

## *Piriformospora indica* alters $\text{Na}^+/\text{K}^+$ homeostasis, antioxidant enzymes and *LeNHX1* expression of greenhouse tomato grown under salt stress

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### ABSTRACT

The utilization of symbiosis with beneficial microorganisms provides a strategy to alleviate salt stress that reduces existing gaps in crops production. The root endophytic fungus *Piriformospora indica* has shown to improve plant growth in diverse plant species under biotic stress, while limited reports have discussed the interaction of *P. indica* with tomato under salt stress. In this study, the impact of *P. indica* on tomato exposed to 200 mM NaCl for one month in soil-free culture was examined. Growth performance, marker osmolytes, antioxidant enzymes and expression of *LeNHX1-4* genes of tomato leaves were measured. Results show that colonization of roots by *P. indica* improved root branching, fresh and dry weight of salt-stressed plants. Likewise, *P. indica* colonization increased levels of chlorophyll *b*, indole acetic acid, catalase and superoxide dismutase in leaves of tomato under salt stress. Meanwhile, *P. indica* reduced the increase of abscisic acid and proline levels when compared to non-colonized plants. Importantly,  $\text{Na}^+/\text{K}^+$  ratios in shoots and roots of colonized plants were lower than in the corresponding non-colonized plants, which may be attributed to the higher  $\text{K}^+$  concentration observed in leaves and roots of colonized plants under saline water irrigation condition. This change in ion homeostasis was combined with an increase in *LeNHX1* transcripts in leaves of colonized plants. Moreover, compared to non-treated plants, colonization with *P. indica* enhanced fruit yield by 22% and 65% under normal and saline water irrigation, respectively. Our study shows that *P. indica* enhances the growth and yield of tomato plants under normal and salt stress conditions, opening up a window of opportunity for its application in desert agriculture.

### 1. Introduction

Tomato (*Solanum lycopersicum* L.) is a vegetable crop that is affected by diverse abiotic stresses. Salinity is one of the major abiotic stress that increasingly obstructs cash crop production in arid and semi-arid areas (Schmöckel et al., 2017). By the year 2050, approximately 50% of the cultivated soils are expected to suffer from salinity unless efficient management strategies are applied (Blumwald and Grover, 2006). High  $\text{Na}^+$  concentrations in soil or irrigation water inhibit nutrient uptake, photosynthesis and protein synthesis; causing severe disruption of plant metabolism and performance (Shabala and Munns, 2012). Plants suffering from salt stress regulate  $\text{Na}^+$  and  $\text{K}^+$  homeostasis through several mechanisms (Jha et al., 2010). The tonoplast  $\text{Na}^+/\text{H}^+$  antiporters

(NHXs) play an important role during ionic homeostasis by activating  $\text{K}^+$  uptake into vacuoles; regulating cell turgor and stomatal function (Barragán et al., 2012), and by  $\text{Na}^+$  sequestration into the vacuole; maintaining pH and intracellular cation homeostasis (Bassil and Blumwald, 2014). The NHX family comprises eight NHX members in Arabidopsis (AtNHX1-8) and six in rice (OsNHX1-6) and maize (ZmNHX1-6) (Yamaguchi et al., 2012). In tomato, four NHX isoforms (*LeNHX1-4*) were reported to contribute to vacuolar  $\text{K}^+$  accumulation (Gálvez et al., 2012), with the vacuolar *LeNHX1* and endosomal *LeNHX2* isoforms being most important (Rodríguez-Rosales et al., 2009). Moreover, sodium toxicity can lead to damage of cellular organelles and generation of reactive oxygen species (ROS) (Chen et al., 2017). These detrimental effects are attenuated by cellular ROS

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scavenging mechanisms such as antioxidant enzymes (e.g. superoxide dismutase, peroxidase and catalases) (Li et al., 2017).

Symbiosis with beneficial soil microorganisms is a natural mechanism that might offer a quicker, cost-efficient and eco-friendly solution to mitigate salinity stress (Andrés-Barrao et al., 2017; de Zélicourt et al., 2018). *Piriformospora indica* is a phytopromotional, biotrophic mutualistic root endosymbiont that can be cultivated axenically. It belongs to the order Sebaciales (Basidiomycota) and has been reported to colonize a broad range of plants comprising monocots and dicots (Varma et al., 1999), provides plants with multifaceted amenities (Unnikumar et al., 2013) and helps plants to overcome both biotic and abiotic stresses (Gill et al., 2016). In this respect, *P. indica* enhances resistance of *A. thaliana* and barely to powdery mildew (Stein et al., 2008; Qiang et al., 2012) and alleviates severity of infections by the pathogenic fungi *Fusarium oxysporum* and *Verticillium dahlia* as well as with the viral pathogen Pepino Mosaic Virus in tomato (Sarma et al., 2011; Fakhro et al., 2010; Sun et al., 2014). In addition, *P. indica* reported to protect plants against salt stress by altering antioxidant enzyme levels, inducing ROS scavenging systems (Baltruschat et al., 2008) and regulating  $K^+/Na^+$  ratios of the colonized plants (Abdelaziz et al., 2017). In tomato, *P. indica* colonization increased photosynthetic pigment content, proline and glycine betaine accumulation in inoculated roots than in non-inoculated roots (Ghorbani et al., 2018). Meanwhile, *P. indicac* promoted 14 metabolites and ions conferring tolerance to salt stress in barley grown under 300 mM NaCl (Ghaffari et al., 2016). Yun et al. (2018) reported that the negative effects of NaCl on *Zea mays* seedlings were alleviated after *P. indica* inoculation, probably by improving stomatal conductance and lower  $K^+$  efflux from roots and increase potassium content in shoots than non-inoculated plants under 200 mM NaCl. Furthermore, rice and wheat, colonized with *P. indica* exhibited better photosynthetic pigment contents and plant growth performance (Zarea et al., 2012; Jogawat et al., 2013). In addition, limited reports discussed the interaction between *P. indica* and tomato under salt stress conditions (Ghorbani et al., 2018). However, the exact mechanisms by which *P. indica* induces salt stress tolerance of plants remain unclear. In this work, we explored the impact of *P. indica* on different agronomical parameters of plants exposed to salt stress, including shoot and root system size, branching, fresh and dry weight as well as on yield and quality of tomato fruits. In addition, physiological changes of marker osmolytes, antioxidant enzymes and the expression of *LeNHX1-4* of tomato plants, which may explain the tolerance mechanisms induced by *P. indica* were explored.

## 2. Material and methods

### 2.1. Plant material and growth conditions

Seeds of tomato (*Solanum lycopersicum* cv. Super Strain B) were surface-sterilized in 70% ethanol for 2 min, immersed in 5% NaClO for 15 min, and then washed 3 times with sterile ddH<sub>2</sub>O. Sterilized seeds were then placed in trays filled with peat moss (Miracle-Gro, USA) for germination. Growing seedlings were supplied with  $1/4$  strength Hoagland solution (Merck, Germany) twice a week. At the stage of the second true leaf development, seedlings were transplanted in pots (35.5 cm in diameter) filled with a mixture of sand, peat moss and vermiculite in 1:1:1 volumes. Twelve pots were used per single treatment and each pot contained two plants. Pots were arranged at 50 x 50 cm distance in plastic greenhouses at the Faculty of Agriculture, Cairo University, Egypt, during the two summer seasons of 2016 and 2017. Greenhouse day/night temperatures were  $28 \pm 2/18 \pm 2$  °C, with 14 h light ( $350\text{--}400 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) and 55–65% relative humidity. The growth medium in each pot was mixed with 2.5 g of  $(\text{NH}_4)_2 \text{SO}_4$ , 1.5 g of  $\text{CaH}_4\text{P}_2\text{O}_8$  and 1.0 g of  $\text{K}_2\text{SO}_4$  before planting. The pots were irrigated daily and fertilized twice a week with  $1/2$  strength Hoagland solution. For salt stress treatments, a dose of 50 mM NaCl was applied with the irrigation water at 10 days after transplanting followed by a

dose of 100 mM NaCl after 3 days. For the subsequent 2 weeks, 200 mM NaCl was applied in 2-day intervals with the irrigation water.

### 2.2. Fungal propagation and inoculation

*Piriformospora indica* (accession DSM 11827, kindly provided by Prof. Ralf Oelmüller, Friedrich-Schiller-University Jena, Germany) was propagated on solid KM medium (Hill and Kafer, 2001) and incubated at 24 °C for 2 weeks. For plant inoculation, 250 ml liquid KM medium was supplied by 5 fungal plugs and incubated for 15 days at 24 °C and 120 rpm on a rotary shaker. Pure white mycelium was collected and washed 3 times with sterile ddH<sub>2</sub>O. For inoculation, 2 g of *P. indica* mycelium was mixed with 100 ml sterilized ddH<sub>2</sub>O to produce a 2% (w/v) *P. indica* suspension ( $\pm 1.5 \times 10^7$  spores/ml). Ten ml of *P. indica* suspension per pot was inoculated to the root zone of tomato plants at 3 and 7 days after transplanting. Pure ddH<sub>2</sub>O without *P. indica* was used to inoculate control plants.

### 2.3. Staining of *P. indica* hyphae in root tissue

Four weeks post inoculation; roots of tomato plants were stained with the chitin specific dye WGA-AF 488 (Molecular Probes, Karlsruhe, Germany) to monitor *P. indica* colonization (Deshmukh et al., 2006), while DAPI was used to stain DNA of intact nuclei according to manufacturer's protocol (Life technology). Confocal fluorescence images were recorded on a multichannel TCS-SP2 confocal microscope (Leica, Bensheim, Germany). WGA-AF 488 fluorescence was excited with a 488 nm laser line and detected at 500–540 nm, while DAPI fluorescence was excited with a 350 nm laser line and detected at 460 nm.

### 2.4. Growth parameters and crop yield

Thirty days after transplanting to pots, each treatment was divided into two sets (12 plants each). In the first set, the number of leaves and branches, fresh weight and dry weight of tomato plants were measured. In the second set, mature fruits were collected gradually and number of fruits per plant were recorded and weighted to calculate the total yield per plant and average fruit weight.

### 2.5. Stomatal conductance and transpiration rate

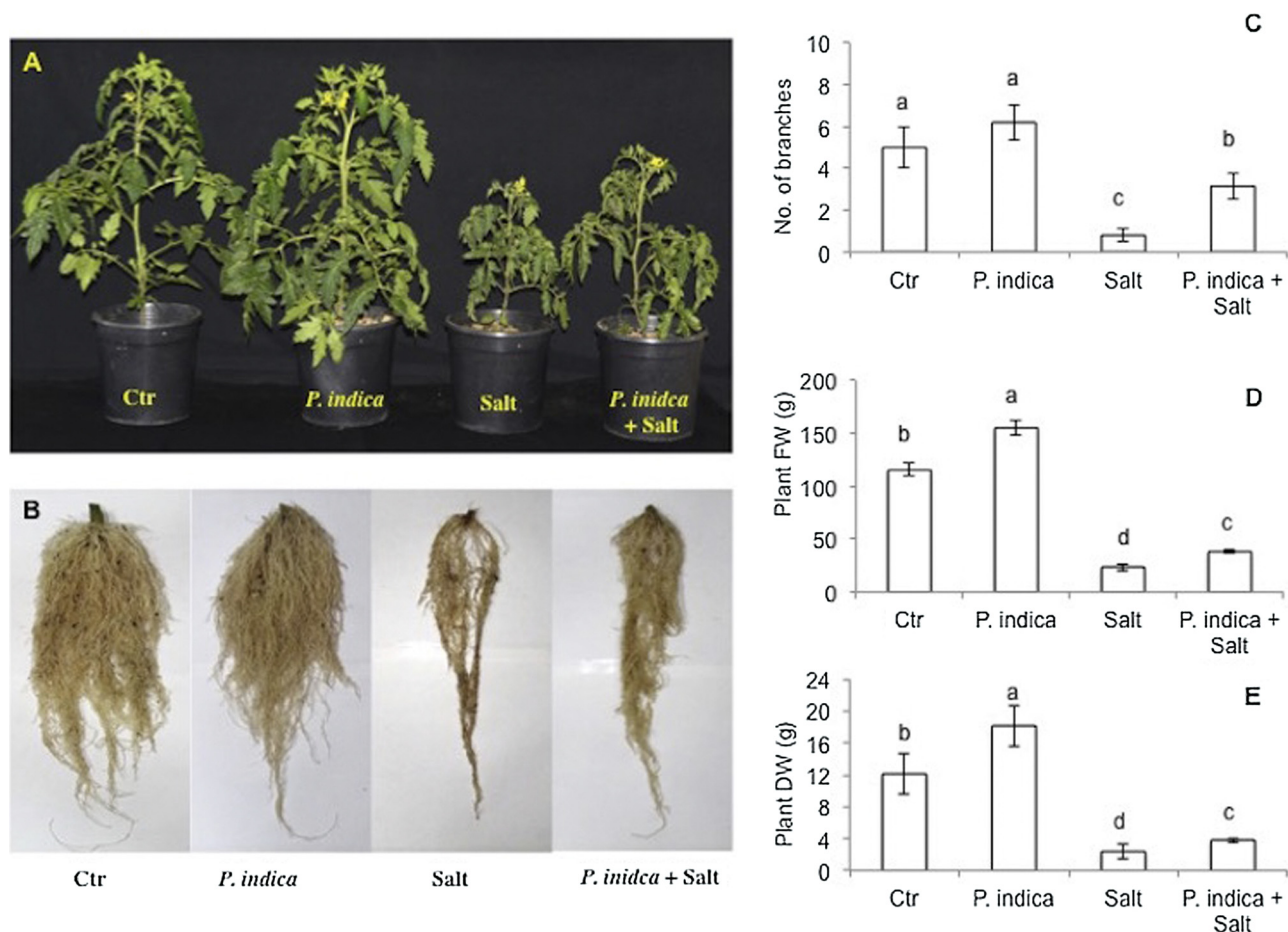
The stomatal conductance and transpiration rate of tomato plants were assessed at the fifth leaf from the top using 12 plants per treatment using LI-1600 steady state Porometer (LI-COR, Lincoln, NE, USA).

### 2.6. Quantification of chlorophyll and proline

Chlorophyll (*a* and *b*) content was measured in the fifth leaf using *N,N*-Dimethylformamide (Moran and Porath, 1980). Free proline was quantified according to Claussen (2005). Approx. 0.5 g of the fifth fresh leaf was ground in 5 ml of 3% (w/v) sulfosalicylic acid solution. The homogenate was filtered and mixed with 2% (v/v) ninhydrin reagent. Pure proline was used as standard and the concentration of proline in the samples was estimated based on the absorbance at 546 nm. Total soluble solids in tomato fruits were measured in fresh juice at room temperature using an Atago digital refractometer (Heidelberg, Germany).

### 2.7. Antioxidant enzymes activity assay

Approx. 0.5 g of the fifth fresh leaf were powdered in liquid nitrogen and homogenized in 5 ml potassium phosphate buffer (100 mM, pH 7.0) containing 0.5% Triton X-100, 2% (w/v) *N*-Vinylpyrrolidinone, 5 mM ethylene diamine tetraacetic acid disodium salt dehydrate and 1 mM ascorbic acid (Polle et al., 1994). Homogenates were then centrifuged at  $12,000 \times g$  for 20 min at 4 °C and the supernatants were used to



**Fig. 1.** Impact of *P. indica* inoculation on growth of tomato plants at 45 days post inoculation under normal and salt stress conditions. Plants were supplied with 50 mM NaCl at 10 days after transplanting, then supplied with a dose of 100 mM NaCl after 3 days followed by continuous doses of 200 mM NaCl at 2days intervals for 2 weeks. Representative images of (A) shoot system and (B) root system of tomato plants, (C) number of branches, (D) total plant fresh weight (FW) and (E) total plant dry weight (DW). Data are means of combined analysis of two growing seasons. Means marked by the same lower-case letter are not significantly different ( $P < 0.05$ ) according to LSD (One-way ANOVA). Error bars represent standard error of the means (SE),  $n = 10$ .

determine the activity of superoxide dismutase (SOD, EC 1.15.1.1) according to [Beauchamp and Fridovich \(1971\)](#); catalase (CAT, EC 1.11.1.6) by [Aebi \(1984\)](#) and ascorbate peroxidase (POX) by [Nakano and Asada \(1981\)](#).

## 2.8. Determination of $\text{Na}^+$ and $\text{K}^+$ contents

Fresh leaves and roots of tomato plants were dried at 100 °C for 24 h and digested in 1% nitric acid at 60 °C for 16 h ([Roy et al., 2012](#)).  $\text{Na}^+$  and  $\text{K}^+$  concentrations were determined with a flame photometer (Jenway Inc., Burlington, USA).

## 2.9. ABA and IAA hormone content analysis

Freeze-dried tomato leaves were ground to fine powder and 10 mg leave of the powder was washed three times with 80% methanol (v/v) and 2,6-bis (1,1-dimethylethyl)-4-methylphenol at 4 °C in the dark. The extract was centrifuged at 4000 rpm, and the supernatant was decanted, adjusted to pH 8.6, and then extracted three times with an equal volume of pure ethyl acetate. The combined alkaline ethyl acetate-extracts were dehydrated over anhydrous sodium sulphate and then filtered. The filtrate was evaporated to dryness under vacuum at 35 °C and re-dissolved in 1 ml absolute methanol. The methanol extract was used after methylation according to [Fales et al. \(1973\)](#). The quantification of the endogenous phytohormones was carried out using Ati-Unicomp gas-liquid chromatography, 610 Series, equipped with flame ionization

detector according to the method described by [Vogel \(1975\)](#). The fractionation of phytohormones was conducted using a coiled glass column (1.5 m x4 mm.) packed with 1% OV-Gases flow rates were 30, 30, 330 ml/min, for nitrogen, hydrogen and air, respectively. Peak identification and quantification of abscisic acid (ABA) and indole-acetic acid (IAA) were performed by using pure standards of the hormones and a Microsoft program to calculate the concentrations of the identified peaks.

## 2.10. Quantitative real-time RT-PCR analysis

Total RNA was extracted from 50 mg of frozen root and leaf materials using a Qiagen RNeasy kit according to the manufacturer's protocol. One microgram of total RNA was used to synthesize cDNA using 200U superscript reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative real time PCR (qPCR) assay was carried out in three technical replicates. SYBR green PCR master mix (Applied Biosystems) was used for amplification in a total volume 20  $\mu\text{l}$  as follows: 10  $\mu\text{l}$  2X SYBR mix, 10 ng cDNA and 300 nM of forward and reverse primers for the targets genes (Table S1) ([Galvez et al. 2012](#)). Relative transcript levels of *LeNHX1*, *LeNHX2*, *LeNHX3* and *LeNHX4* were calculated according to  $2^{-\Delta\Delta\text{Ct}}$  method ([Schmitgen and Livak, 2008](#)) using *LeEF1a* as a reference gene. All experiments were repeated three times, and the mean values and standard errors were determined from the three independent biological replicates. The significance of differences between data sets were evaluated using paired

student's *t*-test.

### 2.11. Statistical analysis

Greenhouse experiments were arranged in a randomized block design based on a factorial experiment. Test of normality of value distribution was carried out (Shapiro and Wilk, 1965) using SPSS v. 17.0 computer package (2008). Combined analysis of variance of a RCBD across the two seasons was computed after carrying out Bartlett's test. Analysis of variance (one-way ANOVA) was performed and LSD (Duncan's test,  $P < 0.05$ ) was calculated to test the significance of differences between means. The student's *t*-test was used for gene expression experiment based on the Kolmogorov-Smirnov test. The significance of differences between data sets was evaluated using paired student's *t*-test.

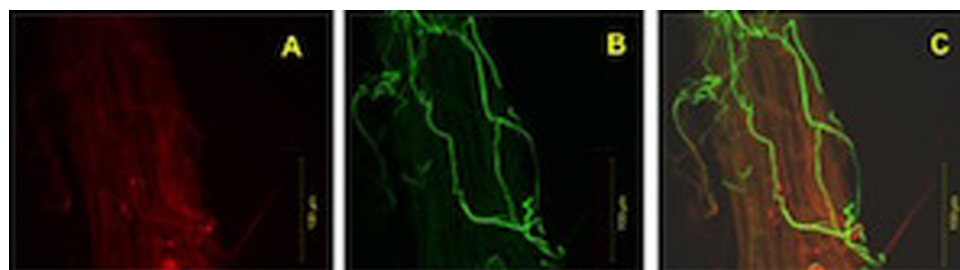
## 3. Results

### 3.1. *P. indica* enhances plant growth under salt stress

Under salt stress conditions, *P. indica* successfully colonized the root surface of tomato plants at 30 days post inoculation (Fig. 2). Colonization of tomato with *P. indica* partially enhanced total fresh and dry weight and branching of plants (Fig. 1A–E) as well as shoot and root dry weight (Fig. S1), in normal condition. Exposure of tomato plants to salt stress severely reduced plant growth in comparison to control plants (Fig. 1). Salt stress decreased plant fresh and dry weight by 84% and 80%, respectively, when compared to control (Fig. 1D,E). In contrast, *P. indica* colonization resulted in an increased fresh weight (26%) and dry weight (33%) when compared to non-colonized plants (Fig. 1D,E). Moreover, inoculation with *P. indica* was able to alleviate the detrimental effects of salt on shoot and root growth and branching (Figs. 1A, C and S1).

### 3.2. *P. indica* colonization affects accumulation of chlorophyll, proline and ROS detoxification under salt stress irrigation of tomato

No difference in total chlorophyll content was observed between colonized and non-colonized plants in normal conditions (Fig. S2). Similar results were obtained for chlorophyll *a* in separate measurements, even under salt stress condition (Fig. 3A). However, a significant increase in the contents of chlorophyll *b* was found in leaves of colonized plants under salt stress treatment (Fig. 3B). Tomato plants were found to accumulate high proline levels in the leaves of salt stressed plants (Fig. 3C). Notably, *P. indica* colonization led to a significant reduction in the accumulation of proline under salt stress conditions. Among the ROS detoxifying enzymes, the activities of superoxide dismutases (SOD), catalases (CAT) and ascorbate peroxidases (POX) were also measured. Although SOD, CAT and POX activity levels did not differ significantly in *P. indica* colonized or non-colonized plants under normal condition (Fig. 3D–F), the levels of SOD and CAT activities were significantly higher in colonized than non-colonized plants under salt stress conditions (Fig. 3).



**Fig. 2.** Monitoring the colonization of *P. indica* on tomato plant roots at 45 days post inoculation. Plants were supplied with 50 mM NaCl at 10 days after transplanting, then supplied with a dose of 100 mM NaCl after 3 days followed by continuous doses of 200 mM NaCl at 2 days intervals for 2 weeks (Side bar 100  $\mu$ m). (A) DAPI (4',6-Diamidino-2-phenylindole) was used to stain intact nuclei. Fungal mycelium on the root surface of inoculated tomato was visualized by the (B) chitin-specific dyes WGA-AF 488. (C) An image overlay is shown.

### 3.3. $\text{Na}^+$ and $\text{K}^+$ homeostasis

One of the strategies that plants use to cope with salt stress is to reduce the accumulation of toxic  $\text{Na}^+$  ions in the root and shoot systems. Another way is to counterbalance the entry of  $\text{Na}^+$  ions into cells is by increasing intracellular  $\text{K}^+$  concentrations. Therefore, it is essential to determine the content of  $\text{Na}^+$  and  $\text{K}^+$  in the roots and shoots under salt stress and control conditions. Here, salt stress was accompanied by a clear increase of  $\text{Na}^+$  concentration; combined with low  $\text{K}^+$  concentrations in shoots and roots of both colonized and non-colonized plants (Fig. 4). When compared to non-colonized plants, plants colonized with *P. indica* accumulated less  $\text{Na}^+$ , but more  $\text{K}^+$ , in both leaves and roots (Fig. 4C,D). This pattern was confirmed by calculating  $\text{Na}^+/\text{K}^+$  ratios in leaves and roots of colonized and non-colonized plants (Fig. S3). Under salt stress, colonization with *P. indica* resulted in a 4-fold reduction of  $\text{Na}^+/\text{K}^+$  ratios in leaves and roots of tomato, respectively (Fig. 4B,D).

### 3.4. Plant hormones

Plant growth and stress tolerance are determined by a number of plant hormones, among which auxins play a key role in growth and abscisic acid (ABA) in abiotic stress adaptation. While salt stress treatment resulted in reduced IAA levels in leaves of non-colonized plants, *P. indica* colonization maintained higher IAA levels under both normal and salt stress conditions. On the contrary, salt stress induced massive ABA accumulation in tomato, but the levels of ABA in colonized plants were much lower than in non-colonized plants (Fig. 5).

### 3.5. $\text{Na}^+/\text{H}^+$ antiporters *LeNHX1-4* transcript levels

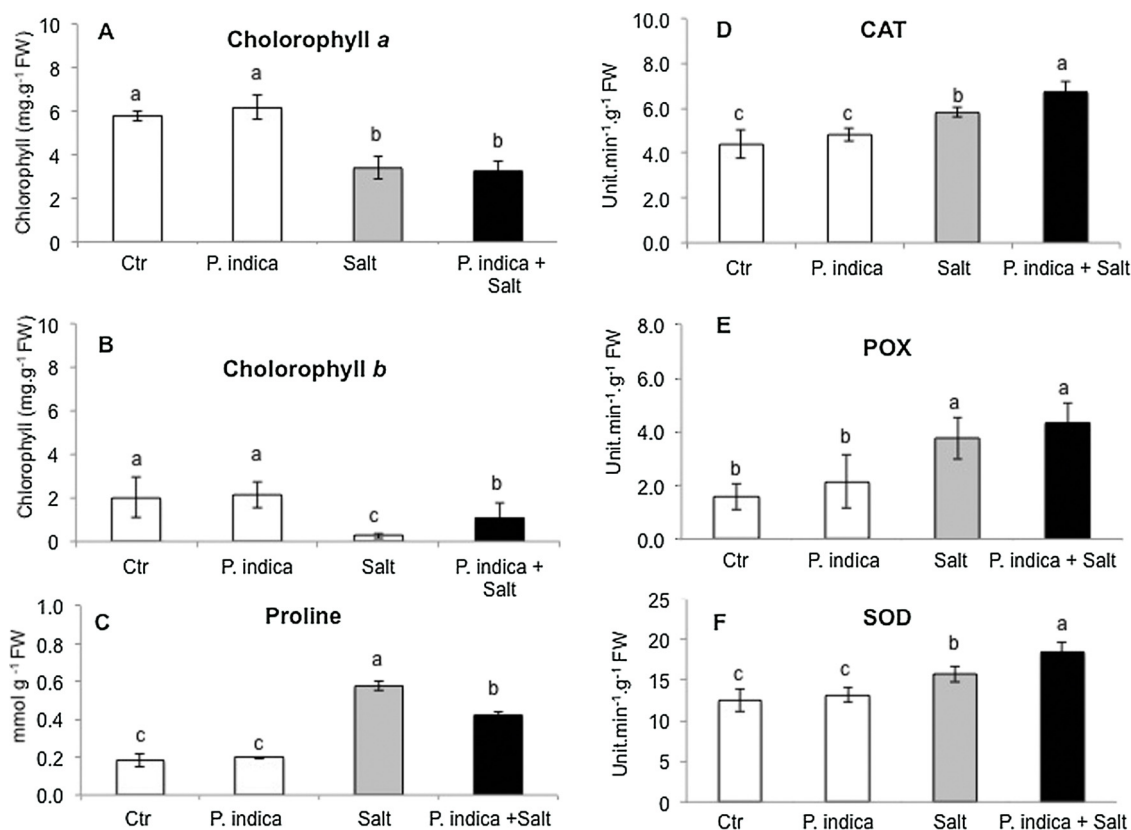
To investigate the impact of *P. indica* colonization on salt stress tolerance via transcriptional regulation of tonoplast  $\text{Na}^+/\text{H}^+$  antiporter genes, data in Fig. 5 shows that *P. indica* colonization did not affect *LeNHX1-4* transcript levels significantly under normal condition. However, under salt stress, *P. indica* colonization induced *LeNHX1* expression 2-folds higher in the leaves of tomato plants compared to non-colonized plants.

### 3.6. Fruit yield

Salt stress has severe affects on the productivity and crop yield of tomato plants (Table 1). Interestingly, salt-stressed plants that were colonized by *P. indica* showed increases in average total yield (65%), number of fruits per plant (37%), fruit weight (43%) and total soluble solids (13%). Likewise, *P. indica* colonization led to favourable effects in absence of salt stress in terms of crop yield (22%) and average number of fruits per plant (24%).

## 4. Discussion

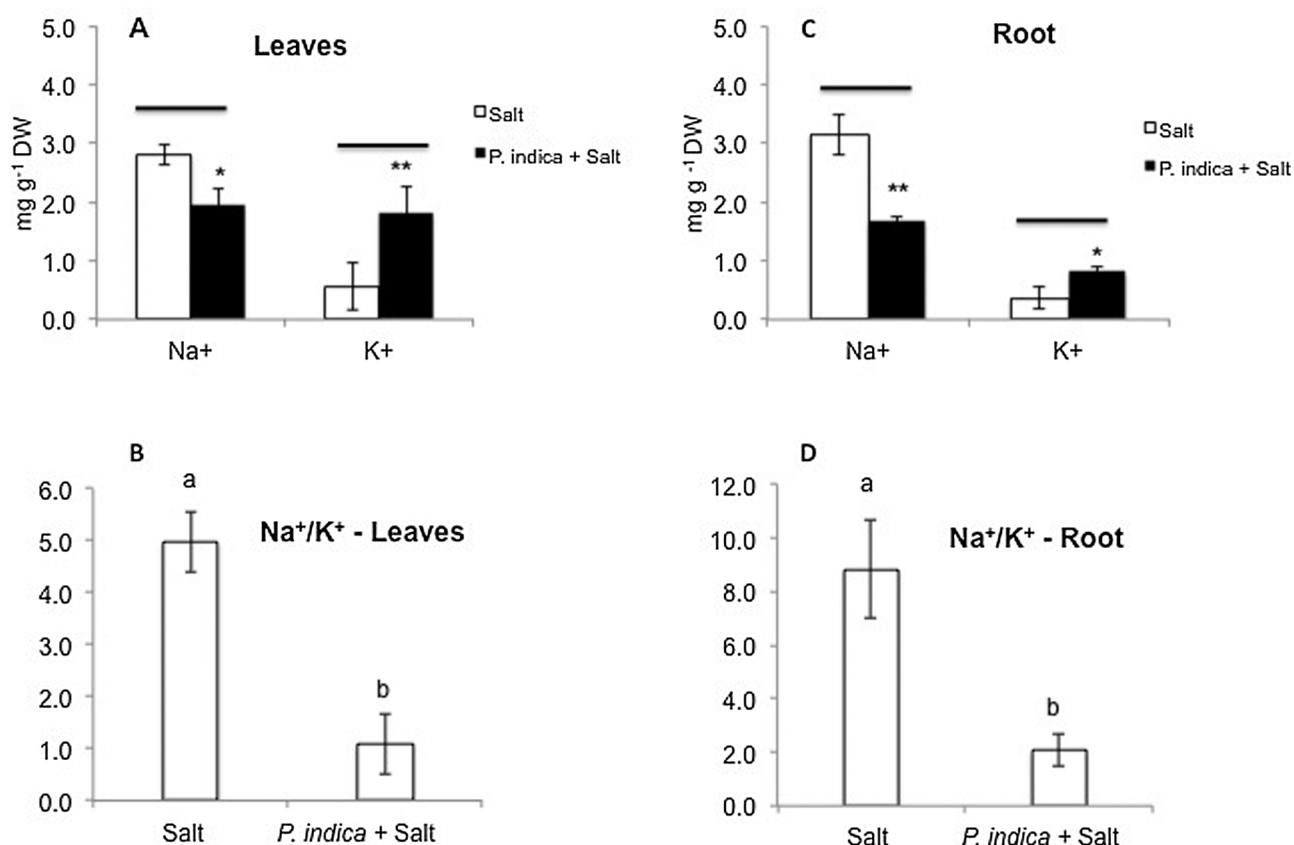
The detrimental effects of salt stress on plant growth have long been intensively studied by researchers and breeding companies to enhance



**Fig. 3.** Contents of (A) chlorophyll a, (B) chlorophyll b and (C) proline, and the activities of (D) catalase (CAT), (E) ascorbate peroxidase (POX) and (F) superoxide dismutase (SOD) in leaves of tomato plants inoculated with or without *P. indica* at 45 days post inoculation under normal and salt stress conditions. Plants were supplied with 50 mM NaCl at 10 days after transplanting, then supplied with a dose of 100 mM NaCl after 3 days followed by continuous doses of 200 mM NaCl at 2 days intervals for 2 weeks. Data are means of combined analysis of two growing seasons. Means marked by the same lower-case letter are not significantly different ( $P < 0.05$ ) according to LSD (One-way ANOVA). Error bars represent standard error of the means (SE),  $n = 8$ .

crop salt tolerance (Fan et al., 2016). Here, we show that growth of tomato plants and the fruit yield are adversely affected when irrigated with saline water. Based on reported beneficial effects of the root-endophytic fungus *Piriformospora indica* on a large variety of plant species, we investigated its potential plant growth and salt stress tolerance promoting effects on tomato in greenhouse conditions. Indeed, tomato colonization with *P. indica* was confirmed and mitigated the adverse effects of salt stress by increasing the number of branches as well as the fresh and dry weight of the plants (Fig. 1). These results are in agreement with earlier investigations that explored the ability of *P. indica* to stimulate growth of colonized plants under severe salt stress conditions (Jogawat et al., 2013; Ghorbani et al., 2018). Our results show a reduction of chlorophyll contents in leaves of tomato by salt stress (Amjad et al., 2014). *P. indica* colonization did not affect the content of chlorophyll a and b in the absence of salt stress, but did increase chlorophyll b levels in the leaves when plants were treated with salt water. Hence, a slight significant increase in the contents of total chlorophyll was found in leaves of salt-stressed plants with *P. indica* colonization (Fig. S2). This might indicate that chlorophyll a could be more sensitive to salinity than Chlorophyll b. These results agree with the previous findings of Mohsen and Ebrahim (2017) who mentioned that salt stress decreases chlorophyll a and b in tomato while a reverse trend was observed upon mycorrhizal inoculation. It seems that *P. indica* alleviated the destructive effects of osmotic stress by increasing the chlorophyll fluorescence, photosystem II activity and water use efficiency (Yang et al., 2014; Saddique et al. 2018). Plants trigger several defence mechanisms to mediate stress tolerance against enhanced production of Reactive Oxygen Species (ROS) and other free radicals that damage plant cells (Tuteja, 2007). Therefore, the antioxidative defence systems were analysed to elucidate if *P. indica* mediates salt tolerance in colonized

plants. In this work, *P. indica*-colonized plants showed significantly higher levels of SOD and CAT enzyme activities than non-colonized plants under salt stress conditions (Fig. 3). These results suggest that the activation of ROS scavenging enzymes may have contributed to the improved salt stress tolerance caused by *P. indica* colonization in tomato (Hosseini et al., 2017). Many plants, including tomato in this study, produce enhanced levels of proline under salt stress (Fig. 3C). Surprisingly, tomato that had been inoculated with *P. indica* showed much lower proline levels under salt stress conditions. These decreases in proline upon *P. indica* colonization may be explained by the observed ABA levels. ABA controls proline biosynthesis (Verslues and Bray, 2006) to handle the potential cytoplasmic osmotic stress caused by increasing NaCl in root zone (Cao et al., 2014). Under salt stress conditions, the massive increase in ABA in non-colonized tomato was reduced by approx. 30% with *P. indica* colonization, suggesting that the plants may have experienced an alleviation of the toxic effects posed by the salinity (Khalid et al., 2018). This clear decrease in ABA content was associated with increasing transpiration rate and stomatal conductance in colonized plants compared to non-colonized plants under stress (Fig. S4). Our results also show that growth enhancement of *P. indica*-colonized tomato plants under normal or salt stress conditions were correlated with the higher IAA levels in tomato leaves. In this respect, it could be suggested that *P. indica* improve plant salt stress tolerance by increasing IAA contents (Hilbert et al., 2013). Recent work with *P. indica* confirmed that fungus-improved maize root growth is correlated with enhanced expression of abscisic acid and auxin functional genes leading to improved plant performance under drought stress (Zhang et al., 2018). The major harmful effect of salt stress is up-regulating  $K^+$  efflux from root cell by either depolarization-activated outward rectifying  $K^+$  (GORK) and ROS-activated non-selective  $K^+$  permeable cation



**Fig. 4.** Na<sup>+</sup> and K<sup>+</sup> content as well as Na<sup>+</sup>/K<sup>+</sup> ratio in leaves and roots of tomato plants inoculated with or without *P. indica* at 45 days post inoculation under normal and salt stress conditions. Plants were supplied with 50 mM NaCl at 10 days after transplanting, then supplied with a dose of 100 mM NaCl after 3 days followed by continuous doses of 200 mM NaCl at 2 days intervals for 2 weeks. Data are means of combined analysis of two growing seasons. Means marked by the same lower-case letter are not significantly different, P < 0.05 (\*) and P < 0.001 (\*\*), according to LSD (One-way ANOVA). Error bars represent standard error of the means (SE), n = 10. Black bar indicates comparison between the two below columns.

channels (NSCC) (Shabala and Pottosin, 2014). In the same time, increased salt in the root zone results in Na<sup>+</sup> toxicity (Anschütz et al., 2014) that decreases K<sup>+</sup> uptake, reduces cell expansion, stomatal opening and photosynthesis rates (Porcel et al., 2016; Shabala et al., 2016). In this work, we confirm that leaves and roots of salt-stressed plants show higher content of Na<sup>+</sup> and lower content of K<sup>+</sup> in non-colonized tomato plants (Ghorbani et al., 2018). Meanwhile, colonizing salt-stressed plants with *P. indica* resulted in 30% and 53% decreases in Na<sup>+</sup> contents associated with 3 and 2 fold increases in K<sup>+</sup> content in the leaves and roots, respectively, when compared to non-colonized plants. Our results are in agreement with Alikhani et al. (2013) who found that Na<sup>+</sup> accumulation in the leaves of *P. indica*-colonized and non-colonized barely plants was increased up to 7 and 13 fold, respectively, under 300 mM NaCl if compared to non-salt conditions. In terms of K<sup>+</sup> content, the same authors also found a decrease by 7% and 2% in non-colonized and colonized plants, respectively, which led to an overall increase in foliar K<sup>+</sup>/Na<sup>+</sup> ratio in colonized plants than non-colonized plants. However, cytosolic K<sup>+</sup> homeostasis is a curial mechanism for salt stress adaptation in a broad range of plant species (Percey et al., 2016). In this respect, it could be suggested that this favourable modulation in Na<sup>+</sup>/K<sup>+</sup> ratio, in leaves and roots of colonized plants is related to the ability of *P. indica* to maintain K<sup>+</sup> retention under salt stress. In connection, the beneficial effects of *P. indica* on salt stress tolerance have been corroborated by deterring the altered expression of some K<sup>+</sup> ion channel genes related to salt stress responses. Abdelaziz et al. (2017) indicated that *P. indica* increases K<sup>+</sup>/Na<sup>+</sup> ratios in Arabidopsis by inducing the expression of genes encoding ion channels; *HKT1*, *KAT1* and *KAT2*. To address if *P. indica* colonization could stimulate other ion channels, we tested the expression levels

of the intracellular antiporter *LeNHXs1-4* genes under our experimental conditions. Here, no differences were observed in the expression of *LeNHX2*, *LeNHX3* or *LeNHX4* (Fig. 5). Interestingly, our results demonstrate that *P. indica* colonization was accompanied by high transcript levels of the *LeNHX1* gene in leaves of tomato under salt stress. Similar results were obtained by Ghazanfar et al. (2016) who reported that mycorrhizal inoculation altered *LeNHX1* transcript levels in leaves of colonized tomato seedlings under salt stress. Furthermore, recent *P. indica* work confirms that *LeNHX1* in leaves of colonized *Brassica campestris* plants presented 2-fold increase under salt stress compared to non-colonized plants (Khalid et al., 2018). Meanwhile, overexpressing *LeNHX2* in tomato showed higher K<sup>+</sup> content in both roots and shoots without difference in Na<sup>+</sup> content compared with untransformed plants under saline stress (Huertas et al., 2013). NHX1 and NHX2 have been reported to play a key role in plant salt tolerance by activating K<sup>+</sup> transport into vacuoles (Barragan et al. 2012) and overexpression of Na<sup>+</sup>/H<sup>+</sup> antiporters in plants renders them more tolerant to salt stress (Shabala, 2017). Consequently, the regulation of NHX proteins in tomato by *P. indica* requires further exploration. Regarding the impact of *P. indica* colonization on tomato yield and quality under saline water irrigation, colonization of tomato roots with *P. indica* enhanced plant fruit yield up to 22% and 65% under normal and salt stress conditions, respectively. Likewise, *P. indica* increased the number of fruits per plant, and a significant increase in fruit weight and total soluble solids (TSS) was observed. These findings show that *P. indica* can enhance tomato yields under salt stress conditions. Moreover, *P. indica* was reported to alleviate the deleterious effects of stress by enhancing water and mineral uptake (Abadi and Sepehri, 2016). As shown in Fig. 2, the better root size caused by *P. indica* colonization might facilitate and

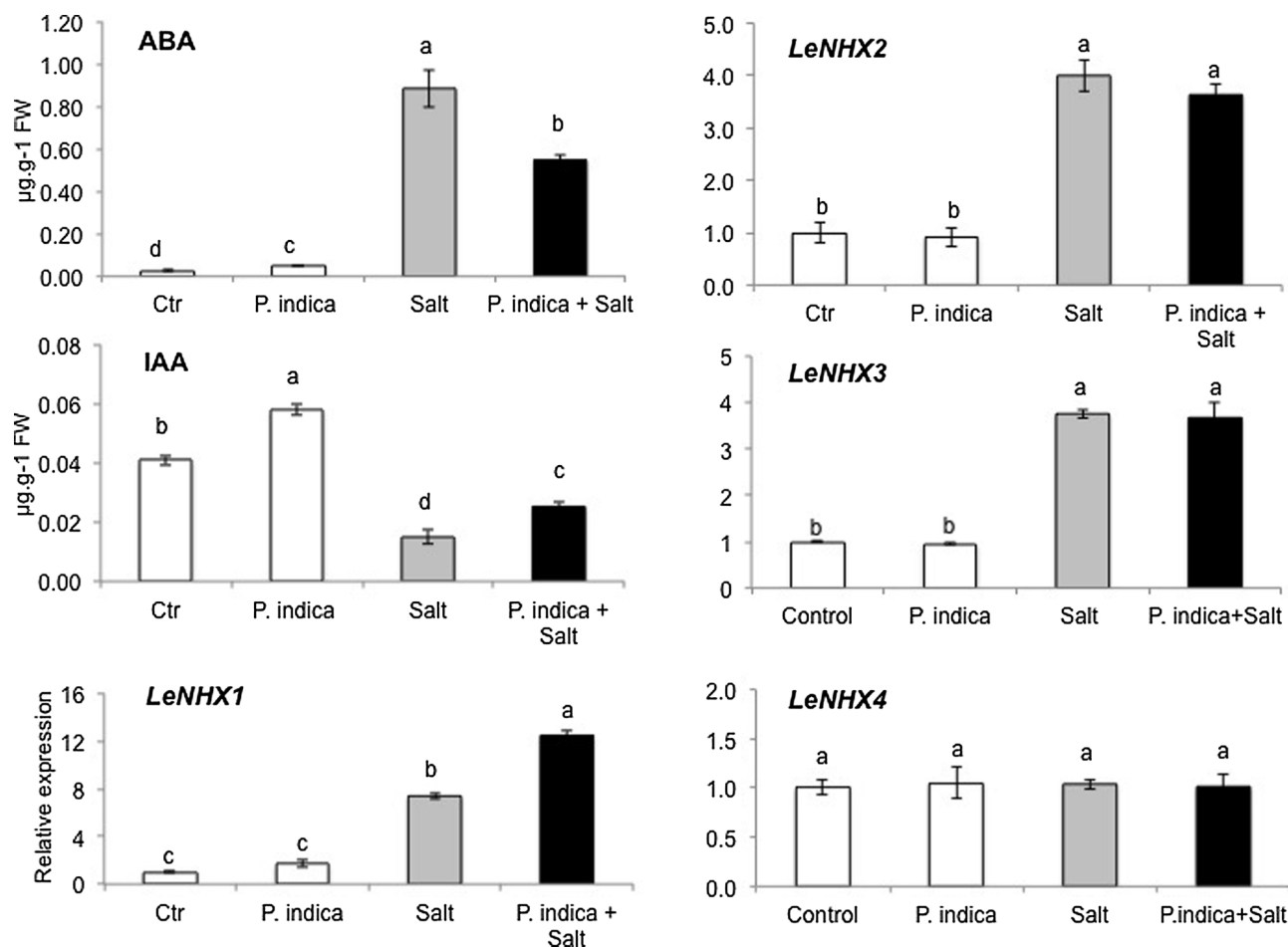


Fig. 5. Hormone quantification of ABA and IAA and relative gene expression of *LeNHX1,2,3,4* in leaves of tomato plants inoculated with or without *P. indica* at 45 days post inoculation under normal and salt stress conditions. Data are means of combined analysis of two growing seasons. Means marked by the same lower-case letter are not significantly different ( $P < 0.05$ ) according to LSD. Error bars represent standard error of the means (SE),  $n = 6$ . The mRNA levels for *LeNHX1,2,3,4* are expressed as a ratio of the value measured in the colonized plant to that in the corresponding non-colonized control (Ctr). Significance of differences ( $P < 0.001$ ) between means was evaluated by Student's *t*-test,  $n = 3$ , based on three independent experiments.

Table 1

Impact of *P. indica* inoculation on total plant fruit yield and its components (fruit weight and number of fruits per plant) as well as total soluble solids (TSS) of tomato fruits under normal and salt stress conditions. Data are means of combined analysis of two growing seasons. Means followed by the same letter are not significantly different ( $P < 0.05$ ) according to LSD (One-way ANOVA).

| Treatments              | Yield (g/plant)   | Fruit weight (g) | Number of fruits per plant | Total soluble solids (TSS) |
|-------------------------|-------------------|------------------|----------------------------|----------------------------|
| Control                 | 934 <sup>b</sup>  | 70 <sup>a</sup>  | 13 <sup>b</sup>            | 5.0 <sup>c</sup>           |
| <i>P. indica</i>        | 1204 <sup>a</sup> | 71 <sup>a</sup>  | 17 <sup>a</sup>            | 5.5 <sup>c</sup>           |
| Salt                    | 88 <sup>d</sup>   | 22 <sup>c</sup>  | 4 <sup>d</sup>             | 8.8 <sup>b</sup>           |
| <i>P. indica</i> x Salt | 252 <sup>c</sup>  | 35 <sup>b</sup>  | 7 <sup>c</sup>             | 10.1 <sup>a</sup>          |

improve nutrient uptake from the rhizosphere (Sun et al., 2010; Das et al., 2014).

## 5. Conclusions

Our study shows that *P. indica* colonization improves tomato growth performance and yield under salt stress by inducing a series of morphological and biochemical events that might together contribute to alleviate the impact of salt stress in tomato. These improvements by *P. indica* are an important step to enable agricultural production on so-far unused land and/or using saline water for irrigation.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.scienta.2019.05.059>.

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