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

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Salinity is global stress that directly affects plant growth and leads to drastic productivity losses. The present study aims to investigate *Trichoderma longibrachiatum* TG1 ability to increase endogenous salicylic acid (SA) and antioxidant activity of wheat seedlings under different salt stress conditions. Wheat seeds were pretreated in TG1 spore suspension (1×10^8 spores ml^{-1}) for 12 h before exposure to different salt stresses. The result showed that SA content increased with increasing salinity at each stress stage, but primarily in TG1-treated seedlings compared with NaCl-treated seedlings. Compared to control, TG1 significantly ($p<0.05$) increased SA content by 27%, 49%, and 61% at 50, 100, and 150 mM NaCl stress, respectively. Similarly, TG1 induced and increased phenylalanine ammonia-lyase (PAL) activity by 18%, 27%, 31%, and 41%, respectively, compared to control. At 150 mM NaCl stress, shoot and root of TG1-treated seedlings superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) increased significantly ($P<0.05$) by 52%, 50%, 73% and 49%, 60%, and 58%, respectively, compared to control. *Trichoderma longibrachiatum* TG1 increased plant salinity tolerance via systemic acquired resistance pathway (SAR) and increased activity of antioxidant enzymes.

Article

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Abstract: Salinity is global stress that directly affects plant growth and leads to drastic productivity losses. The present study aims to investigate *Trichoderma longibrachiatum* TG1 ability to increase endogenous salicylic acid (SA) and antioxidant activity of wheat seedlings under different salt stress conditions. Wheat seeds were pretreated in TG1 spore suspension (1×10^8 spores ml^{-1}) for 12 h before exposure to different salt stresses. The result showed that SA content increased with increasing salinity at each stress stage, but primarily in TG1-treated seedlings compared with NaCl-treated seedlings. Compared to control, TG1 significantly ($p<0.05$) increased SA content by 27%, 49%, and 61% at 50, 100, and 150 mM NaCl stress, respectively. Similarly, TG1 induced and increased phenylalanine ammonia-lyase (PAL) activity by 18%, 27%, 31%, and 41%, respectively, compared to control. At 150 mM NaCl stress, shoot and root of TG1-treated seedlings superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) increased significantly by 52%, 50%, 73% and 49%, 60%, and 58%, respectively, compared to control. *~~Trichoderma~~ longibrachiatum* TG1 increased plant salinity tolerance via systemic acquired resistance pathway (SAR) and increased activity of antioxidant enzymes.

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 health (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 defense responses. SA has been suggested to have great agronomic potential to improve stress resistance of crops of agricultural importance (Miura & Tada 2014). Moreover, ~~salicylic acid~~ 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 salicylic acid (Kadıoğlu & Sağlam 2013; Moharekar *et al*. 2003). Again, it was reported that the synthesis of carotenoids and xanthophylls was induced by ~~salicylic acid~~ 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 ~~salicylic acid~~ 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). Using indole-3-acetic acid (IAA) or 1-aminocyclopropane-1-carboxylate (ACC) producing microbes such as *Trichoderma* to treat seeds is an effective method to increase stress tolerance and crop yield. 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 ~~physiological~~ metabolism and growth, an effective antioxidant capacity is essential (Saghafi et al. 2018).

However, the potential of *Trichoderma* species to increase endogenous ~~salicylic acid~~ and decrease ~~reactive oxygen species~~ of wheat seedlings under salt stress has not been investigated in various studies. The present study aims to investigate the potential of *Trichoderma 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 *Trichoderma 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 was quantified and stored at 4 degrees Celsius.

Salt Concentration Preparation

Salt tolerance was tested at NaCl concentrations of 0, 50, 100, and 150 mM. To one liter of liquid water agar medium, 0 g, 3 g, 6 g, and 9 g of NaCl were added. The solutions were then shaken vigorously to dissolve and autoclaved at 121 ℃.

Plant Material and Treatment

Wheat seeds (*Triticum aestivum* L.) ~~with a~~ cultivar ~~named~~ 'Yongliang 4' were used. According to the method of Gond et al. (2015), the seed was sterilized. Briefly, wheat seeds of equal sizes were surface sterilized with distilled water for 5 minutes to rinse the seedlings from dust and make them smooth for further treatment. Than 1% NaOCl was applied for 10 minutes for surface sterilization. All seedlings were rinsed with sterile water six to ten times after disinfection.

Seeds Treatment

Wheat seeds were soaked in (i) TG1 suspension only, and 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

Trichoderma 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 seeds without NaCl, (ii) TG1-treated wheat seeds with NaCl (50, 100, and 150 mM), (iii) untreated TG1 wheat seeds soaked with sterile water without NaCl, and (iv) untreated TG1 wheat seeds soaked with sterile water with NaCl (50, 100, and 150 mM). The physiological, biochemical, and molecular parameters of wheat seedlings were determined on day 8 after harvest. This was repeated six times with six sample replicates.

Growth Parameters

After 8 days of NaCl treatments, the wheat seedlings were harvested. The shoots and roots of the wheat seedlings were removed, washed three times with distilled water, dried, and weighed. Root length and weight were measured immediately after the 8-day growth period.

To determine the dry weight, all samples of wheat shoots and roots were oven-dried at 105°C for 30 minutes and then held at 80°C to maintain a constant weight before being weighed. Each maintenance and control was performed six times. The relative water content (RWC) of the shoots and roots was measured using Tian et al. (2015).

RWC(%) = (FW-DW)/FW ×100; where RWC represents relative water content, FW represents fresh weight, and DW represents the dry weight

148 **Chlorophyll and carotenoid content determination**

149 Total chlorophyll and carotenoids were extracted with 100% acetone according to the
150 method of (Bojović & Stojanović 2005). The fresh shoot of 0.2 g was homogenized with 10 ml
151 of acetone. The chlorophyll and carotenoid content were evaluated in a two-wavelength
152 spectrophotometer at the absorbance of ~~A~~661.6 nm, 644.8 nm, and 470 nm. This was repeated
153 six times with six sample replicates.

154

155 **Lipid peroxidation and H_2O_2 content determination**

156 The levels of malondialdehyde (MDA) and H_2O_2, a product of lipid peroxidation
157 produced by the thiobarbituric acid reaction and an indicator of oxidative damage to a system,
158 were measured according to the manufacturer's protocol using the assay kits provided (Solarbio,
159 China). The absorbance of each sample was measured at 450 nm, 532 nm, 415 nm, and 600 nm.
160 The content of MDA was expressed as μmol g^{-1} FW. This was repeated six times with six
161 sample replicates.

162

163 **Antioxidant enzymes and Phenylalanine ammonia-lyase (PAL) activity**

164

165 Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and Phenylalanine
166 ammonia-lyase PAL activities were measured respectively using assay kits (Solarbio, China).
167 Absorbance values were measured at 560 nm, 470 nm, 240 nm, and 290 nm respectively.

168

169 **Extraction of total RNA and analysis of gene expression by quantitative real-time reverse**
170 **transcriptase-PCR (qRT-PCR)**

171 Total RNA extraction and analysis of 100 mg wheat seedlings exposed to different levels of
172 NaCl stress was performed according to the methods of Xie et al. (2013) and using PureLink®
173 RNA Mini Kit (Tiangen Biotechnology, Beijing, China). The quantity and quality of isolated
174 RNA were analyzed using a Nano-Drop spectrophotometer at the absorbance of 230 and 260 nm.
175 The A260/A230 ratio indicated that the RNA was free from protein contamination. First-strand
176 cDNA synthesis was performed using Revert Aid TM First Strand cDNA Synthesis Kit (Tiangen
177 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.

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 with six sample replicates.

Endogenous salicylic (SA)

According to Wa[illegible] 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 distilled water to prepare a stock solution. To prepare the working solution, 1 ml of the stock solution was added to 9 ml of deionized water. 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, 100µl 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 $P < 0.05$.

206

207

208 **Results**

209 **Wheat seedling germination and growth**

210 Seed pretreatment with *T. longibrachiatum* TG1 significantly ($p<0.05$) affected the wheat
211 seeds germination (Table 2). Compared to NaCl treatment, TG1 stimulated germination rate
212 (GR), germination potential (GP), and germination index (GI) by 16%, 10%, and 9%,
213 respectively. The concentrations of NaCl also significantly ($p<0.05$) affected wheat
214 seedlings GR, GP, and GI. An increase in NaCl concentration decreased GR, GP, and GI. At
215 150 mM NaCl stress, GR, GP, and GI of NaCl-treated seeds decreased by 33%, 31%, and
216 32%, respectively, compared to control. However, at 150 mM NaCl stress GR, GP, and GI
217 of TG1- treated seeds decreased by 6%, 23%, and 22.9% respectively as compared to
218 control (Table 2).

219 *~~Trichoderma~~ longibrachiatum* TG1 significantly ($p<0.05$) affected the shoot and root
220 length of wheat seedlings (Fig. 1A and B). Shoot and root length of TG1-treated seedlings
221 increased by 20% and 15%, respectively, compared to NaCl-stressed plants. Moreover,
222 shoot and root length were significantly ($p< 0.05$) affected by the different concentrations of
223 NaCl. Shoot and root length decreased with an increase in NaCl concentration. Compared to
224 the control, the shoot and root length of NaCl-treated plants significantly decreased by 56%
225 and 62%, respectively, at 150 mM NaCl stress. However, TG1-treated shoot and root length
226 significantly decreased by 41% and 45%, respectively, at 150 mM NaCl stress compared
227 with the control (Fig. 1A and B). Although TG1 treatment increased leaf area, a clear
228 increase in shoot length and leaf area was observed in TG1-treated seedlings at 100 mM
229 NaCl compared with NaCl-treated seedlings (Fig. 1C).

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235 **Endogenous salicylic acid content and Phenylalanine ammonia-lyase (PAL) activity**

236

237 Wheat seeds inoculated with *T. longibrachiatum* TG1 resulted in a significant ($p<0.05$) increase
238 in SA content and PAL activity (Fig. 2). Compared to NaCl stress, TG1 increased SA content by
239 27% and PAL activity by 18% respectively.

240 Different concentrations of NaCl induced SA and PAL significantly ($p<0.05$). TG1-treated
241 seedlings increased SA -content, and PAL -activity at 150 mM NaCl by 61% and 41%,
242 respectively, compared to control. However, SA -content, and PAL -the activity of NaCl-treated
243 seedlings increased by 19% and 23%, respectively, at 150 mM NaCl compared with the control.
244 This is a consequence of the pre-adaptive effect of TG1 on plants exposed to salt stress through
245 the activation of the salt-dependent signaling pathway. Thus, the activation of defense responses
246 induced by TG1 might be related to its ability to increase the endogenous SA content, which
247 plays an important role in the induction of systemic acquired resistance in plants.

248

249 **Biomass accumulation and relative water content**

250 Total fresh weight (FW) and dry weight (DW) of wheat seedlings were significantly ($p<0.05$)
251 increased by *~~Trichoderma~~ longibrachiatum* TG1 (Table 3). Compare to the NaCl-stress, the
252 shoot FW and DW of TG1-treated seedlings increased by 29% and 24%, respectively. Besides,
253 the root FW, DW, and relative water content (RWC) of TG1-treated seedlings increased by 23%,
254 16%, and 2%, respectively, compared with NaCl-stressed seedlings. NaCl concentration
255 significantly ($p<0.05$) decreased the FW, DW matter, and RWC of wheat seedlings. The shoot
256 and root FW of NaCl-treated seedlings at 150 mM NaCl decreased by 74% and 68%,
257 respectively, compared to the control. However, the shoot and root FW of TG1-treated seedlings
258 at 150 mM NaCl stress decreased by 47% and 58%, respectively, compared to control.

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263 **Chlorophyll and carotenoid contents**

264 Wheat seedlings treated with and without TG1 were examined for pigmentation under
265 salinity stress on day 8. Seedlings pretreated with TG1 significantly ($p<0.05$) increased the
266 pigmentation of wheat seedlings (Table 4). Chlorophyll a, b, total (a+b) and carotenoid
267 content of TG1-treated seedlings increased by 24%, 32%, 28%, and 23%, respectively,
268 compared to NaCl-stressed seedlings. NaCl concentration significantly ($p<0.05$) decreased
269 the pigmentation of wheat seedlings. Chlorophyll a, b, total (a+b) and carotenoid contents of
270 NaCl-treated seedlings at 150 mM NaCl stress decreased by 55%, 73%, 63% and 58%,
271 respectively, compared to the control. However, Chlorophyll a, b, total (a+b) and carotenoid
272 contents of TG1-treated seedlings at 150 mM NaCl stress decreased by 32%, 40%, 35%, and
273 29%, respectively, compared to control. In conclusion, *Trichoderma longibrachiatum* TG1
274 pretreated seedlings showed increased pigmentation compared to NaCl pretreated seedlings
275 (Table 4).

276

277 **MDA and H_2O_2 accumulation**

278 The extent of lipid peroxidation and H_2O_2 content were determined after 8 days of
279 treatment. Salt stress-induced MDA and H_2O_2 content and its effect were significantly
280 ($p<0.05$) attenuated by TG1 (Table 5). The MDA content of shoot and root of TG1-treated
281 seedlings decreased by 26% and 27%, respectively, compared to NaCl-stressed seedlings.
282 H_2O_2 content in the shoot and root of TG1-treated seedlings decreased by 28% and 37%,
283 respectively, compared with NaCl-stressed seedlings. Increasing NaCl concentration
284 significantly ($p<0.05$) increased both MDA and H_2O_2 content. At 150 mM NaCl, MDA
285 content in shoot and root of NaCl-treated seedlings increased by 77% and 69%, respectively,
286 compared to the control. Similarly, the shoot and root H_2O_2 content of NaCl-treated
287 seedlings increased by 60% and 72%, respectively, compared to the control. However, MDA
288 content in shoot and root of TG1-treated seedlings at 150 mM NaCl increased by 70% and
289 57%, respectively, compared to the control. Similarly, H_2O_2 content in shoot and root of

TG1-treated seedlings at 150 mM NaCl increased by 50% and 52%, respectively, compared to the control (Table 5).

Accordingly, TG1 pretreatment decreased the accumulated MDA and H_2O_2 content in both shoot and root compared to non-TG1-treated seedlings under salt stress (Table 5).

Antioxidants enzymes activities

Trichoderma 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 46%, 38%, and 40%, respectively, compared to NaCl stress plants. Similarly, the activities SOD, POD, and CAT increased significantly by 30%, 22%, and 13%, respectively, in the roots compared with the NaCl plants.

An increase in NaCl concentration significantly ($p<0.05$) increased the enzyme activity. Under 150 mM NaCl stress, shoot and root activities of TG1-treated seedlings SOD, POD and CAT significantly increased by 52%, 50%, 73%, and 49%, 60%, and 58%, respectively, compared to the control (Fig. 3). Salt stress-induced and significantly increased the activities of antioxidant enzymes in wheat seedlings, including the activities of SOD, POD, and CAT (Fig. 3). Also, the activities of SOD, POD, and CAT were significantly increased after being treated with TG1 under salt stress compared with the control and NaCl-stressed.

Antioxidant gene expression

*Trichoderma longibrachiatum*TG1 significantly ($p<0.05$) enhanced the up-regulation of SOD, POD, and CAT genes in wheat seedlings under salinity stress (Fig. 4). However, the expression of SOD and POD genes was significantly higher in TG1-treated seedlings at 50 mM NaCl compared with the control. A significant decrease in transcript level was observed in SOD and POD at 100 and 150 mM NaCl, while the transcript level of CAT was high. Similarly, the gene CAT was expressed higher in TG1-treated seedlings at 100 mM NaCl than in the control. Significantly, the genes SOD, CAT, and POD were relatively up-regulated in the salt-tolerant TG1-treated seedlings at all NaCl concentrations compared with the housekeeping gene. This was enhanced by robust antioxidant enzyme activity and scavenging of reactive oxygen species (Fig. 4 A, B, and C).

320

321

322 **Discussion**

323 The application of naturally occurring plant-growth-promoting-rhizobacteria (PGPR) is
324 an emerging technology aimed at ameliorating the negative effects of salinity. Salinity remains
325 the major environmental threat to crop production which hinders plant growth. Salt tolerance in
326 plants is a complex trait that involves the corresponding action of several gene families that
327 perform different functions, such as regulation of stomata water loss, sequestration of ions,
328 metabolic and osmotic modifications, and antioxidant defense (Chakraborty et al. 2019). Plant
329 grafting and transgenic capabilities have been used to develop new cultivars with improved salt-
330 tolerant traits, but increasing the salt tolerance of cultivars has not been successful (Hu et al.
331 2012). Industrial use of transgenic plants to alleviate salinity resulted in the loss of genes, high
332 costs, and other regulatory concerns (Xie et al. 2017).
333 Some *Trichoderma* species are an important class of plant growth-promoting fungi that
334 have been shown to stimulate plant growth and improve salt stress tolerance (Oljira et al. 2020b).
335 In this current study, ~~*Trichoderma*~~ *longibrachiatum* TG1 alleviated salt stress; synthesize
336 phytohormones for plant growth, increased endogenous ~~salicylic acid (~~SA~~)~~, and antioxidant
337 activity of wheat seedlings under different levels of salt stress.
338 *In vitro* experiment, various concentrations of NaCl decreased the germination
339 parameters of wheat seed and growth significantly which corresponds with the hypothesis of the
340 study. Several previous studies have declared the negative effects of salinity on plant seeds
341 germination and growth both *in vitro* and under greenhouse (Azooz et al. 2011). Similarly,
342 sodium chloride treatment has been reported to inhibit root hair growth (Bhat et al. 2020).
343 In contrast to these findings, the germination rate of wheat seedlings increased, which is to be
344 expected when biocontrol agents are used, but conversely, application of salt-tolerant
345 ~~*Trichoderma*~~ *longibrachiatum* TG1 significantly doubled the germination and growth rate in the
346 saline medium. In addition, the application of salt-tolerant ~~*Trichoderma*~~ *longibrachiatum* TG1
347 reduced the deleterious effect of NaCl stress on wheat seedling growth, thereby increasing shoot
348 and root length and improved root hair formation, which is beneficial for stressed plants.
349 Similarly, the application of *T. harzianum* T22 enhanced tomato (*Lycopersicum esculentum* L.)

seed germination under abiotic stresses (Ma et al. 2012). Likewise, *Trichoderma longibrachiatum* T6 promoted wheat seedlings (*Triticum aestivum* L.) growth under NaCl stress by increasing shoot and root length (Zhang et al. 2019).

Salicylic acid (SA) regulates the activities of several enzymes, such as superoxide dismutase and phenylalanine ammonia-lyase, which are the 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 such as POD, SOD, and CAT. 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/reactive oxygen species (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 SAR pathway and scavenging of ROS via increased SOD, POD, and CAT activities.

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, *Trichoderma longibrachiatum* TG1 increased the translocation 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-rhizobacteria 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 *Trichoderma 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 leads 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. Superoxide dismutase, catalase, GPX, GST, and the enzymes of APX, DHAR, and GR are among the enzymatic antioxidants. These enzymes are essential in scavenging 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 (SOD, POD, and CAT). In this regard, *T~~richoderma~~ 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 *~~Trichoderma~~ 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.

Author Contributions: S.B. and T.L. performed the experiments, analyzed data, wrote and edited the manuscript. S.Z.; B.X. and A.C. conceived the idea and revised the manuscript. S.Z. designed the experiment, conceived the idea, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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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

HPLC- High-pressure liquid chromatography

DNA - Deoxyribonucleic acid

SGR - Seed Germination Rate

GI - Germination Index

GP- Germination Potential

NaCl- Sodium Chloride

ITS - Internal transcribed spacer

TEF- Translation elongation factor

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653 the brown algae *Lessonia nigrescens* enhance salt stress tolerance to wheat seedlings by
654 enhancing the antioxidant system and modulating intracellular ion concentration. 10:48.
655

656

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.

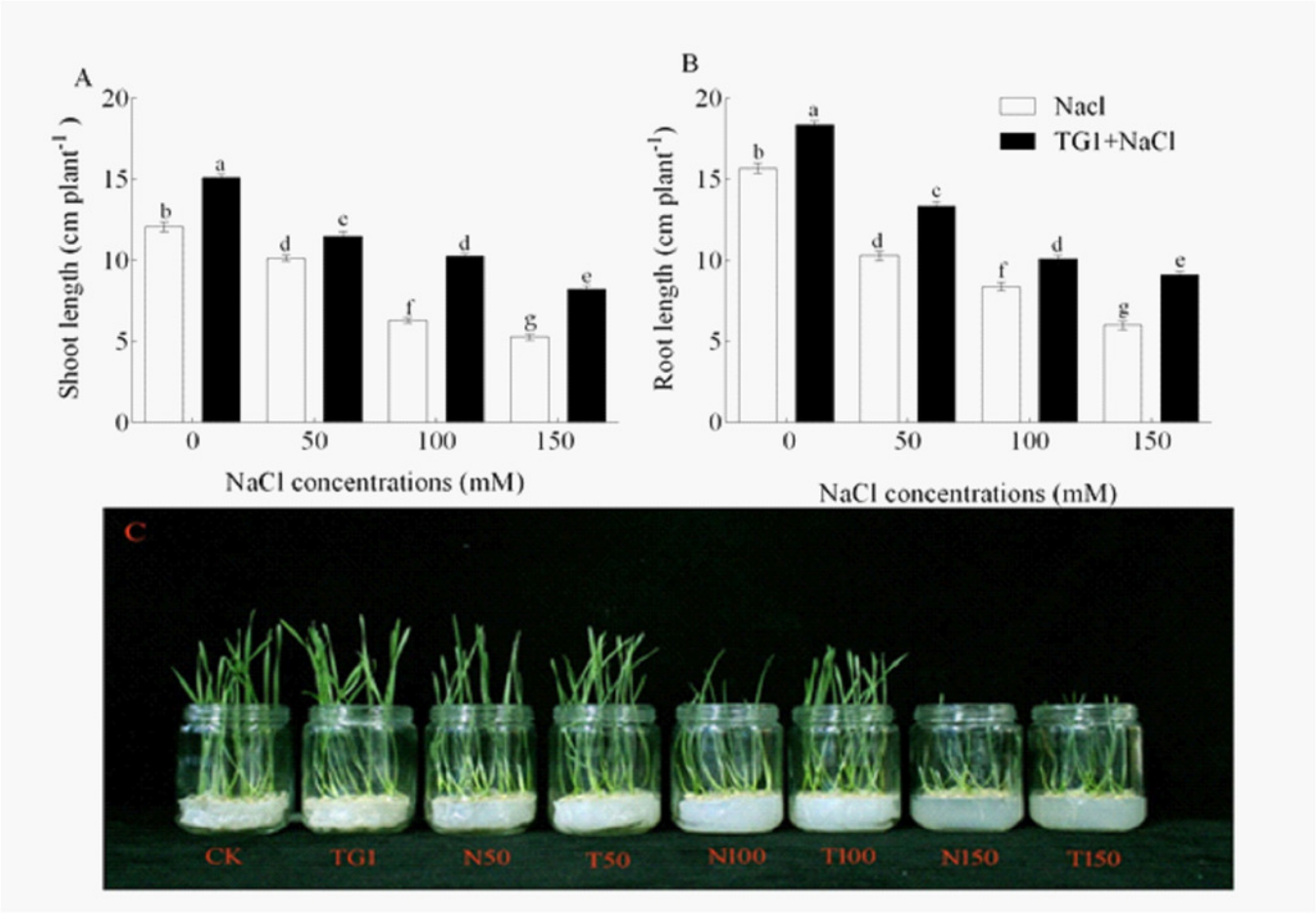
A
Shoot length (cm plant-1)
20
15
10
5
0
b
a
d
e
f
d
g
e
0
50
100
150
NaCl concentrations (mM)
B
Root length (cm plant-1)
Nacl
TG1+NaCl
NaCl concentrations (mM)
C
CK
TG1
N50
T50
N100
T100
N150
T150

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.

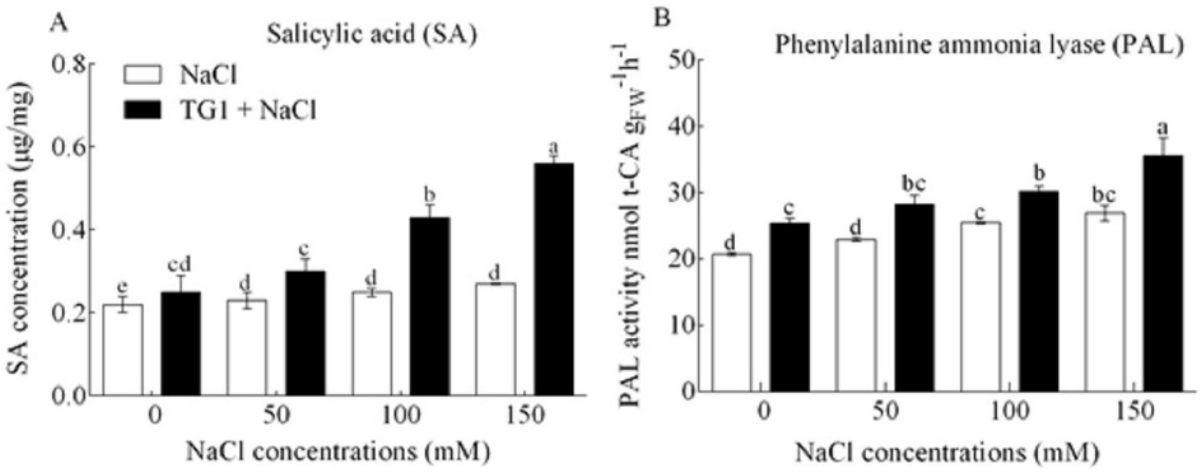

Figure 3

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.

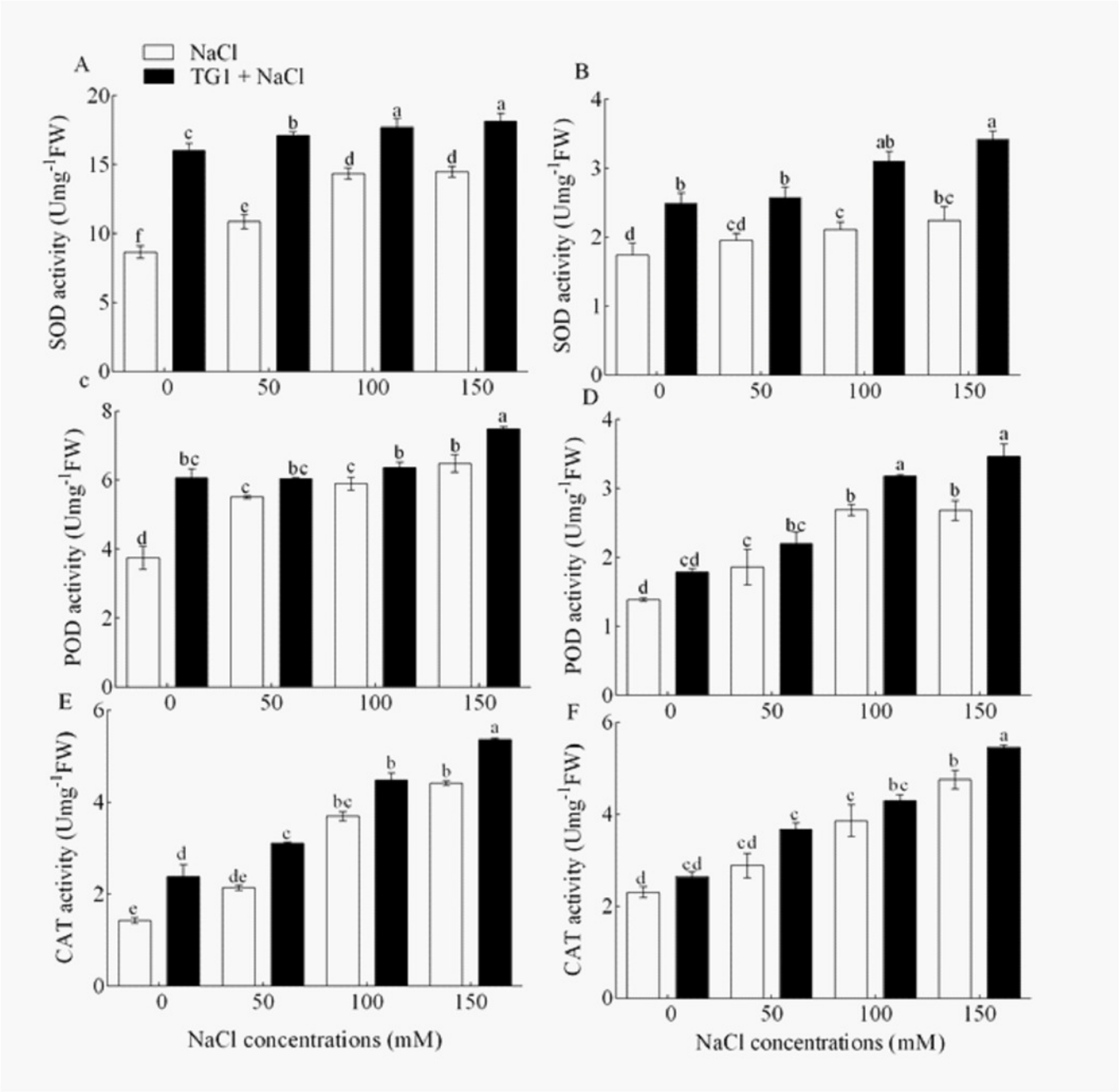
A
NaCl
TG1 + NaCl
SOD activity (Umg-1FW)
B
SOD activity (Umg-1FW)
C
POD activity (Umg-1FW)
D
POD activity (Umg-1FW)
E
CAT activity (Umg-1FW)
F
CAT activity (Umg-1FW)
NaCl concentrations (mM)
NaCl concentrations (mM)

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.

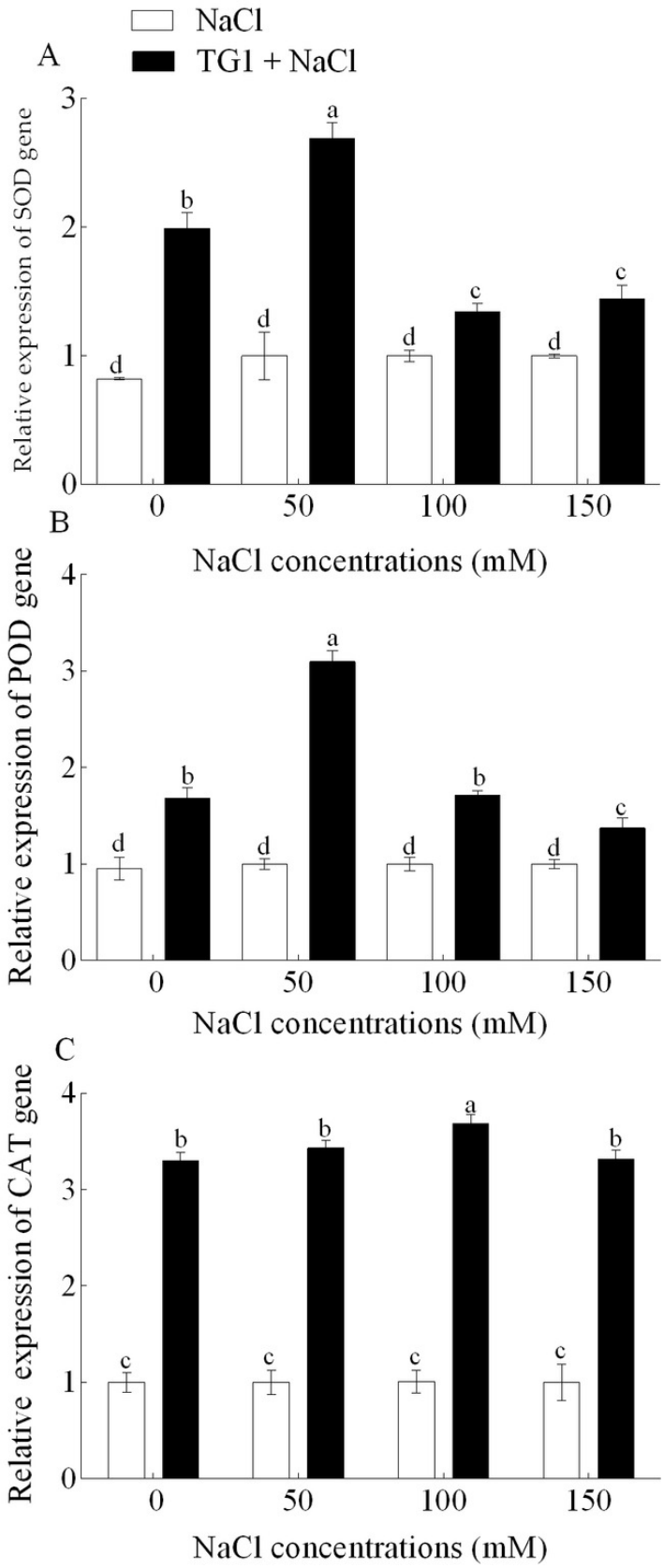
NaCl
TG1 + NaCl
A
Relative expression of SOD gene
B
Relative expression of POD gene
C
Relative expression of CAT gene
NaCl concentrations (mM)
0
50
100
150

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)

Effect of *T. longibrachiatum* TG1 on wheat seeds germination under different salt stress

Data are presented as means ± standard errors of replicates, and germination rate, potential, and indices were determined 3, 5, and 8 days after treatment, respectively. Different letters in the same column indicate significant differences at a level of $P < 0.05$ by Duncan's new multiple range test (n = 6). Control treatment represents wheat seedlings inoculated in distilled water; *Trichoderma longibrachiatum* TG1 treatment represents seedlings inoculated with 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 2 Effect of *T. longibrachiatum* TG1 on wheat seeds germination under different salt stress

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
	Average	**72.5B**	**67.5B**	**45.0B**
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
	Average	**86.25A**	**74.6A**	**49.72A**

Data are presented as means ± standard errors of replicates, and germination rate, potential, and indices were determined 3, 5, and 8 days after treatment, respectively. Different letters in the same column indicate significant differences at a level of $P < 0.05$ by Duncan's new multiple range test (n = 6). Control treatment represents wheat seedlings inoculated in distilled water; *Trichoderma longibrachiatum* TG1 treatment represents seedlings inoculated with 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)

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

Data are presented as mean ± standard error of replicates in a column followed by different letters representing significant difference at $P < 0.05$ (n = 6) based on Duncan's multiple range test using one-way ANOVA. The treatments are detailed in the footnote of Table 2.

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

Treatment	NaCl concentration (mM)	Wheat shoot Fresh weight (mg plant^{-1})	Wheat shoot Dry weight (mg plant^{-1})	Wheat shoot Relative water content (%)	Wheat root Fresh weight (mg plant^{-1})	Wheat root Dry weight (mg plant^{-1})	Wheat root 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	87.17±0.81a
	100	290.33±9.86f	29.83±1.45e	89.57±1.21a	137.77±4.43d	22.40±1.32e	83.68±1.29b
	150	148.67±8.06g	17.67±0.45f	87.99±0.91b	94.07±2.35e	19.83±0.33f	78.91±0.27d
	Average	**357.53B**	**36.34B**	**89.00A**	**185.73B**	**26.52B**	**84.36B**
TG1+ NaCl	0	653.33±4.09a	60.33±2.74a	90.77±0.38a	355.56±4.13a	38.23±0.50a	89.21±0.50a
	50	546.66±5.16c	52.32±1.36c	90.42±0.37a	288.86±4.45b	34.31±0.62b	88.12±0.12a
	100	504.44±6.05d	49.01±1.04c	90.29±0.21a	201.11±5.41c	30.60±0.31c	84.59±1.12b
	150	308.87±6.11f	30.88±3.32e	89.94±5.73a	123.56±5.62d	23.53±0.85e	80.84±0.97c
	Average	**503.33A**	**48.13A**	**90.36A**	**242.27A**	**31.67A**	**85.69A**

Data are presented as mean ± standard error of replicates in a column followed by different letters representing significant difference at $P < 0.05$ (n = 6) 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)

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

Data are presented as mean ± standard error of replicates in a column followed by different letters representing significant difference at $P < 0.05$ (n = 6) based on Duncan's multiple range test using one-way ANOVA. Treatments are detailed in the footnote of Table 2.

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

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
	Average	**1.92B**	**1.44B**	**3.35B**	**2.75B**
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
	Average	**2.53A**	**2.11A**	**4.64A**	**3.58A**

Table 5(on next page)

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

Data are presented as mean ± standard errors of replicates in a column followed by different letters representing significant difference at $P < 0.05$ (n = 6) based on Duncan's multiple range test using one-way ANOVA. Treatments are detailed in the footnote of Table 2.

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

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
	Average	**12.94A**	**6.69A**	**7.69A**	**5.68A**
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
	Average	**9.59B**	**4.90B**	**5.53B**	**3.59B**

Data are presented as mean ± standard errors of replicates in a column followed by different letters representing significant difference at $P < 0.05$ (n = 6) based on Duncan's multiple range test using one-way ANOVA. Treatments are detailed in the footnote of Table 2.