Inter-Genera Colonization of *Ocimum tenuiflorum* Endophytes in Tomato and Their Complementary Effects on Na⁺/K⁺ Balance, Oxidative Stress Regulation, and Root Architecture Under Elevated Soil Salinity

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Endophytic bacilli of ethano-botanical plant *Ocimum tenuiflorum* were screened for salt stress-alleviating traits in tomato. Four promising *O. tenuiflorum* endophytes (*Bacillus safensis* BTL5, *Bacillus haynesii* GTR8, *Bacillus paralicheniformis* GTR11, and *Bacillus altitudinis* GTS16) were used in this study. Confocal scanning laser microscopic studies revealed the inter-genera colonization of *O. tenuiflorum* endophytes in tomato plants, giving insights for widening the applicability of potential endophytes to other crops. Furthermore, in a pot trial under 150 mM NaCl concentration, the inoculated endophytes contributed in reducing salt toxicity and improving recovery from salt-induced oxidative stress by different mechanisms. Reduction in reactive oxygen species (ROS) (sub-cellular H₂O₂ and superoxide) accumulation was observed besides lowering programmed cell death and increasing chlorophyll content. Endophyte inoculation supplemented the plant antioxidant enzyme system via the modulation of enzymatic antioxidants, viz., peroxidase, ascorbate peroxidase, superoxide dismutase, and catalase, apart from increasing proline and total phenolics. Antioxidants like proline have dual roles of antioxidants and osmoregulation, which might also have contributed to improved water relation under elevated salinity. Root architecture, viz., root length, projection area, surface area, average diameter, tips, forks, crossings, and the number of links, was improved upon inoculation, indicating healthy root growth and enhanced nutrient flow and water homeostasis. Regulation of Na⁺/K⁺ balance and water homeostasis in the plants were also evident from the modulation in the expression of abiotic stress-responsive genes, viz., *LKT*1, *NHX*1, *SOS*1, *LePIP*2, *SIERF*16, and *SiWRKY*39. Shoot tissues staining with light-excitable Na⁺ indicator Sodium Green™ Tetra (tetramethylammonium) salt showed low sodium transport and accumulation in endophyte-inoculated plants. All four endophytes exhibited different mechanisms for stress alleviation and indicated complementary effects on plant growth. Furthermore,
INTRODUCTION

Increasing soil salinity has become a major bottleneck in realizing the production potential of crops. A recent report indicated that 11.73 million km² soil area is salt affected, in which Asia, Africa, and Australia are worst affected (Hassani et al., 2020). In saline soils, high osmotic stress and sodium toxicity adversely affect nutrient uptake, mobilization, osmotic balance, membrane integrity, oxidative stress, rate of photosynthesis, and overall growth, thereby seriously limiting crop productivity and limiting sustainable land use (Czarnocka and Karpinski, 2018; Arif et al., 2020; Pan et al., 2020). Breeding-based approaches for the alleviation of salt stress have several bottlenecks (Uga, 2021). The amelioration strategies for reducing soil salinity are cost intensive (Qadir et al., 2001), and the genetically modified crops have a long way to go to realize their widespread usage for salinity tolerance in major crops (Kumar et al., 2020). However, microbial agents, specifically the bacterial endophytes, were found to have salinity tolerance, thus improving capability (Abdelshafy Mohamad et al., 2020; Kushwaha et al., 2020).

Microbes have different mechanisms for abiotic stress management, such as maintaining Na⁺/K⁺ balance (Lee et al., 2016), spatial exclusion of ions via ion transporters (Zhang et al., 2008), restricting Na⁺ uptake in roots (Ashraf et al., 2004; Dodd and Pérez-Alfocea, 2012), osmotic balance (López-Gómez et al., 2019; Nadeem et al., 2020), membrane stability (Bano and Fatima, 2009; Singh and Jha, 2016; Yasin et al., 2018; Chaunhan et al., 2019), improved nutrient uptake (Lee et al., 2015; Albdaiwi et al., 2019; Abdelshafy Mohamad et al., 2020), production of enzymatic (Halo et al., 2015; Fukami et al., 2018) and non-enzymatic antioxidants (Fazal and Bano, 2016; Singh and Jha, 2017), etc. Since all these effects are generally not found in a single endophyte, exploring the endophytes having different complementary mechanisms could pave the way for the inoculants having multiple mechanisms and stress-alleviating potential.

Endophytes exhibit enormous diversity even at the cultivar level (Sahu et al., 2020b); therefore, exploring different plants and their varieties for potential salt stress alleviation is desirable. Holy basil (Ocimum tenuiflorum), also called “Tulsi” locally, is a medicinal shrub having a religious value (Shekhawat and Shah, 2013; Singh and Chaudhuri, 2018; Taufiq and Darah, 2018). The biological control potential of bacterial endophytes from O. tenuiflorum has been previously worked out (Chowdhary and Kaushik, 2015; Sahu et al., 2020a) along with their potential to alleviate oxidative stress. However, there are only scanty reports available for the application of O. tenuiflorum endophytes in abiotic stress alleviation. Since there is a close cross-talk between signaling for biotic and abiotic stresses in plants (Fujita et al., 2006) and oxidative stress arises from both biotic and abiotic stresses, therefore, the present investigation was carried out for exploring O. tenuiflorum endophytes for alleviation of salt stress in tomato.

On the other hand, the horizontal transmission of the endophytes from the surrounding to the system of the plant is crucial for the success of applied endophytic inoculants (Verma et al., 2021b). Magnificent studies have been done on the potential of endophytic inoculants (Egamberdieva et al., 2016; Abdelshafy Mohamad et al., 2020); nevertheless, the adaptability on different crop hosts was least worked out. Endophytic microbes can confer salt tolerance ability to their host plants (Khan et al., 2020). Whether the same kind of protection could be conferred to the plants other than the host needs to be explored for wider adaptability and further commercialization of endophytic strains. The use of endophytes, which are able to successfully colonize the non-native host and improve the plant performance, could be of greater significance for the utility of commercial microbial inoculants on a wide range of crops. Keeping all these considerations, the present study was conducted to explore O. tenuiflorum bacterial endophytes for inter-genera colonization in tomato, their effect, and complementation on salt stress-mitigating strategies of plant.

MATERIALS AND METHODS

Source of Bacterial Endophytes Used in the Study

O. tenuiflorum bacterial endophytes from our previous study (Sahu et al., 2020a), namely, Bacillus safensis BTL5, Bacillus haynesii GTR8, Bacillus paralicheniformis GTR11, and Bacillus altitudinis GTS16 (National Agriculturally Important Culture Collection, ICAR-NBAIM, India accession numbers NAICCC-B-02221–NAICCC-B-02224, respectively), were used for the in vivo studies. Two in vivo experiments were conducted: the first was to confirm inter-genera endophytic colonization in tomato plants in a seedling tray experiment and the second was to evaluate the salt stress alleviation potential of these endophytes in pot trial.

Inter-Genera Colonization Study

Growing Plants in Seedling Trays

Tomato seeds (variety, Pusa Ruby) were surface sterilized by washing in sterile water, followed by dipping in 70% ethanol for 30 s and washing with sterile distilled water. Afterward, the seeds were dipped in 2% sodium hypochlorite (1 min) followed by three sterile distilled water washes, followed by 10-min dip in
2% sodium thiosulfate. The surface-sterilized seeds were treated with the respective bacterial endophyte inoculum (24 h old, 0.2 OD suspension). Six treatments were made, each with 10 replications. The treatment details were as follows: T1 = absolute control, T2 = saline control (150 mM NaCl), T3 = 150 mM NaCl + Bacillus safensis BTL5, T4 = 150 mM NaCl + Bacillus haynesi GTR8, T5 = 150 mM NaCl + Bacillus paralicheniformis GTR11, and T6 = 150 mM NaCl + Bacillus altitudinis GTS16. The seeds were sown in 98-well seedling trays containing sterile sand: soil mixture (1:3). Nutrients were provided through sterile Hoagland solution (1X). The tomato plants were allowed to grow for a period of 3 weeks before harvesting the roots for the colonization studies.

Visualisation of Endophytic Colonization

The roots were gently washed thrice in sterile distilled water and stained with LIVE/DEAD™ BacLight™ bacterial viability stain (Invitrogen, United States) containing SYTO9 and propidium iodide (Stiefel et al., 2015). The roots were stained with 35-µM concentration of SYTO9 and 400 µM of propidium iodide for a period of 30 min in the dark. The excess fluorescent dyes were washed using phosphate buffer saline. The stained roots were mounted on glass slides and visualized under a confocal scanning laser microscope (CSLM; Nikon, Japan) using 488 nm, 543 nm, and TD channels. The images were processed using NIS element 3.2.3 program (Nikon, Japan).

In vivo Evaluation of Salinity Tolerance in Tomato

Soil Preparation and Nursery

Pots were filled with 5 kg of autoclaved soil. The heterogeneity within the pots was nullified by row and column randomization. The nutrients were mixed in a ratio of 100:50:50 kg NPK per hectare using urea, diammonium phosphate, and muriate of potash. The soil in these pots was brought to natural compaction by three cycles of wetting and drying. Salinity was maintained by 150 mM NaCl, and the pots were irrigated till 80% of the field capacity. Tomato (variety, Pusa Ruby) seeds were sown in a planting tray containing soil/sand/cocopit mixture (1:1:1) and kept moistened by sprinkling water. After 30 days, the plants were removed; the roots were gently washed and treated with the respective endophytic inoculant by root dipping. The inoculum (5 mL/L; 24-h-old 0.2 OD culture of respective endophytes) was mixed with 0.5% carboxy methyl cellulose, and the roots were dipped for half an hour and transplanted in the pots. The treatment details were the same as used in the seedling tray experiment with three replications. This experiment was repeated twice for the consistency of the results under a similar experimental setup. Data was recorded 45 days after transplanting (DAT); however, root architecture, length, and dry weight of the plant was measured 75 DAT.

Modulation in Gene Expression

RNA Extraction and cDNA Preparation

The plant sample (100 mg of root, shoot, and leaf) was mixed with 1 ml TRIzol reagent and 10 µl mercaptaethanol and incubated for 5 min at ambient temperature before grinding in liquid nitrogen and further processed as per the instructions of the manufacturer for using PureLink™ RNA mini-Kit (Invitrogen, United States). The total RNA isolated was converted to cDNA using High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, United States) following instructions of the manufacturer. The cDNA was further used for real time quantitative PCR (RT-qPCR) studies.

RT-qPCR Assay

The RT-qPCR based expression analyses of salt stress-responsive genes (Table 1) were carried out using RT-qPCR Detection System (Bio-Rad, United States) and Eva Green SYBR Green Supermix Kit (Bio-Rad, United States) using three technical replicates of each sample. The final concentration of gene-specific primers was maintained at 10 pmol/µl, and internal controls were utilized to determine and normalize the transcript level of mRNA. The final volume of the reaction mixture was maintained to 10 µl [diluted cDNA samples (50 ng/ml), 2 µl; forward and reverse primers (10 µM), 1.5 µl each; and real-time master mix, 5 µl]. The RT-qPCR condition involved denaturation at 95°C for 2 min, 40 repeats at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The $2^{-ΔΔCT}$ method was used for data normalization using the mean CT values of the two endogenous genes, actin and glyceraldehyde 3-phosphate dehydrogenase. The fold accumulation of transcripts was compared by using the mean of the CT values of the three technical replicates from each biological replication with control (Livak and Schmittgen, 2001).

Cellular Osmotic Balance and Membrane Integrity Under Elevated Salinity

Total Phenolics

The accumulation of total phenolic content (TPC) in the plants was assessed by the protocol described by Malick and Singh (1980) and Sadasivam and Manickam (1996). One gram of leaf sample was ground in pestle and mortar using 80% ethanol. The content was centrifuged at 10,000 rpm for 20 min. The supernatant was evaporated, and the precipitate was dissolved in 5 ml distilled water. Then, 200 µl of this content was made up to 3 ml with distilled water, and 0.5 ml of Folin–Ciocalteau reagent was added. Two milliliters of 20% sodium carbonate was added after 3 min and mixed thoroughly. It was kept in boiling water bath for 1 min, and OD was recorded at 650 nm.

Proline Content

Proline content was measured by crushing 0.5 g of leaf sample in 10 ml of 3% aqueous sulphosalicylic acid. In 2 ml of the filtrate, 2 ml each of glacial acetic acid ninhydrin was added and kept in boiling water bath for 1 h. After reaction termination, toluene (4 ml) was mixed and kept at ambient temperature for toluene layer separation. The upper layer was taken, and the absorbance of red color was measured at 520 nm. Calculation was done using a standard curve as described by Sadasivam and Manickam (1996).

Electrolyte Leakage

Ten leaf discs were placed in 25 ml of distilled water and kept for 4 h at ambient temperature to measure the
The same was autoclaved at 121°C for 30 min, and the electrical conductivity was measured. The electrolyte leakage was assessed following the formula given in Khare et al. (2010).

**Effects of O. tenuiflorum Endophyte Inoculation on Enzymatic Reactive Oxygen Species Scavengers**

The activity of peroxidase (PO), catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APx) was measured at 50 DAT as per the protocol given in Sadasivam and Manickam (1996). Briefly, enzyme extract (for all four enzymes) was prepared by grinding 1 g of fresh plant tissue in 12,000 rpm for 15 min. For PO activity, 100-µl enzyme extract was mixed with 50 µl of 20 mM guaiacol solution, and 3 ml of 50 mM phosphate buffer was followed by the addition of 30 µl of 12.3 mM H₂O₂ in a cuvette to start the reaction; the absorbance was recorded at 436 nm. For CAT activity, the reaction mixture was prepared by mixing 100 µl of enzyme extract and 3 ml of 50 mM phosphate buffer, followed by the addition of 30 µl of 12.3 mM H₂O₂ in a cuvette to start the reaction; the absorbance was recorded at 240 nm. For SOD activity, 50 µl of enzyme extract was added to the reaction mixture containing 50 mM phosphate buffer, pH 7-8, 13 mM methionine, 75 mM nitroblue tetrazolium (NBT), 2 mM riboflavin, and 1 mM EDTA. In this mixture, riboflavin was added last, and the tubes were shaken. The reaction was started by placing these tubes under light for 10 min and stopped by covering the tubes with a dark cloth, and the absorbance was read at 560 nm. For APx activity, 1 ml of reaction mixture containing 50 mM KPO₄ buffer (pH 7.0), crude enzyme extract, and 0.35 mM ascorbate were prepared. The reaction was initiated by adding 5 µl of 10 mM H₂O₂. In this reaction mixture, the H₂O₂-dependent oxidation of ascorbate was recorded at 290 nm for 120 s.

### TABLE 1 | List of RT-qPCR primers used in the gene expression analysis.

| S. no. | Genes                        | Primer sets | Sequence (5′–3′)                                               | References                      |
|--------|------------------------------|-------------|---------------------------------------------------------------|----------------------------------|
| 1.     | K⁺ transporter               | LKT1-F      | ACTTGCTCTTCAACCTTTTG                                          | Wei et al., 2017                 |
|        |                              | LKT1-R      | CCACACATTTGTCAATGGC                                           |                                  |
| 2.     | Aquaporin                    | LePIP2-F    | AAGGATTACAAAAGGACC                                              | Shiota et al., 2006              |
|        |                              | LePIP2-R    | ACCAAAAAGCCAAACGAC                                              |                                  |
| 3.     | Na⁺/K⁺ antiporter            | NHK1-F      | TGGCGAGATGGGGCGATG                                               | Wei et al., 2017                 |
|        |                              | NHK1-R      | AACGACTCTCTTCAGGAATGACC                                         |                                  |
| 4.     | Ethylene response factor transcription factor | SiERF 16-F | GCGATTATACAGAAGCCGACTT                                          | Gharsallah et al., 2016         |
|        |                              | SiERF 16-R  | TQAOAAQAGGAAAGATCOGAAATT                                        |                                  |
| 5.     | WRKY transcription factor    | SiWRKY 39-F | GCGGTAATGCGAAAGACAAC                                             | Gharsallah et al., 2016         |
|        |                              | SiWRKY 39-R | TCAAGTCCTGTGATTTACG                                             |                                  |
| 6.     | Salt overly sensitive Na⁺/K⁺ antiporter | SOS1-F    | GTGCAGTACAGATGCTTTTACGG                                         | Wei et al., 2017                 |
|        |                              | SOS1-R      | AGGGCCCAACACAGCCACAG                                            |                                  |
| 7.     | Actin (reference gene)       | Actin-F     | GGAACCTTGAGAAGGAGCTAAG                                          | Wei et al., 2017                 |
|        |                              | Actin-R     | CAACACCAACAGCAACAGAC                                              |                                  |
| 8.     | GAPDH (reference gene)       | gadph-F     | GAAATGCATCTTGACATGACAAAGCTT                                     | Shih et al., 1992               |
|        |                              | gadph-R     | CTGTGAGTAACCCCATTTACCATGACAGC                                   |                                  |

### Accumulation of Reactive Oxygen Species and Programmed Cell Death

#### H₂O₂ Accumulation

Detection of H₂O₂ accumulation was done by staining with 3,3-diaminobenzidine (DAB; Sigma) following the protocol of Sakamoto et al. (2008). Leaf tissue was dipped in DAB solution overnight, and polymerization of DAB could be seen as a brown color deposit after decolorization with ethanol/glacial acetic acid (3:1). The images were captured using a stereomicroscope.

#### Superoxide Accumulation

The superoxide radical (O₂⁻) present in the leaf tissue reacts with nitroblue tetrazolium (NBT; Sigma) and forms blue-colored formazan (Rao and Davis et al., 1999). The NBT solution was prepared by dissolving 0.2 g NBT in 50 mM of sodium phosphate buffer (pH 5.5). The leaf tissues were dipped in this solution and incubated overnight, followed by bleaching with ethanol/glacial acetic acid (3:1) solution. The blue-colored spots, indicating superoxide radicals, appeared, and images were captured using a stereomicroscope.

#### Programmed Cell Death

Programmed cell death (PCD) in leaf tissue due to salt stress was assessed by Evans Blue staining (Baker and Mock, 1994). The leaf tissue was decolorized by boiling in ethanol, followed by dipping for 5–6 h in 0.25% of aqueous solution of Evans Blue for 5–6 h. The development of blue-colored spots indicated PCD. The images were captured using a stereomicroscope.

### Na⁺ Transport in Plant Tissues

The transport of Na⁺ ions in shoot was assessed using Sodium Green™ Tetra (tetrabutylammonium) salt (Invitrogen, United States) by capturing the sodium fluorescence using a confocal scanning laser microscope. Shoot tissues of equal physiological states were taken, and thin sections were prepared using a microtome and stained with Sodium Green™ Tetra (tetrabutylammonium) salt. The stained sections were...
mounted in slides and visualized under a CSLM (Nikon 90i, Japan) using 488-nm channel. The images were processed using NIS element 3.2.3 program (Nikon, Japan). All the treatments were visualized using the same optical adjustment to avoid any capture artifacts in signal strength.

Chlorophyll Content
After 45 days of transplantation, leaves were collected for assessing chlorophyll content as described by Witham et al. (1971). One gram of leaf tissue was crushed in 80% pre-chilled acetone, and the volume was made up to 100 ml with pre-chilled acetone. The absorbance of the supernatant was recorded at 663 and 645 nm using UV-vis 1700 spectrophotometer (Shimadzu, Japan). The amount of chlorophyll present (mg/g) in the leaf tissue was calculated using the following formula:

\[
(1) \text{Chl } a \text{ mg/g of leaf} = 12.7 \left(\frac{A_{663}}{V}\right) - 2.69 \left(\frac{A_{645}}{V}\right) \\
(2) \text{Chl } b \text{ mg/g of leaf} = 22.9 \left(\frac{A_{645}}{V}\right) - 4.68 \left(\frac{A_{663}}{V}\right)
\]

Root Architecture
The entire soil from the pot was washed carefully to harvest the complete root system. The harvested roots were washed thrice with clean water to remove the adhering soil. The roots were placed on the scanning tray filled with sterile water. The roots were scanned using EPSON Expression 12000 XL scanner, and the subsequent analyses of various root parameters [like cumulative length of total root (cm), projection area (cm²), surface area (cm²), average diameter (mm), root volume (cm³), tips, forks, crossings, and number of links] were carried out using WINRHIZO Pro software.

Statistical Analysis
In this study, laboratory experiments were laid out in a completely randomized design with three replications each and repeated thrice. The experiment for inter-genera colonization was carried out with six treatments and 10 replications. The pot experiment was laid out in a randomized complete block design with six treatments and three replications each, and the pot experiment was repeated twice. All the data (except for the gene expression study) were analyzed using statistical formulae in Microsoft Office Excel, and means were compared with Duncan’s multiple range test at \(p \leq 0.05\). Origin program was used for the graphical representation of data. The gene expression data was analyzed and compared based on non-parametric test (Kruskal–Wallis test) using SPSS 20 program.

RESULTS

Inter-Genera Colonization of
O. tenuiflorum Endophytes in Tomato Roots
The confocal scanning laser micrographs of the colonization experiment showed that all the four endophytes could establish themselves in the tomato roots with variable colonization patterns (Figure 1). SYTO9 was present in LIVE/DEAD\textsuperscript{TM} BacLight\textsuperscript{TM} bacterial viability staining kit-stained bacterial cells in yellow-green color based on membrane potential and integrity. Bacterial signals were observed from the epidermis and root hair cells. O. tenuiflorum endophytes Bacillus haynesii GTR8 and Bacillus altitudinis GTS16 were found to colonize in the rhizoplane as well as endosphere, whereas Bacillus paralicheniformis GTR11 and Bacillus safensis BTL5 showed only internal colonization (Figures 1C–E). However, absolute and saline control plants had signals, too, but the intensity was relatively very less (Figures 1A,B).

Modulation in Gene Expression
The treatments were significantly different for all the genes at 1% level of significance except in the case of NHX1, where treatments are significantly different at 5% level of significance. The LKT1 gene was over-expressed in Bacillus altitudinis GTS16-treated plants (17.17-folds), whereas statistically non-significant differences were found among other treatments (Table 2). The NHX1 gene expression was more than fourfolds higher in Bacillus altitudinis GTS16 inoculation and more than twofolds higher in Bacillus safensis BTL5 and Bacillus haynesii GTR8 inoculation, which was significantly higher than both absolute control and saline control. Overexpression of SOS1 by more than ninefolds was recorded in Bacillus haynesii GTR8 and Bacillus altitudinis GTS16 plants. However, there was no significant increase in other treatments. Although overexpression of LePIP2 gene was observed in all the saline treatments, the highest expression was recorded in Bacillus paralicheniformis GTR11. In SlERF16 and SlWRKY39 genes, a higher expression was recorded from Bacillus haynesii GTR8-inoculated treatments. Bacillus altitudinis GTS16 and Bacillus paralicheniformis GTR11 inoculation caused 3.49- and 2.17-folds increased in SlERF16 gene expression, respectively.

Reactive Oxygen Species Accumulation
in Tomato Leaves and Antioxidant System
Cellular Osmotic Balance and Membrane Integrity
Under Elevated Salinity
The applied bacterial endophytes were able to supplement plant machinery for oxidative stress mitigation which were visible in the overall plant growth (Figure 2). The accumulation pattern of TPC was variable among the treatments. Treatment with Bacillus haynesii GTR8 inoculation accumulated a significantly higher TPC. On the contrary, treatments inoculated with Bacillus safensis BTL5 could accumulate lower levels of TPC at par with the absolute control plants (Figure 3A). In case of total proline, endophyte inoculation gave a mixed effect (Figure 3B). The Bacillus altitudinis GTS16- and Bacillus paralicheniformis GTR11-treated plants were having a higher accumulation, whereas Bacillus safensis BTL5 and Bacillus haynesii GTR8 were having a similar accumulation as saline control. However, both salinity and inoculation had significant effects on proline content. On the other hand, high salinity (saline control treatment) lowered the membrane integrity and enhanced the
FIGURE 1 | Confocal scanning laser microscopy images showing the inter-genera colonization of *Ocimum tenuiflorum* bacterial endophytes in tomato roots treated with LIVE/DEAD™ BacLight™ bacterial viability stain. The white arrows indicate live bacterial endophytes in tomato roots in different treatments. (A) absolute control, (B) saline control, (C) BTL5, (D) GTR8, (E) GTR11, and (F) GTS16.
TABLE 2 | Effect of bacterial endophytes on the expression of salt stress-responsive genes under elevated salinity (150 mM NaCl).

| Treatments  | LKT | LePIP | NHX | SOS | SlERF | SlWRKY |
|-------------|-----|-------|-----|-----|-------|--------|
| Absolute control | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Saline control | 1.62 | 1.49 | 0.99 | 2.14 | 0.23 | 0.43 |
| BTL5 | 35.40 | 2.02 | 9.93 | 9.59 | 31.13 | 8.62 |
| GTR8 | 3.24 | 2.02 | 9.93 | 9.59 | 31.13 | 8.62 |
| GTR11 | 1.71 | 3.45 | 9.93 | 9.59 | 31.13 | 8.62 |
| BTL5 inoculation increased the total chlorophyll content compared to the absolute control (Figure 7). Inoculation of endophytes Bacillus altitudinis GTS16 and Bacillus safensis BTL5 could improve the membrane integrity at par with the absolute control and significantly reduced the electrolyte leakage.

Effects of O. tenuiflorum Endophyte Inoculation on Enzymatic Reactive Oxygen Species Scavengers

PO accumulation was highest in T6 (3.45 U mg\(^{-1}\) FW), APx was higher in T4 (0.44 U mg\(^{-1}\) FW), CAT accumulated in highest quantity in T3 (7.38 U mg\(^{-1}\) FW), whereas SOD activity was found to be highest in the Bacillus haynesii GTR8-inoculated (4.64 U mg\(^{-1}\) FW) plants (Figures 4A–D). Inoculation of endophyte Bacillus altitudinis GTS16 remarkably enhanced the production of PO and SOD. Bacillus haynesii GTR8 inoculation was able to significantly enhance the levels of APx, CAT, and SOD than the positive control. Endophyte Bacillus safensis BTL5 could activate more of CAT and PO for alleviating the effects of salinity. Bacillus paralicheniformis GTR11 inoculation chiefly enhanced the levels of APx in tomato plants for reducing the effects of elevated salinity.

Accumulation of Reactive Oxygen Species and Programmed Cell Death

The salinity induced sub-cellular H\(_2\)O\(_2\) accumulation was reduced in the leaves of endophyte-inoculated plants, especially in Bacillus safensis BTL5-, Bacillus haynesii GTR8-, and Bacillus paralicheniformis GTR11-inoculated plants (Figure 5A). Superoxide accumulation was highest in the saline control. However, the accumulation was also observed in other treatments, including endophyte-inoculated plants, but was lower than the absolute control treatment (Figure 5B). Evans Blue staining indicated increased PCD in the saline control and Bacillus safensis BTL5-, Bacillus paralicheniformis GTR11-, and Bacillus altitudinis GTS16-inoculated plants. Least PCD was observed from absolute control and Bacillus haynesii GTR8-inoculated plants (Figure 5C).

Na\(^+\) Transport and Accumulation in Plant Tissues

Sodium Green™ staining of tomato shoot tissues indicated higher sodium transport and accumulation in the saline control treatment (Figure 6B) compared to the absolute control (Figure 6A). However, there were variations in endophyte-inoculated saline treatments (Figures 6C–F). Na\(^+\) transport and accumulation were less in Bacillus haynesii GTR8, Bacillus paralicheniformis GTR11, and Bacillus altitudinis GTS16, whereas Bacillus safensis BTL5 had higher sodium accumulation than other endophyte-inoculated treatments. Bacillus haynesii GTR8 plants had lowest Na\(^+\) transport, which was comparable to the absolute control (Figure 6D).

Effect on Chlorophyll Content

The effects on the chlorophyll content of tomato leaves are shown in Figure 7. Among the endophytes, except Bacillus paralicheniformis GTR11, all three could improve the total chlorophyll content over the saline control. Endophyte Bacillus safensis BTL5 inoculation increased the total chlorophyll content.
FIGURE 2 | Phenotypic observation of the plants under different treatments indicating improvement in root and shoot growth and biomass accumulation. T1, absolute control; T2, saline control; T3, BTL5; T4, GTR8; T5, GTR11; and T6, GTS16.

of the tomato plants at par with the absolute control (Figure 7). In case of Chl a and Chl b also, significantly higher total chlorophyll content was observed in *Bacillus safensis* BTL5 inoculated and absolute control plants, whereas lowest was recorded from saline control plants.

**O. tenuiflorum** Endophytes Modulated Tomato Root Architecture Under Elevated Salinity

Root scanning data for cumulative length of root, projection area, surface area, average root dia, root volume, number of tips, forks, crossings, and links were taken for comparison. The inoculation of endophytes resulted in a robust root system in terms of the cumulative length of the root, projection area, surface area, root volume, number of tips, forks, links, and crossings. However, no significant difference in root diameter was observed among various microbial inoculations and uninoculated controls. The inoculation of *Bacillus paralicheniformis* GTR11 resulted in the highest cumulative length of root (1,378.22 cm), projection area (67.87 cm²), surface area (213.23 cm²), tips (3,208.67), and crossings (1,887.0) among the microbial inoculation treatments (Table 3). It is worthy to note that the root system developed in the endophyte-inoculated tomato plants under salt stress was statistically comparable to un-inoculated plants grown without any stress.

**Effect of Salinity and O. tenuiflorum Endophytes on Plant Growth and Biomass Accumulation**

Shoot length was significantly improved over saline control in all the endophyte-inoculated plants; however, *Bacillus paralicheniformis* GTR11-inoculated plants have the lowest shoot length among endophyte-inoculated plants (Figure 8A). On the other hand, *Bacillus paralicheniformis* GTR11-inoculated plants had significantly higher root length than any other treatments (Figure 8B). Plants inoculated with *Bacillus safensis* BTL5 significantly influenced the root biomass after 45 days of transplanting compared to all other treatments (Figure 8D). The shoot biomass was also higher in the treatments inoculated with *Bacillus safensis* BTL5 but was at par with the absolute control treatment (T1) and treatments with *Bacillus haynesii* GTR8 inoculation (Figure 8C).

**DISCUSSION**

**Inter-Genera Colonization of O. tenuiflorum Endophytes in Tomato Roots**

The results of the colonization study indicated that *O. tenuiflorum* endophytes could enter into tomato roots and colonize both outer and interior surfaces (Figure 1). Complex cross-talk is involved between plant and microorganism that affects endophytic colonization (Lahrmann et al., 2013); thus, exploring the potential endophytes having inter-genera colonization could widen the application of such potential cultures. The bacterial colonization in Figure 1 is indicated with white arrows. Strong colonization competence could have contributed to the successful colonization of *O. tenuiflorum* endophytes in tomato as earlier reported in the case of rice (Sahu et al., 2020a). There are reports indicating that inter-genera colonization contributes to plant fitness (Elbeltagy et al., 2001; Melnick et al., 2008; Abdallah et al., 2020). Endophytes having colonization in a wider range of hosts could be of greater significance for sustaining agriculture productivity.

**Modulation of Gene Expression**

Modulation in the expression of *LKT1*, *NHX1*, *SOS1*, *LePIP2*, *SIERF16*, and *SlWRKY39* genes was observed in tomato plants (Table 2), complementing the stress mitigation. This might be due to the induction of systemic tolerance by the applied endophytes that could have triggered the plants to overproduce the genes involved in salt stress alleviation (Vaishnav et al., 2020).
Out of multiple genes involved in stress signaling, three structural genes, \(LKT1\), \(NHX1\), and \(SOS1\), were chosen for this study since they code for \(K^+\) inward channel, \(Na^+/K^+\) antiporter in vacuolar membrane, and \(Na^+/K^+\) antiporter in roots, respectively (Hartje et al., 2000; Barragán et al., 2012; Wei et al., 2017). The fourth gene, \(LePIP2\), encodes for plasma membrane aquaporins to maintain water homeostasis under salt stress. These four genes could have enhanced potassium influx and water balance and impart salt tolerance in tomato plants as validated in previous literatures (Qin et al., 2016; Wei et al., 2017; Molina-Montenegro et al., 2020). Thus, exploring these genes could provide insights for the induction of systemic tolerance by \(O. tenuiflorum\) endophytes. In plants, different \(WRKY\) and \(ERF\) transcription factors are extensively reported to be involved in drought and salt stress alleviation (Eulgem and Somssich, 2007; Gharsallah et al., 2016; Hong et al., 2018). The upregulation of \(SWRKY39\) and \(SIERF16\) transcription factors in tomato plants could have activated multiple genes involved in ion homeostasis, oxidative stress pathway, photosynthesis, and enzyme activities as reported in different studies pertaining to salt stress (Sharma et al., 2010; Huang et al., 2012; Sun et al., 2015; Gharsallah et al., 2016; Hong et al., 2018). Our results showed that \(Bacillus\) \(haynesii\) GTR8, \(Bacillus\) \(paralicheniformis\) GTR11, and \(Bacillus\) \(altitudinis\) GTS16 inoculation enhanced the expression of \(SIERF16\) and \(SWRKY39\) genes, correlating to the improvement in plant growth and development under elevated salinity. Similar to our results, improvement in salt stress tolerance was also reported in tomato plants over-expressing \(ERF16\) and \(SWRKY39\) transcription factors (Gharsallah et al., 2016).

The expression of genes by endophyte inoculation was variable among the treatments, indicating the different modes of action of the applied inoculants. GTS 16 inoculation upregulated all the genes under study, whereas BTL5 could upregulate only \(NHX1\). This might be due to difference in the modes of action among the applied endophytes. A similar complementation among the different microbial inoculants for improving plant performance was reported earlier (Shang and Liu, 2021). This could be a useful trait for the application of endophytic inoculants in a consortium.

**Cellular Osmotic Balance and Membrane Integrity Under Elevated Salinity**

In the present study, oxidative stress protection could be correlated with enhanced accumulation of proline in the plants inoculated with endophytes \(Bacillus\) \(paralicheniformis\) GTR11 and \(Bacillus\) \(altitudinis\) GTS16 (Figure 3). Proline plays a dual role of antioxidant as well as osmoregulator and is significant in maintaining water homeostasis under stress. In the present study, both proline-induced osmoregulation and maintenance of root structure might play roles in maintaining the water status of salt-affected plants (Hayat et al., 2012; Qurashi and Sabri, 2013; Shafi et al., 2019). Decreased content of proline (as observed in T3 and T4) was also reported as a beneficial trait, indicating lowered salt stress in the plant system (Bhise et al., 2017). Similar effects were observed in total phenolics content (Figure 3A). Increased total phenolics content is reported as a salt stress-alleviating trait (Bhise et al., 2017). Golkar and Taghizadeh (2018) reported that phenolics are the main contributors of antioxidant activity in safflower at the cellular level apart from having a positive correlation with other compatible solutes contributing to the osmotic balance under elevated salinity. Plants of \(Bacillus\) \(altitudinis\) GTS16-inoculated treatment were found to have the lowest electrolyte leakage similar to the...
absolute control treatment, which indicates ion homeostasis and compatible solute production. Higher membrane integrity could be a result of the accumulation of osmoprotectants, production of enzymatic and non-enzymatic antioxidants, and reduced ROS generation. In similar lines, Amanifar et al. (2019) reported a decrease in electrolyte leakage due to the application of arbuscular mycorrhizal fungi under elevated salinity. There are other reports of improving membrane integrity under salt

FIGURE 4 | Effect of bacterial endophytes on enzymatic antioxidants under elevated salinity (150 mM NaCl). (A) Peroxidase, (B) ascorbate peroxidase, (C) catalase, and (D) superoxide dismutase. T1, absolute control; T2, saline control; T3, BTL5; T4, GTR8; T5, GTR11; and T6, GTS16.

FIGURE 5 | Effect of bacterial endophytes on (A) salinity induced sub-cellular H$_2$O$_2$ accumulation by 3,3’-diaminobenzidine, (B) superoxide accumulation by NBT staining, and (C) programmed cell death by Evan’s blue staining under elevated salinity (150 mM NaCl) as revealed. T1, absolute control; T2, saline control; T3, BTL5; T4, GTR8; T5, GTR11; and T6, GTS16.
Effects of *O. tenuiflorum* Endophyte Inoculation on Different Reactive Oxygen Species Scavengers and Programmed Cell Death

Plants harbor antioxidant enzyme systems which protect them from the deleterious effect of very high levels of reactive oxygen species (ROS) generated due to stress (Kasote et al., 2015). The results from the enzymatic antioxidant activity indicated that the mechanisms for salt stress alleviation varied among different *O. tenuiflorum* endophytes (Figure 4). The enhancement in enzyme activities could be due to the triggering of the systemic mechanisms of the plant by endophytes (Sahu et al., 2019) by microbe-associated molecular patterns and other endophytic activities contributing to the induced systemic tolerance. There are various reports of endophytes improving the enzymatic antioxidant activity of plants by induced systemic tolerance (Halo et al., 2015; Vaishnav et al., 2020).

Triggering of PO, CAT, APx, and SOD activities by the induction of systemic tolerance could be a reason for the reduced sub-cellular accumulation of H$_2$O$_2$ and superoxide stress by inoculating stress-alleviating rhizobacteria (Sapre et al., 2021). Modulation of proline content and other compatible solutes due to the application of endophytes could also be one of the reasons for the reduction of ROS and increased membrane integrity.
radicals in tomato leaves (Figure 5). Leaf staining revealed that the antioxidant system was triggered due to endophyte application, which could have contributed to the mitigation of salt stress. The antioxidant defense system of the plant was supplemented by endophyte inoculation, as reported in other studies in tomato (Halo et al., 2015; Sahu et al., 2019), under elevated salinity. Additionally, a lower sign of PCD was observed from *B. haynesii* GTR8-inoculated treatments (*G. T. R. 8, O. tenuiflorum* the inoculation of *Na*+ content of the plant tissue with differential exposures to *NaCl* (Wegner et al., 2011). This Sodium Green™ Tetra (tetramethylammonium) salt is a visible light-excitable *Na*+ indicator and provides a brilliant spatial and temporal resolution of *Na*+ concentrations with sufficient sodium ions than other monovalent cations (Minta and Tsien, 1989). The spectral characteristics give the advantage of low cellular autofluorescence (Zhang and Melvin, 1996). The reduction in *Na*+ concentration of endophyte-inoculated plants could be due to the reduced sodium uptake, higher biomass accumulation, and ion compartmentalization by maintaining the *Na*+/K+ balance in cells. It also reflects the effects of the endophytes on the upregulation of structural (*LKT1, NHX1, SOS1*, and *LePUP2*) and regulatory genes (*SIERF16 and SIWRKY39*) which, in turn, reduces *Na*+ toxicity in the cells.

**Na**+ Transport and Accumulation in Plant Tissues

Reduction in *Na*+ transport and accumulation was found by the inoculation of *O. tenuiflorum* endophytes, especially in *Bacillus haynesii* GTR8, *Bacillus parlicheniformis* GTR11, and *Bacillus altitudinis* GTS16-inoculated treatments (Figure 6). Similar reports with the use of sodium Green™ Tetra (tetramethylammonium) salt have shown a relationship of stress. The antioxidant defense system of the plant application, which could have contributed to the mitigation of salt toxicity (Koevoets et al., 2016; Arif et al., 2019). Improvement in root parameters benefits plants in surviving under salinity (150 mM NaCl). It is well documented that the root system architecture of tomato plants was significantly affected by salinity (Koevoets et al., 2016; Arif et al., 2019). In the present study, inoculation of *O. tenuiflorum* endophytes improved the root architecture (Table 3). Higher root projection area and surface area with a higher number of lateral roots in endophyte-inoculated plants might have helped the plants to mine water and nutrients more efficiently under osmotic stress caused by salinity.
Similar to our results, improvement in the root architecture by endophyte inoculation was also reported by other workers (Maggini et al., 2019; Muthukumar and Sulaiman, 2021; Verma et al., 2021b), specifically, improvement in root surface area (Irizarry and White, 2017; Yakti et al., 2018), root branching (Crush et al., 2004; Irizarry and White, 2017), root diameter (Martinuz et al., 2015), number of tips (Irizarry and White, 2017), etc. The improvement could be due to the production of a number of phytohormones, including IAA by the endophytes, that can modulate the endogenous levels of phytohormones in plants (Verma et al., 2021a). Increased levels of IAA due to the presence of endophytes can increase the root surface area, lateral root, and root hair formation (Vacheron et al., 2013; Zamioudis et al., 2015). Wang et al. (2015) reported that inoculation of endophytic *Bacillus* sp. LZR216 could influence auxin biosynthesis and transport, resulting in increased lateral root growth and reduced primary root elongation. The endophytes evaluated in this study could produce IAA (2.51–4.52 µg ml⁻¹), which might have contributed toward the improvement of the root system architecture of tomato plants. However, the improvement in root growth can be independent of jasmonate, auxin, or ethylene signaling as well—for instance, the increased lateral root growth of *Arabidopsis* due to inoculation of *Bacillus siamensis* YC7012 has been attributed toward the production of volatile compounds (Hossain et al., 2019). Considering the previous findings and the results from the present study, it can be concluded that suitable endophyte inoculation can promote lateral root development and higher root length in tomato plants under high salt stress.

### Effect of Salinity and *O. tenuiflorum* Endophytes on Plant Growth and Biomass Accumulation

The increase in biomass accumulation by endophyte application (Figure 8) indicated the reduced effect of stress in the plants, due to which the plants could grow well and accumulate higher dry matter similar to the reports of Egamberdieva et al. (2016) and Vaishnav et al. (2020). The growth of the plants is

![FIGURE 8](image-url) Effect of bacterial endophytes on the growth and biomass accumulation of tomato plants under elevated salinity (150 mM NaCl). (A) Average shoot length, (B) average root length, (C) average shoot dry weight, and (D) average root dry weight. T1, absolute control; T2, saline control; T3, BTL5; T4, GTR8; T5, GTR11; and T6, GTS16.
basically dependent on the photosynthetic rates and availability of water and nutrients in the soil. The improvement in root architecture, reduction in ROS accumulation, and upregulation of the genes responsible for membrane transporters and other regulatory pathways by the O. tenuiflorum endophytes could have provided osmotic balance in plant cells and helped maintain normal growth under elevated salinity. High salinity negatively affects chlorophyll content and thus the total photosynthesis (Akcin and Yalcin, 2016; Chauhan et al., 2018). Therefore, enhancement in chlorophyll content could also have contributed to total biomass accumulation. Like rhizospheric microorganisms, endophytes could have also played a significant role in nutrient mobilization and uptake which, in turn, can improve the overall growth of plants. Secretion of organic acids, siderophores, by the endophytes helps in the sequestration of nutrients like phosphorus, potassium, zinc, etc. (Verma et al., 2021a). The improved growth and significant enhancement of biomass of pearl millet by Bacillus cereus EPP5 was due to the solubilization of nutrients, production of siderophores, and synthesis of IAA (Kushwaha et al., 2020). Similarly, the inoculation of P-solubilizing, diazotrophic Bacillus methylotrophicus CK to apple resulted in a significant increase in root and shoot growth (Mehta et al., 2014). Ismail et al. (2021) reported that inoculation of IAA-producing and P-solubilizing Bacillus thuringiensis PB2 and Brevibacillus agri PB5 resulted in increased biomass and yield traits in Phaseolus vulgaris. Therefore, the plant growth-promoting traits exhibited by the endophytes used in the present study might have additionally enhanced the performance of the plants under salt stress and improved the biomass accumulation.

**Complementary Roles of O. tenuiflorum Endophytes**

The different endophytes used in this study have shown variable effects on plant growth. Here the effects of applied microbial inoculants on salt stress tolerance could be operated at least in two different modes: (i) endophyte could reduce the effect of salt toxicity (i.e., plant senses less salt stress) by effectively excluding and compartmentalizing the Na⁺ ions (Barragán et al., 2012; Wei et al., 2017), and (ii) once the plant senses salt toxicity, it activates the pathways for protecting plants by the formation of compatible solutes, ROS scavengers, and other compounds which can reduce the ill effects (Qurashi and Sabri, 2013; Halo et al., 2015). The salt stress-alleviating microbes could follow any of the abovementioned paths to complement the mechanism of salt tolerance of plants. Additionally, in mode (ii), since microbes have a vast metabolic diversity, they may supplement different mechanisms of plants as seen in T4; it has significantly enhanced TPC (Figure 3A) but has no effect on proline content among the saline treatments. Inoculation of T3 (Bacillus safensis BTL5 +150 mM NaCl) did not improve TPC and proline but has the lowest electrolyte leakage (Figure 3C), highest chlorophyll content (Figure 7), and highest root and shoot dry weight (Figures 8C,D). It has significantly improved different aspects of root architecture and also has a relatively higher expression of NHX1. It indicates the possibility of mix operating of modes (i) and (ii) in Bacillus safensis BTL5. On the other hand, inoculation of Bacillus haynesii GTR8 did not have much effect on root architecture but had the highest overexpression of LePIP2, SOS1, SIERF16, and SLWRKY39 genes, contributing to ionic balance, water homeostasis, and stress signaling. Additionally, Bacillus paralicheniformis GTR11 inoculation had a significantly higher effect on ascorbate peroxidase activity and thus reduced the accumulation of H₂O₂, and superoxide was observed. BTL5 and GTS16 inoculation had the lowest electrolyte leakage, whereas inoculation with GTR8 and GTS16 had the highest total phenolics and proline content, respectively. It is very crucial that analyzing the results revealed that the endophytes were complementary to each other. All four endophytes found complementing plant machinery for stress alleviation through different mechanisms. The results indicate that these endophytes could be suitable for application as consortium so as to complement each other in improving plant performance under salt stress.

**CONCLUSION**

The results obtained from the present study suggested the protective roles of bacterial endophytes in tomato plants under elevated salinity. These endophytes could modulate the antioxidant machinery, root architecture, and ionic balance of the cells of plants to strengthen them against salt stress. Thus, the application of bacterial endophytes by harnessing the complementary roles of Bacillus safensis BTL5, Bacillus haynesii GTR8, Bacillus paralicheniformis GTR11, and Bacillus altitudinis GTS16 could be of greater significance in promoting plant growth and productivity in saline areas. The added features of the inter-genera colonization of endophytic isolates could add to the applicability and popularity of the strain by not limiting the choice of farmers to apply the inoculants. However, the field efficacy of these inoculants is yet to be tested. Exploring more endophytes having a wider host range could add to the pace of commercial endophytic inoculant development. Further dissecting the effects of endophytes on protein level could give a wider understanding of the mechanisms of the endophytes involved.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

**AUTHOR CONTRIBUTIONS**

PSa conceived the idea and designed the experiments. PSa, SS, HC, BT, and RB performed the experiments. PSa, BS, and US
analyzed the data. PSa, PSh, BS, HS, and AS wrote and edited the manuscript. AB has analyzed the gene expression data. All authors have reviewed the manuscript and have given approval to the final version.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.744733/full#supplementary-material

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