Influence of Pseudomonas spp. on Okra (*Abelomuscus esculantaus* L.) growth parameters and antioxidant activities under soil salinity

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Abstract

In this study total twenty seven gram negative NaCl tolerant rhizobacterial strains were isolated from disturbed soils of Lucknow districts. The 3 selected sampling sites were Bijnor, Pasi Quila and Babasaheb Bhimrao Ambedkar University Campus, Lucknow, India. The isolates have been investigated for different plant growth promoting and biochemical activities. Based on morphological and biochemical tests the total eight (8) rhizobacterial strains were designated as *Pseudomonas* spp. Among PGP traits, all 8 *Pseudomonas* strains were examined for Indole-3-Acetic Acid (IAA) production activity. The bacterialisolate IAA^4 produces maximum 21.16±3.24 µg mL^{-1} of IAA at 100 µg mL^{-1} tryptophan concentration among *Pseudomonas* strains. This strain show excellent P solubilization and N fixatation in qualitative assay. The higher amount of NaCl salt in soils positively reduces paddy plant physiology and growth parameters. The IAA^4 inoculation in okra seeds significantly ($P<0.001$) improved plant growth parameters, green pigments and modulate antioxidant activities under pot experimental study.

Introduction

The generation of disturbed lands and non fertile soils due to environmental stresses is serious concerns for crop cultivation and food security. As the population graph is continuously rising around the globe and the food productivity has not sufficient to feed this rising human population. Soil salinity is one of the major environmental stresses that decline soil fertility and reduce crop productivity in arid and semi-arid regions of the world.[1-3] Salt stress significantly affect plant physiology, morphology as well as metabolisms. Soil salinity hinders photosynthesis, protein synthesis, lipid metabolism and other physical and biochemical mechanisms of plants.[2] The stunted plant height, bluish green younger leaves, scalding and burning on tip and edges of older leaves, poor flowering, fruit and grain productivity are major symptoms due to salinity stresses.[4] Excess salt ions in soil solution adversely affect xylem and phloem passage through osmotic inhibition of water uptake by roots or specific ion effects. It has been proposed that the salinity affected >20% and 50% of the total cultivated and irrigated lands in the world. The higher rate of water percolation in soils and higher rate of evaporation above ground level initiate the process of soil salinisation.[5] The whites salt depositions clearly appears saline affected soil in many parts of the world. This layer of salts in upper crust of soil, not only reduces the soil fertility but it also affects plant growth, microbial community structure and soil microbial biomass.[6,7]

The Okra (*Abelmoschus esculentus* L.) is an annual vegetable crop of *Malvaceae* family. The vegetable is cultivated mostly in tropical and warm temperate region so the world. The edible part of this vegetable is its fibrous mucilaginous fruit containing white seeds. Okra fruit got the status of high nutritional value. This fruit contains very rich level of energy, minerals, fibre, vitamins and carbohydrates. The mucilage produced by Okra plant has also been reported for waste water treatment due to its flocculent properties.[8] The Okra is considered as semi or moderately tolerant of salinity compare to other vegetables.[9] However, salinity reduces its growth and finally productivity. Application of agrochemical to enhance it Okra productivity not only ruin its nutritional properties but also polluted soil fertility. The chemically infected Okra fruit can be directly consumed by humans and portion of agrochemicals has been appears in food chains.

The microbial application technology is green, clean and very safe option of sustainable productivity and agricultural stress management.[10] Application of efficient microbial strain as single of on consortia with other microbes or other organic sources as (Biochars, manures, waste sludge, grain straws, etc.) will be very beneficial for enhancement of soil fertility and restoration of unproductive lands.[11] A number of microbes have been isolated and applied with various crops in recent decades.[12] Soil-plant-microbe interactions under stress agriculture attracts researcher around globe. Plant growth promoting rhizobacteria are free living soil bacteria aggressively colonize plant root areas and promote plant health through various traits.[13] This mechanism is known as direct and indirect plant growth promotion activities. The direct activities are as phytohormone production, mineral solubilization, nitrogen fixation and indirect activities are siderophore production, antibiotics synthesis, enzymes, anti-fungicidal compounds production and antagonism are intentionally reported.[13,14] The PGPRs modulate local and systemic mechanisms in plants to offer defence under adverse soil and environmental conditions. Besides influencing physicochemical properties of rhizospheric soil, PGPR support plant health in stress soils through phytohormones, exopolysaccharides production, biofilms formation and induction of antioxidants and osmoprotectants. The main objectives of this study are:

i) To isolate rhizobacterial strains from plants grown on unproductive soils and analyse salt tolerance efficacy of isolates;
ii) To examine biochemical and plant growth promoting attributes of isolates;
iii) To develop bioformulation with potential strain and analyse its efficiency in Okra plant growth promotion.

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Material and Methods

Selection of sampling sites

The adjoining unproductive area of university was selected for sampling. The plants naturally grown on these soils were collected with roots and rhizospheric soils and transported immediately to the laboratory of further analyses.

Isolation and purification of salt tolerant rhizobacterial strains

The rhizobacterial strains were isolated from the roots of halophytes grown on unproductive soil of above sampling sites. The serial dilution plate technique was applied for rhizobacterial isolation. The end diluted test tube proportion (0.1 mL) was spreaded on nutrient agar [g L^{-1}: Peptone (5), NaCl (5), HM Peptone B (1.5), Yeast extract (1.5), Agar (15), final pH (7.2±0.2) and King's B medium (g L^{-1}: Protease peptone (20), K_2HPO_4 (1.5), $MgSO_4.7H_2O$ (1.5), Agar (20) and final pH (7.2±0.2) and incubated for 24-48 h (30±2°C)]. The NaCl level was further enhanced in both medium by 0-1500mM and incubated for 24-72 h (30±2°C). The rhizobacterial strains were purified through several step colonies streaking process.

Phenotypic characterization of isolates

Phenotypic characterization of all bacterial isolates was done according to Bergey's manual of systematic bacteriology 2010 (Table 1).

Biochemical tests

Catalase

The catalase activity was examined according to protocol of Graham and Parker.[15] About 3% hydrogen peroxide solution was flooded directly on pure bacterial culture. Liberation of oxygen bubbles from culture indicates positive results.

Oxidase

Oxidase test was performed according to protocol of Shields and Catkart.[16] The short piece filter paper was dipped of Kovacs oxidase reagents (1% tetra-methyl-p-phenylenediamine dihydrochloride in water) and dried under aseptic conditions. The pure colony was streak on this sheet and colour change to blue (5-15 minute) shows positive result.

Gram reaction

The smear was prepared on glass slide under partial flame heating with late log phase culture of isolates. The staining of all isolates was performed according to protocol of Coico[17] and examine under phase contrast microscope.

Citrate utilization

Citrate utilization ability of isolates was performed according to Koser.[18] Isolated cultures were streaked on Simmon citrate agar plate [g L^{-1}: $MgSO_4.7H_2O$ (0.2), $NH_4H_2PO_4$ (1), K_2HPO_4 (1), $Na_3C_6H_5O_7$ (2), Bromothymol blue (0.08), agar (15)] and incubated for 24-48 hrs for 30±2°C. The transparent zone around streaked culture shows positive result.

Amylase production test

Amylase production activity was examined according to Rao *et al*.[19] Further the pure colonies was streaked on starch agar [g L^{-1}: meat extract (3), peptone (5), starch (2), agar (15)] and incubated for 24 hours at 30±2°C. After 48 hrs the plate was flooded by iodine solution. The zone formation around the colonies appears for positive result.

Casein hydrolysis

Casein agar hydrolysis was performed according to Wehr and Frank.[20] The rhizobacterial strains were inoculated on Skimmed milk agar [g L^{-1}: skimmed milk (28), casein enzymic hydrolysate (5), yeast extract (2.5), dextrose (1), agar (15)] and incubated for 48 hrs 30±2°C. The zone formation indicates positive result.

Plant growth promoting activities of the isolates

Indole-3-Actic Acid (IAA) production

IAA production test was performed qualitatively as well as quantitatively according to Patten and Glick.[21] The freshly grown isolates culture was inoculated in nutrient broth medium amended with 100 µg mL^{-1} L-tryptophan concentrations and incubated for 48-72 hrs at 30±2°C. The supernatants were collected by centrifugation at 6000 rpm for 15 minutes at 4°C. The supernatant of each culture was mixed with 100 µL of o-phosphoric acid and 4 mL of Salkowski's reagent (50 mL of 35% perchloric acid and 1 mL 0.5M $FeCl_3$). The reaction test tube was placed in the dark for 30 min at room temperature. The development of pink colour indicates potential IAA production. The colour intensity was measured with double beam spectrophotometer and production was estimated by comparing standard IAA curve.

Phosphate (PO_4) solubilization test

P-solubilization efficiency of isolated strains was examined according to Mehta and Nautiyal.[22] The pure colonies of isolates were spot inoculated on freshly prepared NBRIP medium (g L^{-1}: Glucose (10), $Ca_3(PO_4)_2$ (5), $MgCl_2.6H_2O$ (5), $MgSO_4.7H_2O$ (0.25), KCl (0.2), $(NH_4)_2SO_4$ (0.1) and bromophenol blue (0.025) and incubated for 48-96 hrs at 30±2°C. The zone formation around colonies indicates P-solubilization ability of the isolates.

Nitrogen (N_2) Fixation

Nitrogen fixation ability of isolates was examined on Jensen's N agar medium.[23] The isolates was inoculated on Jensen's N medium containing following ingredients Sucrose (20.0), K_2HPO_4, (1.0), $MgSO_4.7H_2O$ (0.5), $FeSO_4.H_2O$ (0.1), NaCl (0.5), Na_2MoO_4 (0.005), $CaCO_3$ (2.0), agar (15) and incubated for 24-48 hrs (30±2°C). Appearance of bacterial colonies on agar medium indicates positive result.

Development of Talc based bio-formulation with potential strain isolate

A talc based bio-formulation was developed with selected rhizobacterial strain as Vidhyasekaran and Muthamilan.[24] Single colony of strain transferred in nutrient broth and put in orbital shaker at 160 rpm for 48

Table 1. Physico-chemical characteristic of saline soil for rhizobacterial isolation.

Study sites	Colour	Moisture (%)	pH	EC (dS m^{-1})	Total soluble salt (EC×640)
Bijnor	Whitish brown	45	7.4	6.7	4288
Pasi Quila	Whitish brown	40	7.6	4.5	2880
BBAU campus	White	36	7.5	5.5	3520

hrs at 30±2°C. The culture obtained at stationary phase was centrifuged at 6000 rpm for 10 minutes and bacterial cells were re-suspended in phosphate buffer saline (100 mM, pH 7.0). The cell concentration was adjusted to10^8 cfu mL^{-1}. One kg of talc powder was taken and the pH was neutralizing by adding CaCO3 at the rate of 15 g kg^{-1} for bio-formulation development. 10 gram of CMC was further added as a sticker agent and the mixture was autoclaved for 30 min for two consecutive times. The 400 mL of 48 hrs grown bacterial suspension containing 9×10^8 cfu mL^{-1} was mixed with sterilize talc powder under aseptic conditions. After drying (approximately 40% moisture content) overnight under sterile conditions, it was packed, sealed and stored at room temperature (25±2°C). This developed bio-formulation was used in this experiment.

Seed procurement

Okra seeds were procured from the local importers and packing was aseptically opened. Okra seeds were surface sterilized using 0.01N NaClO for 10 minutes, and washed twice with sterile H_2O and air-dried in laminar air-flow.

Experimental design

Germination test

Total eighteen (18) sterilized and treated okra seeds were placed in wet cotton containing pertriplates. These Pertri plates were in inoculated for in dark for 5 days at 30±2°C and germinated index was calculated according to formula:

Germination (%) = Number of seeds germinated /Total number of seeds sown for germination × 100

Pot experiment

The pot experiment was performed to examine bacterial efficiency to reduce the negative effect of soil salinity on Okra plant. The pots were filled with saline soils taken from the university campus (pH =7.5 and EC = 6 ds m^{-1}) area. The FYM was used as an organic supplement matter to reduce the soil salinity. The experiment was conducted for six (6) weeks and irrigation was done with non-saline MQ water. The treatments are as follows:

i) Control
ii) Control + FYM
iii) Control + Bacteria (IAA[4])
iv) Control + FYM + Bacteria (IAA[4])

In last day of sixth week the plants were pulled out from pots and analysis of plant growth parameters. The green leaves were analysed for pigment and antioxidant analysis.

Effect on plant health parameters

The Okra plant was sampled after 45 days of inoculation and examined for plant growth parameters.

Plant growth parameters

The plant height, root length and dry weight were measured with standard meter scale according to Kurunc and Unlukara.[25] The green part of Okra plants (leaf) was sampled for pigment status and antioxidant activities examination.

Photosynthetic pigments

The Chl a was analyse in Okra plant according to protocol of Arnon.[26] The fresh leaves was partially crushed and immersed in 80% acetone for overnight. The chlorophyll was extracted in acetone and measured with double beam spectrophotometer. The calculation was done with give formulas as:

Chl a (mg g^{-1} fresh weight) = $12.7(A_{663}) - 2.69(A_{645})$

For Chl b, the chlorophyll extracted acetone was measured at different nm length in spectrophotometer. The calculation was done with formula as:

Chl b (mg g^{-1} fresh weight) = $22.9(A_{645}) - 4.68(A_{663})$

The total amount of chlorophyll was calculated with formula given below:

Total chlorophyll (mg g^{-1} fresh weight) = (*Chl a* + *Chl b*)

Table 2. Morphological features of isolates.

Colony morphology of isolate	Size (cm)	Shape	Edge	Elevation	Texture	Pigment	Colour
IAA1	0.2	Circular	Entire	Convex	Smooth	Yes	Cream
IAA2	0.2	Circular	Entire	Pulvinate	EPS producing	No	Cream-Yellow
IAA3	0.3	Circular	Entire	Pulvinate	EPS producing	Yes	Cream
IAA4	0.3	Circular	Entire	Convex	EPS producing	No	Cream
IAA5	0.4	Circular	Entire	umbonate	Smooth	No	Cream-Yellow
IAA6	0.2	Circular	Entire	Convex	Smooth	No	Cream-Yellow
IAA7	0.6	Circular	Entire	Pulvinate	EPS producing	No	Cream-Yellow
IAA8	0.3	Circular	Entire	Convex	Smooth	Yes	Cream

Table 3. Biochemical activities of isolates rhizobacterial strains.

Isolates morphology	Gram reactions	Microscopic visualization	Catalase activity	Oxidase test	Citrate utilization	Amylase production	Casein hydrolysis
IAA[1]	G -	Rod shape	-	+	++	-	++
IAA[2]	G -	Rod shape	-	-	+++	++	+
IAA[3]	G -	Rod shape	-	+	+	-	-
IAA[4]	G -	Rod shape	+++	++	+++	++	-
IAA[5]	G -	Rod shape	+++	+	+	++	++
IAA[6]	G -	Rod shape	++	+	+++	+	+
IAA[7]	G -	Rod shape	+	-	+	++	+++
IAA[8]	G -	Rod shape	+	+++	+	++	-

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

Antioxidant activities

The Okra plant green leaf (0.2 g) was placed in mortar and pestle with 2 mL of 50 mM ice cold phosphate buffer (pH 7.8) containing 1 mM EDTA and homogenize properly. The homogenate was centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatant was used for CAT and SOD enzyme assays.

Catalase (CAT)

The CAT activity was examined on the basis of decline in H_2O_2 levels. The reaction mixture (3 mL) consisted of 100 mM phosphate buffer (pH 7.0), 0.1 µM EDTA, 0.1% H_2O_2, and 0.1 mL of enzyme extract. The reaction was initiated by adding the enzyme extract to the reaction mixture. The decrease in H_2O_2 levels was determined by measuring the absorbance at 240 nm with a Spectrophotometer, and quantified by using extinction coefficient (36 M^{-1} Cm^{-1}). The leaf CAT activity was calculated using the following formula:

CAT (U mg^{-1} protein) = (absorbance of blank - absorbance of tested sample) × 271 / (60 × volume of enzyme extract) / protein concentration in enzyme extract

Peroxidase (POX)

The POX activity was determined based on the oxidation of guaiacol using H_2O_2. The reaction was initiated by adding 20 µL of the enzyme extract in to 3 mL of reaction mixture (100 mM phosphate buffer (pH 7.0), 20 µL of guaiacol solution, and 10 µL of H_2O_2). The absorbance was measured at 470 nm at time points of reaction initiation and 5 min later with a Spectrophotometer. The POX activity was calculated on the difference in absorbance per minute using the extinction coefficient (26.6 mM^{-1} cm^{-1}) with formula given below:

POD activity (U mg^{-1} protein) = (absorbance of tested sample - absorbance of blank) / 12 × volume of reaction mixture / volume of enzyme extract / reaction time / protein concentration in enzyme extract × 1000

Statistical analyses

The collected data were subjected analysis of variance (ANOVA) performed with software IBM SPSS 20.0. All values are in mean of triplicate ± standard error. The result was considered significant at $p<0.05$. All graphs were prepared in Graph pad Prism.

Results

Physico-chemical properties of soil

The soil collected was whitish in colour and contains enormous amount of soluble salts. These salts are directly responsible for barren properties. The grasses appear on these soils are the member of *Poaceae* family. The physico-chemical properties of these soils are depicted in Table 1. The maximum amount of salinity is found at Bijnor sampling site. Total twenty-seven (27) rhi-

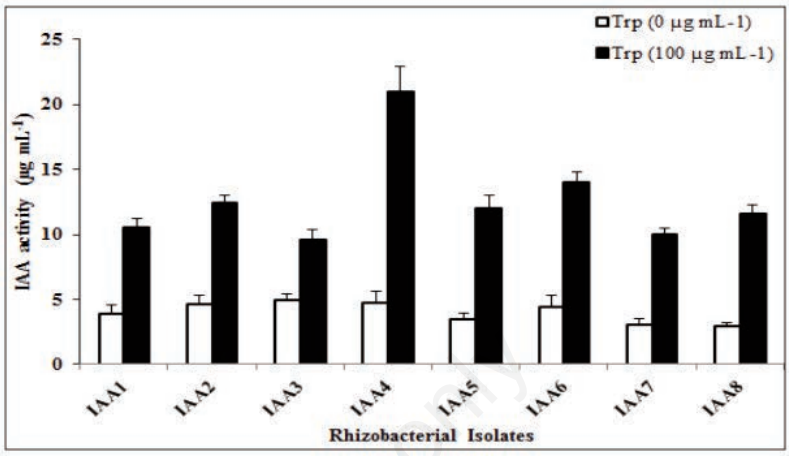

Figure 1. IAA production activity of isolates.

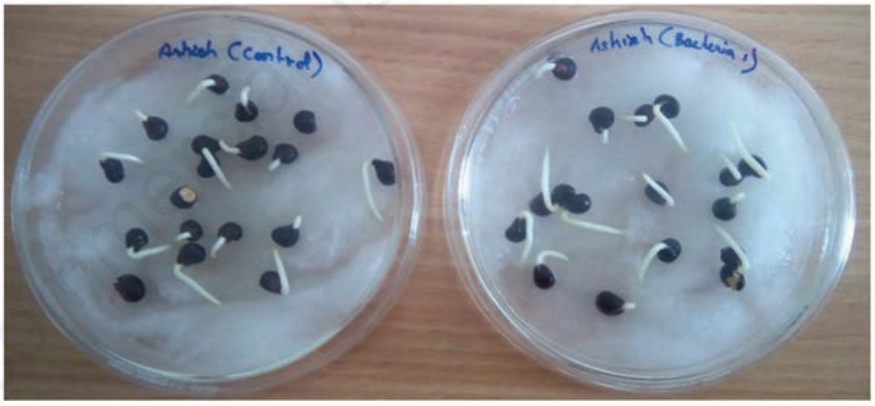

Figure 2. Okra seed germination test.

Figure 3. Impact of treatments on Okra plant.

zobacterial strains were isolated from these sampling sites. Eight (8) bacterial strains having similar colony morphology and showing potential of salt tolerance up to 1600 mM NaCl level has been selected for this study. These strains were designated as (IAA^1, IAA^2, IAA^3, IAA^4, IAA^5, IAA^6, IAA^7 and IAA^8).

Phenotypic, biochemical identification, plant growth promoting traits and selection of potential salt tolerant PGPR strain

The phenotypic properties of these eight salt tolerant strains were presented in Table 2. The various biochemical properties of these strains were depicted in Table 3. Based on biochemical activities these strains were designated as *Pseudomonas* spp.

These eight isolates were analysed for different plant growth promoting properties. The strain IAA^4 was observed highest for IAA production activity with tryptophan amendment. While the strain IAA^3 shows highest production without tryptophan precursor (Figure 1). The strain IAA^4 was shown positive for P solubilization and as nitrogen fixation activity (Table 4). Thus, on the basis of PGPR characteristics strain IAA^4 was found potential bio-agent for bioformulation development.

Impact on Okra seed germination, plant growth parameters, pigment status and antioxidant activities under saline stress soils

The okra seeds inoculated with bacterium (IAA^4) shows improved germination rate by 14.28% compare to control seeds (Figure 2).

The plant height was significantly ($F=17.741$, $P<0.002$) improved in inoculated plants. The maximum plant height was recorded in Control + FYM+ Bacteria (IAA^4) which are 42.74% higher compare to control plants. The root length, fresh weight and dry weight of okra plant was highest is the similar treated pots (Figure 3). Which are 29%, 55.72% and 45.47% higher with their respective control (Figures 4). The F value and significant level (P) of plant growth parameters are depicted in Table 5. The chlorophyll level has been enhanced in the treated plants (Figure 5). The level of Chl *a* (27.87%), Chl *b* (5.52%) and Total Chl *ab* (15.11%) was significantly improved in FYM+ IAA^4 treated okra plants (Table 5).

The healthier and green plant significantly reduces antioxidant enzyme level in okra under saline stressed soils (Table 5). The CAT activity was reduced in FYM + IAA^4 treated plots by 53.84 % and POX by 27.86 % compare to control (Figure 6).

Table 4. Plant growth promoting test of isolates.

Isolates	IAA Activity	P-Solubilization	N_2– Fixation
IAA^1	++	+	-
IAA^2	+++	-	-
IAA^3	+	+	+
IAA^4	+++	+++	++
IAA^5	++	+	+
IAA^6	++	+	-
IAA^7	+	-	+
IAA^8	+	-	+

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

Table 5. ANOVA value and significant level in okra plant growth parameters, pigments and antioxidant enzymes.

Parameters	F-Value	Significance level
Plant height	17.741	<0.002
Root length	1.556	<0.002
Fresh weight	24.803	<0.001
Dry weight	4.478	<0.040
Chl a	6.552	<0.015
Chl b	0.140	<0.009
Chl ab	2.988	<0.009
CAT	11.247	<0.003
POX	13.633	<0.002

Table 6. Pearson correlation analyses between plant growth parameters, pigments and antioxidant enzymes in okra treated with isolated Pseudomonas sp. N=12 (4 treatments×3 replicates).

Parameters	Plant height	Root length	Fresh weight	Dry weight	Chl-a	Chl-b	Chl-ab	CAT
Plant height (cm)	1.000							
Root length (cm)	0.678*	1.000						
Fresh weight (gm)	0.962**	0.622*	1.000					
Dry weight (gm)	0.821**	0.425	0.886**	1.000				
Chl-a (mg g^{-1} fresh weight)	0.623*	0.220	0.774**	0.734**	1.000			
Chl-b (mg g^{-1} fresh weight)	0.224	-0.093	0.250	0.312	0.042	1.000		
Chl-ab (mg g^{-1} fresh weight)	0.584*	0.087	0.707*	0.722**	0.717**	0.727**	1.000	
CAT (U mg^{-1} protein)	-0.852**	-0.552	-0.894**	-0.735**	-0.685*	-0.317	-0.692*	1.000
POX (U mg^{-1} protein)	-0.733**	-0.476	-0.799**	-0.652*	-0.736**	-0.247	-0.679*	.812**

Discussion

For this study the potential rhizobacteria was isolated from extreme environmental conditions as saline soil and reapplied in saline soil as bioinoculants for okra plant growth promotion. This IAA[4] strain exhibited ability to survive successfully in saline conditions (>1600mM NaCl) and positive for the plant growth promoting activities.

Soil salinity has been significantly affected the okra seed germination as well as plant growth. Minhas and Gupta[27] observe 50% reduction in fresh fruit yield of okra at 6.7 ds m^{-1} of salinity. The plant morphology, physiology and metabolism are strongly disturbed under saline stress soils.[28] We observed a very poor plant growth in control okra plants.[29] However, the treatments show their effectiveness in salinity stress management.

The PGP activities by strain IAA[4] as production of efficient level of indole-3-Acetic Acid (IAA), P solubilization and N_2 fixing ability are positively correlated with other studies.[5,6,8] The IAA plays very vital role in seed germination, root formation, signalling, and stress management. The FYM in association with control soils and PGPR strain was found positive in plant height, root length, fresh weight and plant dry weight promotion and significantly reduces negative impact of soil salinity.

The photosynthetic pigments chlorophyll a and the chlorophyll b were significantly poor in underdeveloped plant. The inoculation with strain IAA[4] and FYM significantly improved the plant growth and well developed plant shows good amount on green pigment in his tissues.

Addition of FYM + IAA[4] reduces salinity level and saline stress. The CAT and POX antioxidant enzymes activity was significantly decline in treated pots shows positive plant growth. Our study finds support of other observations.[29]

A positive correlation was significantly observed between plant growth parameters, pigments and antioxidant activities (Table 6). As the plant growth and pigments content was improved the antioxidants activities was decrease in plant. That represents the efficacy of isolate in management of negative effect of soil salinity stress in okra plant.

Conclusions

In this study, we concluded that the isolate IAA[4] designated as *Pseudomonas* spp. efficiently neutralizes the negative impact of soil salinity on okra plant in pot experiment. The *Pseudomonas* spp. significantly

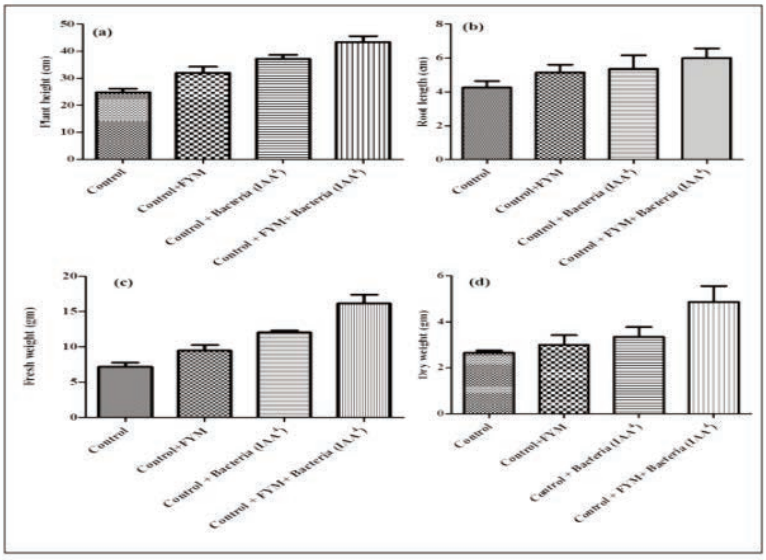

Figure 4. Treatments effect on Okra plant growth parameters (a) plant height (b) root length (c) fresh weight and (d) dry weight.

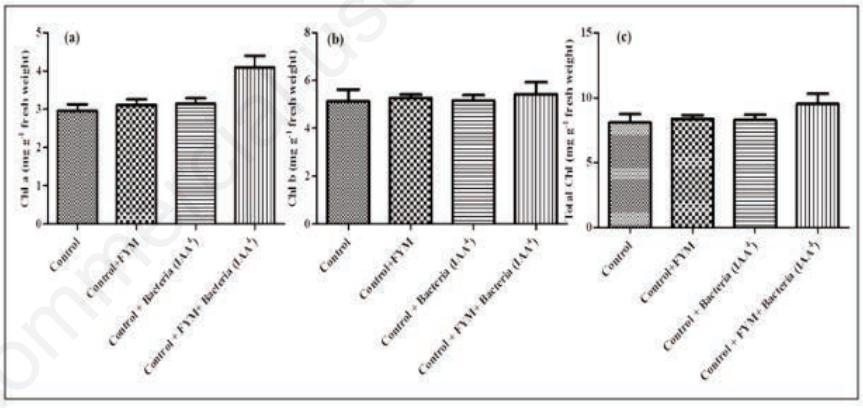

Figure 5. Treatments impact on okra plant pigment contents.

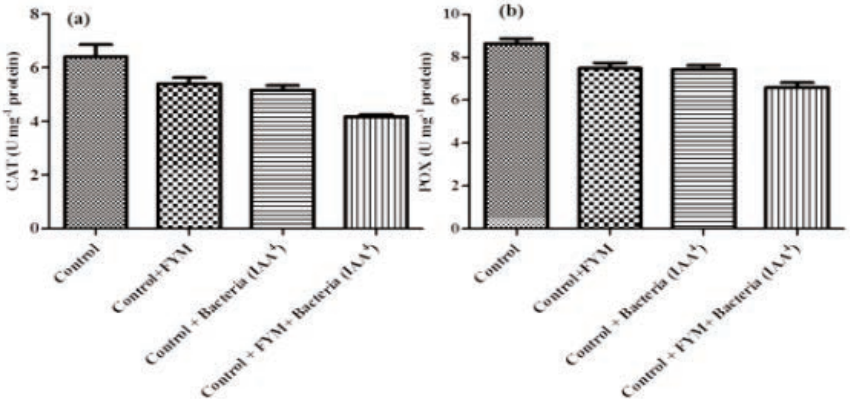

Figure 6. Treatments impact on okra plant antioxidant enzyme activities (a) Catalase (CAT) activity and (b) Peroxidase (POX) activity.

manages stress environments through producing efficient amount of phytohormone, solubilizing P and N fixation activities. The improvement in plant chlorophylls content and reduction in antioxidant enzyme activity was also managed due to the inoculation of isolated bacterial strains. The pot experiment in this study was just a preliminary study to identify efficient bacterial strains however; it could be evaluated in the field also to enhance the growth and yield of other crops under saline conditions.

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