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FULL LENGTH ARTICLE

Yield and yield components of wheat as affected by salinity and inoculation with *Azospirillum* strains from saline or non-saline soil

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KEYWORDS

Azospirillum isolates;
Nutrient uptake;
Salinity tolerance;
Solute accumulation;
Wheat

Abstract In many arid and semi-arid areas of the world where sustainability of agriculture is limited by salinity, use of biological potential may be a key component of sustainable plant production. A greenhouse experiment was used to test the effectiveness of inoculation with *Azospirillum* strains isolated from saline or non-saline soil in alleviating the salinity stress in wheat plants grown with irrigation water with different electrical conductivities (ECw) of 0.7, 4, 8 and 12 dS m^{-1}. Inoculation with the two isolates increased salinity tolerance of wheat plants; the saline-adapted isolate significantly increased shoot dry weight and grain yield under severe water salinity. The component of grain yield most affected by inoculation was grains per plant. Plants inoculated with saline-adapted *Azospirillum* strains had higher N concentrations at all water salinity levels.

1. Introduction

Field salinization is an increasing problem worldwide. 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 is affected by salinity (Qadir et al., 2000). The growing competition for scarce water resources, coupled with laws limiting ground water pumping, has led to the utilization of low quality water in irrigated agriculture (Al Omron et al., 2012). Some keys to agricultural success in semi-arid areas are to use the soil biology potential to maintain soil fertility, and to guard against erosion and water limiting (Zarea, 2010).

Scientists have searched for new salt-tolerant crop plants (Glenn and O'Leary, 1985), developed salt-tolerant crops

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through breeding (Shannon, 1984), and continued to investigate the physiology of genetic alterations involved in salt tolerance (Apse et al., 1999). Other attempts to deal with saline soils have involved the 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).

The application of nitrogen (N) fertilizer to plants growing in arid climate can increase the salt tolerance of plants (Cordovilla et al., 1994). Wallace and Berry (1981) suggested that wheat yield reduction due to increased salinity might not be entirely due to chloride (Cl^-) toxicity, but might be partially due to induced deficiency of NO_3^- caused by the external Cl^- concentration. According to Bernstein et al. (1974), decreased N uptake with increased salinity resulted in reduced plant growth.

The use of plant growth-promoting bacteria and symbiotic microorganisms has proved useful in developing strategies to facilitate plant growth in saline soils (Kohler et al., 2009). 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).

In this study, the following hypotheses were tested: (1) that the deleterious effects of salinity could be ameliorated by *Azospirillum* strains; and (2) that *Azospirillum* strains isolated from saline soil had a higher capacity to alleviate saline stress than strains from non-saline soil.

2. Material and methods

2.1. Pot experiments and types of soil

The pot experiments were carried out inside a greenhouse of the Horticulture Department, Faculty of Agriculture Science in Ilam. Soils were previously heat-sterilized in metal buckets at 121 °C for 1 h on each of three successive days. This is a recognized technique for soil sterilization; since some spore-forming bacteria can tolerate high temperatures, and spores may germinate on the second or third day. 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 was sterilized by autoclaving 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) 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.

2.2. Sterilization of seeds

To eliminate possible contamination, 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 washed three times with sterile distilled water. Pots were sterilized by swabbing thoroughly with 95% (v/v) ethanol.

2.3. Germination of seeds

Seeds were germinated in sterilized dishes containing sterile damp filter paper and sterile distilled water was added at intervals to keep the filter paper and germinating seeds wet. Seeds were incubated at 20 °C for 2–3 d until radicles were 2–3 cm long and root hairs appeared. Ten seedlings per pot were inoculated with *Azospirillum* strains.

2.4. Bacteria used in the study

The inoculum of *Azospirillum* strains was collected from Dr. M.J. Zarea of the Department of Agronomy and Plant Breeding, Faculty of Agriculture, Ilam University, Ilam, Iran. 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, for the isolation of *Azospirillum* sp the method described by Dobereiner and Day (1976) was used. Samples of root pieces washed and treated with 1% chloramine T were placed into tubes, each of which contained 5 mL of nitrogen-free semisolid malate (NFb) medium, and incubated at 35 °C. Paper-like white pellicles were formed at the surface after 3–5 days; the cultures were streaked out on agar plate with the same medium but containing 0.020 g of yeast extract. Colonies of these two species after 1 week were small, dry white and curled. Individual colonies were then re-checked by transforming them into semisolid NFb medium. Pellicle formation in this medium indicated successful isolation (Gunarto et al., 1999). For insurance the cultures that exhibited a positive nitrogenase activity were streaked out on agar–Congo red medium plates (Bacilio et al., 2004). Typical pink, often wrinkled colonies were picked out and streaked out on potato medium. After 1 week pinkish colony was selected and transferred into new semi-solid NFb vial. Further characterized carried out by gram staining, glucose assimilation and biotin requirement were used (Baldani and Dobereiner, 1980; Tarrand et al., 1978). *Azospirillum* strains were kept in agar–Congo red medium (Bacilio et al., 2004), transferred to Okon, Albrecht and Burris liquid medium 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

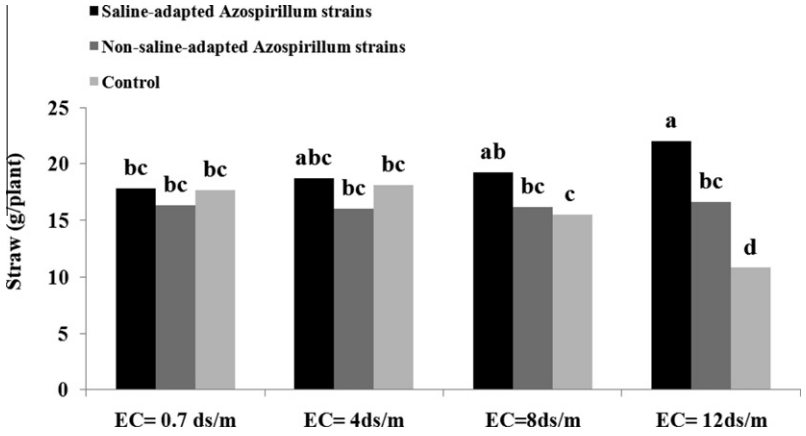

Figure 1 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on straw weight in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

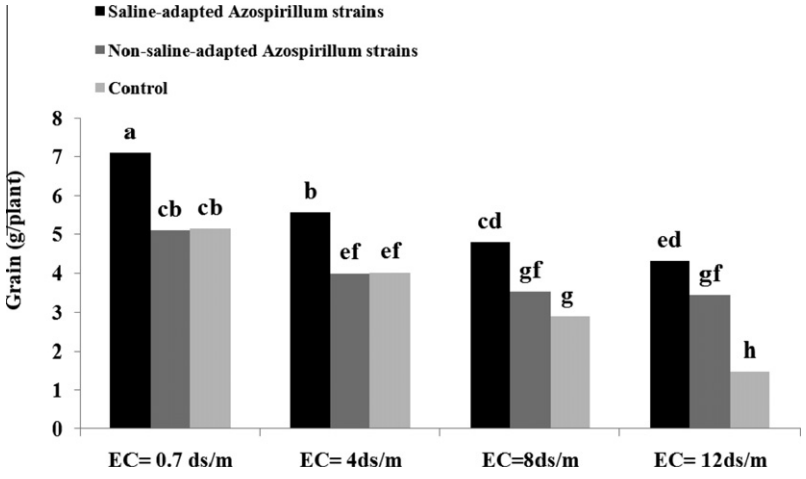

Figure 2 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on grain weight in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

red medium at seven NaCl levels (0, 0.1, 0.2, 0.3, 0.4, 0.5 or 0.6 mol/L), 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. The upper thresholds of the saline-adapted and non-adapted *Azospirillum* strains were 0.2 and 0.4 mol L^{-1} NaCl, respectively. The percentage recoveries of CFU at 0.2 mol L^{-1} NaCl were >95% for saline-adapted strains and remained >50% and 27% at 0.3 and 0.4 mol L^{-1} NaCl, respectively. The percentage recoveries of CFU were >75% for non-adapted *Azospirillum* strains in 0.1 mol L^{-1} NaCl and declined to 15% at 0.2 mol L^{-1} NaCl. No CFU of non-adapted strains was observed at 0.3 mol L^{-1} NaCl. Based on PCR amplification all bacteria were used in this experiment are *Azospirillum* according to 16S rDNA patterns and *brasilense* species according to the biochemical tests (Data not shown).

2.5. Salinity stress treatment

After transplanting the inoculated and non-inoculated seedlings into pots, there were four levels of water salinity applied: (i) non-saline water (NSW) – tap water with ECw = 0.7 dS m^{-1} (control); (ii) mild with ECw = 4 dS m^{-1} (LSW); (iii) moderate with ECw = 8 dS m^{-1} (MSW); and (iv) severe with ECw = 12 dS m^{-1} (SSW). The saline water was diluted to avoid osmotic shock, with 100 mL of each level applied once every 3 d, and with salt treatments continued for 13 weeks.

2.6. Determination of total N, photosynthetic pigments and solute accumulation

Plants were harvested at 120 d after planting. Each plant was decapitated and the shoot systems were then weighed. Total N was determined according to Page et al. (1982). The variations in their solute accumulations (proline and sugars) and photosynthetic pigment contents (chlorophyll *a* and *b*) were measured. Total water soluble carbohydrates were estimated

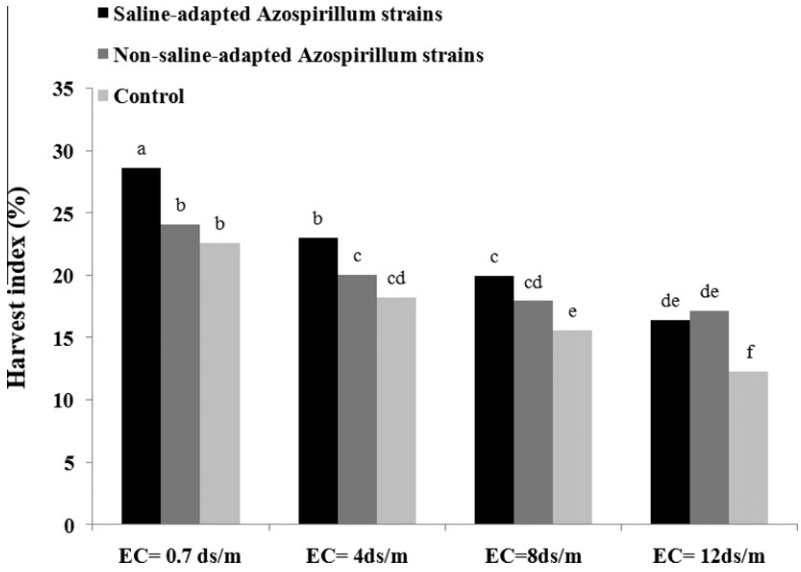

Figure 3 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on harvest index (%) in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

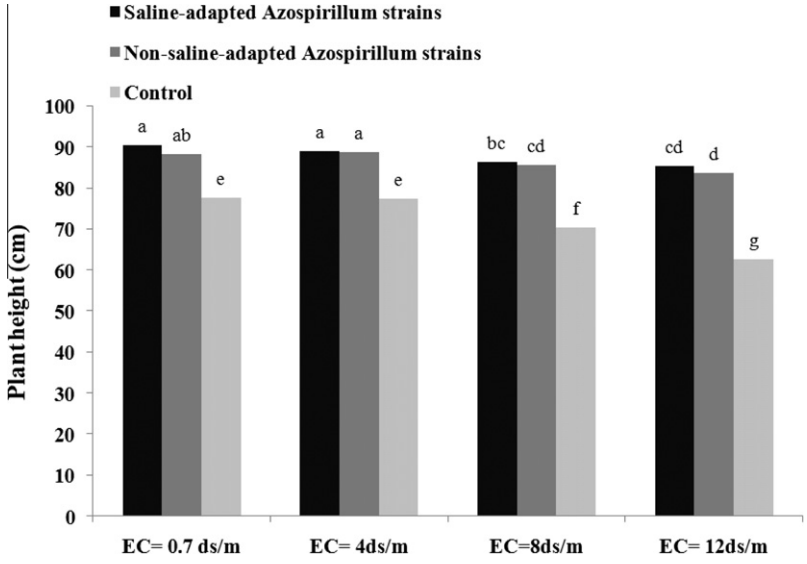

Figure 4 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on plant height (cm) in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

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). 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):

$$\text{Chl } a(mg\ mL^{-1} = 11.6 \times (A663) - 2.16 \times (A645)$$
$$\text{Chl } b(mg\ mL^{-1} = 20.97 \times (A645) - 2.16 \times (A663)$$

2.7. Determination of Yield and yield components

All the plants of different treatments were harvested in the same physiological growth state. Plants were hand-harvested at 5 cm from the above soil level at 180 d after planting. Data on wheat total dry biomass, grain and straw yields, harvest index, tiller and spike numbers per plant, seed number per spike, plant and spike height and weight of 1000 kernels were recorded.

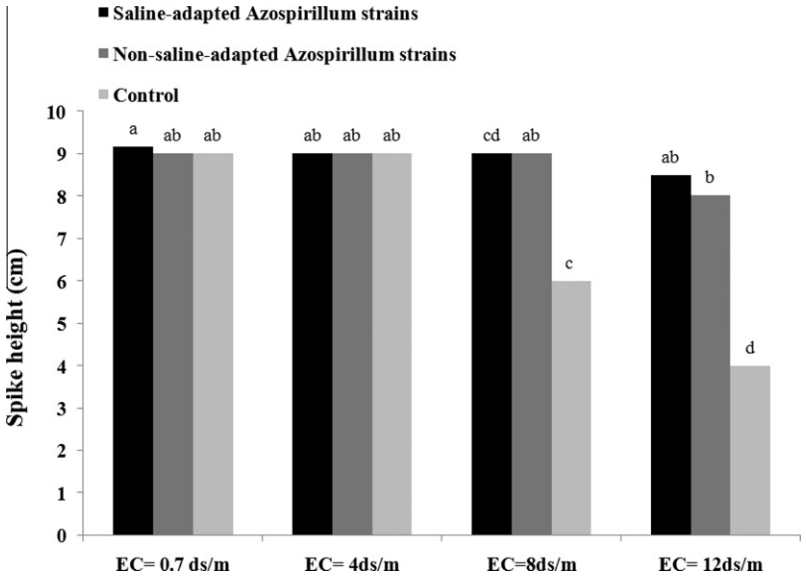

Figure 5 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on spike height (cm) in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

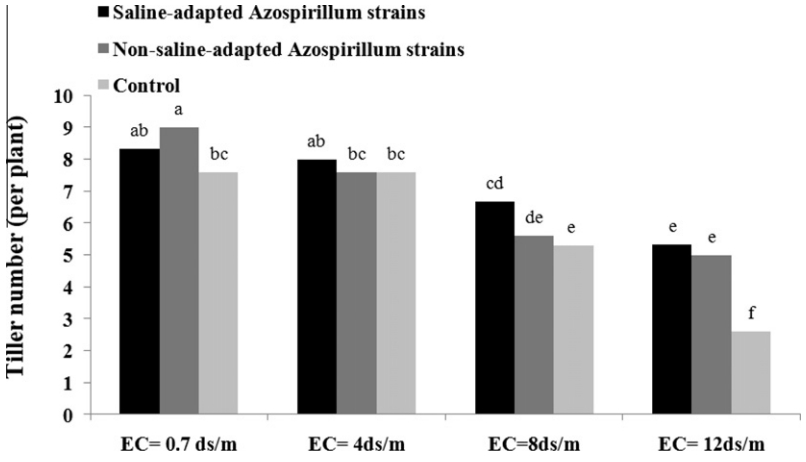

Figure 6 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on tiller number $plant^{-1}$ in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

Spikes were oven-dried at 70 1 °C for 72 h and their dry weights determined. Tiller and spike numbers per plant were recorded from 5 randomly chosen plants. Spike weight per plant was recorded from 5 randomly chosen plants. Dried biomass was calculated corresponding to relative moisture of 86% dry weight (DW).

2.8. Statistical analysis

The experiment consisted of a 4×3 complete factorial design comprising four salinity treatments. The experiment was conducted as a factorial design in randomized blocks with two factors and threefold replication. The first factor had three levels: seedlings inoculated with the 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. 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.Ds. ($P < 0.05$) were used to compare means within and among treatments.

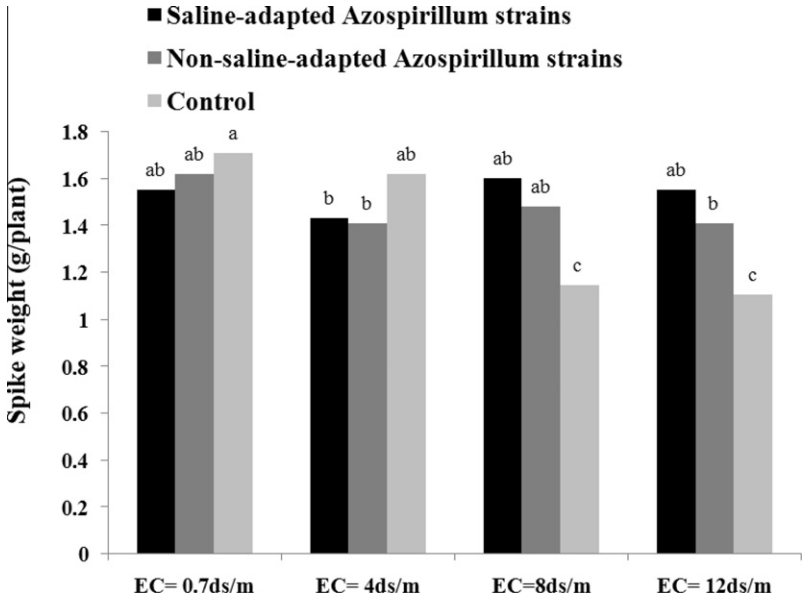

Figure 7 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on spike weight (g plant^{-1}) in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

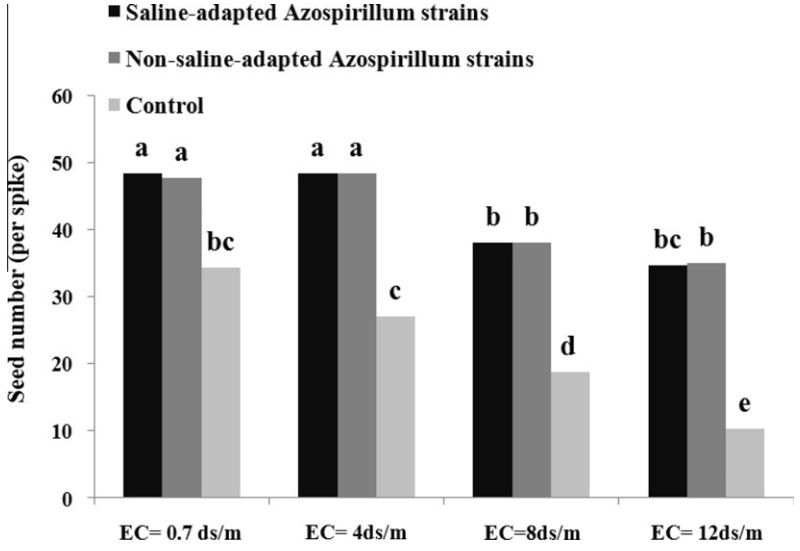

Figure 8 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on seed number (spike^{-1}) in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

3. Results

Apparently, inoculation with saline adapted *Azospirillum* improved growth under salt-stress conditions. Subjecting inoculated wheat plants to salt stress diminished the adverse effects caused by salinity stress, such as reduction of straw weight (Fig. 1), grain yield (Fig. 2), harvest index (Fig. 3), plant and spike height (Figs. 4 and 5), tiller number (Fig. 6), spike weight (Fig. 7), seed number (Fig. 8) and 1000-seed weight (Fig. 9) of wheat. *Azospirillum* inoculation significantly increased total plant dry weight, total number of tillers, and ears; earlier heading and flowering time; number of spikes and grains per spike; grain weight and higher harvest index, both at non-saline water and severe salinity water. Shoot height and fresh and dry weight of wheat seedlings inoculated with saline-adapted *Azospirillum* strains were enhanced, despite the salt stress. Inoculating wheat seedlings with saline-adapted *Azospirillum* strains exposed to severe salt (NaCl) stress significantly reversed part of the negative effects; both stresses reduced the relative elongation rate of shoots. Total yield was significantly reduced by salt impairing grains per plant and 1000-grain mass. The component of grain yield most affected by saline-adapted *Azospirillum* strains inoculation was grains per plant.

Chl *a* and *ab* concentrations in the leaves of wheat plants inoculated with both strains compared with controls were

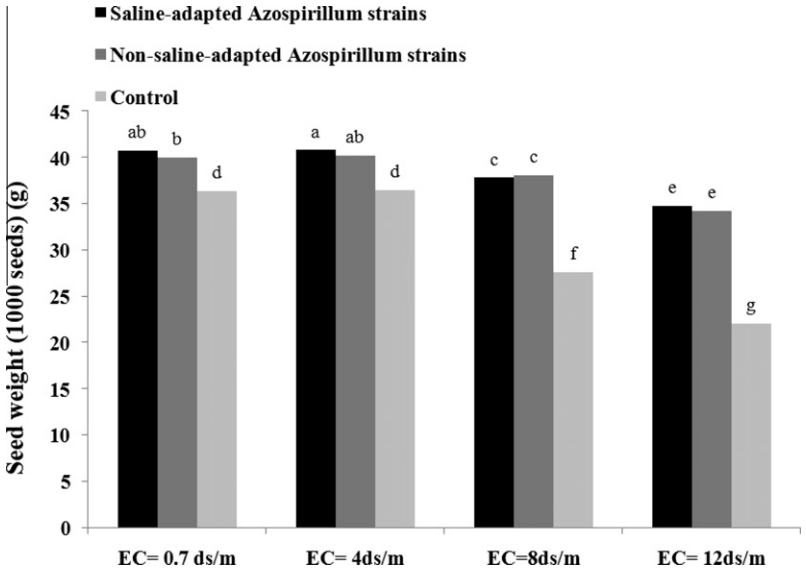

Figure 9 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on 1000-seed weight (g seed^{-1}) in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

Table 1 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on photosynthetic pigments (Chl*a*, *b* and *ab*) and proline accumulation (μmol g^{-1}) fresh weight in 100-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}).

ECw		Chl*a*	Chl*b*	Chl*ab*	Proline
0.7 dS m-1					
	Adapted *Azospirillum sp.*	0.18a	0.05a	0.23a	10.5a
	Non-adapted *Azospirillum sp.*	0.17a	0.04b	0.22a	9.91b
	Control	0.10b	0.04b	0.15b	9.90c
4 dS m-1					
	Adapted *Azospirillum sp.*	0.13a	0.05a	0.18a	13.43a
	Non-adapted *Azospirillum sp.*	0.13a	0.04b	0.18a	12.85b
	Control	0.09b	0.04b	0.13b	9.97c
8 dS m-1					
	Adapted *Azospirillum sp.*	0.11a	0.03a	0.15a	17.46a
	Non-adapted *Azospirillum sp.*	0.12a	0.02b	0.15a	16.56b
	Control	0.07b	0.02b	0.10b	13.12c
12 dS m-1					
	Adapted *Azospirillum sp.*	0.11a	0.02a	0.14a	27.29a
	Non-adapted *Azospirillum sp.*	0.12a	0.01b	0.14a	23.01b
	Control	0.05b	0.02b	0.08b	14.80c
	Salt	***	***	***	***
	Inoculation	***	***	***	***
	Salt × Inoculation	***	***	***	***

Values are means (n = 3) ±S.E. NS: no difference.
*** Significant difference at $P < 0.001$ by ANOVA

higher for irrigation with non-saline water and at mild, moderate and severe salinities (Table 1). Plants inoculated with the salt-adapted isolate had higher Chl *b* concentrations than controls and those inoculated with non-saline-adapted *Azospirillum* at all water salinity levels (Table 1).

Total soluble sugar concentrations were not affected by inoculation of plants with either *Azospirillum* isolate. The increasing NaCl concentrations in irrigation water had no effect on sugar accumulation in plants. The 100-d-old plants inoculated with saline-adapted *Azospirillum* had higher proline accumulation in leaves than controls and plants inoculated with non-saline-adapted *Azospirillum* at all salinity levels (Table 1).

Plants inoculated with salt-adapted *Azospirillum* had higher N concentrations (Fig. 10) and protein contents (Fig. 11) in shoots than controls and plants inoculated with non-saline-adapted *Azospirillum* at severe salinity level. Compared with non-inoculated plants, plants inoculated with the non-saline-adapted isolate had higher N concentrations and protein contents at all water salinity levels (Figs. 10 and 11).

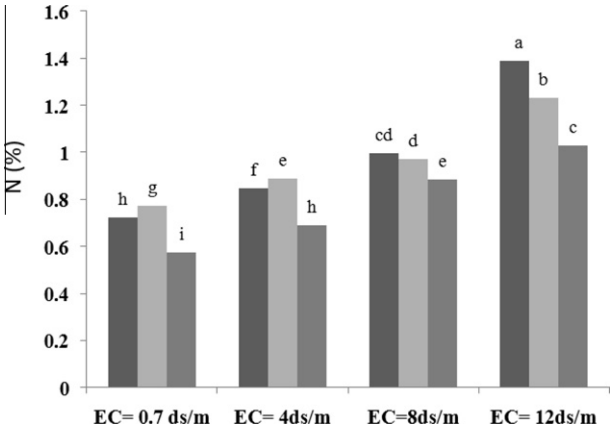

Figure 10 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on N concentration (%) in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

4. Discussion

The wheat plants inoculated with the two *Azospirillum* isolates showed increased salinity tolerance. The saline-adapted isolate significantly increased grain yield at severe water salinity. It has been shown in several crops that the tolerance to salt at one growth stage is not correlated to tolerance at another stage (Shannon, 1997). Therefore, the results presented above may be evident only at maturity growth stages. The saline-adapted *Azospirillum* isolate was more efficient in alleviating salinity toxicity to wheat at severe water salinity. The results suggested *Azospirillum* strains adapted to higher salinity environments may have greater ability to improve the growth of host plants than *Azospirillum* isolates from normal edaphic conditions.

For plants to survive under salt stress, the adjustment of leaf osmotic potential is very important and requires intracellular osmotic balance. Under salt stress, plants accumulate some organic solutes (e.g. proline and soluble sugars) and inorganic ions to maintain osmotic adjustment (Yang et al., 2009). Therefore, better growth of *Azospirillum*-inoculated wheat compared to non-inoculated plants when exposed to water salinity may be a result of increased proline as well as an increase in total N and protein in the leaves of inoculated plants. The damaging effects of NaCl on wheat seedlings can be reduced by inoculation with *A. brasilense* Sp245 (Creus et al., 1997), which partially reversed the negative effects of salt and osmotic stress on the relative elongation rate of shoots – such reduction was accompanied by a higher relative water content. *Azospirillum* can accumulate proline and glutamate 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.

Plants inoculated with saline-adapted *Azospirillum* had higher N concentrations. Salinity stress can decrease N concentrations (Kaya et al., 2009). Plants' metabolism and growth respond to the interaction between salinity and N in order to cope with changes in the environment (Shenker et al., 2003). An apparent increase in salt tolerance has been noted when N levels supplied under saline conditions exceeded those that were optimum under non-saline conditions (Papadopoulos and Rendig, 1983), implying that increased fertilization (especially N) may ameliorate the deleterious effects of salinity (Ravikovitch and Porath, 1967). This may be caused by ion accumulations in leaves, particularly old leaves (Greenway and Munns, 1980). Tabatabaei (2006) concluded that N concentration in the root zone, when increased salinity concentration was experienced by salt-tolerant cultivars, should be increased in order to improve plant growth. In our study, the *Azospirillum* strain isolated from saline soil promoted the growth of wheat plants under saline stress by increasing N concentrations without affecting P, Na and Cl concentrations.

The property of salinity tolerance is not a simple attribute, but it is an outcome of various features that depend on different physiological interactions, which are difficult to determine (Amira and Qados, 2011). In the present study, the symbiotic association between *Azospirillum* (especially the saline-adapted isolate) and wheat plants improved the growth of plants under salinity stress. We conclude that the mechanism underlying *Azospirillum*-inoculated plant growth improvement in wheat irrigated with saline water was associated with photosynthetic pigments and solute accumulation concentrations as well as N concentrations. In the present study, results suggested that *Azospirillum* strains which are adapted to higher salinity environments may have greater ability to improve the growth of

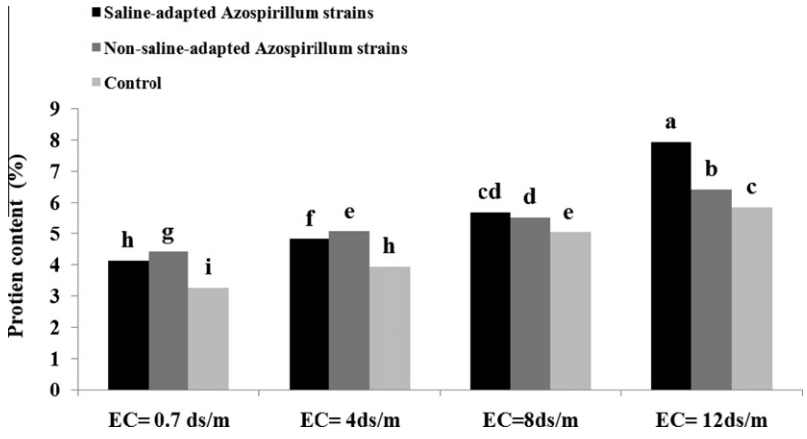

Figure 11 Effect of *Azospirillum* strain isolates from saline soil and from non-saline on protein content (%) in 180-day-old wheat plants under water salinity levels (water electrical conductivity (ECw) of 0.7–12 dS m^{-1}.

host plants than *Azospirillum* isolates from normal edaphic conditions.

Acknowledgement

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