**Trichoderma reesei** improved the nutrition status of wheat crop under salt stress

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**ABSTRACT**

The use of plant endophytes to release salt stress is cheap and quick. In this respect, we have isolated an endophytic fungus (**Trichoderma reesei**) from **Solanum surattense** that promoted the wheat growth under salt stress. The results showed that the fungal inoculated wheat plants had higher chlorophyll a and b, carotenoids, reduced glutathione (GSH), ascorbate, peroxidase, catalase, weight of plant, flavonoids, total soluble protein, stomatal conductance, transpiration rate, and relative water contents. Also, **T. reesei** treated plants showed higher IAA, GA, Ca and K, while lower ABA, H₂O₂, phenol, sugar, proline, electrical conductivity, malondialdehyde (MDA), Na⁺ and Cl⁻. The results concluded that the integrative use of **T. reesei** might help the wheat plants to stand salt stress.

**Introduction**

Salinity as an abiotic stress can affect crop production by inducing osmotic and/or ionic imbalance inside plant cells. Salt stress limits the seed growth and germination (especially wheat) by inducing toxicity, reduction in water availability, immobilization of stored reserves and modifying the structural organization of proteins (Ibrahim 2016). To combat salinity stress, tolerant variety can be developed through traditional breeding or advanced molecular techniques, but the former is time consuming, while the latter is highly expensive. In the current scenario of quick expansion of human population and demands for food at a steady rate, it is necessary to discover the mechanisms that are cheap and quick. In this regard, one of the alternative ways to achieve normal plant growth under salt stress is the efficient utilization of plant promoting endophytes (Bilal et al. 2018; Ikram et al. 2018).

Plants are considered as rich reservoir of endophytes, mainly fungi and bacteria (Hussain et al. 2015; Hamayun et al. 2017; Ismail et al. 2018; Mehmood et al. 2019). The endophytic fungi and bacteria (Rhizobium, Pseudomonas, Bacillus, Azobacter, **Trichoderma**, Aspergillus, etc.) reside in the host tissues without any negative effects. In recent years, various studies have demonstrated the positive role of endophytic fungi in growth promotion of host under stress (Kang et al. 2012; Rehman et al. 2017). In symbiotic mode, endophytic fungi can promote plant growth, increase nutrient uptake, plant fitness by water use efficiency, discouraging pathogenic attacks and curtailing abiotic stresses (Hussain et al. 2018; Mehmood et al. 2018; Jan et al. 2019). **Endophytes can help in** phosphorus and potassium solubilization, and provide higher amounts of nitrogen. These nutrients can promote stem diameter and length, number of leaves, number of seeds and seed dry weight. Also, endophytes having 1-aminocyclopropane-1-carboxylate (ACC) deaminase can encourage the host plants to grow normal under stress. The ACC deaminase converts the ethylene precursor (1-aminocyclopropane-1-carboxylate) to ammonia and 2-oxobutanoate and blocks the ethylene signaling pathway (Hussain et al. 2018; Ismail et al. 2018). Some of the endophytes can induce salt tolerance in vulnerable host plant species by increasing the levels of antioxidants (Hamayun et al. 2017) or controls osmo and stomatal regulation (Hardoim et al. 2015). In short, endophytic fungi produce a range of important bioactive metabolites (flavonoids, ACC deaminase and polyphenols, etc.) and plant hormones (gibberellins, indole acetic acid, cytokinins, etc.) that enable crop plants to stand firm against various stresses, including salinity (Hamayun et al. 2017; Bilal et al. 2018; Hussain et al. 2018; Ikram et al. 2018).

Among the endophytic fungi, **Trichoderma sp.** (soil-borne filamentous fungi) has been known for its potential to help host plant species against biotic and abiotic stresses (Contreras-Cornejo et al. 2016; Nieto-Jacobo et al. 2017). Many **Trichoderma sp.** can ably secrete indole-3-acetic acid (IAA) to control various aspects of plant growth under saline conditions (Contreras-Cornejo et al. 2016). Moreover, **T. atroviride** and **T. virens** has promoted the initiation of lateral roots and root hairs in **Arabidopsis** plants under normal and saline conditions (Contreras-Cornejo et al. 2014). Keeping the potential benefits of endophytes in view, the present study has been designed to isolate endophytic fungi from **S. surattense** and to screen out the growth promoting species.

**Materials and methods**

**The Solanum surattense** plants were gathered from the saline area of district Kohat, wheat variety Bhakkar-2000

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(KJ672075) were generously provided by the National Agriculture Research Center (NARC), Islamabad. All the chemicals were purchased from Sigma-Aldrich.

**Isolation of endophytic fungi**

The collection of *S. surattense* plant samples were performed in saline areas of the Kohat District (33°35′13′′ N, 71°26′29′′ E), Pakistan. From the collected plants, 5 plants were washed thoroughly in the laboratory under the tap water. Roots from the cleaned plants were detached, cut into 25 small pieces. Ethanol (70%) treatment for 2 min, followed by 1% perchloric acid for 30 sec was used to sterilize the segments. The sterilized segments were then washed with ddH2O to remove residues of ethanol and perchloric acid (Khan et al. 2008). The outer layer of the sterilized root segments were removed and 5 segments per plate were placed on Hagem medium (0.5 g/l KH2PO4, 0.5 g/l MgSO4·7H2O, 0.5 g/l NH4Cl, 5 g/l glucose, 80 mg/l streptomycin, 1 g/l FeCl3, and 15 g/l agar; pH 5.6 ± 0.2) to isolate endophytic fungi. Parafilm was used to seal the plates and the plates were put in an incubator at 27°C for one week to allow endophytic growth. The emerged fungal colonies were separated and cultured on potato-dextrose agar medium plates. Plates having individual fungal colonies were incubated for another 7 days at 25°C and the process was repeated till the collection of pure cultures or colonies. Czapek culture broth (10 g/l peptone, 10 g/l glucose, 0.5 g/l MgSO4·7H2O, 0.05 g/l KCl, 0.01 g/l FeSO4·7H2O; pH 7.3 ± 0.2) as a growth medium was used to collect fungal biomass and culture filtrates. The broth having the fungal isolate after incubation was transferred to shaking incubator, operated at 120 rpm and 30°C. The broth was further incubated for 7 days in shaking incubator. The harvesting of the culture filtrate (CF) was done by centrifugation at 4000 × g at 4°C for 15 min. The collected pellet and supernatant were then lyophilized (ISE Bondiro Freeze Dryer) and kept at ~70°C.

**Screening of isolated strains for salinity stress tolerance**

The resistance of isolated strains against various salt (NaCl) concentrations (0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mM) was checked by allowing the isolates to grow in Hill and Kafer broth medium (Hill and Kafer 2001) supplemented with NaCl. The cultures in a salt medium were incubated at 27°C in a shaker (120 rpm) for 7 days and the growth of isolates was measured in terms of fresh and dry weight by collecting fungal biomass as described by Kumar et al. (2009). The resistant strain was then checked for various growth parameters and applied to wheat plants under salt stress.

**HPLC analysis of indole-acetic acid (IAA)**

The salt-resistant strain was cultured in the Czapek media with or without tryptophan (0.1 g/l). The media were then shifted to an incubator, operated at 30°C for 7 days. The concentration of IAA in the culture medium was analyzed by a high-performance liquid chromatography (HPLC). Chromatograms were acquired by injecting 20 µl of the filtrate into a 5 µm reverse phase column (Waters Associates µBondapak C18, 250 mm × 4 mm). Isocratic conditions were kept to separate the components of the sample. MeOH and water in a ratio of 80:20 v/v were used as a mobile phase. The flow rate of the mobile phase was adjusted to 1.0 ml/min. The sample components were detected by ultraviolet (UV) detector at 280 nm. Standard IAA was used to quantify IAA in the culture media.

**Supplementation of various L-tryptophan concentrations to media**

IAA production of the selected fungal isolate was analyzed with elevated levels of L-tryptophan. The fungal isolate was allowed to grow in Czapek broth supplemented with elevated tryptophan concentrations, i.e. 100, 500, and 1000 µg/ml. The culture in the flask was then incubated for 7 days at 28°C and 120 rpm. The IAA was identified and quantified by HPLC.

**Supplementation of nitrogen and carbon sources to media**

Fungal isolate was allowed to grow in Czapek broth augmented with nitrogen and carbon sources, i.e. peptone (10 g/l) + sucrose (5 g/l) and yeast (6 g/l) + glucose (5 g/l). The culture in the flask was then incubated for 7 days at 28°C and 120 rpm. The IAA was identified and quantified by HPLC.

**Phosphate solubilization**

The isolated fungal strain was cultured on Pikovskaya agar media (10.0 g/l dextrose, 0.5 g/l yeast extract, 0.50 g/l ammonium sulfate, 5.0 g/l calcium phosphate 0.2 g/l potassium chloride, 0.0001 g/l manganese sulfate, 0.1 g/l magnesium sulfate, 0.0001 g/l ferrous sulfate and 15 g/l agar) and incubated at 27°C for 48 h to observe a clear zone around the tested strains (Wahyudi et al. 2011).

**Siderophores activity**

In the case of siderophores, appearance of orange halos by the isolated endophyte indicates siderophores production. The siderophores activity was determined by the inoculation of endophyte on Chrome Azurol S (CAS) plates by the method of Schwyn and Neilands (1987).

**Ammonia production**

Fungal isolate CGF-11 was evaluated for ammonia production in peptone water. Fungal cultures were transferred to glasstubes containing 10 ml of peptone water. The tubes were then placed in preset (27°C) incubator for 48–72 h. After incubation each tube was added with 0.5 ml of Nessler’s reagent. The appearance of brown-yellow color upon the addition of Nessler’s reagent indicated a positive test for ammonia production (Joseph et al. 2012).

**Enzymatic activity of the isolated endophyte**

The methodology of Østergaard and Olsen (2011) was applied for l-aminocyclopropane-1-carboxylic acid deaminase (ACC) activity with some modifications adopted from Shaharoona et al. (2006). A standard curve was plotted by using α-ketobutyrate (10–200 µmol). The concentration of
produced α-ketobutyrate by the culture filtrates was determined at 540 nm.

Catalase activities of fresh culture isolate were detected on glass slide method as described by Mac Faddin (1976). Production of gas bubbles upon the addition of H2O2 revealed the existence of catalase.

The oxidase enzyme was observed by using the Kovac’s Reagent as described by Steel (1961). The appearance of black color demonstrated the presence of oxidase enzyme.

**Extraction of DNA from the isolated strains**

Extraction of DNA from the selected strain was performed, according to the well-established protocol of Khan et al. (2008). The purity of the extracted DNA and its quantity was measured by Thermo Scientific Nano Drop spectrophotometer at 260 nm (Chen and Kuo 1993).

**Identification of fungal isolate**

Selected endophytic fungal strain was identified by amplifying their ITS region of 18S rDNA with universal primers ITS-1 (5′-TCC GTA GGT GAA CCT GCGG-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Lee Taylor and Bruns 1999). A 20 ng of gDNA as template was mixed with a 30 µl of EF-Taq (Solgent, Korea) and the mixture was placed in a PCR machine. The conditions of PCR were: 95°C for 2 min; 35 cycles (95°C for 1 min, 55°C for 1 min and 72°C for 1 min); 72°C for 10 min. The PCR products along with DNA markers (DNA ladder) were then loaded onto an agarose gel and subjected to electrophoresis for 30 min. The gel was developed by using 0.01 g/ml ethidium bromide stain and examined under UV lamp.

**Sequencing of isolated strains**

A purified PCR products of 1600 bp were sequenced with 18S rDNA region by utilizing universal primers ITS-1 (5′-TCC GTA GGT GAA CCT GCGG-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Lee Taylor and Bruns 1999). A Big Dye terminator cycle sequencing kit v.3.1 was used for that purpose. Both PCR sequencing and amplification were analyzed by an automated DNA sequencing system (Applied Biosystems, Foster City, USA) at the Macrogen, Inc., Seoul, Korea.

The obtained PCR product was initially sequenced and then subjected to a homology search by using online tool, BLAST (http://blast.ncbi.nlm.nih.gov/Blast).

**Potential of culture filtrate and biomass of selected entophytic fungi to ameliorate the salinity stress of wheat**

For the pot experiment, soil (200 g) was sterilized and transferred to the pots. Healthy seeds of wheat variety Bhakkar-2000 (KJ672075) provided by NARC Islamabad were surface sterilized with 70% ethanol for 1 min, followed by dipping in 1% perchloric acid for 30 s. The seeds were rinsed thrice with ddH2O to remove traces of ethanol and perchloric acid. The experiment was carried out in triplicate and each replicate comprised of 10 pots with 4-seedlings (total = 4 × 10 × 3 = 120 seedlings per treatment). All the pots were kept in greenhouse at 30°C, 80% of relative humidity under 12 h light and 12 h dark regime. After one week of growth, the culture filtrates (20 ml) as T. reesei inoculum and 3 g of T. reesei mycelium was applied to the pots labeled as fungal associated plants. Pots without active culture biomass and culture filtrate were assigned control. At 3 leaf stage the plants were subjected to different concentration of NaCl (0, 60, 120, 180 mM) and with (TR/Trp) or without (TR) exogenous L-tryptophan (1000 µg).

The four treatments were defined as:
- Control = no NaCl added
- Low concentration = 60 mM NaCl
- Moderate concentration = 120 mM NaCl
- High concentration = 180 mM NaCl

Salinization was done at three leaf stage that took 3 weeks. After the fourth weak, plants were harvested and analyzed for various physicochemical parameters.

**Extraction of antioxidant enzymes**

After 30 days of sowing, wheat plants were harvested and stored at −70°C before investigating the enzyme activities. The enzymatic activity exhibited by various enzymes were analyzed by using spectrophotometer.

Peroxidase (POD) activity was determined by the method outlined by Gorin and Heidema (1976). The activity of ascorbate (APX) through ascorbic acid oxidation by the method of Asada (1987). Catalase (CAT) activity was observed by following the method of Chandlie and Scandalios (1984). Reduced glutathione (GSH) activity was determined by measuring the oxidation of NADPH according to the well-established method of Ellman (1959).

**Chlorophyll contents**

Chlorophyll contents of wheat leaves were quantified by chlorophyll meter (Spad-502 plus, Japan). MacKinney equations were used to determine the Chl a, Chl b, and carotenoids (Sestak 1971). Fully expanded fresh leaves from fungal inoculated and non-inoculated wheat plants were detached. The detached leaves were homogenized in 2 ml of acetone (80%). The final volume of the homogenate was brought to 7 ml with acetone. The absorbance was then observed at 480, 645, and 663 nm.

**Total flavonoids**

Calorimetric method was followed to measure the total flavonoids in the sample (Chang et al. 2002). Fresh leaves (0.25 g) were put into deionized water (1.25 ml). A 0.75 ml of sodium nitrite solution (5%) was then added to it and left for 6 min at room temperature. After incubation, the mixture was supplemented with 0.15 ml of AlCl3 (10%). The mixture was incubated for another 5 min at room temperature. Finally, 0.5 ml of sodium hydroxide (1 M) was added to the mixture and the volume was set to 2.5 ml with distilled water. The mixture was kept for 30 min at 25°C and the absorbance was determined at 510 nm; quercetin was used as a standard.

**Total phenols**

Total phenolics were estimated by the modified Folin–Ciocalteu colorimetric method (Cai et al. 2004). Fresh leaves (0.2 g)
were added to 0.5 N Folin–Ciocalteu reagents and left for 4 min at room temperature. The reaction was then neutralized with saturated Na₂CO₃ (75 g/l). The tubes were placed in the boiling water bath for 60 s and the absorbance was observed at 650 nm; gallic acid was used as a standard.

**Total sugar**

Total sugar was determined by the method as described by Bates et al. (1973). In this method, fresh leaves (0.2 g) were homogenized in 4 ml of water. The homogenate was then incubated for 30 min at 100°C in a water bath. The boiled mixture was cooled at room temperature and centrifuged for 10 min at 12,000 rpm. The supernatant was transferred to a new tube and supplemented with 10 ml of water and 0.2 ml of an anthrone solution (0.5 g anthrone dissolved in 500 ml of 80% sulfuric acid solution). The mixture was kept at 100°C for 10 min and the absorbance was finally read at 620 nm.

**Total protein**

Fresh leaf samples (0.2 g) were homogenized in 4 ml of phosphate buffer (0.01 M, pH = 7.0) in order to determine the protein in the sample. The homogenate was then centrifuged at 12,000 rpm for 10 min and the supernatant was collected. The collected supernatant (2 ml) was then supplemented with 4 ml of Coomassie brilliant blue G250 solution. The absorbance of the mixture was measured at 595 nm.

**MDA content**

Malondialdehyde (MDA) content was estimated by the procedure of Heath and Packer (1968). Fresh leaf samples (0.2 g) were homogenized in 10 ml of 10% trichloroacetic acid. The homogenate was centrifuged at 12,000 rpm for 10 min and the supernatant was carefully pipetted into a new tube. Thiobarbituric acid (2 ml of 0.6%) was added to the supernatant (2 ml) and the mixture was incubated in a water bath for 15 min at 100°C. The mixture was cooled and centrifuged at 12,000 rpm for 10 min. The absorbance of the supernatant was calculated at 532, 600, and 450 nm.

**Proline content**

The method of Bates et al. (1973) with some minor changes was carried out to monitor the proline content. Fresh leaf samples (0.2 g) were homogenized in 5 ml of 3% aqueous sulphosalicylic acid. The homogenate was centrifuged for 10 min at 12,000 rpm. To 2 ml of the supernatant, added 2 ml of acid ninhydrin and 2 ml of glacial acetic acid. The mixture was boiled in a water bath at 100°C for 60 min. The boiled mixture was then toluenized with 4 ml of toluene. The absorbance of chromophore was measured at 520 nm.

**Physiological analyses**

Gas exchange attributes and transpiration rate was measured as described by Ashraf et al. (2003). H₂O₂ was measured by the method of Bernt and Bergmeyer (1974). Electrolyte leakage was estimated by the method of Lutts et al. (1996), using an Electrical Conductivity Meter (HM EC-3M).

Root and shoot length was measured with a scale, whereas analytical balance was used to measure the fresh weights of shoots and roots. The dry weight of the roots and shoots were measured after drying the tissues in a forced draft oven at 70°C for 48 h.

The relative water content (RWC) was calculated by adding 1 g fresh roots and shoots to 50 ml of distilled water in a 100 ml flask. The contents of the flask were left for 4 h at room temperature. The turgid sample was then dried in an oven at 70°C for 48 h and the dry weight was calculated. The RWC was determined as:

\[ RWC (%) = \left[ \frac{(FW - DW)}{(TW - DW)} \right] \times 100 \]

where FW = fresh weight; DW = dry weight; TW = turgid weight

**Estimation of IAA, GA3 and ABA**

The purification of isolated plant hormones was done by using HPLC according to the method of Kettner and Dörfling (1995). Fresh leaves (1 g) were crushed at 4°C in 80% methanol and butylated hydroxy toluene (an antioxidant). Centrifugation of the extract was carried out at 3000 rpm after 72 h. The supernatant was collected in a tube and portioned against the ethylacetate (1/4th volume of the extract) at a pH of 2.5–3. The ethyl acetate fraction was collected and dried in rotary thin film evaporator (RFE). The residues obtained after drying were re-dissolved in 100% methanol. The methanolic solution was filtered through a Millipore filter (0.45 μm) and was subjected to HPLC (Agilent 1100). The HPLC assembly was equipped with variable UV detector and C18 column (39 × 300 mm) (Bondapack Porasil C18, 37/50 μm, Waters, Eschborn, BRD). The mobile phase consisted of methanol and water (30:70; v/v). Flow rate of the mobile phase was kept at 1500 μL/min with a run time of 20 min/sample. The identification of plant hormones was based on the comparison with the retention time of standards.

**Mineral analyses**

Leaves sample (0.25 g), previously dried in an oven was taken in a 50 ml flask. A 6.5 ml of acid solution consisting of nitric acid, sulfuric acid, perchloric acid (5:1:0.1) was added to it. The mixture was digested in a fume hood on a hot plate. Appearance of white fumes indicated the complete digestion of the sample. After the digestion, the contents of the digestion flask were allowed to cool. The cooled digest was collected in 50 ml volumetric flasks and the volume was set to 50 ml with distilled water. The extract was passed through the Whatmann filter paper before quantifying the mineral elements by Atomic Absorption Spectrophotometer (Shimadzu AA-670) (Allen et al. 1974).

**Microscopic analysis**

The roots segments of host wheat plants inoculated with *T. reesei* were sectioned after receiving salinity stress. The sub-segments/sections were then cleaned with sodium hypochlorite (3%) for 5 min to ensure the aseptic condition. The root were then acidified with 10% HCL and stained in 90% lactic acid for 24 hrs. The root segments
were finally subjected to a light microscope (Stemi SV 11 Apo, Carl zeiss) to observe active colonization (Hamayun et al. 2010).

**Statistical analysis**

The experiments comprised of 4 plants per pot, while each treatment comprised of five replicates. The recorded data were analyzed by SPSS-20 at $p < 0.05$. The significant means were separated by Tukey’s HSD test. The graphical sketches were drawn using 6.01 versions GraphPad Prism (San Diego, California, USA).

**Results**

**Screening of isolated strains for salinity stress tolerance**

A total of four strains of endophytic fungi (CGF-1, CGF-11, CGF-111 and GF-1111) were isolated from the root of halophytic plant *S. surattense* cultured on Hagem media (results not shown). Among all the isolated strains, the strain CGF-11 was only selected for further experiments on the basis of resistance to salt stress.

Response to salt tolerance by endophytic CGF-11 was noticed in the form of visible fungal growth at various concentrations of applied NaCl (0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mM). The CGF-11 strain has resisted NaCl stress till 200 mM, whereas the recorded % inhibition of fungal biomass on dry and fresh weight basis have been 18% and 20%, respectively. At 250 mM of NaCl concentration, a drastic changes in fungal biomass (50% inhibition) has been noted on both dry and fresh weight basis (Figure 1(A)).

**Production of IAA in various medium by endophytic fungi**

Endophytic fungi (CGF-11) isolated from *S. surattense* has been tested for plant hormones production on Hagem media supplemented with various sources (yeast, malt, sucrose, glucose, tryptophan). The IAA produced by CGF-11 on various sources have been detected calorimetrically, which has been confirmed and quantified by HPLC technique. The amount of IAA varied, depending on the different sources used in the medium. Fungal strain (CGF-11) yielded significantly ($p \leq .05$) higher concentrations of IAA on medium supplemented with 500 µg of tryptophan, followed by sucrose, yeast, malt and glucose extract, respectively (Figure 1(B)).

**Identification of the fungal strain CGF-11**

BLAST analysis showed sequence homology of isolate CGF-11 with *T. reesei* QM6a, having 99% sequence homology in the MP Dendrogram. The isolate ‘CGF-11’ was finally identified as *T. reesei* through phylogenetic analysis and sequence homology. ITS rDNA sequence has been submitted to NCBI GenBank with an Accession No. KY100257.1.

**Growth promoting activities of T. reesei**

The isolated fungal strains *T. reesei* exhibited the ability to secrete ammonia and solubilize phosphate. Also, *T. reesei* has demonstrated ACC deaminase and siderophores activities in a Petri plate assays (Figure 2).

**Physicochemical changes in T. reesei associated wheat plants under salinity stress**

**Effect on oxidative stress**

The level of peroxidase, reduced glutathione (GSH), ascorbate and catalase activity have been significantly affected ($p \leq .05$) by the *T. reesei* associated (TR and TR/Trp) in comparison to control plants (Figure 3). The peroxidase and ascorbate activity have been increased in all treatments with increased salinity, but slower trends have been noticed in TR/Trp plants, followed by TR plants and control (Figure 3(A,D)). Moreover, TR/Trp and TR treated plants displayed reverse trends in GSH and catalase activity as compared to the control. Certainly, as soon as the plants exposed to the salinity, a significant ($p \leq .05$) decrease in catalase and increase in GSH activity has been noticed in TR/Trp and TR plants unlike control (Figure 3(B, C)).

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**Figure 1.** Salt tolerance ability and IAA production by isolated CGF-11 strain. (a) represents salt resistance assay of endophytic strain CGF-1 cultivated under various concentrations of NaCl; (b) represents production of IAA by CGF-11 on media supplemented with different nutrient sources; Suc = sucrose; Glu = glucose; Trp = tryptophan. Means followed by different letters are significantly different at $p \leq .05$. Each bar shows ±SE of means.
The effect of *T. reesei* association with wheat plants (TR/Trp and TR) under salinity stress was determined by assessing the growth attributes of wheat plants after 30 days of germination. The result revealed a significant (*p* ≤ .05) increase in shoot and root lengths in *T. reesei* inoculated wheat plants as compared to the control. Under salt stress (60, 120 and 180 mM of NaCl) conditions, the endophytic strain facilitated the host wheat plants to grow normal roots and shoots (Figure 4(A, B)). Similarly, the dried and fresh weight of shoots and roots of TR/Trp plants have been significantly (*p* ≤ .05) higher, followed by TR plants, whereas the control plants have lower shoots and roots weight at all tested salt stress concentrations (Figure 4(C–F)).

### Chlorophyll and carotenoid contents

Total chlorophyll (Chl), Chla and Chlb contents of non-inoculated wheat plants have significantly (*p* ≤ .05) decreased with increase in salinity, whereas minor changes in the Chl, Chla and Chlb have been noticed in TR/Trp and TR plants (Figure 5(A–C)). Similarly, carotenoid contents of TR/Trp and TR plants have been less affected by salinity stress as compared to the control plants (Figure 5(D)).

### Effect of *T. reesei* on water contents and stress mitigation in wheat plants

Control, TR and TR/Trp treated wheat plants differed significantly (*p* ≤ .05) in water content (Figure 6(A)). *T. reesei* inoculated wheat plants retained higher water contents, when exposed to salt stress (Figure 6(A)). The H$_2$O$_2$ content and electrolytic leakage (EL) has barely affected in TR/Trp plants. The control plants on the other hand released significantly (*p* ≤ .05) higher electrolytes and have higher H$_2$O$_2$ content during stress conditions (Figure 6(B,C)). Although, salinity has negatively affected the transpiration rate, but *T. reesei* helped the host plant to overcome this effect. The transpiration rate of *T. reesei* associated wheat plants was far better at various levels (60, 120, 180 mM) of salt stress (Figure 6(D)). Similarly, the control plants under salinity stress had significantly (*p* ≤ .05) reduced stomatal conductance whereas, promising results have been observed in plants associated with *T. reesei* under salt stress (Figure 6(E)).
Figure 4. Effect of T. reesei on growth parameters of wheat plants under salt stress. (A) represents Shoot length (SL); (B) represents root length (RL); (C) represent shoots fresh weight; (D) represent roots fresh weight; (E) represent shoots dry weight; (F) represent roots dry weight; Ctrl = control plants; TR = T. reesei inoculated plants; TR/Trp = plants inoculated with T. reesei harvested from a medium supplemented with 500 µg of tryptophan. Means followed by different letters are significantly different at p ≤ 0.05. Each bar shows ±SE of means.

Figure 5. Effect of T. reesei on chlorophylls and carotenoids contents of wheat plants under salt stress. (A) represents total chlorophyll (Chl) contents; (B) represents chlorophyll a (Chla) contents; (C) represents chlorophyll b (Chlb) contents; (D) represents carotenoid contents; Ctrl = control plants; TR = T. reesei inoculated plants; TR/Trp = plants inoculated with T. reesei harvested from a medium supplemented with 500 µg of tryptophan. Means followed by different letters are significantly different at p ≤ 0.05. Each bar shows ±SE of means.
Biochemical analyses of plants
The mitigating response in *T. reesei* inoculated wheat plant under salt stress was evaluated for lipid peroxidation. Significantly (*p* ≤ .05) lower concentrations of malondialdehyde (MDA), total soluble sugar, proline and polyphenol have been noticed in wheat plants associated with *T. reesei* as compared to control plants (Figure 7(A–D)). Conversely, the total soluble proteins and flavonoids in TR/Trp and TR plants under salt stress conditions has been significantly (*p* ≤ .05) higher than that of control plants (Figure 7(E,F)).

Phytohormones contents
The recorded ABA concentration in TR/Trp plants has been significantly (*p* ≤ .05) lower, followed by TR plants. However, the ABA contents of the non-inoculated control plants increased with an increase in salinity (Figure 8(A)). Furthermore, higher concentrations of GA were noticed in plants inoculated with *T. reesei* (Figure 8(B)). The TR/Trp helped the wheat plants to resist 60, 120 and 180 mM of NaCl by releasing higher amounts of GA. Likewise, the concentration of IAA in *T. reesei* associated plants have been high, where the IAA contents of control plants have significantly (*p* ≤ .05) fallen with the salt stress (Figure 8(C)).

Nutrients analysis under stress condition
The results have shown lower concentrations of Na⁺ and Cl⁻ ions before salt stress irrespective of endophytes association. However, after inductions of salt stress the levels of Na⁺ in non-inoculated wheat plants increased significantly (*p* ≤ .05) as compared to endophytic associated wheat plants (Figure 9(A)). Similar, pattern of distribution has been noticed for chlorine ions (Figure 9(B)), suggesting the role of endophytes in inhibiting the transportation of Na⁺ and Cl⁻ ions across roots and leaves. Moreover, salinity has induced a decrease in Ca²⁺ and K⁺ concentrations in leaves of non-inoculated endophytic plants (Figure 9(C,D)).

*T. reesei* association with wheat plants
Study of the wheat roots inoculated with endophytes under the light microscope displayed an active association of *T. reesei* (Figure 10).

Discussion
Plants are susceptible in their response to mediate salt stress, amplifies ionic imbalance inside organs, arresting the plant growth, productivity and creates water deficiency. Various reports have favorably demonstrated the role of plant beneficial *Trichoderma* spp. The species in symbiotic relationship

Figure 6. Plant water potential and stress mitigation of *T. reesei* inoculated wheat plants. (A) represents RWC (relative water content); (B) represents EC (electrical conductivity); (C) represents *H₂O₂* (hydrogen peroxide); (D) represents TR (transpiration rate); (E) represents SC (stomatal conductance); Ctrl = control plants; TR = *T. reesei* inoculated plants; TR/Trp = plants inoculated with *T. reesei* harvested from a medium supplemented with 500 µg of tryptophan. Means followed by different letters are significantly different at *p* ≤ .05. Each bar shows ±SE of means.
Figure 7. Effect of endophyte T. reesei on biochemical contents of host plants under salinity stress. (A) represents MDA content; (B) represents proline content; (C) represents Tot. sol. sugar; (D) represents phenol; (E) represents Tot sol. ptn.; (F) represents flavonoids contents; MDA = malondialdehyde; Tot. sol. sugar = total soluble sugars; Tot. sol. ptn = total soluble proteins; Ctrl = control plants; TR = T. reesei inoculated plants; TR/Trp = plants inoculated with T. reesei harvested from a medium supplemented with 500 µg of tryptophan. Means followed by different letters are significantly different at $p \leq .05$. Each bar shows ±SE of means.

Figure 8. Effect of T. reesei on phytohormones of wheat plants under salt stress. (A) represents IAA content; (B) represents GA content; (C) represents ABA content; IAA = indole acetic acid; GA = gibberellins; ABA = abscisic acid. Ctrl = control plants; TR = T. reesei inoculated plants; TR/Trp = plants inoculated with T. reesei harvested from a medium supplemented with 500 µg of tryptophan. Means followed by different letters are significantly different at $p \leq .05$. Each bar shows ±SE of means.
can enable the host plants to cope with stress, gain biomass and improve plant metabolism (Rubio et al. 2017). *Trichoderma* spp. is known to produce a large array of organic substance that boosts plant growth (Kumar et al. 2017). Therefore, the isolated of endophytic fungi (CGF-11) from halophytic plant *S. surattense* in the current study was used to investigate its effect on host wheat plants. The culture filtrate of isolated fungi was primarily screened for IAA production. From the results, we observed that CGF-11 strain produced higher amounts of IAA on media supplemented with 500 µg of tryptophan (Figure 1(B)). The evaluation of isolated CGF-11 strain through phylogenetic analysis revealed highest similarity with *T. reesei*, having 99% bootstrap support value. In the past, several reports indicated that endophytic *Trichoderma* spp. lives in symbiotic relation with host roots that secrete bioactive compounds and phytohormones to help the host plant under stress conditions (Kumar et al. 2017). The analysis of IAA production capability by *T. reesei* has been confirmed through HPLC. The results of the present study are in line with previous finding in which phytohormones secreting *Trichoderma* spp. encouraged the growth of host plants under salinity (Kumar et al. 2017). *Trichoderma* spp. can also increase the plant capacity to tolerate salinity directly by alleviating toxicity levels of the salts in plant or indirectly by improving plant growth (Zhang et al. 2016). Likewise, the isolated *T. reesei* in the current study has also helped the host wheat plants against salt stress and alleviated salt toxicity.

The enzymatic properties of the *Trichoderma* spp. might also improve host plant nutrients acquisition, thus helping the host plants to defy salt stress (Zhang et al. 2016). In the current study, *T. reesei* also showed enzymatic properties in support of host plants to withstand salt stress (Figure 3). The endophytic fungal association certainly helps the host plants to develop specialized antioxidant systems during stress in order to reduce the ROS toxicity in their cells (Zhang et al. 2016). In fact, high ROS concentrations are detrimental to cells that can lead to plant death. The enzymatic system can remove ROS toxicity, through catalase or peroxidase or by regeneration of AA and GSH. Both POD and CAT...
activities have greatly reduced in the CGF-11 associated salt stressed plants, which mean that the said endophyte has provided the strength to the plants under salinity. These enzymes might eliminate H$_2$O$_2$ from mitochondria and regulated the responses to stress in plants. Symbiotic- interaction of *Trichoderma spp.*, with host plants can improve its physiological processes and thus increase plant tolerance to oppose stressful situations (Contreras-Cornejo et al. 2016). In the present work, IAA producing strain *T. reesei* has been tested to study its role in host plant tolerance against various degrees of salt stress (60, 120, 180 mM). Growth attributes, like fresh and dry weight of wheat shoot and root, shoot and root lengths, chlorophyll, and carotenoids were higher in *T. reesei* inoculated plants (Figures 4–6). Likewise, the rate of photosynthesis and photosynthetic activities are also affected, when plants are grown under stress condition (Kalaji et al. 2016). Our results also demonstrated a decrease in Chla and Chlb in control plants with increase in NaCl concentration, while marginal changes have been observed in *T. reesei* associated plants. In fact, chlorophyll content and plant growth are correlated; therefore, the reduction in chlorophyll contents indicate photosynthetic damages (Lee et al. 2013). Similarly, carotenoid contents have a key role in ROS scavenging and membrane protection in stress (Das and Roychoudhury 2014) that might be weakened, when the plant undergoes stress situations. The presence of salts in higher concentrations can cause ionic imbalance that leads to disturbed homeostasis (Gupta and Huang 2014). Abiotaic stresses also cause loss in water holding capacity of plant tissues, which affects leaf water content, retarded growth and development (Munns and Tester 2008). Measurement of relative water contents (RWC) in plant cells helps to indicate cellular volume as well as level of response to stress environment (Tátrai et al. 2016). The present findings suggested that the fungal inoculated plants had higher water content, which is in close agreement with that of previous finding (Zhang et al. 2016). Salinity can cause higher electrolyte discharge inside plant tissues that further damages or displace cellular membrane stability (Kang et al. 2012). The outcomes of this study have revealed a lower electrolyte leakage in *T. reesei* inoculated wheat plants. On the contrary, MDA (an aldehydes lipid breakdown product) represents injuries and membrane damages at the cellular level in plants under severe stress (Zhang et al. 2016). However, low levels of lipid peroxidation with reduced cellular damages have been observed in *T. reesei* treated plants under stress. Correspondingly, osmo-protectants, such as proline can be accumulated inside the plants under saline conditions, preventing ionic and osmotic imbalances (Gharsallah et al. 2016). Though, accumulation of proline in *T. reesei* associated plants exposed to higher salt suggested a decrease in the ionic influx in cells in order to maintain osmotic balance. Furthermore, higher amounts of flavonoid and phenolic compounds have been observed in *T. reesei* associated plants under salt stress, which is in agreement with the study of Mona et al. (2017). The produced flavonoids and phenols exhibit central functions in various aspects of plant life, including growth, reproduction, resistance to pathogens and protect the plant against abiotic stress (Mona et al. 2017). Under salt stress conditions, plant cells accumulate different types of osmolytes to adjust the intracellular osmotic potential and avoid cell injury. Among many different kinds of osmolytes, soluble sugars are one of the major types. The key role of soluble sugars during stress includes carbon storage, osmo-protection, and hunting of the reactive oxygen species (Gupta and Huang 2014). Higher contents of soluble protein in *T. reesei* associated wheat plants have been recorded, which demonstrated an enhanced capacity of the host plants to stand abiotic stress conditions. Moreover, both leaves and roots of control plants had elevated concentrations of Na$^+$ and Cl$^-$ ions as compared to *T. reesei* associated plants. The higher concentrations of Na$^+$ and Cl$^-$ might affect the absorption and transport of other ions, mainly Ca$^{2+}$, Mg$^{2+}$ and K$^+$ (Guimaraes et al. 2012). Additionally, higher levels of ABA in plants indicate stress, while higher GA indicates an improved water and nutrient uptake. The presence of lower concentration of ABA and higher concentrations of IAA and GA in *T. reesei* inoculated plants confirmed the function of endophytes to protect the host from stress.

**Conclusions**

*T. reesei* inoculation and its symbiotic-interaction with wheat plants have promoted the growth and strengthen the host plants against the adverse effects of salinity. *T. reesei* as an endophyte has secreted phytohormones, including IAA and GA inside the host plant that improved the productivity and quality of economically important wheat crop under stress conditions.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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