Trichoderma longibrachiatum TG1 increases endogenous salicylic acid content and antioxidants activity in wheat seedlings under salinity stress

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Several studies have reported the deleterious effects excessive salt stress on *Triticum aestivum* L. seedlings. Seed pretreatment with exogenous salicylic acid (SA) enhance plants to tolerate salt stress. Herein, the present study aims to investigate the potential of using the plant-growth-promoting *Trichoderma longibrachiatum* TG1 to increase endogenous SA; induce salt tolerance, and enhance the antioxidants activity in wheat seedlings under different salt stresses. Wheat seeds were pretreated in TG1 spore suspension before exposure to different salt stresses. Compared to NaCl stress across the four concentrations (0, 50, 100, and 150 mM), the TG1 treated wheat seedlings increased SA content and phenylalanine ammonia-lyase activity (PAL) by an average of 55.87% and 24.10% respectively. In addition, the TG1-treated seedlings increased superoxide dismutase (SOD), peroxidases (POD), and catalase (CAT) enzymatic activities in the shoot by an average of 47.68%, 23.68%, and 38.65%; the root by an average of 43.87%, 23.59%, and 16.93%, respectively compared to NaCl-stressed seedlings. Compared to 0 mM NaCl of TG1-treated seedlings, SOD, POD and CAT transcription levels increased by an average of 0.92, 1.23 and 1.05-fold respectively, across the various levels of NaCl concentrations. Our results indicated that seeds pretreatment with plant-growth-promoting *T. longibrachiatum* TG1 could increase endogenous SA of plants and promote seedling growth under salt stress by improving enzymatic antioxidant activities and gene expression

Article

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Abstract: Several studies have reported the deleterious effects excessive salt stress on *Triticum aestivum* L. seedlings. Seed pretreatment with exogenous salicylic acid (SA) enhance plants to tolerate salt stress. Herein, the present study aims to investigate the potential of using the plant-growth-promoting *Trichoderma longibrachiatum* TG1 to increase endogenous SA; induce salt tolerance, and enhance the antioxidants activity in wheat seedlings under different salt stresses. Wheat seeds were pretreated in TG1 spore suspension before exposure to different salt stresses. Compared to NaCl stress across the four concentrations (0, 50, 100, and 150 mM), the TG1 treated wheat seedlings increased SA content and phenylalanine ammonia-lyase activity (PAL) by an average of 55.87% and 24.10% respectively. In addition, the TG1-treated seedlings increased superoxide dismutase (SOD), peroxidases (POD), and catalase (CAT) enzymatic activities in the shoot by an average of 47.68%, 23.68%, and 38.65%; the root by an average of 43.87%, 23.59%, and 16.93%, respectively compared to NaCl-stressed seedlings. Compared to 0 mM NaCl of TG1-treated seedlings, SOD, POD and CAT transcription levels increased by an average of 0.92, 1.23 and 1.05-fold respectively, across the various levels of NaCl concentrations. Our results indicated that seeds pretreatment with plant-growth-promoting *T. longibrachiatum* TG1 could increase endogenous SA of plants and promote seedling growth under salt stress by improving enzymatic antioxidant activities and gene expression.

Keywords: *Trichoderma* spp; wheat; salinity stress; antioxidants; salicylic acid.

Introduction

Wheat (*Triticum aestivum* L.) is one of the major cereal crops grown in arid and semi-arid regions and provides 20% of the total dietary calories and proteins that promote human nutrition (Shiferaw et al. 2013). Globally, sodium chloride (NaCl) stress negatively affects subsistence and commercial crop production, resulting in an annual loss of 65% of crop yield (Husen et al. 2018). For major agricultural commodities such as wheat, 70% yield loss has been recorded due to salinity stress (Etesami & Beattie 2018). Salinity, as the only abiotic stress, suppresses plant growth and root development in a dose-dependent manner by blocking auxin signaling (Contreras-Cornejo et al. 2014), triggering dehydration, nutrient deficiency, membrane dysfunction, and oxidative stress, leading to tissue damage or early senescence (Hossain et al. 2017; Wu & Wang 2012). Salinity affects plant growth and development and hinders seed germination.

Salicylic acid (SA) is a naturally occurring phenolic compound. In the regulation of plant growth, production, maturation, and defense responses, SA plays an important role. SA plays an important role in the response to abiotic stresses, including drought, low temperature, and salt stress, in addition to plant defense responses (Khan et al. 2015). SA has been suggested to have great agronomic potential to improve stress resistance of crops of agricultural importance (Miura & Tada 2014). Moreover, SA greatly increased the size and mass of plantlets compared to the untreated control when applied exogenously to wheat seedlings (Shakirova, 2007). However, in contrast to these findings, a decrease in chlorophyll content was observed in plants pretreated with SA (Kadıoğlu & Sağlam 2013; Moharekar *et al*. 2003). Again, it was reported that the synthesis of carotenoids and xanthophylls was induced by SA and the rate of deep oxidation was also increased with a concomitant decrease in chlorophyll pigments and chlorophyll a/b ratio of wheat and moong (Afshari et al. 2013). Moreover, when SA was applied exogenously, leaf emergence and growth of leaves and roots of barley plants were delayed in a dose-dependent manner (Pancheva et al. 1996). In *Funaria hygromatica*, dose-dependent inhibition of bud formation was also observed when SA was administered exogenously (Christianson & Duffy 2002). Also, exogenous application of SA was found to alter nutrient status, resulting in reduced

uptake of phosphate and potassium by roots, and this reduction was found to be pH-dependent, indicating higher protonated SA type activity (Hayat & Ahmad 2007).

Trichoderma species play an important role in salinity reduction. They have antimicrobial ~~potentials~~ to colonize different substrates under different environmental conditions (Fu *et al.* 2017). A Seed pretreatment with *Trichoderma* species increases indole-3-acetic acid (IAA) or 1-aminocyclopropane-1-carboxylate (ACC) contents in plants under stress and induces stress tolerance leading to an increase in plant growth (Zhang et al. 2019) . The accumulation of reactive oxygen species (ROS) is a well-known consequence of salt stress (Saghafi et al. 2018). Plants develop scavenging mechanisms that include both enzymatic and non-enzymatic antioxidants to effectively mitigate the ROS damage. The major enzymatic systems for ROS scavenging mechanisms, superoxide dismutase (SOD), peroxidases (POD), and catalase (CAT), are also important parameters for assessing salt resistance in plants. These ROS scavenging mechanisms, mediated by antioxidant enzymes, are the first line of defense against salt stress and directly reflect the effects of salt stress on plants. To maintain the balance between ROS development and interception and to mitigate the negative effects of salt stress on plant metabolism and growth, an effective antioxidant capacity is essential (Saghafi et al. 2018).

However, the potential of *Trichoderma* species to increase endogenous SA and decrease ROS of wheat seedlings under salt stress has not been investigated in various studies. The present study aims to investigate the potential of *T. longibrachiatum* TG1 to induce salt tolerance, increase endogenous SA and enhance the antioxidant activity of wheat seedlings under various salt stress conditions.

Materials and Methods

Fungal Inoculum Preparation

The salt tolerance *T. longibrachiatum* TG1 was obtained from Gansu Agricultural University's Laboratory of Plant Pathology. The species were cultured on potato dextrose agar (PDA) in Petri dishes for several days at 25 ℃. The conidia suspensions TG1 was prepared according to the method of Zhang et al. (2014). A conidia suspension of 1.0×10^8 spore per mL was quantified and stored at 4 ℃.

Salt Concentration Preparation

The salt assay was performed according to Zhang et al. (2016) formulation with minor modifications where one liter of liquid medium water agar (WA) was prepared. Then 0 g, 2.922 g, 5.844 g, and 8.766 g NaCl were added to achieve NaCl concentrations of 0, 50, 100, and 150 mM. The solutions were then shaken vigorously to dissolve and autoclaved at 121℃.

Plant Material and Treatment

Wheat seeds (*Triticum aestivum* L.) cultivar 'Yongliang 4' were used. According to the method of Gond et al. (2015), the seed was sterilized. Briefly wheat seeds of uniform size were surface-sterilized for 10 minutes with a 1 % NaOCl solution, then thoroughly washed six times with distilled water.

Seeds Treatment

Wheat seeds were soaked in (i) TG1 suspension only, and (ii) sterile water only for 12h. Seeds were air-dried overnight under aseptic conditions before sowing, according to Zhang et al. (2019).

Seeds Germination under Salt Stress

T. longibrachiatum TG1-treated wheat seeds and the control seeds were exposed to 0, 50, 100, and 150 mM NaCl in 9-cm Petri dishes. The dishes were covered with a layer of absorbent cotton and blotting paper. The Petri dishes were incubated at 25±1℃ at 16/8h light/dark photoperiod. The germination parameters were calculated according to the formula of (Niu et al. 2013).

Wheat Seedling Growth

The *in vitro* experiment was conducted at a normal temperature of 25 ℃ with a 16/8 h light/dark photoperiod. Twenty wheat seedlings of comparable size were planted in transparent glass pots of 8 cm height and diameter containing 100 mL of sterilized water agar. The experimental setups were divided into the following treatments: (i) TG1-treated wheat seedlings without NaCl, (ii) TG1-treated wheat seedlings with NaCl (50, 100, and 150 mM), (iii) sterile water treated seedlings without NaCl, and (iv) sterile water treated seedlings with NaCl (50, 100, and 150 mM). The physiological, biochemical, and molecular parameters of wheat seedlings were determined on day 8 after treatment. This was repeated six times.

121

122 **Growth Parameters**

123 After 8 days of NaCl treatments, the wheat seedlings were harvested. The shoots and
124 roots of the wheat seedlings were removed, washed three times with distilled water, dried, and
125 weighed. Shoot, root length and weight were measured using a meter rule and weighing balance.
126 To determine the dry weight, all samples of wheat shoots and roots were oven-dried at 105°C for
127 30 minutes and then held at 80 °C to maintain a constant weight before being weighed. Each
128 maintenance and control was performed six times. The relative water content (RWC) of the
129 shoots and roots was measured using Tian et al. (2015). RWC (%) = (FW-DW)/FW ×100;
130 where RWC represents relative water content, FW represents fresh weight, and DW represents
131 the dry weight.

132 **Chlorophyll and Carotenoid Content Determination**

133 Total chlorophyll and carotenoids were extracted with 100% acetone according to the
134 method of (Bojović & Stojanović 2005). The fresh shoot of 0.2 g was homogenized with 10 mL
135 of acetone. The chlorophyll and carotenoid content were evaluated in a two-wavelength
136 spectrophotometer at the absorbance of 661.6 nm, 644.8 nm, and 470 nm. This was repeated six
137 times.

138 **Lipid peroxidation and H_2O_2 Content Determination**

139 At 8 days after wheat seeds treatment, the shoot and root samples were used for both
140 oxidants investigations. Oxidants activity such as MDA and H_2O_2 were investigated according to
141 the manufacturer's protocol using the assay kits provided (Solarbio, China). The absorbance of
142 the MDA sample was measured at three different wavelengths 450 nm, 532 nm, and 600 nm, and
143 H_2O_2 at 415 nm using spectrophotometer (EPOCH2 Plate Reader, BioTek, America). The
144 content of MDA and H_2O_2 were expressed as μmol g^{-1} FW. This was repeated six times.

145 **Antioxidant Enzymes and PAL activity**

146 At 8 days after wheat seeds treatments, the shoot and root samples were used for
147 antioxidants investigations. The antioxidants activity of SOD (EC 1.15.1.1), POD (EC 1.11.1.7),
148 PAL (EC 4.3.1.5), and CAT (EC 1.11.1.6) were measured according to the manufacturer's
149 protocol using the assay kits provided (Solarbio, China). SOD was measured at 560 nm, POD at
150 470 nm, PAL at 290 nm, and CAT at 240 nm respectively using a spectrophotometer (EPOCH2
151 Plate Reader, BioTek, America). This was repeated six times.

Extraction of Total RNA and Analysis of Gene Expression by Quantitative Real-Time Reverse Transcriptase-PCR (qRT-PCR)

Total RNA extraction and analysis of 100 mg wheat seedlings exposed to different levels of NaCl stress was performed according to the methods of Xie et al. (2013) and using PureLink® RNA Mini Kit (Tiangen Biotechnology, Beijing, China). The quantity and quality of isolated RNA were analyzed using a Nano-Drop spectrophotometer at the absorbance of 230 and 260 nm. The A260/A230 ratio indicated that the RNA was free from protein contamination. First-strand cDNA synthesis was performed using Revert Aid TM First Strand cDNA Synthesis Kit (Tiangen Biotechnology, Beijing, China). Total RNA was adjusted to the same concentration using RNase-free water. Specific primers for the SOD, POD, and CAT genes and the internal control actin gene were used to amplify amplicons specific for wheat seedlings (Table 1).

The qRT-PCR was performed in a reaction tube with 20 µl reaction volume using Heff SYBR® Green Master Mix reaction mixture with 1 µl cDNA solution and 10µM primers. The primers used in the experiments were designed according to the wheat EST sequences of the candidate proteins available in NCBI using Primer Express 5.0 software to amplify the target genes. The relative expression of (SOD, POD, CAT, and actin) genes was determined using the $2^{-\Delta\Delta Ct}$ formula of (Livak & Schmittgen 2001). This was repeated six times.

Endogenous SA Determination

According to Warrier et al. (2013), the determination of salicylic acid in wheat leaves was carried out by spectrophotometric method where 0.1 g SA was dissolved in 100 mL of water to generate a stock SA (0.1 g $HOC_6H_4CO_2H$, and 100 mL H_2O_2) solution. There were 1000 parts per million (ppm) in this solution. To prepare the working solution of 10 mL, 1.0 mL of the stock solution were added to enough water (9 mL). This solution had a concentration of 100 ppm (µg/mL).Using liquid nitrogen and 1 mL of the working solution, 0.1 g of the leaf sample was homogenized. Centrifuge for 10 minutes at 10000 g. The supernatant was kept on ice. With 0.1% freshly prepared ferric chloride, 100 µL of the supernatant was mixed. Spectrophotometry at 540 nm absorbance, SA was measured relative to the standard solution.

Statistical analysis

The data was subject to one-way ANOVA using the SPSS package (SPSS V16.0, SPSS, Inc., Chicago, IL, USA). Treatment effects were determined using Duncan's multiple range test and the significances were expressed at $P < 0.05$.

Results

Wheat Seeds Germination and Growth

Seed ~~pretreated~~ with *T. longibrachiatum* TG1 significantly ($p<0.05$) affected the wheat seeds germination (Table 2). Compared to sterile water treatment, TG1 stimulated germination rate (GR), germination potential (GP), and germination index (GI) by an average of 20.52%, 10.77%, and 10.79%, respectively across the four NaCl concentrations (0, 50, 100 and 150 mM). An increase in NaCl concentration decreased GR, GP, and GI. At 150 mM NaCl stress, GR, GP, and GI of NaCl-treated seeds decreased by 32.94%, 32.25%, and 31.31%, respectively, compared to control. However, at 150 mM NaCl stress GR, GP, and GI of TG1- treated seeds decreased by 5.88%, 22.93%, and 22.91% respectively as compared to control (Table 2). Also, *T. longibrachiatum* TG1 significantly ($p<0.05$) affected the shoot and root length of wheat seedlings (Fig. 1A and B). Similarly, across the four NaCl concentrations, wheat seedlings with TG1 treatment increased the shoot height and root length by an average of 39.45% and 29.72% respectively compared to NaCl-stressed seedlings. Although TG1 treatment increased leaf area, a significant increase in shoot length and leaf area was observed in TG1-treated seedlings at 100 mM NaCl compared with NaCl-treated seedlings (Fig. 1C).

Endogenous SA Content and PAL Activity

Wheat seeds inoculated with *T. longibrachiatum* TG1 resulted in a significant ($p<0.05$) increase in SA content and PAL activity (Fig. 2). Compared to NaCl stress across the four concentrations (0, 50, 100, and 150 mM), the TG1 treated wheat seedlings increased SA content and PAL activity by an average of 55.86% and 24.10% respectively.

Biomass Accumulation and Relative Water Content

Total fresh weight (FW) and dry weight (DW) of wheat seedlings were significantly ($p<0.05$) increased by *T. longibrachiatum* TG1 (Table 3). ~~Compare~~ to the NaCl-stressed seedlings, the shoot FW and DW of TG1-treated seedlings increased by an average of 56.85% and 43.27%, respectively across the four NaCl concentrations (0, 50, 100 and 150 mM). Besides, the root FW and DW of TG1-treated seedlings increased by an average of 32.80% and 21.19%

respectively, compared with NaCl-stressed seedlings across the four NaCl concentrations. The RWC of the shoot and root of TG1-treated seedlings increased by 2.14% and 2.58% at 150 mM NaCl compared to NaCl-stressed seedlings.

Chlorophyll and Carotenoid Contents

Wheat seedlings treated with and without TG1 were examined for pigmentation under salinity stress on day 8. Seedlings pretreated with TG1 significantly ($p<0.05$) increased the pigmentation of wheat seedlings (Table 4). Chlorophyll a, b, total (a+b) and carotenoid content of TG1-treated seedlings increased by an average of 32.89%, 58.13%, 42.14%, and 34.85%, respectively, compared to NaCl-stressed seedlings across the four NaCl concentrations (0, 50, 100 and 150 mM). *T. longibrachiatum* TG1 pretreated seedlings showed increased pigmentation compared to sterile water treated seedlings (Table 4).

MDA and H_2O_2 Accumulation

The ~~extents~~ of the oxidants were determined on day 8 after treatment. Salt stress-induced MDA and H_2O_2 content and its effect ~~were~~ significantly ($p<0.05$) attenuated by TG1 (Table 5). The MDA content of shoot and root of TG1-treated seedlings decreased by an average of 26.89% and 26.63%, respectively, compared to NaCl-stressed seedlings across the four NaCl concentrations (0, 50, 100 and 150 mM). H_2O_2 content in the shoot and root of TG1-treated seedlings decreased by an average of 30.20% and 37.54%, respectively, compared to NaCl-stressed seedlings across the four NaCl concentrations. TG1 pretreatment decreased the accumulated MDA and H_2O_2 content in both shoot and root compared to non-TG1-treated seedlings under the salt stresses (Table 5).

Antioxidants Enzymes Activity and Expression

T. longibrachiatum TG1 significantly ($P<0.05$) induced antioxidant activities (Fig. 3). Enzyme activities in the shoots of TG1-treated seedlings SOD, POD and CAT increased significantly by an average of 47.68%, 23.68%, and 38.65%, respectively, compared to NaCl-stressed plants across the four NaCl concentrations (0, 50, 100 and 150 mM). Similarly, the activities SOD, POD, and CAT increased significantly by an average of 43.87%, 23.59%, and 16.93%, respectively, in the roots compared with the NaCl-stressed seedlings across the four NaCl concentrations.

241 *T. longibrachiatum*TG1 significantly ($p<0.05$) enhanced the up-regulation of SOD, POD,
242 and CAT genes in wheat seedlings under salinity stress (Fig. 4 A-C). Compared to 0 mM NaCl
243 TG1-treated seedlings, SOD, POD and CAT transcription levels increased by an average of 0.92,
244 1.23 and 1.05-fold respectively, across the various levels of NaCl concentrations. Significantly,
245 the genes SOD, CAT, and POD were relatively up-regulated in the salt-tolerant TG1-treated
246 seedlings at all NaCl concentrations compared with the control.
247

248 **Discussion**

249 Some *Trichoderma* species are an important class of plant growth-promoting fungi that
250 have been shown to stimulate plant growth and improve salt stress tolerance (Oljira et al. 2020b).
251 In this current study, *T. longibrachiatum* TG1 alleviated salt stress; ~~synthesize~~ phytohormones
252 for plant growth, increased endogenous SA, and antioxidant activity of wheat seedlings under
253 different levels of salt stress.
254 *In vitro* experiment, various concentrations of NaCl decreased the germination
255 parameters of wheat seed and growth significantly which corresponds with the hypothesis of the
256 study. Several previous studies have confirmed the negative effects of salinity on plant seeds
257 germination and growth both *in vitro* and under greenhouse (Azooz et al. 2011). Similarly,
258 sodium chloride treatment has been reported to inhibit root hair growth (Bhat et al. 2020).
259 In contrast to these findings, the germination rate of wheat seedlings increased, which is to be
260 expected when biocontrol agents are used, but conversely, application of salt-tolerant *T.*
261 *longibrachiatum* TG1 significantly doubled the germination and growth rate in the saline
262 medium. In addition, the application of salt-tolerant *T. longibrachiatum* TG1 reduced the
263 deleterious effect of NaCl stress on wheat seedling growth, thereby increasing shoot and root
264 length and improved root hair formation, which is beneficial for stressed plants. Similarly, the
265 application of *T. harzianum* T22 enhanced tomato (*Lycopersicum esculentum* L.) seed
266 germination under abiotic stresses (Ma et al. 2012). Likewise, *T. longibrachiatum* T6 promoted
267 wheat seedlings growth under NaCl stress by increasing shoot and root length (Zhang et al.
268 2019).
269 SA regulates the activities of several enzymes, such as SOD and PAL, which are the
270 main components of induced plant protection against biotic and abiotic stresses. This study

showed that plants combat salinity by using their endogenous SA when not supplied externally. Again, endogenous SA increases in proportion to an increase in stress. Several previous studies revealed salinity tolerance in rice seedlings was caused by the endogenous SA level and the activity of the SA biosynthetic enzyme benzoic acid 2-hydroxylase (Sawada et al. 2006). The results suggest that SA plays a role in the salinity response. In agreement with our studies, several reports suggest that SA induces a plant immune system that can respond to various stresses (Chojak-Koźniewska et al. 2018; Ramakrishna & Kumari 2017). However, co-inoculation of wheat seeds with TG1 increased endogenous SA content twofold in both NaCl stress and normal seedlings. Endogenous levels of SA are increased to induce SAR following an attack by an unconditional environmental factor. Stomatal closure is facilitated by an increase in endogenous SA levels. This study showed that pretreatment of wheat seedlings with *T. longibrachiatum* TG1 increases PAL activity and enhances salinity tolerance. There is some evidence that peroxidase and PAL are important enzymes involved in plant defense against stressors, which is consistent with our findings (Ghazalbash et al. 2018a; Rani & Pratyusha 2013) and that SA is known to stimulate these enzymes in plants (Ghazalbash et al. 2018b). Previous studies have shown that the application of SA treatments increases the activity of the PAL enzyme (Golkar et al. 2019). In comparison with this study, TG1 was found to increase the content of endogenous SA in wheat seedling leaves and significantly increased the activity of PAL and antioxidant enzymes. Recent data have shown that exogenous SA -treated wheat plants had a significant increase in hydrogen peroxide and tend to be associated with increased superoxide dismutase and decreased catalase activity, which SA can also generate oxidative stress/ROS (ROS) in plants (Horváth et al. 2007). The exogenous SA applications could increase or decrease ROS in plants. Compared with the exogenous application of ROS-generating SA, the induced SA content of *T. longibrachiatum* TG1 reduced ROS via the enzyme activities. Therefore, another mechanism of TG1 improvement in salinity tolerance of seedlings could be the SA pathway and scavenging of ROS.

In this study biomass production increased in wheat seedlings subjected to TG1 treatment, suggesting that wheat seedling cells and tissues were protected from salt damage as a result of the increased endogenous salicylic acid. The effects of salinity stress on plant growth were observed as stunted growth of seedlings with reduced biomass and leaf area (Guo et al. 2018;

Zhao & Zhang 2015). Previous studies reported that *Trichoderma* isolates TRC3 significantly increased the physiological parameters such as shoot and root length, leaf area, and total biomass, stem and leaf fresh weight of maize seedlings at all stress levels. Similarly, in this present work, *T. longibrachiatum* TG1 increased the translocation of nutrient and water uptake in both saline and non-saline media, induced production of growth-promoting phytohormones in a balanced ratio, which specifically increased both fresh and dry biomass of wheat seedlings across stress levels. This finding agrees with those reached by Zou et al. (2019), who reported that the application of *T. longibrachiatum* H9 effectively stimulated plant growth by stimulating signaling pathways related to phytohormones on the roots of cucumber plants. Moreover, these findings were again supported by those of Kumar et al. (2017) who found that Plant growth-promoting fungi use various mechanisms to promote plant growth, particularly the provision of nutrients and securing minerals.

In this current work, pigment reductions were observed under salinity stress. A decrease in plant productivity is detrimental due to reduced leaf turgor and leaf surface area in saline conditions (Qin et al. 2010; Shah et al. 2017). From previous studies, it was revealed that salinity stress reduces stomatal opening and CO_2 absorption, which in turn is associated with a decrease in stomatal conductance. In general, salinity affects photosynthetic processes in plants by altering organelle ultrastructure, the concentration of various pigments and metabolites, and enzymatic activities (Stefanov et al. 2016). However, co-inoculation of TG1 reduces the various effects and thus increases the chlorophyll and carotenoid content of seedlings in both saline and non-saline media, which serves an important photo-protective function by dissipating excess energy into the cells. This finding was similar to the literature of Shukla et al. (2012) who indicated that biopriming of seeds with five salt-tolerant isolates of *T. harzianum* (Th-13, Th-14, Th-19, Th-33, and Th-50) on the response of rice (*Oryza sativa* L.) to different levels of salt stress alleviated the stress condition and significantly increased shoot and root length, fresh weight, number of leaves, and total chlorophyll content.

In addition, there was an increase in both H_2O_2 and MDA contents in shoots and roots of NaCl-stressed plants at each concentration that leads to seedling death and necrosis. This was due to high accumulation of reactive oxygen species (ROS) in both the shoot and root of wheat seedlings. The plant can activate antioxidant protection mechanisms by inducing both enzymatic and nonenzymatic antioxidants to detoxify ROS (Shan et al. 2010). MDA, H_2O_2, proline,

ascorbate, and glutathione are nonenzymatic antioxidants that help to scavenge ROS (Shan et al 2010). In many plant species, such as *Arabidopsis* and tomato, *Trichoderma* species can enable the antioxidant protection mechanism to recycle oxidized ascorbate and thus improve plant tolerance to abiotic stresses. Similarly, TG1-treated wheat seedlings accumulated low H_2O_2 and MDA contents in both shoot and root with or without NaCl by doubling the activities of antioxidant enzymes. In this regard, *T. longibrachiatum* TG1 expanded its antioxidant enzyme machinery as a means to maintain osmotic balance and metabolic homeostasis in wheat seedlings under salt stress and enhanced tolerance to oxidative stress.

This finding was in support of those of Ibrahim et al. (2016) who reported that seed priming activates pre-germination metabolic processes and allows radicle emergence, enhances antioxidant system function and membrane repair during germination and emergence under stress.

The expression of salt stress-responsive genes and proteins in salinity-affected plants is reprogrammed by the plant-fungus interaction, resulting in precise stress reduction metabolism as a defense mechanism (Malmierca et al. 2012). Previous studies revealed that exogenous salicylic acid treatment increased the transcripts of genes encoding ascorbate and glutathione cycle enzymes (Kang et al. 2002; Chen et al. 2011), and overexpression of these genes conferred increased resistance to salt and chilling stress (Duan et al. 2012). In addition, variations in the expression of complete gene families associated with abscisic acid (ABA), ion transport, and antioxidants were observed when wheat seeds were inoculated with salt-tolerant *Dietzia natronolimnaea* (Bharti et al. 2016). Similarly, sustained up-regulation of antioxidant mechanism was detected in NaCl-treated roots of salt-tolerant barley 'California Mariout (Achatz et al. 2010). These findings suggest that antioxidants may play a role in both inherited and endophyte-mediated tolerance of plants to salinity. Similarly, in this study, the transcription levels of the genes SOD, POD, and CAT increased significantly under NaCl stress and were up-regulated, consistent with the corresponding antioxidant enzyme activity of *T. longibrachiatum* TG1, indicating that antioxidant genes play an important role against oxidative stress.

However, once the ROS produced by plants exceeds the scavenging capacity of antioxidant enzymes, the antioxidant system is destroyed, therefore the SOD and POD transcript level declined as the salinity increased, but the CAT gene overexpressed across the salinity levels. These findings were supported by those of Luan et al. (2020), who showed that the *Trichoderma*

isolate ThTrx5 conferred salt tolerance to *Arabidopsis* by triggering stress response signals, and that overexpression of the genes SOD, POD, and CAT increased the root length and fresh weight of ThTrx5 transgenic plants.

Conclusion

Our results provide a basis for future incorporation of biological control agents into management strategies to control salinity through reduced exogenous salicylic acid applications and encouraged the use of microbes that can increase endogenous phytohormones and SA for plant treatments to control both biotic and abiotic stresses that pose a threat to current agricultural systems.

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Abbreviations

IAA - Indole-3-acetic acid (IAA)
ROS - Reactive Oxygen Species
MDA - Malondialdehyde
H_2O_2 - Hydrogen peroxide
PGPR- Plant growth-promoting rhizobacteria
RNA-Ribonucleic acid
ACC -1-aminocyclopropane-1-carboxylate
SOD - Superoxide dismutase
POD - Peroxidase
CAT - Catalase
DNA - Deoxyribonucleic acid

SGR - Seed Germination Rate

GI - Germination Index

GP- Germination Potential

NaCl- Sodium Chloride

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Figure 1

plant growth

Fig. 1. Length of shoot (A) and root (B) of wheat seedlings after pretreatment with TG1 under different salt concentrations. (C) Wheat seedlings in salt-water agar media for 8 days (CK, N50, N100, and N150 were seedlings treated with distilled water and subjected to NaCl stress, where the appended number represents the salt concentration; TG1,T50, T100, and T150 were seedlings treated with *Trichoderma* and subjected to NaCl stress). Data are presented as the mean of 6 replicates followed by different letters representing a significant difference at P< 0.05 (n = 6) based on Duncan's multiple range test. The line bars represent the standard errors of the means. Treatments are listed in the footnote of Table 2.

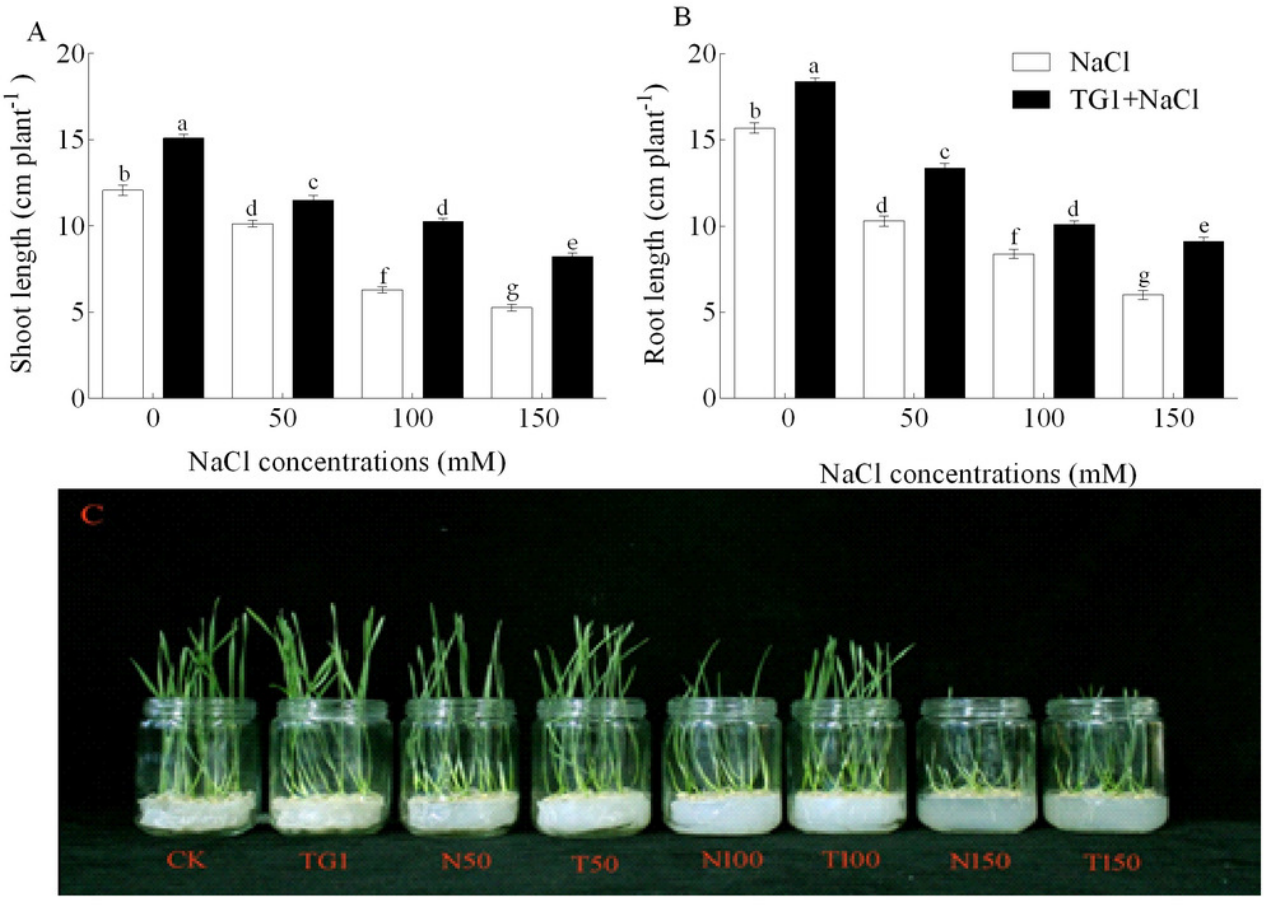

Figure 2

Salicylic acid and PAL activity

Fig. 2 Changes of SA concentration (A) and PAL activity (B) of wheat seedlings under control and salinity stress for 8 days. Different lower case letters indicate significant differences at $P< 0.05$ compared to control group. Treatments are detailed in the footnote of Table 2.

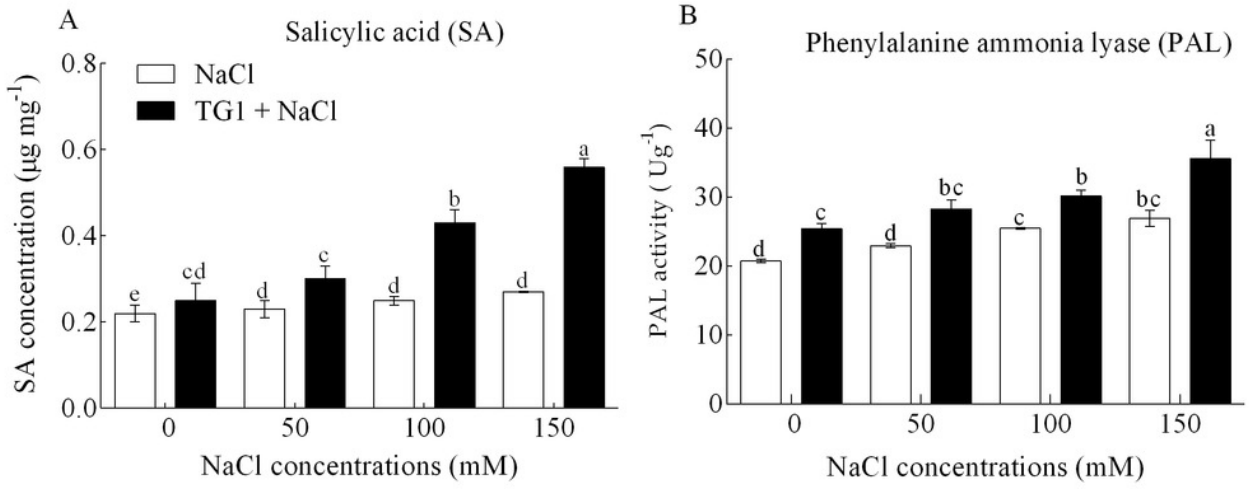

Figure 3

Antioxidants activity

Fig. 3. Changes of antioxidant enzymes activities in the shoot and roots of wheat seedlings under control and salt stress, where A, C, and E are the SOD, CAT and POD activities in shoots, and B, D, and F in roots. Data represent the standard errors of the means (n =6). Different lower case letter indicate significant differences at $P < 0.05$. Treatments are detailed in the footnote of Table 2.

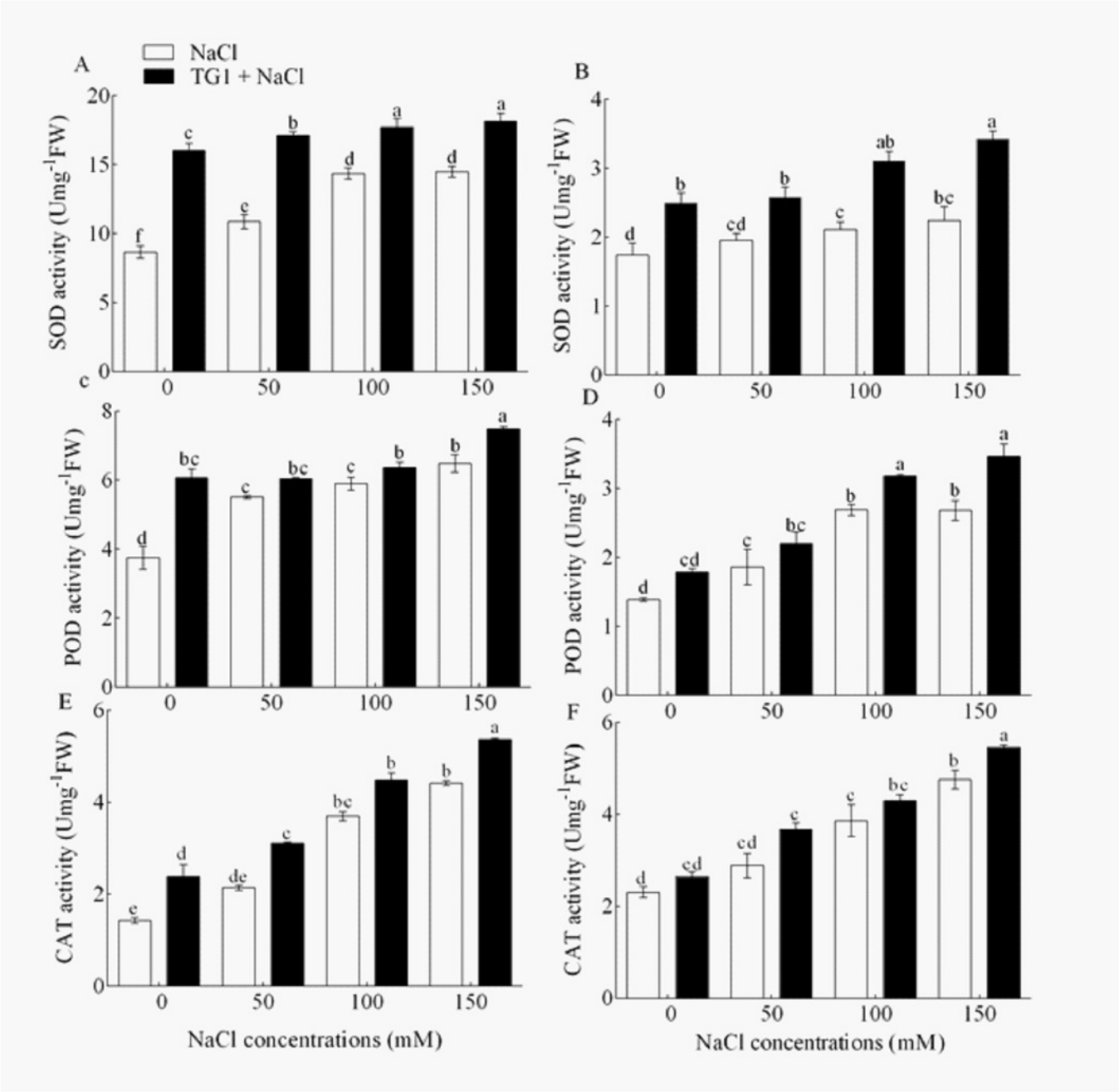
NaCl
TG1 + NaCl
A
B
C
D
E
F
SOD activity (Umg-1FW)
POD activity (Umg-1FW)
CAT activity (Umg-1FW)
NaCl concentrations (mM)
0
50
100
150

Figure 4

Antioxidant gene expression

Fig. 4. Changes of expression patterns of SOD (A), POD (B), and CAT (C) in shoots of wheat seedlings under control and salt stress. The bars represent standard errors of the means (n=6). Different lower case letters indicate significant differences at $P < 0.05$. Treatments are detailed in the footnote of Table 2.

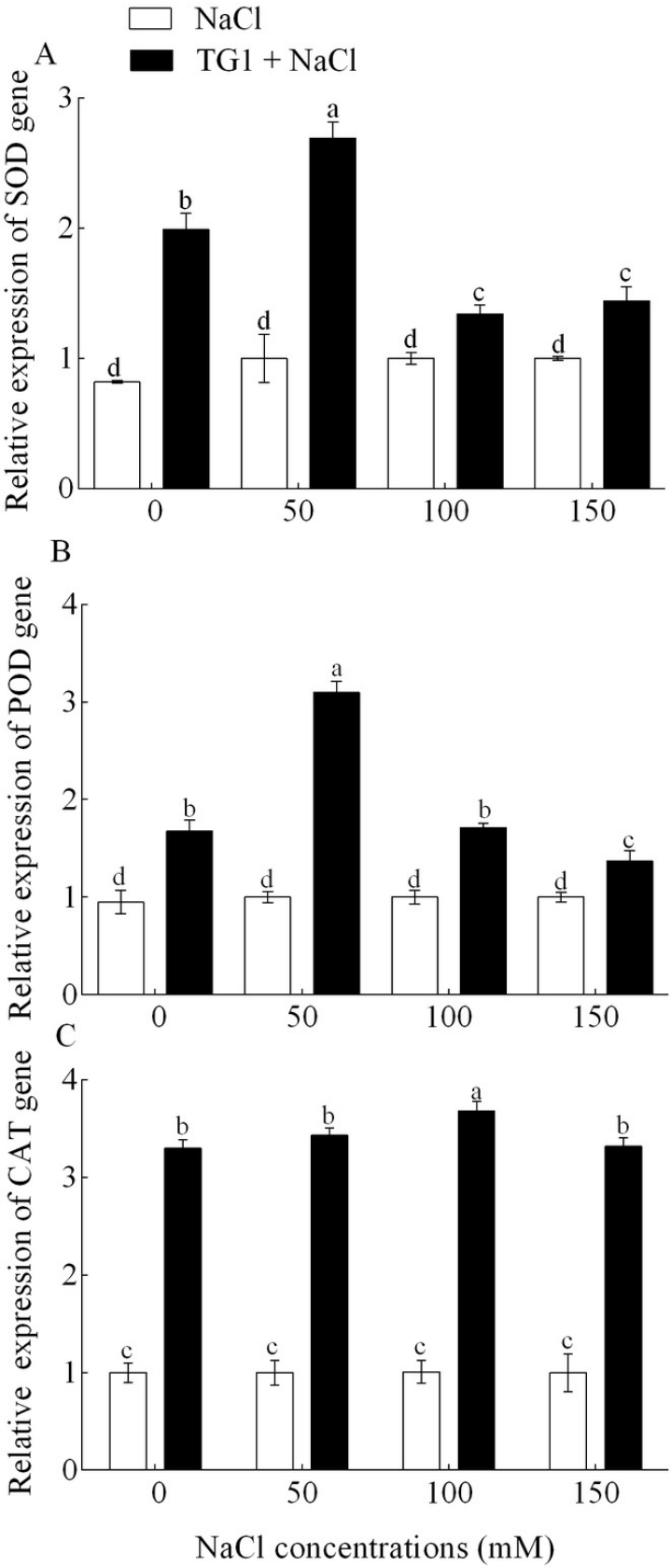
NaCl
TG1 + NaCl
A
Relative expression of SOD gene
B
Relative expression of POD gene
C
Relative expression of CAT gene
NaCl concentrations (mM)

Table 1(on next page)

Genes for PCR

List of genes used for PCR works

Table 1 Genes and primers sequences used for RT-PCR

Genes		Primers sequence (5'-3')
SOD	F	GAAGAACCTCAAGCCTATCAGCG
	R	CAGAGGGTGCTTTACAAGGATCT
POD	F	GCCGTTGAGATTACTGGTGGAC
	R	GTCTTCCTGATGCTACCAAGGG
CAT	F	GCTGGGGTCAACACTTACATGC
	R	GAGGAAGCTATCAGAGTTGGAGGA
Actin	F	GCTCCTAGAGCTGTATTCCCAAGT
	R	CAGTCGAAACGTGGTATCTTGACT

Table 2(on next page)

Track changes of table 2

Track changes of table 2

Table 2 Effect of *T. longibrachiatum* TG1 on wheat seeds germination under different salt stresses

Treatments	NaCl concentration (mM)	Germination rates (%)	Germination potential (%)	Germination index (%)
NaCl	Control	85.00±0.03b	80.00±2.89b	53.33±1.93b
	50	75.00 ±0.05d	68.33±1.67d	45.56±1.11cd
	100	73.00±0.09d	66.65±1.65d	44.44±1.11d
	150	57.00±0.029e	55.00±2.84f	36.63±1.93e
TG1+ NaCl	0	93.00 ±0.03a	85.00±2.89a	56.66±1.92a
	50	90.00±0.08a	76.67±1.67c	51.11±1.10b
	100	82.00±0.02bc	75.00±0.00c	50.00±0.00bc
	150	80.00±0.00c	61.66±2.31e	41.11±2.94d

Data are presented as mean ± standard error of replicates in a column followed by different lower case letters representing significant difference at $P < 0.05$ based on Duncan's multiple range test using one-way ANOVA. The control treatment represents wheat seedlings inoculated in distilled water; *T. longibrachiatum* TG1 treatment represents seedlings inoculated in 1×10^8 spores mL^{-1} TG1 suspension for 12 h; both treated seeds were grown in saline agar at 0, 50, 100, and 150 mM NaCl.

Table 3(on next page)

Track changes of table 3

Track changes of table 3

Table 3 Effect of *T. longibrachiatum* TG1 on biomass and relative water content of wheat seedlings under different salt stresses

Treatment	NaCl concentration (mM)	Wheat shoot			Wheat root		
		Fresh weight (mg plant^{-1})	Dry weight (mg plant^{-1})	Relative water content (%)	Fresh weight (mg plant^{-1})	Dry weight (mg plant^{-1})	Relative water content (%)
NaCl	Control	582.22±4.44b	57.17±0.44b	90.18±0.09a	293.31±5.21b	36.11±0.58b	87.67±0.24a
	50	408.89±4.41e	40.72±1.88d	90.04±0.49a	217.78±5.71c	27.75±0.33d	87.26±0.81a
	100	290.33±9.86f	29.83±1.45e	89.73±1.21a	137.77±4.43d	22.40±1.32e	83.74±1.29b
	150	148.67±8.06g	17.67±0.45f	88.11±0.91b	94.07±2.35e	19.83±0.33f	78.92±0.27d
TG1+NaCl	0	653.33±4.09a	60.33±2.74a	90.77±0.38a	355.56±4.13a	38.23±0.50a	89.25±0.50a
	50	546.66±5.16c	52.32±1.36c	90.43±0.37a	288.86±4.45b	34.31±0.62b	88.12±0.12a
	100	504.44±6.05d	49.01±1.04c	90.28±0.21a	201.11±5.41c	30.60±0.31c	84.78±1.12b
	150	308.87±6.11f	30.88±3.32e	90.00±5.73a	123.56±5.62d	23.53±0.85e	80.96±0.97c

Data are presented as mean ± standard error of replicates in a column followed by different lower case letters representing significant difference at $P < 0.05$ based on Duncan's multiple range test using one-way ANOVA. The treatments are detailed in the footnote of Table 2.

Table 4(on next page)

Track changes of table 4

Track changes of table 4

Table 4. Effect of *T. longibrachiatum* TG1 on the chlorophyll and carotenoid contents of wheat seedlings under different salt stresses

Treatment	NaCl concentration (mM)	Chlorophyll a (mg g^{-1})	Chlorophyll b (mg g^{-1})	Total chlorophyll (mg g^{-1})	Carotenoid (mg g^{-1})
NaCl	control	2.63±0.04b	2.26±0.02b	4.89±0.59b	3.71±0.09b
	50	2.14±0.03d	1.76±0.02c	3.89±0.06c	3.17±0.02c
	100	1.71±0.03g	1.10±0.04e	2.81±0.06f	2.58±0.08d
	150	1.19±0.01h	0.62±0.04f	1.81±0.05g	1.54±0.05e
TG1+NaCl	0	3.74±0.01a	3.31±0.06a	7.05±0.06a	4.91±0.04a
	50	2.53±0.09c	2.23±0.18b	4.76±0.17b	3.62±0.09b
	100	2.05±0.02e	1.54±0.01cd	3.59±0.03d	3.15±0.05c
	150	1.80±0.05f	1.36±0.08d	3.16±0.12e	2.63±0.07d

Data are presented as mean ± standard error of replicates in a column followed by different lower case letters representing significant difference at $P < 0.05$ based on Duncan's multiple range test using one-way ANOVA. The treatments are detailed in the footnote of Table 2.

Table 5(on next page)

Track changes of table 5

Track changes of table 5

Table 5 Effect of *T. longibrachiatum* TG1 on MDA and H_2O_2 content in wheat seedling under different salinity stresses

Treatment	NaCl concentration (mM)	MDA content in Shoot (μmol g^{-1} FW)	MDA content in root (μmol g^{-1}FW)	H_2O_2 content in Shoot (μmol g^{-1} FW)	H_2O_2 content in the root (μmol g^{-1} FW)
NaCl	Control	4.73±0.06f	3.36±0.07g	4.16±0.01e	2.59±0.01e
	50	12.46±0.13c	5.39±0.06e	7.47±0.08c	4.65±0.03d
	100	14.81±0.65b	7.27±0.09c	8.63±0.07b	6.37±0.13b
	150	20.25±0.09a	10.74±0.03a	10.53±0.16a	9.12±0.03a
TG1+NaCl	0	3.50±0.12g	2.55±0.08h	2.44±0.04f	1.49±0.02f
	50	8.79±0.06e	3.66±0.06f	5.47±0.84d	2.68±0.11e
	100	10.55±0.09d	5.63±0.02d	5.92±0.03d	4.82±0.01d
	150	15.52±0.63b	7.76±0.06b	8.29±0.25bc	5.38±0.08c

Data are presented as mean ± standard error of replicates in a column followed by different lower case letters representing significant difference at $P < 0.05$ based on Duncan's multiple range test using one-way ANOVA. The treatments are detailed in the footnote of Table 2.