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Prospecting of Bacterial Endophytes Imparting Salinity Stress Tolerance and Plant Growth Promotion in Tomato (*Solanum lycopersicum*)

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ABSTRACT: Salinity is a severe abiotic factor that harms plant growth and productivity. In recent years, it has been clear that there are microorganisms (fungi and bacteria) that could coexist symbiotically with plants and provide tolerance to abiotic stress. In the current investigation, 58 bacterial endophytes that were isolated from the cold deserts of the Himalayas were in-vitro tested for salinity resistance using different concentrations of NaCl. Nine of the 58 isolates demonstrated tolerance to salinity up to 2.0 M NaCl. After further investigation, it was discovered that the isolates could fix solubilize substances such phosphate, HCN, NH_3, siderophore, proline, IAA, gibberellic acid, abscisic acid, and salicylic acid. Additionally, pre-germinated seeds of Tomato (var. Arka Saurabh) seedlings were tested using a standardization of NaCl (117mM) in paper towel. in which two endophytes (NBE 20 and NBE 23) significantly increased seedling length compared to uninoculated seeds. The endophytes were identified as *Enterobacter cloacae* and *Enterobacter asburiae* by 16S rRNA gene sequence. In greenhouse studies, inoculation with *Enterobacter asburiae* significantly increased the plant growth attributes at 4 dS m^{-1} NaCl, followed by *Enterobacter cloacae* in comparison to control plants. Hence, the present study manifests theses isolate as the potent bacterial endophytes in imparting salt tolerance and growth promotion in Tomato.

Keywords: Bacterial endophytes, NaCl, Tomato (*var.* Arka saurabh), *Enterobacter cloacae, Enterobacter asburiae.*

INTRODUCTION

Salinity is a key abiotic element that affects agricultural yield in many parts of the world. And its affects more than 500-800 million hectares worldwide, or roughly 6% of the world's arable land, according to the FAO (Food and Agriculture Organization) (Munns, 2005). Soil salinity is a growing concern in India, affecting thousands of hectares of irrigated land. Soil salinization occurs when the chloride, sulphate, and nitrate ions of Na^+, K^+, Ca^{2+}, and Mg^{2+} mix, resulting in poor soil quality and its changes the physical qualities of the soil, compromising its health and rendering it unsuitable for agriculture. Soil salinity is a serious agricultural issue, mostly affecting dry and semiarid locations where there is insufficient rainfall to remove salts from the plant's root zone. These areas make up 25% of the earth's surface (Thorne and Peterson, 1954).

Salt stress inactivates photosynthetic and respiratory electron transport (Allakhverdiev *et al*., 1999). Increased ion stress has a physiological and molecular impact on cellular processes, resulting in cell death. Plants fight these negative impacts by activating a variety of biochemical and metabolic processes, including osmolyte accumulation, hormone modulation, ion homeostasis maintenance, and enhanced activity of ROS (Reactive Oxygen Species) scavengers (Golldack *et al*., 2011). The electrical conductivity (EC- dS/m) of the extract of a soil saturated paste determines whether the soil is saline (> 4.0), sodic (4.0), or saline-sodic (> 4.0).

Tomato (*Solanum lycopersicum*) is one of the most widely grown vegetables in the world and second-largest crop in India, covering 53.40 hectares. It's the most salt-sensitive of all the crops (Negrao *et al*., 2011). As a result, a variety of adaptations and mitigation methods are necessary to ensure sustainable agriculture. The need for salt-tolerant tomato varieties that can also withstand a variety of other stresses puts a lot of pressure on breeders to better understand the physiology and genetic regulation of salt tolerance (Khan *et al*., 2012). Breeding methods, on the other hand, are costly, time-consuming, and need a long period of time. Endophytes can help plants overcome abiotic stress and flourish.

Endophytes are bacteria or fungi that infiltrate plants without causing apparent disease signs (Wilson, 1995). The distribution of endophytes in the plant components (leaves, stalks, roots, flowers, and fruits) is numerous in nature, and endophytes colonise practically every portion of the plant (leaves, stems, roots, flowers, and fruits). Many of them have the ability to create vital biochemical components that aid in the defence of plants against illnesses and insect attacks (Abutana *et*

al., 2015). They give a host with advantages like as heat tolerance, salt resistance, and resistance to plant diseases or animal feeding (Redman and Rodriguez 2008).

Thus, endophytes that create a symbiotic relationship with plants give biotic and abiotic stress tolerance. In view of the foregoing, the current inquiry was carried out with the following goals in mind. Bacterial endophytes were screened for salinity stress tolerance *in vitro*. characterization of bacterial endophytes for plant growth promotion, 16SrRNA-based molecular characterisation of chosen endophytes and assessment of selected endophytes for salt stress resistance in tomato.

MATERIALS AND METHODS

Collection of bacterial endophytes

The School of Ecology and Conservation (SEC) Laboratory, Department of Crop Physiology, University of Agricultural Sciences (UAS), Gandhi Krishi Vignana Kendra (GKVK), Bengalore (560 065) maintains 58 bacterial endophytes isolated from plants growing in harsh environments of the north Himalayan cold deserts of Pangong, Changla, and Namika La regions. On nutrient agar media, these bacteria were revived.

Screening of bacterial endophytes for salt tolerance

In liquid cultures, the selected bacterial strains were evaluated for salt tolerance. Each bacterial strain was inoculated with five ml of Nutrient broth (NB) supplemented with NaCl at 0.5 M, 1.0 M, 1.5 M, 2.0 M, and 2.5 M concentrations. As a control, the standard NB was used. A spectrophotometer was used to measure the optical density (OD_{600}) of the cells (Viscardi *et al*., 2016).

Characterization of bacterial endophytes isolates for plant growth promotion

Phosphate solubilization. Pikovskaya's agar was used to assess the qualitative solubility of tricalcium phosphate in the isolated isolates. On the surface of Pikovskaya agar medium, selected bacterial isolates were spot inoculated. After 72 hours at 28ºC, the phosphate solubilizing activity was calculated. Solubilization of inorganic phosphates was indicated by the formation of a clear zone surrounding the colony (Gour, 1980).

Hydrogen cyanide production. Isolates were streaked in each nutrient agar plates amended with 4.4g/l glycine except one, which served as control. Whatman filter paper strip impregnated with an alkaline picric acid solution was placed in the upper lids of inoculated petri plates under aseptic conditions. The plates were incubated for 24 h at 28ºC. A change in color yellow to orange of Whatman filter paper impregnated with alkaline picric acid was observed indicating HCN production by isolates (Bakker and Schipper 1987).

Siderophore production. The 24h bacterial isolates were spot inoculated on nutrient agar amended with universal Chrome Azurol S (CAS) reagent after 24h at 30ºC clear orange color zone is appeared indicates the siderophore production (Schwyn and Neilands 1987).

Ammonia production. In peptone water, bacteria isolates were evaluated for ammonia production. Freshly developed cultures were injected in 5 ml peptone water in each tube separately and incubated at 28ºC for 48 to 72 hours. In each test tube, Nesselers reagent was added. The transformation of brown to yellow colour was a favourable indicator of ammonia production (Geetha *et al*., 2014).

Proline production. In nutrient broth with stress (2.5 M NaCl) and without stress, the amount of proline accumulated in bacterial isolates was calculated. Inoculated broths were cultured for 48 hours and then quantified using Mishra *et al.* (2011)'s technique. The extracted proline was then separated and transferred to new tubes, and the absorbance was measured at 520 nm using a spectrophotometer and the result was expressed as µg of proline ml^{-1} of bacterial culture (Ceylan *et al*., 2012)

Quantification of IAA, Gibberellic acid (GA), Abscisic acid (ABA) and Salicylic acid (SA) using HPLC

The cultures were inoculated in 20 mL nutrient broth containing with stress (2.5 M NaCl) and without stress flasks incubated for 7 days at 30 °C. They were centrifuged at 6000 rpm for 10 minutes after incubation, and the supernatant was collected and adjusted to pH 2.8 using 1 N HCl solution. In a 100 mL conical flask, the acidified supernatant was added to an equal amount of diethyl ether and incubated at 4 °C for 4 hours. The collected solvent phase (top layer) was allowed to evaporate. After membrane sterilisation, 2 to 3 mL of HPLC grade methanol was added to the evaporated samples, which were then kept at -20 °C for high performance liquid chromatography (Patten and Glick 2002).

Evaluation of selected bacterial endophytes for salinity stress tolerance in tomato

Standardization of NaCl concentration for tomato. The paper towel technique was used to determine the NaCl tolerance threshold of tomato seedlings. In sterile distilled water, several concentration of NaCl solution were made (25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 175 mM) (Muddarsu and Manivannan 2017). For each concentration, two germination papers were obtained, and 500 ml of NaCl solutions of various concentrations were applied to each germination paper, with the surplus solution being discarded. The germination paper was kept under control by soaking it in distilled water. The pre-germinated tomato seeds were then put on germination paper and incubated in the growth chamber at 30°C for 7 days. Seedling shoot and root lengths were measured after 7 days of incubation. Using statistical software IBM SPSS statistics 2.0 (https://www.ibm.com/in-en/analytics/spss-statistics-software), the lethal concentration (LC 50) of NaCl was estimated for the shoot, root, and seedling length.

In-vitro inoculation bacterial endophytes to salt sensitive tomato seedlings

Tomato seeds were surface sterilised and kept at room temperature for germination. The sprouting seeds were

then treated for 3 hours with a bacterial solution containing 8×107 CFU/ml population (Walitang *et al*., 2017). The corresponding control was treated with sterile distilled water as a treatment. The endophytes treated seedlings were subsequently exposed to salt stress by being placed on germination paper amended with 117mM NaCl (LC 50 value) and incubated for 7 days at room temperature. Each treatment had two replications, with each replication including ten seedlings. At 7-day intervals, root and shoot lengths were measured.

Molecular identification of bacterial endophytes using 16S rRNA gene sequence

The alkaline lysis approach was used to extract the whole genomic DNA of the bacterial endophyte (Sambrook and Fristsol Maniatis 1989). The NCBI (http://www.ncbi.nlm.nih.gov) primers for 16S rRNA sequence were custom synthesised by Sigma-Aldrich (Sigma, USA) and diluted appropriately for the PCR reactions (26 bp forward primer 5' GTTAGATCTTGGCTCAGGACGAACGC 3' and 24 bp reverse primer 5' GATCCAGCCGCACCTTCCGATACG 3'). PCR was carried out in a 20µl reaction mixture including 2.0µ l of 1X PCR Taq buffer with MgCl2 (1.5 mM), 2.0µl of 10 mM dNTP's mix (200 M), 0.5µl of forward and reverse primers, 0.3µ l of Taq DNA Polymerase (1U Genei Bengaluru), 1.0µl of Template DNA, and 13.7µl of sterile distilled water. An initial denaturation step at 96°C for 4 minutes was followed by 35 amplification cycles of 94°C for 1 minute, 60°C for 30 seconds, and 72°C for 1 minute, followed by a final extension step at 72°C for 10 minutes. Then the amplified DNA product was then electrophoresed on a 1% agarose gel and recorded with a gel documentation system. Chromgene Biotech Pvt. Ltd., Bengaluru, Karnataka, sequenced the amplified product using a gel elution kit (The Gene JETTM Gel Extraction Kit, Thermo Scientific). Using NCBI GenBank, the sequences were analysed for homology.

Evaluation of Endophytes treated tomato plants under greenhouse conditions

The pot experiment was carried out at the University of Agricultural Sciences, GKVK campus, Bengaluru-56065, at the Department of Agricultural Microbiology. Prior to the experiment, the physico-chemical parameters of the soil were examined. (Soil pH 7.32, Electrical conductivity 0.65 dS/m, Available K_2O 39 kg /ha, Exchangeable Na 0.35 meq/lt) The amount of salt needed to keep the EC at 4 dS/m was estimated ($CaCl_2.2H_2O$ 1225 mg/lt NaCl 384 mg/lt $MgSO_4.7H_2O$ 319 mg/lt $MgCl_4.6H_2O$ 1549 mg/lt) and salinity stress was induced in the pot experiment.

Plastic pots with a capacity of 5 kg were filled with a 2:1:1 mixture of soil, sand, and farm yard manure (FYM) respectively. Arka saurabh tomato seedlings were treated with bacterial suspension before they germinated. These seeds were placed in pro-trays with autoclaved coir pith and let to grow for 25 days. The seedlings were then gently moved to the containers. The plants were stressed with salt using the Karnal technique (Tomer and Minhas 2005). By introducing a solution of dissolved salts in sterile distilled water to the individual pots, a salt stress of 4 dS/m was maintained. Two seedlings in each pot were maintained. The growth parameters like plant height, number of leaves and number of branches, and Physiological parameters like chlorophyll content, proline content, Relative water content, electrolyte leakage as influenced by endophytes under salt stress condition were recorded at 45 days after transplantation.

Re-isolation and confirmation of inoculated bacterial endophytes

Individual tomato plant sections (root, stem, and leaf) were collected and utilised for reisolation of endophytes on nutrient agar media. The re-isolated bacteria were identified based on colony shape, and the existence of the same injected isolate was confirmed using the 16S rRNA gene sequence.

Statistical analysis. The data was analysed using the WASP: 2.0 (Web Agri Stat Package 2) statistical programmes (www.icargoa.res.in/wasp2/index.php) and the Duncan Multiple Range Test was used to differentiate the means (DMRT).

RESULT AND DISCUSSION

Screening of bacterial endophytes for salt tolerance

Fifty-eight bacteria isolated from different regions of Himalayan cold desert were screened for salt tolerance using different concentrations of NaCl. Of which 9 bacterial isolates (PBE 4, PBE 6, PBE 8, PBE 15, CBE 12, NBE 7, NBE 20, NBE 21, NBE 23) showed tolerance at 2.0 M NaCl. This includes 4 isolates (PBE 4, PBE 6, PBE 8, PBE 8, PBE 15) from Pangong region, 1 isolates (CBE 12) from Changla (Table 2) and 4 isolate (NBE 7, NBE 20, NBE 21, NBE 23) from Namkila La (Tasmiya and Earanna *et al.*, 2023). Other 49 isolates grown up to 1.5 M NaCl and did not show any growth at 2.0 M indicating that they are susceptible to increased concentration. This may be due to increased ionic influxes, oxidant imbalances, cell division impairment, membrane degeneration and decreased activity of superoxide dismutase (Munns and Tester, 2008)

Ramadoss *et al*. (2013) screened eighty-four halotolerant bacterial strains at different NaCl concentrations and 16S r RNA gene sequence confirmed that, isolates belonged to *Bacillus* and *Halobacillus*. Among all, five isolates (SL3, SL32, SL35, J8W, and PU62) showed growth at 20 percent NaCl but all grew well in 5 percent NaCl.

Characterization of bacterial endophytes isolates for plant growth promotion

Bacterial endophytes were characterized for plant growth promotion like phosphate solubilization which is a primary elements needed for plant growth and involved in normal development and maturity. Siderophore and HCN production are one of the mechanisms of biocontrol activity and ammonia production are useful for the plant growth directly or indirectly. Out of 9 salt tolerant bacterial endophytes, 7

endophytes showed phosphate solubilization, 5 and 4 endophytes were able to produce HCN and siderophore productions, and ammonia was able to produce by 7 endophytes (Table 1). Result obtained are similar to Maheshwari *et al*. (2019) reported that endophytes isolated from nodules and roots of *Cicer arietinum* and *Pisum sativum* plants. Out of 84 isolates, 14 were found to produce siderophore and quantitative analysis was conducted. Ten best siderophore producers (above 65 % siderophore units) were characterized for the type of siderophore produced. Most of them produced hydroxamate and carboxylate type of siderophores. All of them were producing ammonia and indole-3-acetic acid (IAA). Isolate CPFR10 was found to be positive for all the PGP traits *viz*., ammonia, organic acid, HCN and IAA production.

Proline accumulation

Osmolytes concentration increases in the plants during abiotic stress and are mainly involved in monitoring homeostasis of cellular contents in them. In the case of proline, maximum production was observed by the isolate NBE 23 (38.28 µg mL^{-1}) followed by NBE 20 (33.40 µg mL^{-1}) and NBE 7 (30.77 µg mL^{-1}) without stress. Under the salt stress 2.0 M NaCl) maximum production of proline was found in the isolate NBE 23 (28.20 µg mL^{-1}) followed by NBE 20 (25.66 µg mL^{-1}), PBE 7 (22.26 µg mL^{-1}) (Table 2). Results obtained are similar to the results of Danish *et al*. (2020), where under the severe salt stress the inoculation of *Pseudomonas aeruginosa* led to the proline accumulation of 15.97 µmol g^{-1} of fresh weight (F.W.) compared to the inoculation with Enterobacter cloacae (13.73 µmol g^{-1} of F.W.), *Achromobacter xylosoxidans* (12.95 µmol g^{-1} of F.W.) and *Leclercia adecarboxylata* (15.73 µmol g^{-1} of F.W.) in maize plants.

Quantification of IAA, Gibberellic acid (GA), Abscisic acid (ABA) and Salicylic acid (SA) using HPLC

Phytohormones play a vital role in the development of plants. The quantification of gibberellic acid, abscisic acid and salicylic acid production by the selected 9 endophytic bacterial isolates through high performance liquid chromatography (HPLC) is presented in the Table 3.

Indole-3-acetic acid being the most common type of auxin, it regulates various aspects of plant development and growth. Under non-stressed condition and without supplementation of L-tryptophan the maximum quantity of IAA was produced by endophytic bacterial isolate NBE 7 (21.89 µg mL^{-1}) followed by NBE 23 (16.77 µg mL^{-1}) and PBE 8 (15.09 µg mL^{-1}). While under the amendment of L-tryptophan the maximum IAA production was noticed by isolate NBE 23 (143.60 µg mL^{-1}) followed by NBE 20 (115.80 µg mL^{-1}) and NBE 7 (95.22 µg mL^{-1}) isolate. Goswami *et al*. (2014) reported bacteria *Kocuria turfanensis* 2M4, that was found to be dependent on L-tryptophan for producing IAA and could produce 38 µg mL-1 of IAA in the presence of 600 µg mL^{-1} of tryptophan.

Gibberellic acid stimulates cell division and elongation in plants. Moumita *et al*. (2019) stated the stimulation of glyoxalase I and II, that reduced the methylglyoxal concentration and thereby the plants could withstand against the salt stress in wheat seedlings. In the present study under non stress condition gibberellic acid was found to have produced in a higher quantity by the endophytic bacterial isolate NBE 23 (55.95 µg mL^{-1}) followed by NBE 20 (43.30 µg mL^{-1}) isolate. While under stress condition GA production noticed by isolates NBE 23 (76.80 µg mL^{-1}) followed by NBE 20 (63.53 µg mL^{-1}. The results obtained are similar with the production of gibberellic acid (0.108 mg mL^{-1}) by *Bacillus siamensis* BE 76 isolated from the banana plant (*Musa* spp.) as reported by Amawade and Pathade (2015).

Abscisic acid regulates salt stress by mediating osmotic stress tolerance that aid in stomatal closure, thereby reducing the transpirational water loss. The maximum quantity of ABA was produced by the endophytic bacterial isolate under non stress condition, NBE 23 (3.33 µg mL^{-1}) followed by NBE 20 (2.56 µg mL^{-1}) isolate. And stress condition, NBE 23 (5.68 µg mL-1) and NBE 20 (5.34 µg mL^{-1}). The results obtained are similar to results reported by Shahzad et al. (2017), where the production of varying concentration of ABA (0.32 ± 0.015 to 0.14 ± 0.030 ng mL^{-1}) under normal and saline condition by the bacterial endophyte *Bacillus amyloliquefaciens* RWL^{-1} was noticed.

Salicylic acid provides systemically acquired resistance in plants against diseases. La *et al.* (2019) noticed the amelioration of negative effects of decreased osmotic potential, chlorophyll and carotenoid content by the pretreatment of salicylic acid in *Brassica rapa*. The higher quantity of salicylic acid was produced by the isolate NBE 7 (30.9 µg mL^{-1}) followed by NBE 23 (25.9 µg mL^{-1}). The results obtained are in parallel with the results of Gupta (2020), in which he reported the production of salicylic acid (27.3 mg L^{-1}) by the rhizospheric soil bacteria *Rubrivivax gelatinosus*.

Evaluation of selected bacterial endophytes for salinity stress tolerance in tomato

Standardization of NaCl concentration for tomato. Tomato seedlings were screened for salt stress using NaCl at 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM, 175 mM concentrations. The length of root and shoot was decreased with increased NaCl concentrations. The untreated tomato seedlings showed highest seedling length whereas lowest seedling length was observed with increased concentrations. LC_{50} value of NaCl concentration was found to be 117 mM (Plate 1, Fig. 1). The results are in agreement with Jogawat *et al*. (2013) who reported that *Piriformospora indica* in association with rice seedlings screened for drought stress using NaCl showed decreased root and shoot length with increased concentrations.

Table 1: Characterization of selected bacterial endophytes for Plant growth promoting traits.

Sr. No.	Bacterial endophytes	Plant growth promoting traits			
		Phosphate solubilisation	HCN	Siderophore	Ammonia production
1	PBE 4	-	+	-	-
2	PBE 6	+	-	-	+
3	PBE 8	-	-	+	-
4	CBE 12	+	+	+	+
5	PBE 15	+	-	-	+
6	NBE 7	-	-	-	+
7	NBE 20	+	+	+	+
8	NBE 21	+	+	-	+
9	NBE 23	+	+	+	+

Note: *Ammonia production: (+) yellow colour formation (-) absence of yellow colour
*HCN production: (+) reddish brown colour (-) no reddish brown colour
*Siderophore production: (+) organe zone (-) absence of zone.
* Phosphate solubilisation: (+) Zone around the colony (-) absence of zone

Table 2: Proline accumulation of selected bacterial endophytes under without salt stress and stressed induced conditions.

Bacterial endophytes	Proline accumulation ((µg ml^{-1})	
	Without NaCl	With 2.0 M NaCl
PBE 4	5.84^{i}	10.96^{g}
PBE 6	12.29^{g}	7.84^{i}
PBE 8	28.39^{d}	22.26^{c}
CBE 12	10.73^{h}	20.98^{e}
PBE 15	14.22^{f}	9.67^{h}
NBE 7	30.77^{c}	21.78^{d}
NBE 20	33.40^{b}	25.66^{b}
NBE 21	22.39^{e}	20.92^{f}
NBE 23	38.28^{a}	28.20^{a}
CD (P<0.05)	0.01	0.01

Note: Means with same superscript in a column do not differ significantly as per Duncan Multiple Range Test (DMRT)

Table 3: IAA, Gibberellic acid, abscisic acid and salicylic acid production by endophytic bacterial isolates.

Bacterial endophytes	Indole-3-acetic acid (µg mL^{-1})				Gibberellic acid (µg mL^{-1})		Abscisic acid (µg mL^{-1})		Salicylic acid (µg mL^{-1})	
	Without stress		With stress (2.0M NaCl)							
	Tryptophan (-)	Tryptophan (+)	Tryptophan (-)	Tryptophan (+)	Without stress	With stress	Without stress	With stress	Without stress	With stress
PBE 4	4.57^{h}	26.34^{h}	1.21^{i}	5.93^{i}	19.87^{g}	38.7^{f}	0.16^{h}	0.23^{i}	12.33^{h}	26.50^{h}
PBE 6	9.97^{e}	60.22^{f}	3.11^{g}	14.99^{g}	21.34^{f}	30.92^{g}	0.35^{g}	0.44^{h}	10.93^{i}	22.16^{i}
PBE 8	15.09^{c}	98.90^{c}	5.55^{d}	36.38^{c}	10.90^{h}	14.73^{h}	0.88^{d}	0.99^{d}	23.30^{d}	33.77^{e}
CBE 12	7.76^{f}	89.80^{e}	4.44^{e}	19.98^{e}	30.90^{d}	57.05^{d}	0.68^{e}	0.80^{e}	20.90^{f}	27.90^{g}
PBE 15	4.56^{i}	11.34^{i}	3.01^{h}	8.06^{h}	21.30^{f}	44.50^{e}	0.45^{f}	0.64^{g}	22.30^{e}	30.20^{f}
NBE 7	21.89^{a}	95.22^{d}	6.79^{c}	27.62^{d}	23.40^{e}	38.70^{f}	0.99^{c}	1.30^{c}	30.90^{a}	37.77^{c}
NBE 20	14.45^{d}	115.89^{b}	7.06^{b}	41.21^{b}	43.30^{b}	63.53^{b}	2.56^{b}	5.34^{b}	24.30^{c}	39.90^{b}
NBE 21	6.78^{g}	52.89^{g}	4.25^{f}	15.66^{f}	33.20^{c}	60.83^{c}	0.35^{g}	0.70^{f}	15.60^{g}	34.90^{d}
NBE 23	16.77^{b}	143.60^{a}	8.62^{a}	46.70^{a}	55.95^{a}	76.80^{a}	3.33^{a}	5.68^{a}	25.90^{b}	40.70^{a}
CD(P<0.05)	0.01	0.04	0.01	0.01	0.03	0.01	0.01	0.01	0.08	0.04

Note: Means with same superscript in a column do not differ significantly as per Duncan Multiple Range Test (DMRT)

In-vitro inoculation bacterial endophytes to salt sensitive tomato

Out of nine bacterial endophytes, two endophytes (NBE 20 and NBE 23) inoculated seedlings recorded maximum seedling length. Both isolates showed significantly highest length which was selected for characterization (Table 4). NaCl induced 117mM salt stress seedlings showed less seedling growth compared to control (without NaCl) seedlings. Nautiyal *et al.* (2013) reported that *Bacillus amyloliquefaciens* SN13 aided in plant growth by increasing shoot and root length at 200mM NaCl stress from 34.76 and 7.76 cm in control plants to 52.43 and 12.10 cm at the same stress level in inoculated plant.

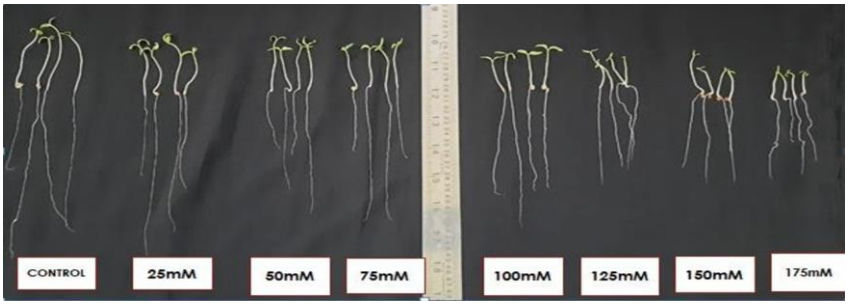

Plate 1: Effect of different concentrations of NaCl on seedling growth of tomato at 7 days after germination.

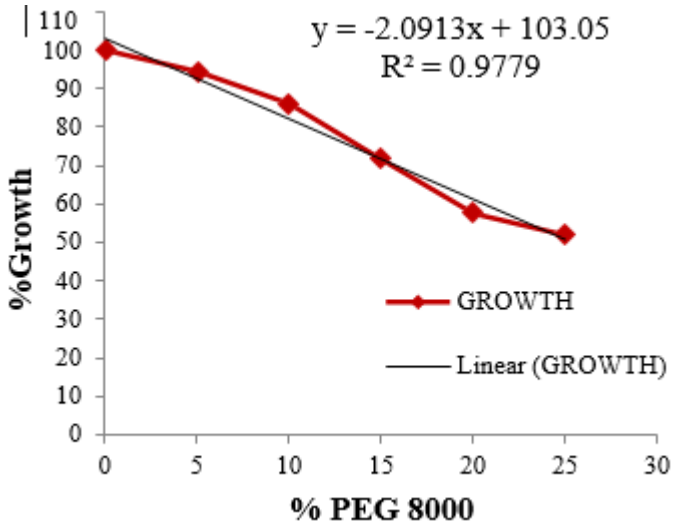

Fig. 1. LC50 value of PEG-8000 concentration for tomato seedlings.

In-vitro inoculation bacterial endophytes to salt sensitive tomato

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Molecular identification of bacterial endophytes using 16S rRNA gene sequence.

In the present study two effective endophytic bacterial isolates were identified through 16S rRNA gene sequence. The genes encoding for 16S rRNA in prokaryotes are most widely used in molecular taxonomy, because they are 1) sufficiently conserved, 2) functionally constant, 3) universally distributed and 4) have adequate length to provide a view of evolution encompassing all living microorganisms (Madigan *et al.*, 2009). Therefore, in the present study the two endophytic bacterial isolates were identified based on the homology of their sequence. The 16S rRNA partial gene sequence of the NBE 20 with 1395 bp (Fig. 2) showed 99.42 % homology with *Enterobacter cloacae* available in the NCBI data base and NBE 23 with 1258 bp (Fig. 3) showed 99.57% homology with *Enterobacter asburiae* available in the NCBI data base.

Thus, the bacteria were identified as *Enterobacter asburiae* and Enterobacter *cloacae*.

Similarly, Figueiredo *et al.* (2009) identified the endophytic bacteria associated with Brazilian sweet corn using 16S rRNA gene sequence and revealed that *Bacillus subitilis* and *B. pumilis* as the most frequently occurring species (15 and 12 isolates, respectively) followed by *B. licheniformes* (7 isolates), *B. cereus* (5 isolates) and *B. amiloliquefascens* (3 isolates). Krishnan *et al*. (2012) reported that the seeds and the endocarp of papaya fruits harbour Acinetobacter and Enterobacter species through 16S rRNA gene sequence.

Table 4: Effect of inoculation of bacterial endophytes on seedling length of tomato with and without salinity stress (117 mM NaCl) after 7 days of germination.

Endophytes	Without Salt Stress		With Salt Stress at 117 mMNaCl Concentration	
	Root Length(cm)	Shoot Length(cm)	Root Length (cm)	Shoot Length (cm)
Control	11.66^{c}	9.60^{c}	7.50^{c}	5.20^{cd}
PBE 4	8.53^{f}	6.10^{e}	4.80^{g}	4.00^{f}
PBE 6	9.46^{e}	6.10^{e}	6.30^{e}	4.00^{f}
PBE 8	6.53^{g}	5.70^{f}	5.40^{f}	4.90^{e}
CBE 12	9.16^{e}	5.70^{f}	7.60^{c}	5.00^{d}
PBE 15	9.50^{e}	6.60^{e}	5.40^{f}	2.80^{g}
NBE 7	9.30^{e}	5.00^{g}	5.50^{f}	5.00^{d}
NBE 20	12.20^{b}	10.10^{b}	8.00^{b}	6.20^{a}
NBE 21	11.20^{d}	7.60^{d}	7.00^{d}	5.70^{b}
NBE 23	13.20^{a}	11.00^{a}	9.40^{a}	5.50^{bc}
CD (P<0.05)	0.38	0.34	0.35	0.21

Note: Means with the same superscript in a column do not differ significantly as per DuncanMultiple Range Test (DMRT)

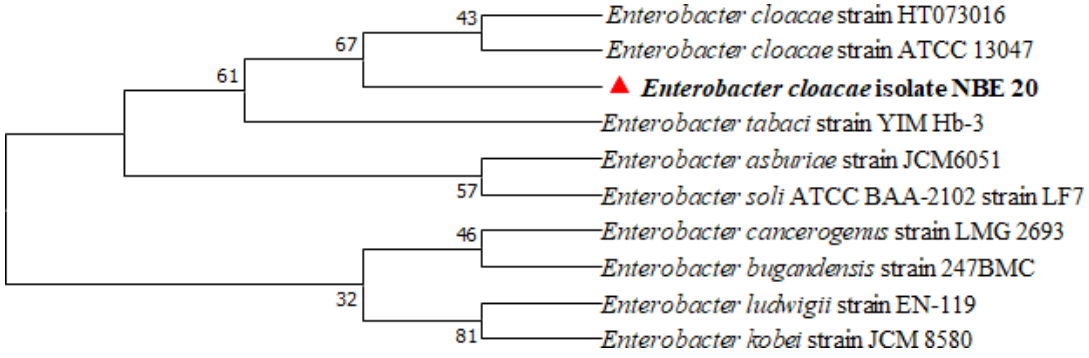


Fig. 2. Phylogenetic tree of *Enterobacter cloacae* isolate NBE 20.

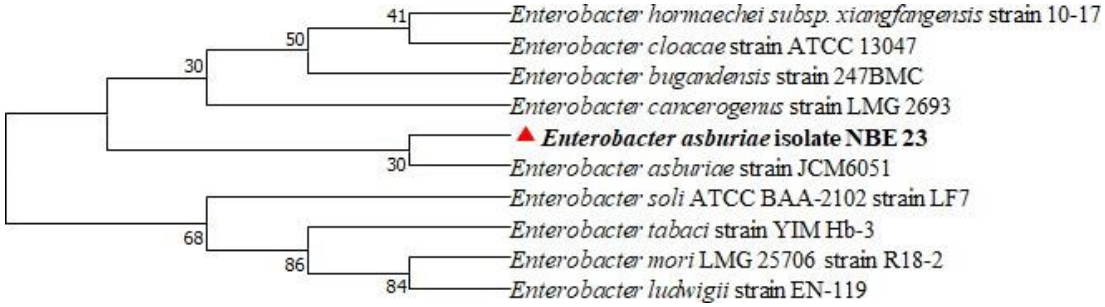


Fig. 3. Phylogenetic tree of *Enterobacter asburiae* isolate NBE 23.

Evaluation of Endophytes treated tomato plants under greenhouse conditions

The two endophytes *Enterobacter asburiae* NBE 23 and *Enterobacter cloacae* NBE 20 performed better under in-vitro condition for salt stress were selected for evaluating them under greenhouse conditions. These bacteria were treated to pre-germinated tomato seeds. The Growth and physiological parameters recorded for plant height, number of leaves and number of branches, chlorophyll content, proline content, RWC and electrolyte leakage on 30 DAT, 60 DAT, 90 DAT and 140 DAT

Plants inoculated with *E. cloacae* and *E. asburiae* showed significantly increased plant height. And average number of leaves per plant was significantly increased in *E. asburiae* inoculated plants which was followed by *E. cloacae* under both normal as well as salt stress as compared to un-inoculated plants. The number of branches also increased in *E. asburiae* inoculated plants compared to un-inoculated plants (Fig 4; Plate 2). Increased growth parameters in endophyte inoculated plants under salt stress could be due to production of phytohormones (Khan *et al.*, 2012). *E. cloacae* and *E. asburiae* inoculation significantly increased the number of fruits and fruit yield compared to un-inoculated plants (Table 5).

Carotenoid and RWC were significantly enhanced in *E. asburiae* treated plants compared to uninoculated plants (Table 6). The proline content did not differ between treatments under normal conditions but under salt stress, *E. asburiae* inoculated plants showed significantly increased proline content. The *E. asburiae* treated plants also showed less electrolyte leakage compared to un-inoculated plants. This was attributed to increased photosynthetic pigments and accumulation of osmolytes by endophyte mediation (Yandigeri *et al.*, 2012). The catalase and peroxidases play a vital role in scavenging ROS (Abogadallah, 2011). Treatment of bacterial endophyte to salt sensitive tomato seedlings showed significant increase in activity of these enzymes (Fig 5). The symbiotic association of bacterial endophytes with host plants is attributed to upregulation of the activity of catalase and peroxidases to scavenge ROS under salt stress (Choudhury *et al.*, 2017). Amjad *et al.* (2017) reported that *Bacillus pumilus* and *Exiguobacterium* sp. inoculated to tomato plants under salinity stress increased plgant growth, biomass, and photosynthetic rate.

Table 5: Effect of bacterial endophytes on yield parameters of tomato under saltstress (4 dS/m).

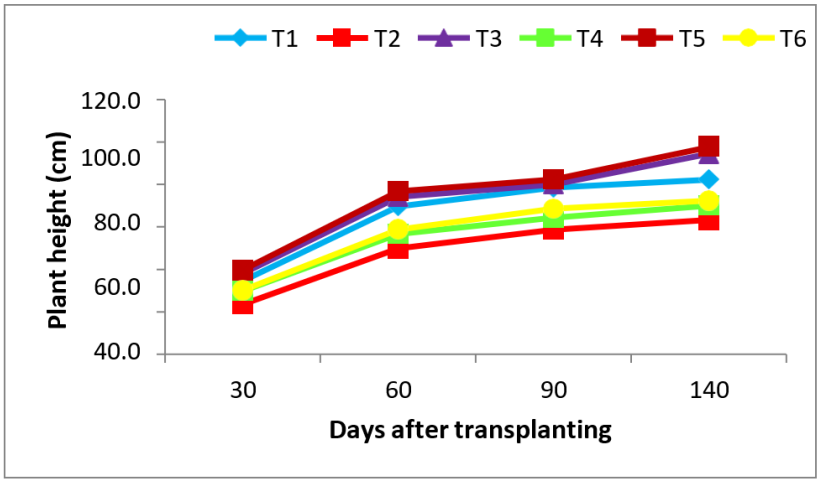
Treatments	No. of fruits/plant	Fruits yield/plant(g)
T_1= Control	21.94^{c}	735.35^{c}
T_2= Salt stress (4 dS/m)	12.31^{f}	369.45^{f}
T_3= *E. cloacae*	24.60^{b}	861.37^{b}
T_4= Salt stress (4 dS/m) + *E. cloacae*	17.60^{e}	616.35^{e}
T_5= *E. asburiae*	25.36^{a}	896.40^{a}
T_6= Salt stress (4 dS/m) + *E. asburiae*	19.60^{d}	686.33^{d}
CD (P<0.05)	0.61	17.53

Note: Means with the same superscript in a column do not differ significantly as perDuncan Multiple Range Test (DMRT)

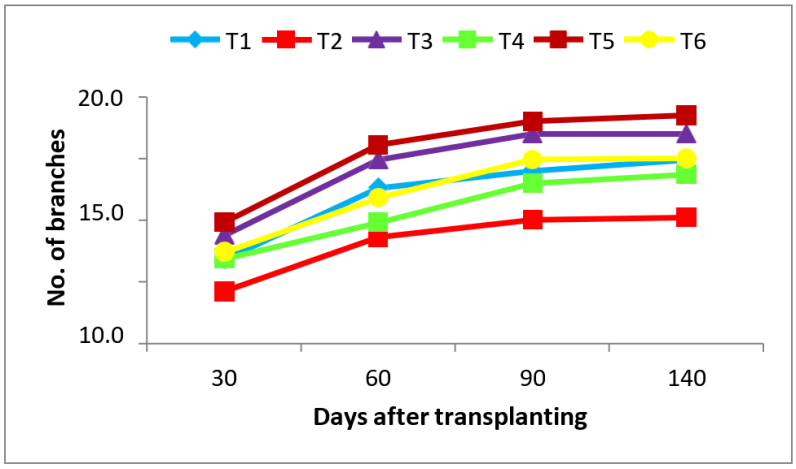
Table 6: Effect of bacterial endophytes on physiological parameters of tomatounder salinity stress (4 dS/m).

Treatments	Chl a(mg/gFW)	Chl b (mg/g FW)	Total chl(mg/g FW)	Carotenoid content (mg/g FW)	RWC(%)	Proline content (µmol/g FW)	Electrolyte leakage (%)
T_1= Control	0.74^{a}	0.32^{ab}	0.92^{a}	0.31^{b}	87.90^{d}	6.36^{d}	35.45^{b}
T_2= Salt stress (4dS/m)	0.55^{c}	0.25^{c}	0.72^{c}	0.27^{c}	71.50^{e}	10.25^{c}	55.60^{a}
T_3= *E. cloacae*	0.74^{a}	0.34^{b}	0.92^{a}	0.34^{a}	89.60^{b}	6.35^{d}	32.21^{d}
T_4= Salt stress (4 dS/m) + *E. cloacae*	0.63^{b}	0.35^{b}	0.74^{c}	0.30^{b}	87.60^{d}	11.84^{b}	35.50^{b}
T_5= *E. asburiae*	0.75^{a}	0.40^{a}	0.95^{a}	0.35^{a}	90.80^{a}	6.35^{d}	30.15^{e}
T_6= Salt stress (4 dS/m) + *E. asburiae*	0.65^{b}	0.35^{b}	0.84^{b}	0.31^{b}	88.80^{c}	11.93^{a}	34.80^{c}
CD (P<0.05)	0.04	0.04	0.03	0.02	0.41	0.05	0.30

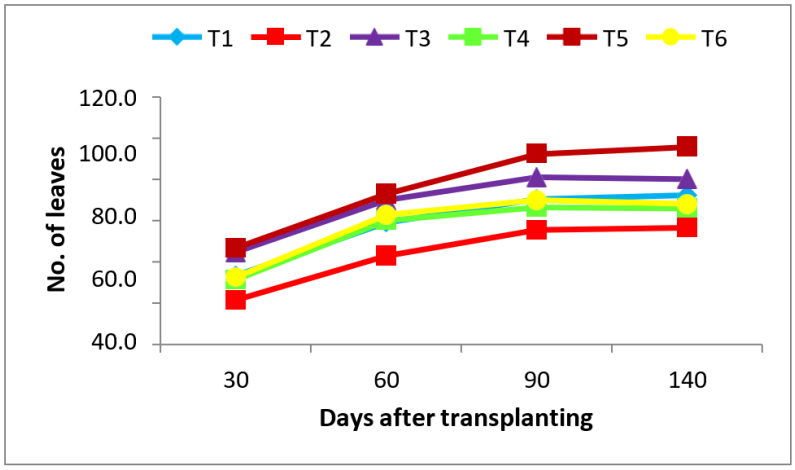
Note: Means with the same superscript in a column do not differ significantly as perDuncan Multiple Range Test (DMRT)



(a)



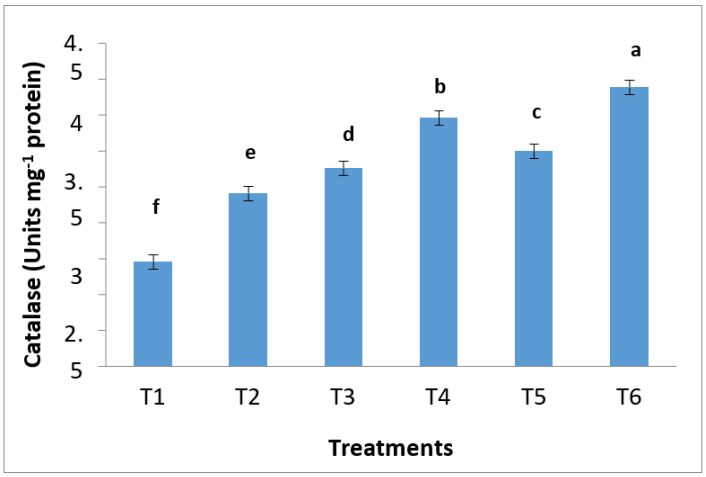
(b)



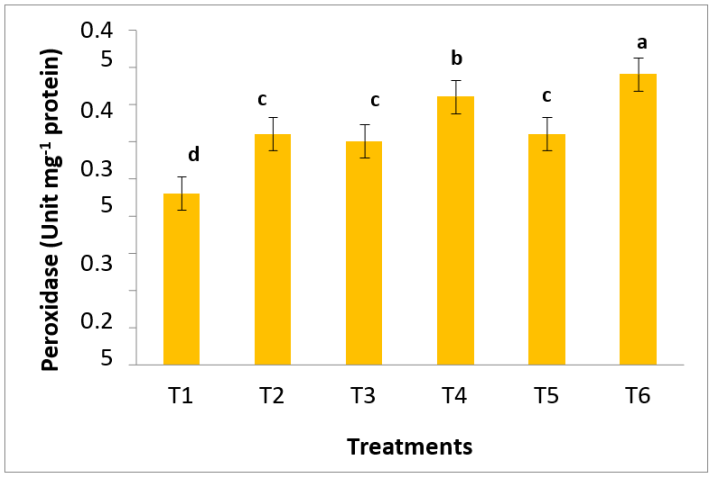
(c)

Note: T1=Control, T2= Salt stress (4dS/m), T3=*E. cloacae*, T4=Salt stress (4 dS/m) + *E. cloacae*,T5=*E. asburiae*, T6=Salt stress (4 dS/m) + *E. asbur*

Fig 4. Effect of bacterial endophytes on (a) Plant height (b) No. of branches (c) No.of leaves of tomato under salt stress (4 dS/m).



(a)



(b)

Note: T1=Control, T2= Salt stress (4dS/m), T3=*E. cloacae*, T4=Salt stress (4 dS/m)+ *E. cloacae*,T5=*E.asburiae*, T6=Salt stress (4 dS/m)+ *E. asburiae*

Fig. 5. Effect of bacterial endophytes on biochemical parameters (a) Catalase and(b)Peroxidase of tomato under salt stress (4 dS/m).

Control *E. cloacae* Salt stress (4 dS/m) Salt stress (4 dS/m) + *E. cloacae*

Plate 2: Effect of bacterial endophytes *E. cloacae* and *E. asburiae* on growth oftomato under normal and salt stress (4dS/m) condition at 60 DAT.

CONCLUSION

Therefore, presence of inoculated bacterial endophytes within inoculated plants were confirmed through re-isolation. Hence, this study suggests the inoculation of endophytes is necessary to confer salinity tolerance. Furthermore, it can be conferred that out of two bacterial endophytes, *Enterobacter asburiae* collected from harsh condition presented superior salinity tolerance to tomato plant under salt stress and these findings can be explored in other agricultural crops.

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