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Effect of *Piriformospora indica* and *Azospirillum* strains from saline or non-saline soil on mitigation of the effects of NaCl

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ABSTRACT

Salinity toxicity is a worldwide agricultural and eco-environmental problem. The intent of this study was to determine the salt tolerance of *Piriformospora indica* and strains of *Azospirillum*, isolated from non-saline and saline soil, as well as to determine their affect on the tolerance of wheat to soil salinity. In this study, an experiment was conducted to investigate the salt stress tolerance abilities of the endophytic fungi, *P. indica*, and *Azospirillum* strains, isolated from non-saline and saline soil, at five NaCl levels (0, 0.1, 0.2, 0.3, 0.4, 0.5 mol L^{-1}). Additionally, a greenhouse experiment was conducted to test the effects of these selected microorganisms under increasing salinity levels on seedling growth, solute accumulation (proline and sugars), and photosynthetic pigments (Chl *a*, *b*, *ab*) of seedling wheat. *Azospirillum* strains were isolated in Iran from the root of field-grown maize from non-saline soil with an EC = 0.7 dS m^{-1} and from saline soil with an EC = 4.7 dS m^{-1}. Plants were irrigated with non-saline water–tap water with an electrical conductivity water (ECw) value of 0.2 dS m^{-1}, as well as low, moderate and severe saline water-irrigation with saline water with an ECw of 4 dS m^{-1}, 8 dS m^{-1} and 12 dS m^{-1}, respectively. The upper threshold of *P. indica* salinity tolerance was 0.4 mol L^{-1} NaCl in both liquid and solid broth medium. The upper thresholds of the salt adapted and non-adapted *Azospirillum* strains were 0.2 and 0.4 mol L^{-1} NaCl, respectively. The results indicated a positive influence of the organisms on salinity tolerance, more with the saline-adapted *Azospirillum* strains than the non-adapted strains. *P. indica* was more effective than the *Azospirillum* strains. These results could be related to a better water status, higher photosynthetic pigment contents and proline accumulation in wheat seedlings inoculated with *P. indica*. The benefits of both isolates and *P. indica* depended on two factors: water salinity and growth stage of the host plant. Inoculation with the two isolates increased salinity tolerance of wheat plants; the saline-adapted *Azospirillum* strains showed better performance with respect to improved fresh and dry weights at 80 and 100 days after sowing under both non-saline and saline conditions. When compared to plants inoculated with non-saline-adapted *Azospirillum* strains, those inoculated with adapted *Azospirillum* strains had much better performance with respect to the presence of photosynthetic pigment (Chl *a*, *b* and *ab*) and proline accumulation. Overall, these results indicate that the symbiotic association between *P. indica* fungus and wheat plants improved wheat growth, regardless of the salinity. It is concluded that the mechanisms for protecting plants from the detrimental effects of salinity by *P. indica* fungus and *Azospirillum* strains may differ in their salinity tolerance and influence the uptake of water, photosynthetic pigment contents and proline accumulation in wheat seedlings.

1. Introduction

The problem of soil drought and salinity present one of the greatest obstacles to enabling agriculture to meet the need of the world's growing population (Killham, 1994). Shannon (1997) estimated that 10% of the world's cropland and as much as 27% of the irrigated land may already be affected by salinity, and one-third of the world's arable land resources are affected by salinity (Qadir

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et al., 2000). Many keys to agricultural success in arid and semi-arid areas are to use adequate plant species and to use the soil biology potential to maintain soil fertility, and to guard against erosion and water stress (Zarea, 2010).

Scientists have searched for new salt-tolerant crop plants (Glenn and O'Leary, 1985), developed salt-tolerant crops through breeding (Shannon, 1984), and continue to investigate the physiology of genetic alterations involved in salt tolerance (Apse et al., 1999). Other attempts to deal with saline soils have involved leaching of excessive salts (Hamdy, 1990) or desalinizing sea water for use in irrigation (Muralev et al., 1997). Although these approaches have been successful, most are beyond the economic means of developing nations (Cantrell and Linderman, 2001). Plant breeding may be available for some plant species in these areas, but not for all crops being grown (Cantrell and Linderman, 2001). An experimental alternative is to alleviate salt stress by inoculating crops seeds and seedlings with various plant growth-promoting bacteria (PGPB), such as Rhizobium and *Azospirillum* spp. and also with mycorrhizal fungi.

The use of PGPB and symbiotic microorganisms has proved useful in developing strategies to facilitate plant growth in saline soils (Kohler et al., 2009). In general, inoculation with PGPB can enhance germination, seedling emergence and modify growth and yield of various cereal and non-cereal crops (Zahir et al., 2004). Regarding *Azospirillum*, the most researched associative bacterium (Bashan and Holguin, 1997), stress conditions appear to emphasize its growth-promoting effects on plants (Barassi et al., 2000). The damaging effects of NaCl on wheat seedlings were reduced by inoculation with *Azospirillum brasilense* Sp245 (Creus et al., 1997), which partially reversed the negative effects on the relative elongation rate of shoots. Such reduction was accompanied by higher relative water contents (Creus et al., 1997). However, for several crops the tolerance to salt at one growth stage is not correlated to tolerance at another stage (Shannon, 1997).

Piriformospora indica, which belongs to the Sebacinales in Basidiomycota (Oelmüller et al., 2009), is a newly described root endophyte (Verma et al., 1998) with AMF-like characteristics (Varma et al., 2001). Moreover, in contrast to AMF which are obligate endosymbionts, *P. indica* has the added advantage of being able to grow in axenic cultures – it is cultivable *in vitro* (Varma et al., 1999). *P. indica* is a wide-host root-colonizing endophytic fungus which allows the plants to grow under extreme physical and nutrient stress (Oelmüller et al., 2009). The fungus can be cultivated on complex and minimal substrates (Oelmüller et al., 2009). *P. indica* has a vast geographical distribution and is reported from Asia, South America and Australia (Oelmüller et al., 2009). Since *P. indica* was isolated from a desert, it is likely that the fungus may confer drought tolerance to plants (Oelmüller et al., 2009).

Fungi tend to be amongst the most tolerant microbes to water stress (Killham, 1994). The improved growth of mycorrhizal plants has been attributed to enhanced nutrient uptake, particularly of N and P and subsequent increased growth (Jeffries et al., 2003). However, in some cases plant salt tolerance was not related to P concentration (Ruiz-Lozano and Azcon, 2000). Thus, it has been proposed that salt tolerance mechanisms, such as enhanced osmotic adjustment and leaf hydration, increased intrinsic water use efficiency, reduced oxidative damage or improved nutritional status, can explain the contribution of AM symbioses to the salinity resistance of host plants (Augé, 2001).

The intent of this study was to determine the salt tolerance of *P. indica* and salinity adapted and non-adapted strains of *Azospirillum*, as well as to determine their affect on the tolerance of wheat to soil salinity. The organisms were tested over a range of soil salinity levels in relation to plant growth and accumulation of solute accumulation (proline and sugars), and photosynthetic pigments. It was expected that *P. indica* and *Azospirillum* strains able to survive at higher salinity would have a greater ability to improve the growth of host plants than species or isolates cultured under normal edaphic condition.

2. Material and methods

2.1. In vitro P. indica salt tolerance test

The inoculum of *P. indica* was collected from Dr. E. Mohammadi Goltapeh of the Department of Plant Pathology, Faculty of Agriculture, Tarbiat Modarres University, Tehran, Iran. *P. indica* was prepared as follows: circular agar discs inoculated with spores of *P. indica* were placed onto Petri dishes containing modified Hill and Kafer medium (Varma et al., 2001) and then incubated in an inverted position for seven days at 28 °C in the dark. Next, five fully-grown fungi agar discs (5 mm in diameter) were inoculated into individual 500-mL Erlenmeyer flasks containing 250 mL of Hill and Kafer broth (Varma and Oelmüller, 2007). The flasks were then incubated at 28 °C with constant shaking at 100 rpm on a rotary shaker. The influence of NaCl on growth was determined by cultivating the isolate *P. indica* in a modified Hill and Kafer broth medium (Varma and Oelmüller, 2007) at seven NaCl levels (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mol L^{-1}), with non-NaCl serving as a control. Briefly, discs of inoculums (5 mm diameter) were cut from the leading edge of actively growing colonies on modified solidified Hill and Kafer broth medium agar plates (Varma et al., 2001) and then inoculated into 9-cm diameter Petri dishes containing 20 mL modified Hill and Kafer broth medium and one of the aforementioned levels of NaCl. Cultures were incubated at 25 °C in the dark for 14 days and each treatment had seven replications. Radii were measured on two perpendicular axes bisecting the center of the colony and the mean was calculated. Proportional growth in the various salt treatments was obtained by dividing the diameter of fungus in each salt-amended medium by its average growth in control media. The influence of NaCl on *P. indica* biomass yield was determined by growing the fungus in a liquid modified Kafer liquid medium (Varma and Oelmüller, 2007) at seven NaCl levels (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mol L^{-1}), using non-NaCl as control. The final pH was adjusted to 6.5 after autoclaving for 15 min at 121 °C. Five fully-grown fungus agar discs (5 mm in diameter) were then inoculated into individual 500-mL Erlenmeyer flasks containing 250 mL of Kafer broth with seven replications. Next, the flasks were incubated at 28 °C with constant shaking at 100 rpm on a rotary shaker. The cultures were then incubated for 14 days. The mycelial mats were then removed from the liquid media, dried overnight at 80 °C and weighed using an electron scale. Finally, the Na concentration was measured by the flame photometry (Ryan et al., 1996).

2.2. Bacteria used in the study

Saline-adapted *Azospirillum* strains were isolated from roots of field-grown maize from a typical saline soil (EC = 4.7 dS m^{-1}) of Khuzestan Province, an arid area in southwest Iran. Non-saline-adapted *Azospirillum* strains were also isolated from roots of field-grown maize from a non-saline soil (EC = 0.7 dS m^{-1}), typical of Lurestan Province, a semi-arid area of western Iran. Briefly, samples of root pieces washed and treated with 1% chloramine T were placed into tubes, each of which contained 5 mL of nitrogen-free semi-solid malate (NFb) medium (Döbereiner and Day, 1976). These enrichment cultures were incubated at 37 °C for 72 h. A white, dense, undulating, diffuse pellicle was then observed 1–4 mm below the surface. The cultures exhibited a positive nitrogenase activity and they were streaked out on agar–Congo red

medium (Cáceres, 1982) plates. Typical pink, often wrinkled colonies were picked out and transferred into semi-solid NFb medium. Pellicle formation in this medium indicated successful isolation. Purified colonies were transferred to a nutrient agar slant for storage and use for further studies. *Azospirillum* strains were kept in agar–Congo red medium (Cáceres, 1982), transferred to Okon, Albrecht and Burris liquid medium (Okon et al., 1977) containing 0.1% NH_4Cl, and incubated at 35 °C with orbital agitation (100 rpm). The bacterial culture was centrifuged at 4000 rpm for 5 min at 2 °C and the sediment was re-suspended in sterilized tap water. The bacterial suspension contained 10^7 CFU mL^{-1}. *In vitro* salt tolerance testing was used to establish the salt tolerance of each of the isolates. The influence of NaCl on the recovery of suspension containing 10^2 CFU mL^{-1} salinity-adapted or non-adapted *Azospirillum* strains was determined by growing the isolates in agar–Congo red medium at seven NaCl levels (0, 0.1, 0.2, 0.3, 0.4, 0.5 or 0.6 mol L^{-1}), with non-NaCl as control. Of each *Azospirillum* strain, 1 mL containing 10^2 CFU mL^{-1} was inoculated into a 9-cm diameter Petri dish containing 20 mL of agar–Congo red medium. Cultures were incubated at 35 °C in darkness for 10 d, with seven replications per treatment. The percentage recoveries of *Azospirillum* strains were measured and the means calculated.

2.3. Pot experiments

Wheat seeds (*Triticum aestivum* cv. *sardari*) were surface sterilized by immersion in 70% alcohol for 30 s, followed by immersion in 2% sodium hypochlorite for 2 min, and then washing three times with sterile distilled water. Seeds were inoculated by immersion in a total imbibition volume of phosphate buffer (control) or bacterial inoculum containing 10^7 bacterial cells $seed^{-1}$ for 3 h, after which they were immediately placed into flasks containing 100 mL of Okon, Albrecht and Burris liquid medium. Non-inoculated treatments received the same amount of autoclaved inoculum. Mycelium from liquid culture was washed with water three times to completely remove the medium, and then crushed with 5–10 short pulses (5 s) of a blender. For inoculation of wheat plants, 3 g of crushed mycelium was added to 300 g of substrate before sowing. The pot experiments were carried out in a greenhouse of the Horticulture Department, Faculty of Agriculture Science in Ilam. Soils were previously three times heat-sterilized in metal buckets at 121 °C for 1 h on each of three successive days. Wheat was grown in a sterilized 2:1:1 mixture of expanded clay, sand and cattle manure in early April 2010. Of the mixture, 3.7 kg were sterilized by autoclaving three times at 120 °C for 20 min on three consecutive days, and placed in 4-L plastic pots. Soil had total N 0.65%, available phosphorus (P) 14.3 mg kg^{-1}, potassium (K) 58.9 mg kg^{-1}, organic matter 0.7% and pH 7.3 (soil:water, 1:1). The soil electrical conductivity (ECe, electrical conductivity of solution extracted from a water-saturated soil paste) was 0.7 dS m^{-1}. Salinity was determined by measuring ECe conductivity of soil paste extracts with a conductivity meter according to Rhoades (1982). To avoid reducing colonization by *Azospirillum* strains, N was broadcast on all pots and incorporated below the soil surface at a rate of 6 g N pot^{-1} as urea at 20 d after sowing. The addition of phosphate fertilization results in a delay in infection as well as a decrease in the percentage of infection of roots by mycorrhizae. Therefore, to avoid any reducing roots colonization by *P. indica*, P was broadcast on all pots and incorporated below the soil surface at a rate of 3 g P pot^{-1} as triple superphosphate at 20 d after sowing. Specifically, seedlings up to 100 days old were cultivated under a day–night cycle of 14 h (27 °C) and 10 h (18 °C). The experiment was conducted in a greenhouse with an average temperature of 25–30 °C and a relative humidity of 50–60%. The average midday photosynthetically active radiation was 250 μE m^{-1} s^{-1}.

2.4. Greenhouse experimental design and salt stress treatments

The experiment consisted of a 4 × 4 complete factorial design comprising four salinity treatments. The experiment was conducted as a factorial design in randomized blocks with two factors and seven-fold replication. The first factor had four levels: seedlings inoculated with the *P. indica*, salinity adapted and non-adapted *Azospirillum* strains, and a non-inoculated treatment. The second experiment had four levels of irrigation water management treatments: (i) non-saline water (NSW)–tap water with ECw = 0.63 dS m^{-1}, and low, moderate and severe saline water (SW)-irrigation with saline water with (ii) ECw = 4 dS m^{-1} (LSW), (iii) ECw = 8 dS m^{-1} (MSW) and ECw = 12 dS m^{-1} (SSW). At the initiation of the salinity treatment, NaCl concentration was gradually increased by ECw = 0.6 dS m^{-1} at 3-d intervals until reaching the required salinity of NaCl for each concentration.

2.5. Colonization assessment, determination of fresh and dry weights, photosynthetic pigments and solute accumulation

The presence of *P. indica* was monitored microscopically throughout the vegetation period. Fungal structures were visualized using 0.01% acid fuchsin-lactic acid (Kormanik and McGraw, 1982) or monitored after Trypan blue staining. Root colonization was evaluated by the grid intersect method followed by staining of about 100 root segments (1 cm each) with Trypan blue or Chlorozal black E. The percentage of root colonization was determined with a grid line intersect method (Giovanetti and Mosse, 1980) under a microscope (40×). The percentage of root colonization was calculated as follows:

$$\text{Percentage root colonization} = \frac{\text{Colonized root number}}{\text{Total root number}} \times 100$$

Plants were harvested at 60, 80, and 100 d after planting. Each plant was decapitated and the shoot systems were then weighed. The variations in their solute accumulations (proline and sugars) and photosynthetic pigment contents (chlorophyll *a* and *b*) were measured. In addition, dried biomass was calculated corresponding to a relative moisture of 86% dry weight (DW). Total water soluble carbohydrates were estimated as described by Thimmaiah (2004). Proline was determined by the method of Bates et al. (1973), and expressed as μmol g^{-1} fresh weight (FW) of leaf. The amount of total soluble sugars was estimated in fresh leaf material using the anthrone method (Thimmaiah, 2004). Photosynthetic pigments (Chl *a* and *b*) concentrations were measured on fresh fully expanded leaves. Fresh tissue (1.0 g) was extracted with 90% acetone, and read using a UV/visible spectrophotometer at 663, 645 and 750 nm wavelengths. Absorbance at 750 nm was subtracted from the absorbance at the other two wavelengths, to correct for any turbidity in the extract, before Chl *a* and *b* concentrations were calculated using the formulae below (Strain and Svec, 1966):

$$\text{Chl } a\left(\text{mg mL}^{-1}\right) = 11.6 \times (A663) - 2.16 \times (A645)$$

$$\text{Chl } b\left(\text{mg mL}^{-1}\right) = 20.97 \times (A645) - 2.16 \times (A663)$$

2.6. Statistical analysis

Differences among treatments were analyzed for main effects (salinity and microorganisms) and their interaction by a two-way ANOVA using the SAS software package (SAS Institute, 2000). Treatment effects were considered significant at $P < 0.05$. L.S.D.s ($P < 0.05$) were used to compare means within and among treatments.

3. Results

3.1. Salt threshold of Azospirillum strains and Piriformospora indica

The upper thresholds of the salinity adapted and non-adapted *Azospirillum* strains were 0.2 and 0.4 mol L^{-1} NaCl, respectively. The percent recovery of CFU at 0.2 mol L^{-1} NaCl was over 95% for salinity adapted *Azospirillum* strains, and remained over 50% and 27% in the 0.3 and 0.4 mol L^{-1} NaCl treatments, respectively. The percent recovery of CFU was greater than 75% for non-adapted *Azospirillum* strains in the 0.1 mol L^{-1} NaCl treatment, and declined to 15% in the 0.2 mol L^{-1} NaCl treatment. No non-adapted *Azospirillum* strains were observed in the 0.3 mol L^{-1} NaCl group.

P. indica had a greater threshold for salinity tolerance than both salinity adapted and non-adapted *Azospirillum*. Additionally, *P. indica* could grow under salinity levels as high as 0.4 mol L^{-1} NaCl (Fig. 1). *P. indica* immediately entered the linear growth phase in the absence of salt stress, with the mycelium suffusing the entire plate after 5 days of culture. However, the growth of the fungus was reduced by salt stress (Fig. 1), and increasing NaCl concentrations led to decreased growth rates. Additionally, the dried biomass yields showed a sizable decline with increasing NaCl concentration. Specifically, the biomass yield at high concentrations of NaCl (0.4 mol L^{-1}) was only 28 μg L^{-1} medium, while it was 275 μg L^{-1} medium in the control. The growth was stunted at high concentrations of NaCl (0.5 mol L^{-1}) in both liquid and solid broth medium. The hyphae Na^+ concentration significantly increased with increasing salt concentrations. Specifically, the Na^+ concentrations in the control were significantly lower ($P < 0.01$) than in all NaCl treatments, while the highest hyphae Na^+ concentration was observed in the 0.4 mol L^{-1} NaCl treatment.

3.2. Wheat growth

The percentage root colonization with *P. indica* was not affected by water salinity. The percentage *P. indica* colonization of the cortex was 67% in 60-day-old wheat plants. The benefits of the organisms depended on two factors: water salinity and growth stage of the host plant. Plants colonized by *Azospirillum* strains, especially saline-adapted *Azospirillum* strains, showed accelerated reproduction and growth rates under both salinity and non-salinity irrigation specifically, 80-day-old plants inoculated with saline-adapted *Azospirillum* strains were at their flowering stage, while those inoculated with non-salt *Azospirillum* strains were at their spike emergence stage (Data not shown). At this growth stage, non-inoculated 80-day-old seedlings were in their stem elongation stage, while *P. indica*-inoculated plants were in their grain filling stage (Data not shown).

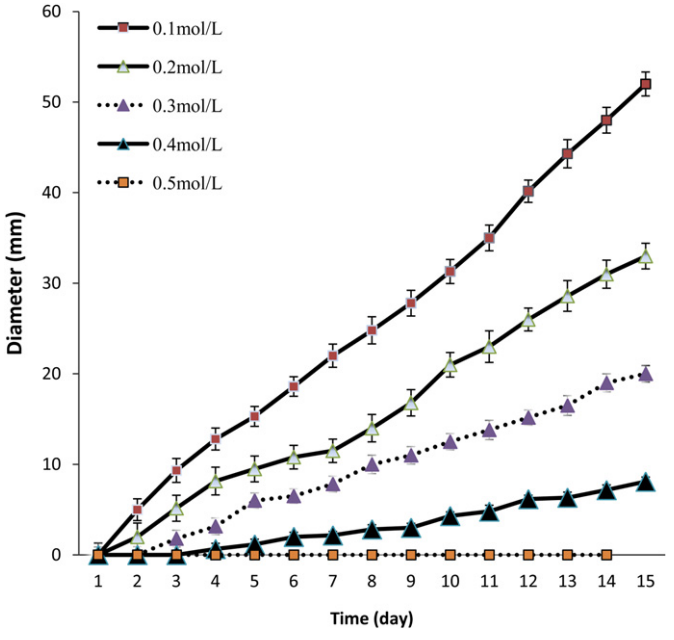

Fig. 1. Dose–response curves for the growth models of *Piriformospora indica* in the presence of NaCl at a range of concentrations in solidified Kafer medium over 15 days. $P < 0.05$. Values are mean ± SE ($n = 7$).

Microorganisms inoculation, salinity and their interaction had no effect on fresh matter yields at 60 days after sowing (Table 1). There was difference in shoot dry matter yields between 60-day-old inoculated and non-inoculated wheat seedlings. Non-salinity adapted *Azospirillum* strains showed better performance with respect to improved dry weights at 60 days after sowing under non-saline, low and moderate water salinity conditions (Table 2). Under severe water salinity conditions (ECw = 12 dS m^{-1}), the shoots of 60-day-old seedlings inoculated with *P. indica* had the highest dry weight (Table 2). The colonized plants generally had higher fresh and dry shoot matter yields than non-colonized plants at 80 and 100 days under both non-saline and saline conditions (Tables 3 and 4). Shoot fresh matter weights of 80-day-old seedlings as well as shoot fresh and dry matter weights of 100-day-old seedlings decreased in plants grown under saline irrigation when compared to non-saline irrigation, regardless of inoculation with *P. indica* or *Azospirillum* (Tables 3 and 4). Shoot dry matter weights of 80-day-old seedlings increased in plants grown under saline irrigation with an ECw of 8 dS m^{-1}, regardless of inoculation with *P. indica* or *Azospirillum* (Table 3). Water-irrigation with saline water with an ECw of 8 dS m^{-1} increased the shoot dry matter yields of 80-day-old seedling wheat inoculated with *P. indica* (Table 3). The shoots of 80- and 100-day-old seedlings inoculated with *P. indica* had the highest fresh and dry weight (Tables 3 and 4). The shoots of plants inoculated with adapted and non-adapted *Azospirillum* strains had greater fresh and dry weights at 80 days when compared to non-colonized plants (Table 3). Salinity adapted *Azospirillum* strains showed better performance with respect to improved fresh and dry weights at 80 and 100 days after sowing under both non-saline and saline conditions (Tables 3 and 4).

3.3. Effect on photosynthetic pigments and solute (proline and sugars)

The leaves of inoculated plants had generally higher photosynthetic pigment contents than the leaves of non-inoculated plants. The leaf photosynthetic pigments contents were lower for plants grown under saline conditions than those grown under non-saline conditions. There was no interaction between the bacterial inoculation and *P. indica* colonization with either of the water salinity regarding photosynthetic pigments (Chl *a* and *ab*) in 80-day-old wheat plants (Table 1). Salinity decreased Chl *a*, *b* and *ab* in 80- and 100-day-old wheat plants (Tables 3–5). Photosynthetic pigments (Chl *a*, *b* and *ab*) increased in response to inoculation with microorganisms (Tables 3–5). Specifically, 80- and 100-day-old plants inoculated with *P. indica* had the highest concentrations of Chl *a*, *b* and chl *ab* (Tables 3–5). The saline-adapted *Azospirillum* strains inoculated plants presented had higher contents of Chl *a*, *b* and *ab* in 80-day-old wheat plants (Tables 3 and 5). 100-day-old plants inoculated with salinity adapted *Azospirillum* strains had higher photosynthetic pigments (Chl *a*, *b* and *ab*) contents in leaves of 100-day-old seedlings sown under both non-saline and saline conditions (Table 4).

Leaf concentrations of proline were generally higher for inoculated plants than non-inoculated plants. Salt treatment resulted in

Table 1
A two-way ANOVA of the effects of microorganisms inoculation (M), water salinity (S) and their interaction on all parameters studied in 60, 80- and 100-day-old wheat plants.

Dependent variable	Mean square				*F*-values		Interaction (M × S)
	Error	Main factors		Interaction (M × S)	Main factors		
		M	S		M	S	
60-day-old-seedling							
Fresh weight	3.60	10.37	5.87	5.97	2.88^{ns}	1.62^{ns}	0.36^{ns}
Dry weight	0.12	0.095	0.930	0.47	0.75^{ns}	7.26***	3.68***
80-day-old-seedling							
Fresh weight	0.28	36.49	81.66	1.47	128.02***	286.47***	5.18***
Dry weight	0.01	0.45	0.10	0.11	37.95***	8.69***	10.00***
Chlorophyll *a*	0.0004	0.0041	0.0119	0.0006	10.05***	28.78***	1.62^{ns}
Chlorophyll *b*	0.0001	0.0016	0.0069	0.0002	13.51***	56.57***	2.44*
Chlorophyll *ab*	0.0008	0.0110	0.0384	0.0012	12.60***	43.83***	1.37^{ns}
Proline content	2.39	29.98	63.60	14.98	12.52***	26.56***	6.26***
100-day-old-seedling							
Fresh weight	0.19	93.56	140.83	1.85	480.29***	722.93***	9.50***
Dry weight	0.02	4.48	8.65	0.26	198.75***	383.44***	11.79***
Chlorophyll *a*	0.0001	0.0469	0.0172	0.0007	399.53***	147.10***	6.48***
Chlorophyll *b*	0.00003	0.00330	0.00618	0.00013	93.46***	174.83***	3.92***
Chlorophyll *ab*	0.00017	0.07285	0.04454	0.00110	409.76***	250.53***	6.22***
Proline content	2.05	243.88	969.13	51.59	118.51***	470.91***	25.07***

Note: Data represent Mean square and *F*-values.
***Indicates significant at $P < 0.0001$.
*Indicates significant differences at $P < 0.05$ for each treatment.
^{ns}Indicates no significant differences at $P < 0.05$ for each treatment.

higher proline levels, although the differences in sugar concentrations were not significant under saline conditions or between inoculated and un-inoculated plants (Data not shown). The levels of proline in the leaves increased in response to inoculation with microorganisms. Proline accumulation was considerably lower for non-inoculated plants. Plants inoculated with *P. indica* showed greater proline accumulation in leaves (Tables 3 and 4). Additionally, 80- and 100-day-old plants inoculated with salinity adapted *Azospirillum* strains had higher proline accumulation in leaves sown under non-saline and saline conditions (Tables 3 and 4). Increasing NaCl concentrations in irrigation water led to increased proline accumulation in plants, with the highest salt concentration producing the greatest proline accumulation in the leaves (Tables 3 and 4). Total soluble sugars concentrations were not affected by inoculation with *P. indica* or salinity adapted and non-adapted *Azospirillum*. Increasing NaCl concentrations in irrigation water had no effect on sugar accumulation by the plants (Data not shown).

Table 2
Effects of *Piriformospora indica* and *Azostrainsirillum* isolates from saline or non-saline soil on shoot dry weight in 60-day-old wheat plants at different water salinity levels (ECw of 0.7–12 dS m^{-1}).

Treatment	60-day-old
	Shoot dry weight (g plant^{-1})
0.7 dS m^{-1}	
Piriformospora indica	1.77 ± 0.16cde
Adapted *Azospirillum* strains	1.67 ± 0.36def
Non-adapted *Azospirillum* strains	2.00 ± 0.34abcde
Control	1.63 ± 0.22ef
4 dS m^{-1}	
Piriformospora indica	1.85 ± 0.12bcde
Adapted *Azospirillum* strains	1.63 ± 0.08ef
Non-adapted *Azospirillum* strains	2.06 ± 0.30abc
Control	1.36 ± 0.08f
8 dS m^{-1}	
Piriformospora indica	2.06 ± 0.09abc
Adapted *Azospirillum* strains	2.03 ± 0.01abcd
Non-adapted *Azospirillum* strains	2.26 ± 0.33a
Control	1.78 ± 0.12bcde
12 dS m^{-1}	
Piriformospora indica	2.35 ± 0.16a
Adapted *Azospirillum* strains	2.10 ± 0.35ab
Non-adapted *Azospirillum* strains	2.08 ± 0.15abc
Control	1.762 ± 0.28cde
LSD $P \leq 0.05$	0.36

Values (mean ± SE, $n = 7$) within a column followed by the same letter are not significantly different at the $P \leq 0.05$ level for the given salinity (Least Significant Difference test).

4. Discussion

4.1. Effects of NaCl concentration on the growth rate of P. indica and CFU of salinity adapted and non-adapted Azospirillum strains

P. indica was more tolerant to salt stress than *Azospirillum* strains. The Basidiomycotina, which are involved in most of the ectomycorrhizal symbioses, appear to have a tolerance to water stress, based solely on the salt concentration of a liquid culture medium, of up to about −70 bar (−7 MPa) (Killham, 1994). The *P. indica* (belongs to the Sebacinales in Basidiomycota) exhibited significantly higher growth rates and biomass yield at lower NaCl concentrations. However, the growth rates declined with increasing NaCl concentration, but not the biomass yields. These findings indicate that the density of the hyphae increased with increasing NaCl concentration, which appears to be a strategy for salt-resistant fungal species to relieve osmotic pressure. These findings agree with the results of a study conducted to investigate hyphal development of arbuscular-mycorrhizal fungi that showed that high salinity could reduce hyphal development, inhibit hyphal branching and elevate hyphal diameter (Juniper and Abbott, 1993; Tang et al., 2009). Osmotic pressure forces the fungus to produce cells of higher density with more compact biomass, which leads to an increase in Na− concentration within the cells to reduce osmotic pressure (Tang et al., 2009). The results of the present study showed that the endophytic fungus *P. indica* can withstand a high salt tolerance of 0.4 mol L^{-1} NaCl via modified physiological and biochemical attributes that allows them to withstand a wide range

Table 3
Effects of *Piriformospora indica* and *Azostrainsirillum* isolates from saline or non-saline soil on shoot fresh and dry weight, proline accumulation and photosynthetic pigments (Chl *b*) accumulation in 80-day-old wheat plants at different water salinity levels (ECw of 0.7–12 dS m^{-1}).

Treatment	Shoot fresh weight (g $plant^{-1}$)	Shoot dry weight (g $plant^{-1}$)	Proline (μmol g^{-1})	Ch *b* (mg mL^{-1})
0.7 dS m^{-1}				
Piriformospora indica	13.64 ± 0.29a	1.42 ± 0.10cd	3.20 ± 0.26ef	0.07 ± 0.005a
Adapted *Azospirillum* strains	12.0 ± 0.70bc	1.36 ± 0.10cdef	3.63 ± 0.13efd	0.08 ± 0.010ab
Non-adapted *Azospirillum* strains	12.32 ± 0.30b	1.28 ± 0.03ef	2.24 ± 0.18f	0.07 ± 0.005bc
Control	11.59 ± 0.02c	1.34 ± 0.06cdef	3.84 ± 0.98efd	0.05 ± 0.007ed
4 dS m^{-1}				
Piriformospora indica	10.93 ± 0.84d	1.56 ± 0.06b	4.76 ± 0.72cde	0.06 ± 0.006cd
Adapted *Azospirillum* strains	10.62 ± 0.41d	1.37 ± 0.09cde	5.55 ± 0.47c	0.07 ± 0.007bc
Non-adapted *Azospirillum* strains	9.98 ± 0.12e	1.25 ± 0.03fg	4.66 ± 0.61cde	0.05 ± 0.005efd
Control	8.53 ± 0.16 h	1.09 ± 0.03h	3.44 ± 0.26efd	0.04 ± 0.006efg
8 dS m^{-1}				
Piriformospora indica	10.97 ± 0.312d	1.81 ± 0.01a	5.90 ± 0.98c	0.04 ± 0.002efg
Adapted *Azospirillum* strains	9.77 ± 0.094 ef	1.43 ± 0.10c	7.82 ± 0.71ab	0.04 ± 0.004fghi
Non-adapted *Azospirillum* strains	9.32 ± 0.23 fg	1.39 ± 0.04cde	4.97 ± 2.79cd	0.04 ± 0.012ghi
Control	7.80 ± 0.39 i	1.16 ± 0.059gh	3.57 ± 0.22efd	0.04 ± 0.009fgh
12 dS m^{-1}				
Piriformospora indica	9.96 ± 0.44e	1.32 ± 0.04efd	9.23 ± 1.59a	0.05 ± 0.007ef
Adapted *Azospirillum* strains	9.11 ± 0.15g	1.36 ± 0.13cdef	8.18 ± 1.63a	0.04 ± 0.004ghi
Non-adapted *Azospirillum* strains	7.65 ± 0.24i	1.33 ± 0.04cdef	6.23 ± 0.50bc	0.03 ± 0.012i
Control	6.63 ± 0.04j	1.30 ± 0.09ef	3.53 ± 0.61efd	0.03 ± 0.008hi
LSD $P \leq 0.05$	0.56	0.11	1.61	0.01

Values (mean ± SE, $n = 7$) within a column followed by the same letter are not significantly different at the $P \leq 0.05$ level for the given salinity (Least Significant Difference test).

of environmental saline stress. Nevertheless, Na+ concentrations increased in *P. indica* as much as in the growing medium.

It is likely that the *Azospirillum* strains isolated from highly saline conditions had higher salt tolerance. Several reports suggest that osmotolerance in *Azospirillum* is strain-specific and depends on the extent of the salinity of the habitat from where the strain is isolated. A variety of *Azospirillum* strains, isolated from the salt affected soils in Brazil, were more salt tolerant than *Azospirillum* species isolated from other habitats and thus showed adaptation to their saline environment. Because microbial cell must maintain an internal water potential similar to that of its environment solutes must be accumulated within the cell (Tripathi et al., 1998). Stress solutes (e.g. NaCl, KCl) may be taken up from the soil environment or may be synthesized by the cell in order to obtain compatibility with intracellular enzymes (Killham, 1994). In order to adapt to fluctuation in soil salinity/osmolarity the bacteria of the genus *Azospirillum* accumulate compatible solutes such as glutamate, proline, glycine betaine and trehalose (Tripathi et al., 1998).

4.2. Wheat growth, shoot water content and solute accumulation in leaves of plants inoculated with P. indica and Azospirillum strains

The percentage *P. indica* colonization of the wheat cortex was not affected by water salinity or *Azospirillum* inoculation, which

Table 4
Effects of inoculation with *Piriformospora indica* and *Azostrainsirillum* isolates from saline or non-saline soil on shoot fresh and dry weight, proline accumulation and photosynthetic pigments (Chl *a*, *b* and *ab*) accumulation in 100-day-old wheat plants at different water salinity levels (ECw of 0.7–12 dS m^{-1}).

Treatment	Shoot fresh weight (g $plant^{-1}$)	Shoot dry weight (g $plant^{-1}$)	Proline (μmol g^{-1})	Photosynthetic pigments (mg mL^{-1})		
				Ch *a*	Ch *b*	Ch *ab*
0.7 dS m^{-1}						
Piriformospora indica	18.92 ± 0.14a	4.16 ± 0.11a	11.74 ± 0.13gh	0.21 ± 0.005a	0.07 ± 0.001a	0.29 ± 0.005a
Adapted *Azospirillum* strains	15.97 ± 0.25c	3.46 ± 0.05c	10.50 ± 0.12hi	0.18 ± 0.014c	0.05 ± 0.009b	0.23 ± 0.008c
Non-adapted *Azospirillum* strains	15.52 ± 0.16c	3.41 ± 0.04c	9.91 ± 0.52i	0.17 ± 0.008c	0.04 ± 0.003bcd	0.22 ± 0.008d
Control	13.37 ± 0.02ef	2.82 ± 0.03e	9.90 ± 0.19i	0.10 ± 0.003g	0.04 ± 0.005bc	0.15 ± 0.008f
4 dS m^{-1}						
Piriformospora indica	16.90 ± 0.4c	3.65 ± 0.14b	14.71 ± 0.17e	0.20 ± 0.000b	0.07 ± 0.001a	0.28 ± 0.001b
Adapted *Azospirillum* strains	13.72 ± 0.66e	2.81 ± 0.09e	13.43 ± 0.18ef	0.13 ± 0.003e	0.05 ± 0.001b	0.18 ± 0.003e
Non-adapted *Azospirillum* strains	12.91 ± 0.06fg	2.62 ± 0.09f	12.85 ± 0.99fg	0.13 ± 0.002ed	0.04 ± 0.004cd	0.18 ± 0.006e
Control	12.43 ± 0.55gh	2.54 ± 0.10fg	9.97 ± 0.67i	0.09 ± 0.002h	0.04 ± 0.001ed	0.13 ± 0.003h
8 dS m^{-1}						
Piriformospora indica	14.31 ± 0.52d	2.99 ± 0.24d	18.41 ± 0.21d	0.16 ± 0.002c	0.04 ± 0.003cbd	0.22 ± 0.003d
Adapted *Azospirillum* strains	12.32 ± 0.23hi	2.46 ± 0.16fgh	17.46 ± 0.53d	0.13 ± 0.004ed	0.03 ± 0.001ef	0.16 ± 0.002f
Non-adapted *Azospirillum* strains	11.85 ± 0.46i	2.42 ± 0.16gh	16.87 ± 0.26d	0.11 ± 0.004fg	0.03 ± 0.002gh	0.15 ± 0.004fg
Control	10.65 ± 0.42j	2.11 ± 0.03j	13.12 ± 0.48fg	0.07 ± 0.001i	0.02 ± 0.001hi	0.10 ± 0.002i
12 dS m^{-1}						
Piriformospora indica	12.60 ± 0.11gh	2.35 ± 0.06hi	30.63 ± 1.38a	0.14 ± 0.003d	0.03 ± 0.001fg	0.18 ± 0.003e
Adapted *Azospirillum* strains	10.87 ± 0.25j	2.29 ± 0.02hi	27.29 ± 3.50b	0.12 ± 0.008f	0.02 ± 0.002ij	0.14 ± 0.002gh
Non-adapted *Azospirillum* strains	10.62 ± 0.33j	2.22 ± 0.08ij	23.01 ± 0.35c	0.11 ± 0.003fg	0.01 ± 0.002j	0.13 ± 0.004h
Control	8.75 ± 0.17k	1.82 ± 0.040k	14.8 ± 0.21e	0.05 ± 0.007j	0.02 ± 0.004ij	0.08 ± 0.008j
LSD $P \leq 0.05$	0.49	0.16	1.569	0.011	0.006	0.013

Values (mean ± SE, $n = 7$) within a column followed by the same letter are not significantly different at the $P \leq 0.05$ level for the given salinity (Least Significant Difference test).

Table 5
Effects of *Piriformospora indica* and *Azostrainsirillum* isolates from saline or non-saline soil and different water salinity levels (ECw of 0.7–12 dS m^{-1}) on photosynthetic pigments (Chl *a* and *ab*) accumulation in 80-day-old wheat plants.

Treatment	Ch *a* (mg mL^{-1})	Ch *ab* (mg mL^{-1})
Piriformospora indica	0.12 ± 0.003a	0.19 ± 0.008a
Adapted *Azospirillum* strains	0.11 ± 0.007ab	0.18 ± 0.012ab
Non-adapted *Azospirillum* strains	0.11 ± 0.011b	0.16 ± 0.016b
Control	0.09 ± 0.009b	0.14 ± 0.013c
LSD ($P \leq 0.05$)	0.01	0.0157
0.7 dS m^{-1}	0.13 ± 0.002a	0.21 ± 0.007a
4 dS m^{-1}	0.12 ± 0.005b	0.18 ± 0.008b
8 dS m^{-1}	0.09 ± 0.008c	0.14 ± 0.011c
12 dS m^{-1}	0.09 ± 0.009c	0.13 ± 0.013c
LSD ($P \leq 0.05$)	0.01	0.0153

Values (mean ± SE, $n = 7$) within a column followed by the same letter are not significantly different at the $P \leq 0.05$ level for the given salinity (Least Significant Difference test).

may have been related to the high salt tolerance of the *P. indica* (Oelmüller et al., 2009). The results indicated a positive influence of the organisms on salinity tolerance, more with the saline-adapted *Azospirillum* strains than the non-adapted strains. *P. indica* was more effective than the *Azospirillum* strains. These results could be related to a better water status, higher photosynthetic pigment contents and proline accumulation in wheat seedlings inoculated with *P. indica*. Our results indicate that the symbiotic association between *P. indica* and wheat plants led to improved wheat growth, regardless of salinity. The results showed that wheat root colonization with *P. indica* increased the proline accumulation. Proline accumulation is a sensitive physiological index of the response of plants to salt and other stresses (Peng et al., 2008). For plants to survive under salt stress conditions, adjustment of leaf osmotic potential is very important, and this process requires intracellular osmotic balance. Under salt stress, plants accumulate some organic solutes (proline, soluble sugars, and so on) and inorganic ions to maintain higher osmotic adjustment (Yang et al., 2009). Therefore, better growth of *P. indica*-inoculated wheat compared to non-inoculated plants when exposed to water salinity may be a result of increased proline in the leaves of colonized plants. Greater concentrations of proline in leaves may have enabled *P. indica*-inoculated plants to maintain higher leaf water potential during stress and kept plants protected against oxidative stress. The increased shoot biomass of seedlings inoculated with *P. indica* may be related to the increase in water uptake. It has been previously shown that *P. indica* induces antioxidants: the amount of ascorbic acid, the ratio of reduced to oxidized ascorbate and the activity of dehydroascorbate reductase were elevated in barley roots (Waller et al., 2005). Root colonization by *P. indica* increased plant growth and attenuated the NaCl-induced lipid peroxidation, metabolic heat efflux and fatty acid desaturation in leaves of the salt-sensitive barley cultivar Ingrid (Baltruschat et al., 2008).

It is likely that the *Azospirillum* strains isolated from a saline environment can confer salinity tolerance to wheat. Since *Azospirillum* can adapt to edaphic conditions, it is able to promote plant growth under saline stress. Differences in the behavior and efficiency of *Azospirillum* may be due to the origin of *Azospirillum*. Our results indicate that the symbiotic associations with *Azospirillum* improved wheat growth. These results could be related to a better water status, higher photosynthetic pigment contents and proline accumulation in wheat seedlings inoculated with *Azospirillum*. It has been shown that the damaging effects of NaCl on wheat seedlings could be reduced by inoculation with *A. brasilense* (Creus et al., 1997; Bacilio et al., 2004). In wheat seedlings, *A. brasilense* Sp245 inoculation partially reversed the negative effects produced by salt and osmotic stress on the relative elongation rate of the shoots. This reduction was accompanied by a higher relative water content and water content (Creus et al., 1997). *Azospirillum* could also accumulate proline in response to NaCl (Bashan and Holguin, 1997), and promote proline accumulation in maize exposed to water stress (Casanovas et al., 2003), thus acting as an osmoprotectant. Additionally, *Azospirillum* has been shown to restrict Na^+ influx into roots, to accumulate proline and glutamate in response to NaCl (Bashan and Holguin, 1997), and to promote proline accumulation in maize exposed to water stress (Casanovas et al., 2003).

Mutualistic interactions between microbes and agroforestrical, horticultural and medicinal plants have long generated a great deal of attention since mutualists can improve the growth, biomass and seed production of crops grown on poor soil with little input of chemical fertilizers and pesticides (Oelmüller et al., 2009). This study showed that the endophytic fungus *P. indica* can withstand high salt levels. It has been shown in several crops that tolerance to salt at one growth stage is not correlated to tolerance at another stage (Shannon, 1997). The benefits of *P. indica* and both isolates depended on two factors: water salinity and growth stage of the host plant. The results of the present study suggested that *Azospirillum* strains able to survive at higher salinity may have a greater ability to improve the growth of host plants than species or isolates from areas with normal edaphic conditions. The application of bacteria and fungi under field conditions has shown to be quite different in comparison to lab experiments using sterile soils or nutrient solutions. Results were difficult to repeat even when experiments were performed identically (Bashan, 1986). In general, shortly after the bacteria are introduced into the soil, the bacterial population declines progressively (Bashan and Levanony, 1988). Some failures derived from the use of biofertilizers containing PGPR may be related to interspecific genetic interaction by the rhizobacteria and the host plant (Figueiredo et al., 2010). Previous studies have documented phenotypic variation within cultivars with respect to health and nutrition of plants from microbial inoculation (Remans et al., 2008). Phenotypic variation among cultivars may be partly due to genetic variation and suggested that the breeding of the host was performed in conjunction with PGPR in biofertilizers (Remans et al., 2008). Another strategy to reduce the effects of phenotypic variation can be the use of biofertilizers with more than two isolates in their composition. Numerous recent studies show a promising trend in the field of inoculation technology. Mixed inoculants (combinations of microorganisms) that interact synergistically are currently being devised. Plant studies have shown that the beneficial effects of *Azospirillum* on plants can be enhanced by co-inoculation with other microorganisms. Mixed inoculation with diazotrophic bacteria and arbuscular-mycorrhizal fungi creates synergistic interactions (Bashan, 1998). A major problem for massive use of PGPR has been formulated for its commercial use (Figueiredo et al., 2010). Several substances have been used in experimental formulations such as lactose, peptone, gum arabic and xanthan, cellulose, and others (Schisler et al., 2004). Inoculation techniques should be practical, economical, and easy to accomplish for the farmer; the formulated product should deliver sufficient inoculum to the plant, must be competitive with existing commercial standards, and must possess a long shelf life (Bashan, 1998).

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