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Original Article

EVALUATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA TO IMPROVE TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) PRODUCTIVITY AND RESILIENCE UNDER SALINITY STRESS

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Abstract: The usage of plant growth promoting rhizobacteria (PGPR) is an eco-friendly approach to neutralize stressful conditions and boost plants resistance. The present research work was intended to isolate halotolerant PGPR to improve tomato (Lycopersicon esculentum Mill.) growth and resilience against salt stress. A total 107 PGPR strains were isolated from the rhizosphere of Kesudo (Butea monosperma Lam.), Kawaria (Cassia tora L.) and Arjun (Terminalia arjuna Roxb.) plants and evaluate their plant growth promoting abilities. BLAST analysis of 16S rRNA sequences identified these isolates as Arthrobacterglobiformis (NAT3), Bacillus subtilis (NBM3), Bacillus thuringiensis (LAT2), Bacillus amyloliquefaciens (NBM6) and Bacillus megaterium (LAT4). The cultures showed significant plant growth promoting activities, such as Indole 3 acetic acid, phosphate solubilisation, sideropore units and ACC deaminase activity. Tomato plants grown from PGPR strains treated seeds exhibited significantly greater germination percentage, seedling growth, plant height, fresh weight, dry weight and leaf area than PGPR non-treated control plants. In addition, salt-stressed tomato plants inoculated with PGPR strains also shows significant increase in total soluble sugar, proline and total chlorophyll content than non-inoculated plants, and the activity of several antioxidant enzymes (SOD, CAT, APX and GR) were also increased in PGPR treated plants under salt stress. These findings suggest that the above described halotolerant PGPR strains have great potential to improve tomato productivity and tolerance under salt stress by eliminating the harmful effects of salt stress on plant growth.

Key words: Rhizobacteria, *Lycopersicon esculentum*, Salinity stress

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INTRODUCTION

High salinity is an abiotic stress that reduces crop productivity in arid and semi-arid regions of the world [1]. It is estimated that, worldwide, 800 million hectare of land and 32 million hectare of agricultural land are salt-affected [2]. India has about 8.1 million hectare salt affected soils out of which 3.1 million hectare is coastal saline soil [3], and 2.8 million hectare is sodic soil and the rest 2.2 million hectare is inland saline soil [4]. Unfortunately, farm lands are still being damaged by salt and becoming more saline due to agricultural practices such as saline water irrigation [5] and excessive fertilization [6]. Soil salinity is a major abiotic factor that limits the growth and development of most crop plants [7] in different ways such as osmotic effects, nutritional disorders and specific-ion toxicity [8]. Soil salinization, which is the excess accumulation of salt in the soil, often results in yield decline in agricultural production systems. Even relatively low salinity (electrical conductivity < 1.0 dsm^{-1}) can result in yield decline in some widely used crops such as rice and tomato [7]. Therefore, the development of salt-tolerant plant is a much-required scientific goal. However, efforts have only been met with limited success, and only a few major genetic determinants of salt tolerance have been identified [9].

Interestingly, as an alternative to breeding and genetic manipulation, plant salt tolerance can also be improved by the application of salt-tolerant microorganisms. The beneficial microorganisms, such as plant growth-promoting rhizobacteria (PGPR), that inhabit the rhizosphere have been investigated for their potential to alleviate salt stress. These studies have shown that the majority of PGPR, including strains of *Agrobacterium, Azospirillum, Bacillus, Pseudomonas*, and *Rhizobium* species, increase the salt tolerance of their host plants [10-12]. In addition, these PGPR can stimulate plant growth through a variety of mechanisms, including the fixation of atmospheric nitrogen, solubilization of phosphate, and production of phytohormones (e.g., indole- 3-acetic acid (IAA), gibberellin, cytokinin, and abscisic acid), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and exopolysaccharide (EPS). For example, treatment with the ACC producing bacterium *Pseudomonas mendocina* has been shown to enhance the uptake of essential nutrients by salt stressed lettuce [13] and treatment with the EPS producing bacteria *Bacillus pumilus* and *Exiguobacteriumoxidotolerans* has been shown to increase the height and bacoside -A content of salt stressed brahmi (*Bacopamonnieri*) [11]. Thus, the application of PGPR can ameliorate salt stress in crop plants, promote plant growth [14], and control diseases [15]. Indeed, PGPR have been reported to improve the growth of a variety of crop species, inclu-ding tomato [16], red pepper [17], maize [18], mung bean [10], and lettuce [13], growing under saline conditions. In addition, PGPR also help plants resist salt stress by increasing the activity of both antioxidant enzymes and non-enzymatic antioxidants [19].

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop next to potato grown around the world. Tomato has an excellent nutritional profile owing largely to its balanced mixture of minerals (potassium, calcium, phosphorous, iron and zinc), vitamins (A, B1, B2, B6, biotin, folic acid, nicotinic acid, pantothenic acid, C, E, and K) and antioxidants such as carotenoids and polyphenolic compounds [20]. It has been proved that salt stress in tomato plants can be alleviated by a PGPR *Achromobacterpiechaudii*ARV8 [21]. Nowadays, much of the agricultural land and coastal land of Gujarat has become saline due to faulty and excessive irrigation practices. Hasty usage of different pesticides and agrochemicals has worsened the problem further. Hence, this study was conducted to reveal the behavior of the selected PGPR under salinity stress condition and their role in enhancing growth of tomato.

MATERIALS AND METHODS

Isolation and identification of microorganisms and collection of soil samples from different points: Three plants Kesudo (*Butea monosperma* Lam), Kawaria (*Cassia tora* L.) and Arjun (*Terminalia arjuna* Roxb.) from forest region of Jessore, Banaskantha district of Gujarat, were selected for the isolation of bacteria from rhizosphere. Rhizosphere soil samples were collected at the depth of 10 cm carefully by uprooting the root system and placed in a cool box for transport to laboratory and stored at 4°C.

Isolation of bacteria: Soil suspensions were prepared by suspending 2 gm of soil in 20 ml of sterile distilled water in 50 ml Erlenmeyer flasks. The flasks were incubated in an orbital shaker at 150 rpm for 1 h. One ml of soil suspension was added to 9 ml

portions of sterile distilled water in glass tubes. Serial dilutions were prepared by further transfers of 1 ml suspension to 9 ml sterile distilled water as 10^{-1} to 10^{-6} dilution. These dilutions were used for inoculation on plates. The 200 µl aliquots from different dilutions were transferred and spread onto nutrient agar plates, luria agar plates, minimal agar plates and trypton soya agar plates. All the plates were incubated in incubator at 37°C and morphologically different colonies appearing on the medium were isolated and sub cultured for further analysis. One hundred seven isolates were obtained, pure culture of these isolates were prepared and maintained on slants according to media.These bacterial cultures were stored at 4°C in refrigerator for further use.

Gram's staining: Smears of each bacterial isolates were separately prepared on a clean glass slide and heat-fixed after drying. One drop of crystal violet solution was put onto the smear and allowed to react for 45 seconds. Excess stain was washed off with sterile water. Then one drop of Gram's iodine solution was put and allowed to react for 45 seconds. Then it was washed with water followed by dipping in absolute alcohol in a 100 ml beaker for 1 minute. Thereafter, one drop of safranine (counter stain) was applied over the smear, and allowed to react for 1 minute. It was washed gently with sterile water, air dried, mounted in glycerine and examined under oil immersion.

Plant growth promoting attributes: Different tests of plant growth promoting properties were performed as described below:

1. IAA production [22]: Bacterial isolates were grown on nutrient broth supplied with 500 µg/ml of DL-tryptophan at 30°C for 48 h. fully grown cultures were centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was taken for estimation of IAA. Then, supernatant (2 ml) was mixed by adding orthophosphoric acid (2 drops) and Salkowski reagent (4 ml). Development of pink colour indicates IAA production. Optical density was measured at 560 nm.

2. Determination of phosphate solubilization: Bacterial isolates were grown in 50 ml Pikovaskaya's broth containing 100 mg of tricalcium phosphate and the amount of soluble phosphorus released was estimated on 7th day after inoculation (DAS). The culture medium was centrifuged at 10,000 rpm for 10 min and the clear supernatant was used for soluble phosphorus estimation by Oslen et al. [23] method.

3. Estimation of siderophore production: Succinate (iron free) medium was used for the production of siderophore with slight modification. It contained K_2HPO_4 6.0 g; KH_2PO_4 3.0 g $(NH_4)_2SO_4$ 1.0 g; $MgSO_4.7H_2O$ 0.2 g in 1 liter of distilled water. 0.5 ml of old culture of each test isolate was inoculated in 100 ml medium in flasks and incubated at 30°C for 72 h on rotary shaker conditions. Cell free supernatant was harvested by centrifugation at 10,000 rpm for 20 minutes at 4°C. The supernatant was used for estimation of siderophores.

For quantitative estimation of siderophores, CAS (Chrome azurol S) liquid assay method was used [24]. 0.5 ml of 72 h old cell free supernatant was mixed with 0.5 ml CAS assay solution (1.5 ml of 1 mM $FeCl_3.6H_2O$ in 10 mM HCl + 7.5 ml of 2 mM CAS stock solution dissolved in 50 ml of HDTMA (hexa-decyltrimethyl-ammonium bromide) in mixing cylinder, add 30 ml piperazine solution into it and final volume was made to 100 ml with distilled water), 10 µl shuttle solution (0.2 M 5-sulfosalicylic acid) was added. Color intensity of the solution was recorded with UV-VIS spectrophotometer at 630 nm against reference after 10 minutes at room temperature. Siderophore production was observed in terms of reduction in blue color as percent siderophore units (% SU).

$$\% SU = \frac{Ar - As}{Ar} \times 100$$

Ar = Absorbance of reference at 630 nm
As = Absorbance of supernatant at 630 nm

4. ACC deaminase activity: ACC deaminase activity was assayed according to a modified methods of Belimov et al. [25] and Shaharoona et al. [26], which measures the amount of α-ketobutyrate produced upon the hydrolysis of ACC. The number of µmol of α-ketobutyrate produced by this reaction was measured by comparing the absorbance (540 nm) of a sample to a standard curve of α-ketobutyrate which was ranging between 10 and 200 µmoL. Stock solution of 100 mmoL L^{-1} α-ketobutyrate was prepared in 0.1 moL L^{-1} of Tris-HCl, (pH 8.5) and stored at 4°C. Just prior to use, the stock solution was diluted with the same buffer to make 10 mmoL L^{-1} of solution from which a standard concentrations curve was generated. In a series of known α-ketobutyrate concentrations, 2 ml of the 2,4-dinitrophenylhydrazine reagent was added, the contents were

vortexed and incubated at 30°C for 30 minutes, during which the α-ketobutyrate was derivitized as aphenylhydrazine. The color of phenyl hydrazine was developed by the addition of 2 ml, 2 moL L^{-1} of sodium hydroxide, the absorbance of the mixture was measured after mixing by using spectrophotometer at 540 nm.

For determining ACC deaminase activity, bacterial isolates were grown in rich tryptic soy broth medium (TSB) for 18 h at 28°C. The cells were then harvested by centrifugation, washed with 0.1 M Tris-HCl (pH 7.5), and incubated for another 18 h in minimal medium containing 3 mM ACC as the sole source of nitrogen. The bacterial cells were collected by centrifugation [27] and suspended in 5 ml of 0.1 moL L^{-1} of Tris-HCl, pH 7.6, and transferred to microcentrifuge tube. The contents of the tubes were centrifuged at 16,000 rpm for 5 min and supernatant was removed. The pellets were suspended in 2 ml 0.1 moL L^{-1} Tris HCl, pH 8.5. Thirty µL of toluene were added to the cell suspension and vortexed for 30 s. Toluenized cells (200 µL) were placed in a fresh micro centrifuge tube, 0.5 moL L^{-1} ACC (20 µ L) was added to the suspension, vortexed, and incubated at 30°C for 15 min, following the addition of 0.56 moL L^{-1} HCl (1 ml), the mixture was again vortexed and centrifuged at 13,000 rpm for 5 min at room temperature. 2 ml of the supernatant was vortexed together with 1 ml of 0.56 moL L^{-1} hydrochloric acid.Immediately, 2 ml of the 2,4-dinitrophenyl-hydrazine reagent (0.2 % 2,4-dinitrophenylhydrazine in 2 moL L^{-1} HCl) was added to the glass tube, and the contents were vortexed and then incubated at 30°C for 30 min. following the addition and mixing of 2 ml of 2 moL L^{-1} of NaOH, the absorbance of the mixture was measured by using spectrophotometer at 540 nm [26]. The cell suspension without aminocyclopropane- 1-carboxylic acid was used as negative control and with $(NH_4)_2SO_4$ (0.2 % w/v) as positive control.

Molecular characterization of bacterial isolates: Molecular characterization of most efficient bacterial isolates was done by sequencing of their 16S rRNA gene. Out of one hundred seven bacterial strains, five bacterial strains were selected for 16S rRNA on the basis of PGPR traits. After that, five best performing strains were identified by 16S rRNA sequencing.

Effect of inoculation of PGPR on physiological and biochemical parameters of tomato under saline condition:

PGPR strain and inoculum preparation: Five different PGPR strains were used in this study: *Arthrobacter globiformis* NAT3, *Bacillus subtilis* NBM3, *Bacillus thuringiensis* LAT2, *Bacillus amyloliquefaciens* NBM6, and *Bacillus megaterium* LAT4. Active cultures of PGPR strains were prepared from nutrient broth and luria broth.

Plant material and study area: Seed of tomato S-22 (*Lycopersicon esculentum* Mill.) (physical purity: min. 98%, inert matter: max. 2%, pure seed: min. 98%, moisture: max. 6% and germination: min. 70%) were collected on 23 January 2018 from the R.K. seed farms (Regd.), Delhi. The whole experiment was performed in a green house of the botanical garden of Hemchandracharya North Gujarat University, Patan (23°50' N Lat, 72°07' E Long) in Gujarat. For the emergence and growth of seedlings, surface soil (vertisol) from the top 15 cm, which is predominant in north region of Gujarat, was collected from a nearby agriculture field.

Salinization of soil: Surface soil was collected, autoclaved, air dried, and passed through a 2 mm mesh. Sodium chloride (NaCl) amounting 7.8 g was then thoroughly mixed with the 3 kg soil to give interstitial soil water salinity of 4 dsm^{-1}. For measurement of soil salinity, a soil suspension was prepared in distilled water with a 1:2 soil: water ratio [28]. The soil suspension was shaken vigorously and allowed to stand overnight. After that the conductivity of the soil suspension was measured with a conductivity meter (Systronic; Model 307). Control soil had a conductivity of 0.3 dsm^{-1}.

Experimental Design: Ten(10) polythene bags (20.5 cm wide and 41 cm long) were each filled with 3 kg of soil for twelve (12) treatments. Total 120 bags were then kept under natural light and temperature in an uncontrolled greenhouse. The healthy tomato seeds were surface sterilized with 0.1 % $HgCl_2$ for 1 min and rinsed six times with sterile distilled water, and then seeds were soaked for 30 min in respective active bacterial culture. For control plants, seeds were soaked in sterile water forthe same period of time. Then seeds were shade dried for 30 min. After shade drying, twenty seed were gently pressed to a depth of about 10-15 mm in each bag on 24 January 2018. Then 30 ml of active culture was

added in each bag except control treatment. 100 ml tap water was provided on alternate days to wet the soil surface. The experiment was conducted in completely randomized block (CRD) design with ten (10) replicates and was repeated three (3) times.

Seedling growth: Two seedlings that established first were left in each bag and others were uprooted as they appeared. The experiment was terminated after 4 months. Twenty plants grown for each treatment were then washed with tap water to remove soil particles that adhered to roots. Morphological characteristics of each seedling were recorded. Seed germination (%) was recorded for 30 days after sowing. Shoot height of plant was measured by using a scale from tip of plant to the end of stem. Root length of plant was measured by using a scale from collar region to the end of root. Fresh weight of shoot and root were measured with the help of weighing machine immediately after harvest. Excess moisture on the shoot and root was blotted with tissue paper before measuring shoot and root fresh weight. Dry weight of shoot and root were measured with the help of weighing machine after drying in hot air oven at 40 °C for 5 days, when constant weight was reached. Leaf area was marked out on graph paper.

Biochemical Parameters

Organic solutes (soluble sugars and proline): Total soluble sugars content was estimated by the phenol sulfuric method [29]. A 100 mg leaves were hydrolyzed by keeping it in a boiling water bath for 3 h with 5 ml of 2.5 N HCl and then neutralized with solid sodium carbonate until the effervescence ceased. Then, the volume was made up to 100 ml and centrifuged. After that, supernatant aliquots of 0.1 and 0.2 ml were taken in separate test tubes and made to 1 ml. Then, 1 ml of phenol solution followed by 5 ml of 96 % sulfuric acid were added to each test tube, shaken well, and placed in a water bath at 25-30 °C for 20 min. Chromophore was read at 490 nm. The amount of total carbohydrate was calculated using the standard curve of glucose.

Proline content was determined according to Patel et al. [30]. Proline was extracted from 500 mg of plant leaves by grinding in 10 ml of 3 % sulfosalicylic acid and the mixture was then centrifuged at 10,000 × g for 10 min. An aliquot of 2 ml of supernatant was taken in a test tube in which an equal volume of freshly prepared ninhydrin solution was added. Tubes were incubated for 30 min in a water bath at 90 °C. After incubation, the reaction was terminated in an ice bath. Then, the reaction mixture was extracted with 5 ml toluene with continuous stirring for 15 min. The tubes were allowed to stand for 20 min in the dark for the separation of the supernatant of the toluene and aqueous phases. The toluene phase was then carefully collected into a test tube and absorbance was measured at 520 nm. The concentration of proline was calculated from a standard curve using the following equation: (µg proline in extract/111.5) /g of sample = µmol g^{-1} of fresh tissue.

Chlorophyll content: The chlorophyll content was measured according to the method of Arnon [31]. About 1 g of leaves were cut in to small pieces and homogenized in a precooled mortar and pestle using 80 % (V/V) acetone. A pinch of $CaCO_3$ was added while grinding. The extract was centrifuged at 3000 rpm for 15 min and made up to 25 ml with 80 % (V/V) acetone. The clear solution was transferred to a colorimeter tube and the OD was measured at 645 nm and 663 nm, against an 80 % acetone blank in spectrophotometer. The levels of chlorophyll 'a' and chlorophyll 'b' were determined using the equation given below:

Chlorophyll 'a' (µ/g/ml) = (12.7 × O.D. at 663 nm) – (2.69 × O.D. at 645 nm)

Chlorophyll 'b' (µ/g/ml) = (22.9 × O.D. at 645 nm) – (4.08 × O.D. at 663 nm)

Total chlorophyll (µ/g/ml) = (20.2 ×O.D. at 645 nm) + (8.02 × O.D. at 663 nm)

The chlorophyll content was expressed as mg chlorophyll per gram fresh weight of the tissue.

Antioxidant enzymes: Plant leaves extractions were prepared for the analysis by homogenizing 200 mg of plant material in 2 ml of 0.2 M potassium phosphate buffer (pH 7.8 with 0.1 mM EDTA). The homogenate was centrifuged at 15,000 × g for 20 min at 4 °C. Afterwards, tissue extract was stored in -20 °C and used within 48 h to determine different antioxidant enzymatic activity.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured using a modified NBT (nitro blue tetrazolium) method described by Beyer and Fridovich [32]. The 2 ml assay reaction mixture containing 50 mM phosphate buffer (pH 7.8), 2 mM EDTA, 9.9 mM L-methionine, 55 µM NBT, and

0.025 % Triton X-100 was taken in a test tube. Then 40 µl of diluted (×2) plant sample and 20 µl of 1 mM riboflavin were added, and the reaction was initiated by illuminating the sample under a 15-W fluorescent tube. During the 10 min exposure, the test tubes were placed in a box lined with aluminum foil that was at a distance of approximately 12 cm from the light source. Duplicate tubes with the same reaction mixture were also kept in the dark and used as blanks. One unit of SOD was defined as the amount of enzyme per milligram of protein sample causing 50 % inhibition of the rate of NBT reduction at 560 nm.

Catalase (CAT; EC 1.11.1.6) activity was determined according to Aebi and Lester [33]. The 3 ml assay mixture contained 2 ml plant leaves extract (diluted 200 times in 50 mM potassium phosphate buffer, pH 7.0) and 10 mM H_2O_2. The decomposition of H_2O_2 was followed as a decrease in absorbance at 240 nm. The extinction coefficient of H_2O_2 (40 mM^{-1} cm^{-1} at 240 nm) was used to calculate the enzyme activity.

Ascorbate peroxidase (APX; EC 1.11.1.11) was estimated using method of Nakano and Asada [34]. The 1 ml assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM H_2O_2, and 10 µl of plant leaves extract. H_2O_2 was added last to initiate the reaction and the decrease in absorbance was recorded for 3 min. APX activity was determined from the decrease in absorbance at 290 nm due to oxidation of ascorbate in the reaction. The extinction coefficient of 2.8 mM^{-1} cm^{-1} for reduced ascorbate was used in calculating the enzyme activity of APX.

Glutathione reductase (GR; EC 1.8.1.7) activity was assayed according to Smith et al. [35]. A 10 µl aliquot of plant leaves extract was used in the assay along with 0.75 mM DTNB (Ellman's reagent; 5,5'-dithio-bis-(2-nitrobenzoic acid)), 0.1 mM NADPH, and 1 mM GSSG in a total of 1 ml assay volume. GSSG was added last to initiate the reaction and the increase in absorbance was recorded for 3 min. The increase in absorbance at 412 nm was measured when DTNB was reduced to TNB by GSH in the reaction. The extinction coefficient of TNB (14.15 mM^{-1} cm^{-1}) was used to calculate the activity of glutathione reductase.

Statistical analysis: Data collected from the experiment were subjected to the analysis of variance (ANOVA) and the means were separated using Tukey's multiple range test.

RESULTS

Isolation and identification of bacterial strains: One hundred seven (107) bacterial strains were isolated from Kesudo (*Butea monosperma* Lam.), Kawaria (*Cassia tora* L.) and Arjun (*Terminalia arjuna* Roxb.) rhizosphere soil, which were found to be a gram negative and gram positive type of bacteria.

Plant growth promoting attributes: All one hundred seven strains were screened for plant growth promoting attributes and found 5 highly potent PGPR strains (NAT3, LAT2, NBM3, NBM6 and LAT4). All five strains are able to produce indoleacetic acid (IAA), siderophore, and ACC deaminase. Except of NAT3 strain, all four strains can be solubalize in phosphate (Table 1).

Molecular characterization: Five of the best performing strains according to their PGPR traits were *Arthrobacter* and *Bacillus* group. NAT3 was found to be *Arthrobacterglobiformis*, NBM3 was identified as *Bacillus subtilis*, LAT2 was identified as *Bacillus thuringiensis*, NBM6 was identified as *Bacillus amyloliquefaciens*, whereas LAT4 was identified as *Bacillus megaterium*, on the basis of 16S rRNA sequencing and comparing the sequences by BLAST (ncbi.nlm.gov.blast/Blast.cgi) database.

Effect of PGPR on plant physiological parameters under saline conditions: The present study investigated the effect of PGPR on the growth of tomato plants grown under normal and saline conditions. The tomato plants treated with each of the PGPR strains possessed significantly higher shoot height, root length, leaf area and germination percentage. The shoot height of plant was significantly higher in NAT3, NBM3, LAT2 and LAT4 treated plant than untreated plant under 4 dsm^{-1}saline stress condition. As same as the root length of plant was significantly higher in NAT3, LAT2, NBM6 and LAT4 treated plant than non-inoculated plant under 4 dsm^{-1} saline stress condition (Table 2).

Furthermore, also in non-saline condition the shoot height of plant was significantly higher in NAT3, NBM3 and LAT4 treated plant than non-inoculated plant and the root length of plant was significantly higher in NAT3, NBM3 and LAT2 treated plant than non-inoculated plant. However, leaf area also

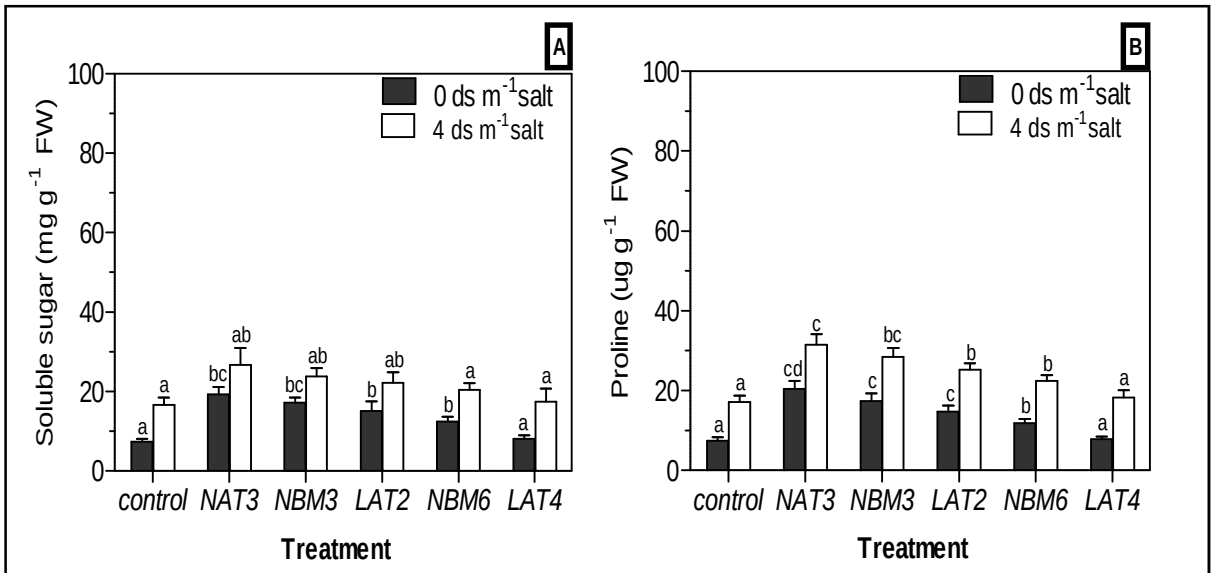

Fig. 1: Effects of plant growth promoting rhizobacteria (PGPR) on the organic solutes of leaf extracts of tomato plant under saline condition. (A) Soluble sugars (B) Proline. Data were analysed by One-way ANOVA Tukey'smultiple range test (P<0.05).

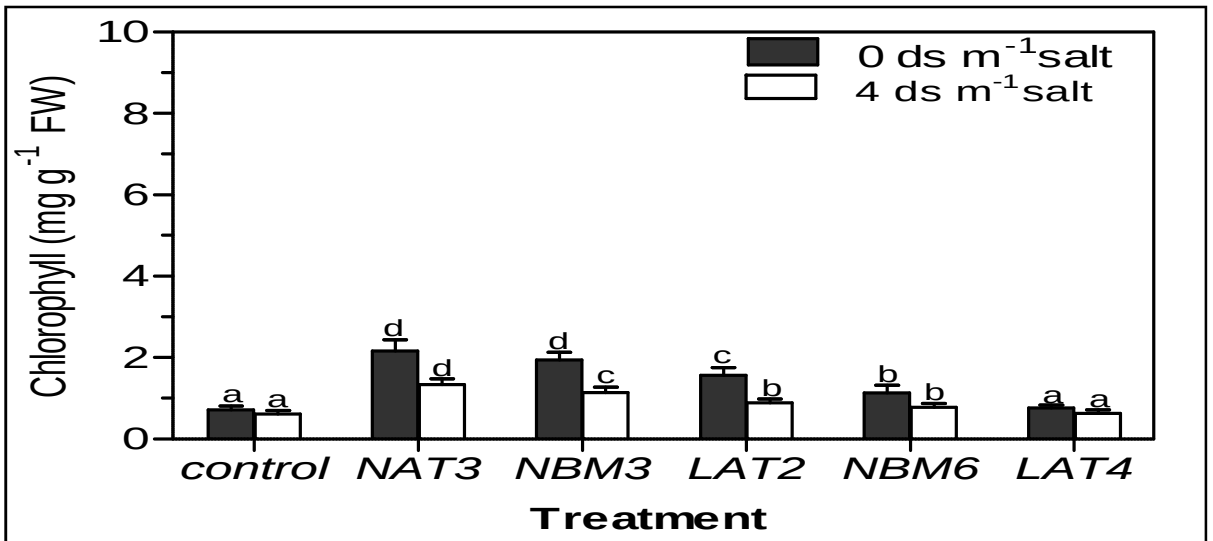

Fig. 2: Effects of plant growth promoting rhizobacteria (PGPR) on the Chlorophyll content of leaf extracts of tomato plant under saline condition. Data were analysed by One-way ANOVA Tukey'smultiple range test (P<0.05).

affected of tomato plant in both grown conditions (saline 4 dsm^{-1} and non-saline 0 dsm^{-1}) when treated with different microorganism. Leaf area of tomato significantly increased when treated with NAT3 and LAT2 strains under saline condition, as same as, leaf area was increased significantly when treated with NAT3 and NBM3 strains under non-saline condition. Germination % of tomato plant was also increased in both saline and non-saline conditions when treated with all five PGPR strains (Table 2).

The tomato plant treated with each of the PGPR strains possessed significantly greater shoot fresh weight, shoot dry weight, root fresh weight and root dry weight. The shoot fresh weight of plant was significantly greater in NAT3, NBM3, LAT2, NBM6 and LAT4 treated plant than non-inoculated plant under 4 dsm-1 saline stress condition. As same as the shoot dry weight of plant was significantly greater in NAT3, NBM3, LAT2, NBM6 and LAT4 treated plant than untreated plant under 4 dsm^{-1} saline stress condition. Furthermore, also in non-saline condition the shoot fresh weight of plant was significantly greater in NAT3 and NBM3 treated plant than untreated plant and the shoot dry weight of plant was significantly greater in NAT3 treated plant than untreated plant. The root fresh weight of plant was significantly greater in NAT3, NBM3, LAT2 and NBM6

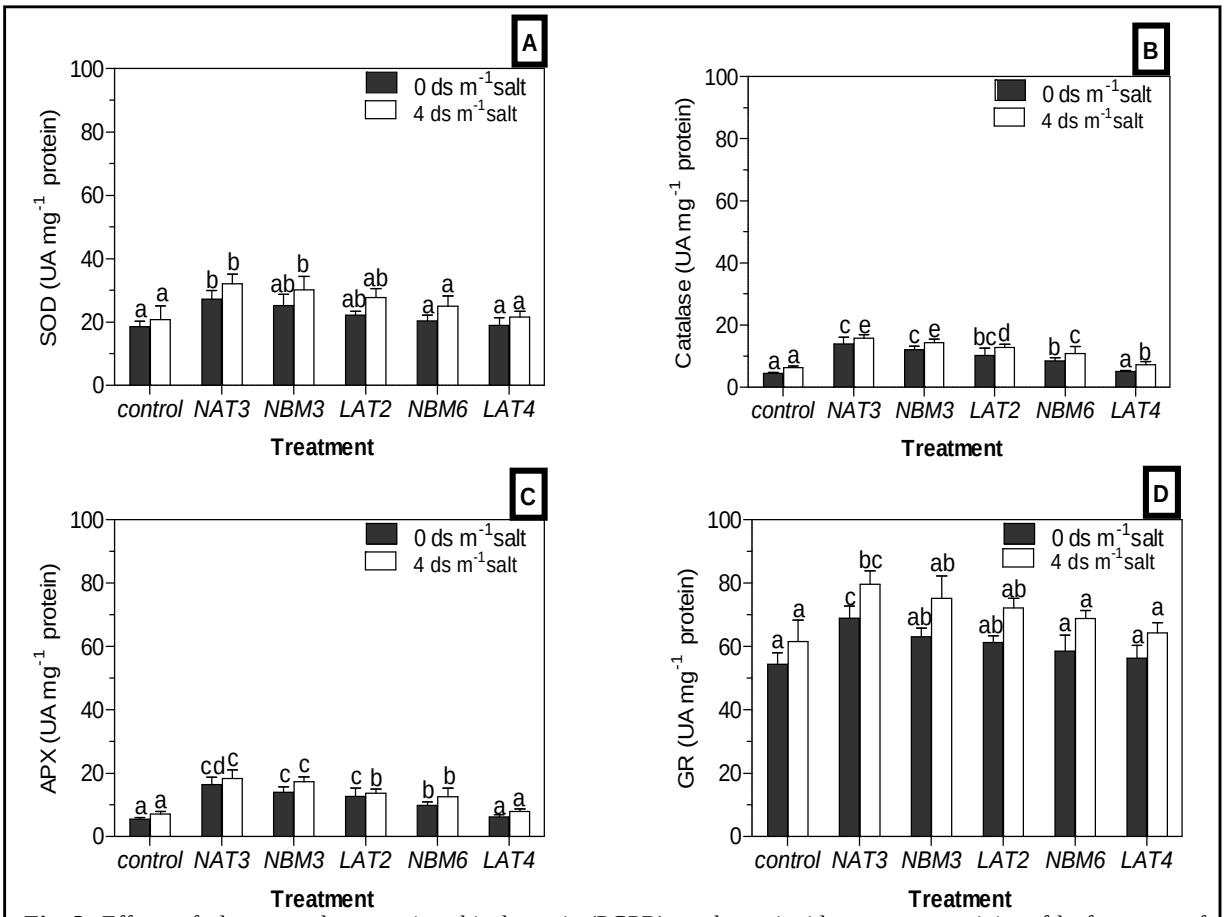

Fig. 3: Effects of plant growth promoting rhizobacteria (PGPR) on the antioxidant enzyme activity of leaf extracts of tomato plant under saline condition. (A)Superoxide dismutase (SOD) activity (B) Catalase activity (C) Ascorbate peroxidase (APX) activity (D) Glutathione reductase (GR) activity. Data were analysed by One-way ANOVA Tukey'smultiple range test ($P<0.05$).

treated plant than untreated plant under 4 dsm^{-1} saline stress condition. As same as the root dry weight of plant was significantly greater in NAT3 and NBM3 treated plant than untreatedplant under 4 dsm^{-1} saline stress condition. Furthermore, also in non-saline condition the root fresh weight of plant was significantly greater in NAT3 and NBM6 treated plant than untreated plant and the root dry weight of plant was significantly greater in NAT3 and NBM3 treated plant than untreated plant (Table 3).

Effect of PGPR on proline and soluble sugar under saline condition: Proline and soluble sugar are common organic solutes in higher plants and accumulate in response to stress. In the present study, the proline content of leaves from PGPR inoculated and non-inoculated tomato plant growing under non saline and saline conditions. Both the proline and soluble sugar content were significantly higher in the PGPR treated plants than in the untreated control plants, and the inoculated plants accumulated greater levels of proline and soluble sugar under saline condition than non-saline condition. The soluble sugar content of salt stressed tomato plant inoculated with three strains NAT3, NBM3 and LAT2, was significantly increased, whereas, in normal condition plant inoculated with strains NAT3, NBM3, NBM6 and LAT2, was increased significantly. However, the proline content of salt stressed and non-salt stressed tomato plant inoculated with four strains NAT3, NBM3, NBM6 and LAT2, it was significantly increased (Fig. 1).

Effect of PGPR on chlorophyll content under saline condition: The Chlorophyll content of leaves was determined for tomato plants grown under both normal and saline conditions. Inoculation with four PGPR strains (NAT3, NBM3, LAT2, and NBM6) resulted in chlorophyll contents that were greater than that of the non-inoculated plants, regardless of growing conditions. Among the five PGPR strains, the effect of LAT4 on total chlorophyll content was not significant (Fig. 2).

Table 1: Plant growth-promoting rhizobacteria (PGPR) characteristics of the selected isolates.

Isolates	IAA production (µg/ml	Phosphate solubilization (mg of P released from 100 mg of tricalcium phosphate)	% Siderophore Units (SU)	ACC deaminase activity (nmoL α-ketobutyrate $mg^{-1} h^{-1}$)	Gen Bank Accession No.
Arthrobacterglobiformis NAT3	34.2	ND	45.3	514.2	KF853104
Bacillus subtilis NBM3	22.1	15.04	52.8	448.3	KF853106
Bacillus thuringiensis LAT2	9.5	4.91	68.7	304.2	KF853125
Bacillus amyloliquefaciens NBM6	35.6	13.44	ND	192.5	KF853107
Bacillus megaterium LAT4	10.95	8.5	50.4	502.3	KF853126

Table 2: Effects of plant growth-promoting rhizobacteria on the shoot height, root length, leaf area and germination % of tomato plants under saline conditions. Data were analysed by One-way ANOVA Tukey'smultiple range test (P<0.05).

NaCl concentration	Treatment	Shoot height (cm)	Root length(cm)	Leaf Area (cm^2)	Germination (%)
0 dsm^{-1}	Non-inoculated	45.76 ± 2.4a	12.44 ± 1.7a	6.91 ± 0.5a	79
	NAT3	85.76 ± 5.2d	22.24 ± 2.1c	9.12 ± 0.9c	96
	NBM3	68.96 ± 5.7c	18.08 ± 1.5b	8.37 ± 0.8b	94
	LAT2	65.76 ± 4.7bc	17.76 ± 0.8b	8.05 ± 0.4ab	89
	NBM6	58.72 ± 6.3b	15.36 ± 1.8ab	7.59 ± 0.6a	86
	LAT4	51.68 ± 2.9b	12.78 ± 1.4a	7.06 ± 0.4a	81
4 dsm^{-1}	Non-inoculated	37.9 ± 1.1a	10.68 ± 1.2a	5.72 ± 0.3a	63
	NAT3	68.05 ± 4.2d	18.37 ± 1.5d	7.53 ± 0.6c	77
	NBM3	57.11 ± 4.6c	14.93 ± 1.1c	6.92 ± 0.6bc	75
	LAT2	52.69 ± 4.6bc	14.66 ± 1.7c	6.65 ± 0.2b	71
	NBM6	46.6 ± 5.1b	12.7 ± 0.8b	6.27 ± 0.3ab	68
	LAT4	42.61 ± 1.8b	10.74 ± 0.7a	5.76 ± 0.3a	64

Table 3: Effects of plant growth-promoting rhizobacteria on the shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight of tomato plants under saline conditions. Data were analysed by One-way ANOVA Tukey'smultiple range test (P<0.05).

NaCl concentration	Treatment	Shoot fresh weight (gm)	Shoot dry weight (gm)	Root fresh weight (gm)	Root dry weight(gm)
0 dsm^{-1}	Non-inoculated	9.58 ± 0.8a	3.44 ± 0.4a	0.59 ± 0.07a	0.21 ± 0.02a
	NAT3	12.14 ± 1.2b	4.36 ± 0.5b	1.03 ± 0.13b	0.37 ± 0.04c
	NBM3	10.94 ± 1.0ab	3.92 ± 0.5ab	0.85 ± 0.11ab	0.30 ± 0.03b
	LAT2	10.76 ± 1.1a	3.85 ± 0.3ab	0.74 ± 0.12ab	0.26 ± 0.03ab
	NBM6	10.41 ± 0.7a	3.72 ± 0.3ab	0.69 ± 0.09b	0.24 ± 0.02a
	LAT4	9.94 ± 0.9a	3.53 ± 0.3a	0.61 ± 0.08a	0.22 ± 0.02a
4 dsm^{-1}	Non-inoculated	5.74 ± 0.5a	2.05 ± 0.1a	0.49 ± 0.03a	0.16 ± 0.01a
	NAT3	10.18 ± 0.8d	3.65 ± 0.3c	0.82 ± 0.09c	0.29 ± 0.02c
	NBM3	9.04 ± 0.7cd	3.24 ± 0.2bc	0.67 ± 0.05bc	0.23 ± 0.02b
	LAT2	8.94 ± 0.7c	3.2 ± 0.5bc	0.58 ± 0.05b	0.20 ± 0.04ab
	NBM6	8.72 ± 0.3bc	3.12 ± 0.4bc	0.56 ± 0.06ab	0.19 ± 0.03ab
	LAT4	7.55 ± 0.5b	2.95 ± 0.2b	0.50 ± 0.06a	0.17 ± 0.01a

Effect of PGPR on antioxidant enzyme activity under saline condition: In the present study, the activity of four antioxidant enzymes (SOD, CAT, APX and GR) from the extracts of leaves from both PGPR inoculated and non-inoculated tomato plants grown under normal and saline conditions. Under such conditions, inoculation with all five PGPR strains activity of all four antioxidative enzymes increased. More specifically, the SOD enzyme activity of tomato plant inoculated with NAT3, NBM3 and LAT2 were significantly increased under both saline and non-saline conditions compared to control condition. The catalase enzyme activity of tomato plant inoculated with all five strains were significantly increased in saline condition and in non-saline condition, it was significantly increased by treated with four strains NAT3, NBM3, NBM6 and LAT2. Furthermore, the APX enzyme activity of tomato plant inoculated with NAT3, NBM3, NBM6 and LAT2 were significantly increased under both saline and non-saline conditions compared to control condition. Whereas, the GR enzyme activity of tomato plant inoculated with NAT3, NBM3 and LAT2 were significantly increased under both saline and non-saline conditions compared to control condition. These results suggest that the enhanced antioxidant enzyme activity of PGPR-inoculated plants could contribute to the plants' improved tolerance of salt stress (Fig. 3).

DISCUSSION

In present study we analyse the effect of PGPR inoculation on the growth, antioxidant enzyme activity, organic solutes like soluble sugar, proline content and chlorophyll content of tomato plant grown under non-saline and 4 dsm^{-1}salt stressed conditions. Under stressful conditions, plant growth and nutrient uptake have been shown to decrease [36,37]. However, the present study reveals that PGPR application can ameliorate the negative effects of saline soil on plant growth. Under saline conditions, both the fresh and dry weights of tomato plants inoculated with PGPR (*Arthrobacterglobiformis* NAT3, *Bacillus subtilis* NBM3, *Bacillus thuringiensis* LAT2, *Bacillus amyloliquefaciens* NBM6 and *Bacillus megaterium* LAT4) were greater than control plants (Table 3). Previous research has also shown that PGPR strains can alleviate the detrimental effects of saline soil on the growth of lettuce, maize, pepper, and wheat [36, 38]. Vivas et al. [39] also reported enhanced root and shoot growth of lettuce plant when inoculated with Bacillus species under dry salt stress conditions. Mi-Seon et al. [40] also reported that under saline conditions, both the fresh and dry weights of pepper plants inoculated with PGPR (*M. oleivorans* KNUC7074, *B. iodinum* KNUC7183, and *R. massiliae* KNUC7586) were greater than those of non-inoculated control plants. In the present work it was also found that the tomato plants treated with each of the PGPR strains possessed significantly higher shoot height, root length, leaf area and germination percentage (Table 2). Tank and Saraf [16] reported that pot studies conducted on tomato plants under 2 % NaCl stress proved that C4 and T15 strains were the best growth promoters. C4 showed 50 % enhancement in shoot height and root length as compared to NaCl added untreated plants as well as in absence of NaCl. Jha and Subramanian [41] reported that PGPR inoculated plants under saline conditions showed 16 % higher germination in paddy rice *(Oryza sativa* L.) 'GJ-17' under green house condition. Woitke et al. [42] reported that tomato plant treated with *Bacillus subtilis* exhibited increased leaf area per plant under saline condition as compared to NaCl added untreated plant.

Furthermore, it has been suggested that phosphate nutrition is a limiting factor for the growth of salt-stressed plants [43]. In the present study, four of the PGPR strains (LAT2, NBM3, NBM6 and LAT4) were able to solubilize phosphate (Table 1) and hence improved phosphate nutrition,which may have partially accounted for the ability of the PGPR treated plants to overcome salinity stress. Indeed, previous studies have reported that phosphate-solubilizing organisms are associated with increased plant phosphate content [44,45]. It has been suggested that the depressive effect of salinity on plant growth is related to reductions in endogenous levels of hormones [46,45]. Therefore, the application of additional natural phytohormones, such as bacterial auxins, could positively affect plant development under high salinity conditions [47]. Moreover, in this study used all five strains were able to produce IAA (Table 1).

Previous studies have also revealed that PGPR release metal chelating substances, such as iron chelating siderophores, into the rhizosphere and have suggested that siderophore producing bacteria influence the plant uptake of various metals, including Fe, Zn, and Cu [48]. Interestingly, the PGPR strains used in the present study all five strains can produce siderophore (Table 1), which suggests that PGPR and other microorganisms can also affect plant stress

tolerance by influencing the bioavailability of metal ions required by their host plants, as reported by Dimkpa et al. [49]. The root elongation might be attributed to the decreased ethylene levels due to the presence of the selected PGPR containing ACC deaminase producing efficiency. It may be postulated that inoculation with these rhizobacterial strains might have decreased endogenous ethylene levels because of ACC deaminase activity, thereby resulting in the formation of longer roots. This also subsequently results in promoting shoot height. Similar elongation in root length and shoot height of maize under 6 dsm^{-1} NaCl stress in the presence of PGPR was observed by Nadeem et al. [50]. In this study used all five strains were able to produce ACC deaminase (Table 1).

Increased soluble sugar content is another important defense strategy for plants facing salinity stress [36], and in the present study, we found that the contents of both proline and soluble sugar were enhanced in the PGPR inoculated tomato plants under saline conditions. Therefore, it is likely that the PGPR strains promoted plant growth under salinity stress by enhancing metabolic defense strategies. Proline accumulation is an adaptive response by plants to both general stress and salinity, since it mediates osmotic adjustment at the cellular level, thereby protecting intracellular macromolecules from dehydration, and also because it serves as a hydroxyl radical scavenger [44].

Han and Lee [37] reported that PGPR, such as *Serratia* and *Rhizobium* species, enhance the growth, nutrient uptake, and chlorophyll content of lettuce grown under different levels of soil salinity. The present study also revealed that PGPR inoculation enhanced the chlorophyll content of tomato leaves. Similar results were reported by Mi-Seon *et al*. [40], who observed that inoculation with all three PGPR strains (*M. oleivorans* KNUC7074, *B. iodinum* KNUC7183, and *R. massiliae* KNUC7586) resulted in chlorophyll contents that were greater than that of the non-inoculated plants. Salt stress induces the formation of reactive oxygen species (ROS), which can cause severe oxidative damage to plants. Antioxidant systems play an important role in protecting plants [51] and animals [52] from oxidative stress and involve a variety of antioxidant enzymes, including superoxide dismutase (SOD), dehydroascorbate reductase (DHAR), glutathione reductase (GR), ascorbate peroxidise (APX), catalase (CAT), and guaiacol peroxidase (GPX) [53]. In plant systems, enzymes and redox metabolites act in synergy to detoxify ROS. For example, both APX and GPX catalyze the conversion of H_2O_2 to water, and CAT converts H_2O_2 to oxygen and water. In the present study, the activities of antioxidant enzymes (SOD, CAT, APX, and GR) in the leaf extracts of PGPR inoculated tomato plants were significantly greater than those observed for the non-inoculated control plants, regardless of growing conditions (i.e., normal or saline). Our results support those of Gururani et al. [54], who also reported that the activities of ROS scavenging enzymes, such as APX, CAT, DHAR, GR, and SOD, were enhanced in PGPR inoculated potato plants exposed to various stressors (salt, drought, and heavy metals) [54]. Furthermore, increased SOD, APX, and CAT activities were also observed in salt stressed okra plants treated with the PGPR *Enterobacter* sp. UPMR18 [19].

The present study shows that PGPR strains can improve the growth and development of plants under salt stress by solubilizing phosphate and by producing ACC deaminase, siderophore, and IAA. The tolerance of tomato seedlings to salt stress was also correlated with elevated levels of ROS scavenging enzymes (SOD, CAT, APX, and GR), as well as the accumulation of proline and soluble sugar, which function as osmoregulants. In this study to identify efficient strains isolated from the rhizosphere of Kesudo, Kawaria and Arjun plants, that enhance the growth of tomato under non stressed and salt stressed conditions. Therefore, the present study suggests that PGPR can alleviate the deleterious effects of salt stress on plants, possibly by functioning as elicitors that enhance plant tolerance to various abiotic stresses. Future research should focus on determining the mRNA expression profiles associated with such tolerance mechanisms in tomato.

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