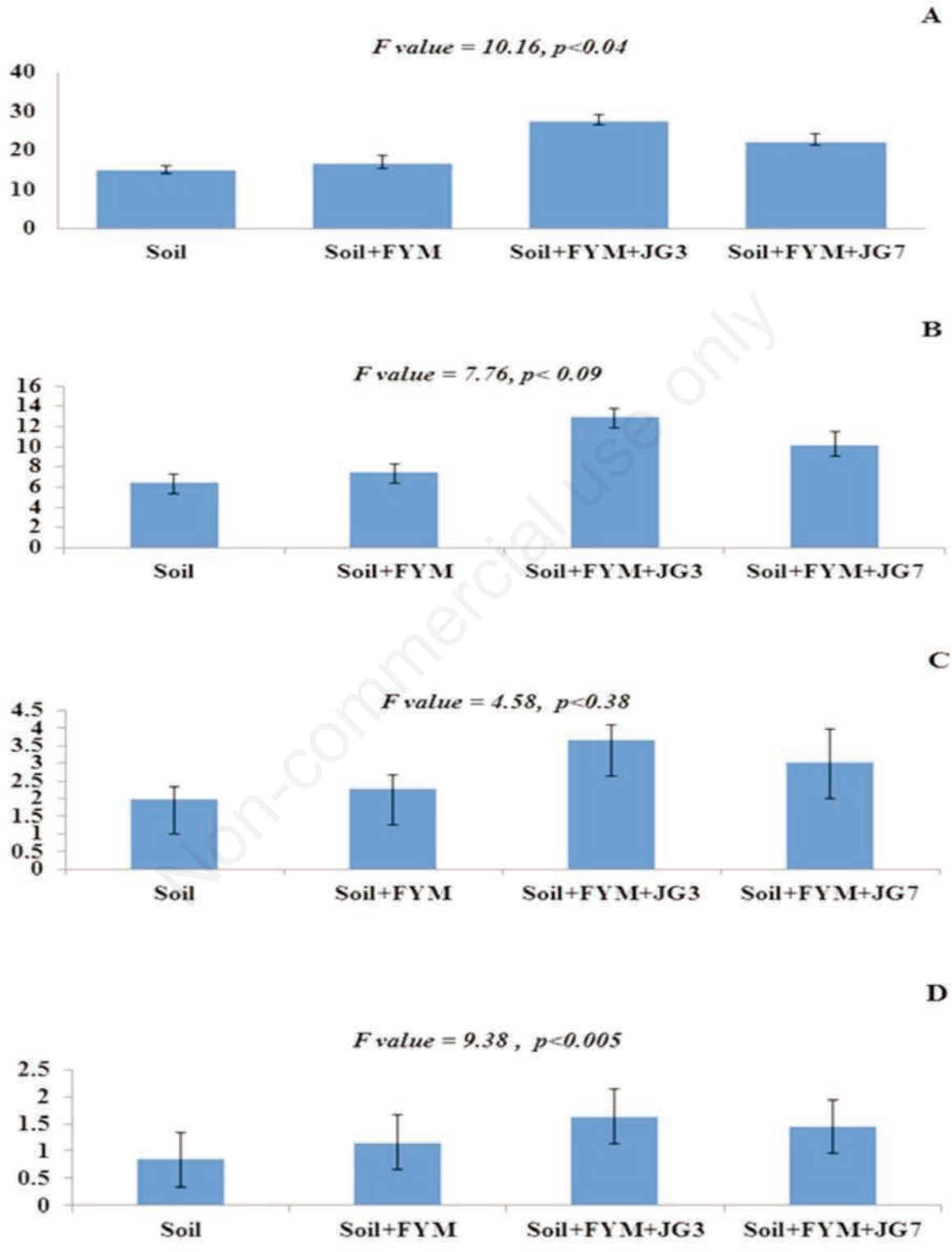


Figure 6. Inoculation effect on plant morphology (A) Plant height (B) Root length (C) Fresh weight (D) Dry weight.

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