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Full Length Article

Biocontrol and Salinity Tolerance Potential of *Azospirillum lipoferum* and its Inoculation Effect in Wheat Crop

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Abstract

Azospirillum lipoferum strain (GQ 255949) exhibits the ability to mitigate stress conditions. The present study was carried out to evaluate the biocontrol and salt tolerance potential of *A. lipoferum* and to examine its ability to mitigate salt stress in wheat crop when inoculated. *A. lipoferum* has shown strong biocontrol potential against *Aspergillus niger* and *Pseudomonass spp.* Strain exhibited the survival efficiency uptill 150 mM NaCl and also showed production of different osmolytes such as proline, soluble protein, soluble sugars and Super oxide dismutase while growing under saline conditions. Although salinity affected growth and metabolism of wheat, *A. lipoferum* inoculation significantly ameliorated its effects by improving the germination and plant growth, particularly under higher salinity levels (50 mM, 100 mM and 150 mM NaCl). Maximum impact of inoculation was observed at 150 mM NaCl. Chlorophyll content and membrane stability improved with inoculation resulting in 38.6% and 15.3% at 150 mM NaCl. Highest increase in concentration of osmolytes was 35.6% in proline, 28% in soluble sugars, in inoculated plants as compared an un-inoculated wheat plant under saline condition. It is concluded from the present findings that *A. lipoferum* strain (GQ 255949) has potential to promote growth of wheat plants under saline conditions.

Keywords: *Azospirillum*; Salinity; wheat; Osmotic stress; Abbreviation: SOD= superoxide dismutase

Introduction

Salinity is one of the major threats to plant biodiversity, especially for crops in arid regions (Munns, 2002; Flowers, 2004; Schleiff, 2008). Soil salinity is the scourge of buildup in salt level, extensive irrigation and application of chemical fertilizers which results in local accumulation of salt. On the whole, chlorides, sulfates and bicarbonates of different ionic species are major constituents of salinity in soil (Tripathia *et al.,* 1998). Salinity has several negative and limiting effects on plant growth, such as biomass reduction, osmotic and mineral imbalance, ion built up inside cellular compartments, shorter height, fewer or smaller leaves and slower growth rates (Parida and Das, 2005). Salt stress reduces water potential and causes imbalance in ion homeostasis and ion toxicity. This altered water status and ion build-up in plants is responsible for growth reduction in early stages which results in limited plant productivity (Hagemann and Eradmann, 1997; Hayashi and Murata, 1998).

To promote plant growth condition in saline soils use of PGPR is one of eminent technology (Bacilio *et al.,* 2004). Involvement of beneficial microorganisms in agriculture had played an important role in the replacement or reduction of chemicals which were previously used for enhancement of plant growth (Burdman *et al.,* 2000; Dobbelaere *et al.,* 2003). Bacteria and fungi established themselves as suppressors of pathogens and plant growth promoters and this valuable knowledge have been exploited extensively in agricultural biotechnology (Berg, 2009).

Growth promotion exerted by PGPRs is mostly related to or result of several important metabolic processes such as nitrogen fixation, release of metabolites, release of hormones like cytokinins, auxins and Gibberellins. Glick (1995); Glick *et al.* (1999); Tortora *et al.* (2011). On the other hand, these microbes interact with pathogenic soil microbes through an indirect mechanism by releasing antimicrobial substances, induction of resistance factor and competition for iron and colonizing sites (Whipps, 2001). In previous years *Azospirillum lipoferum* genus was used as model bacteria to study the positive association between cereals and bacterial population. Several types of phytohormones are secreted by *A. lipoferum* in the vicinity of the roots. These phytohormones are physiologically active and assist the plant to grow in stress conditions (Costacurta and Vanderleyden, 1995). The aim of the present study was to evaluate the salinity tolerance and biocontrol activity of *A. lipoferum* and also to investigate the mitigation ability of strain for salinity in wheat plants.

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Materials and Methods

Salinity tolerance of *A. lipoferum* and production of osmolytes salt tolerance was evaluated by preparing LB broth provided with different salt concentration of 25–200 mM NaCl. Incubation was carried out at 28±2ºC for 48 h (Upadhyay *et al.,* 2012; Ramados *et al.,* 2013). Culture broth was centrifuged at 1000 rpm for 10 min. The pellet was used for protein estimation according to Lowery *et al.* (1951) and for proline estimation supernatant was used (Bates *et al.,* 1973). Total soluble sugars were determined according to Dubios (1951).

Evaluation of Anti-Bacterial and Antifungal Activity

Agar well diffusion method was used to evaluate antifungal (Murthy *et al.,* 2009) and antibacterial activity of *A. lipoferum* (Hamdali *et al.,* 2008; Serabani *et al.,* 2011). Two bacterial strains *Pseudomonas spp., Escherichia coli* and two fungal strains *Aspergillus niger* and *A. terus* were used.

Germination Experiment

Germination experiment was designed design to check the effect of inoculation on Wheat seedling (under different salinity levels) after inoculation with and without *A. lipoferum* with three replications. Surface sterilized Seeds were inoculated with *A. lipoferum* strain. (Rueda-Puente *et al.,* 2007) and un-inoculated seeds were soaked in liquid broth. Each *A. lipoferum*-inoculated or un-inoculated seeds uniformly distributed on a double wattmann filter paper in sterilized petri dishes adequately provided with 0, 50, 100, 150 mM NaCl and 0 indicates control with distilled water. Germination was noted each day and seed was considered as germinated when seedling length reached up to 3 mm.

$$\text{Germination (\%)} = \frac{\text{Total seeds germinated}}{\text{Total No. of seeds}} \times 100$$

For calculation of germination index method and formula of (ISTA, 2005) was followed.

Germination index = number of seedlings emerged on day 'd'/days after planting. By using the method of (Abdul-Baki and Anderson, 1973) seedling vigor index was estimated.

Promptness index (P.I.) was calculated as:

P.I. = {nd2 (1.0) +nd4 (0.75) +nd6 (0.50) +nd8 (0.25)}

Where nd2, nd4, nd6 and nd8 are the seeds germinated on day 2, 4, 6 and 8, respectively (Noreen *et al.,* 2007).

Pot Experiment

Earthen pots with capacity of 10 Kg were filled with sterilized soil and placed in a glass house. Seeds of wheat varieties NARC 2009, NARC 2011 were collected from National Agricultural Research Centre (NARC). Wheat seeds were surface sterilized using 0.1% mercuric chloride (Nosheen *et al*., 2011). Seed inoculation was carried out using *A. lipoferum* strain before sowing. Salt stress was induced by increasing salt concentration (50, 100 and 150 mM) in irrigation water at vegetative stage for 9 days. Sowing was done on 20 October 2013. For plant analysis plants were harvested at vegetative stage before heading.

Treatments

T0	Well watered and control	T4	*Azospirillum* inoculated and well watered
T1	Uninoculated and 50 mM NaCl	T5	*Azospirillum* inoculated and 50 mM NaCl
T2	Un-inoculated and 100mM NaCl	T6	*Azospirillum* inoculated and 100 mM NaCl
T3	Un-inoculated and 150 mM NaCl	T7	*Azospirillum* inoculated and 150 mM NaCl

Plants were examined to evaluate the effect of inoculation on morphological, biochemical and physiological parameters under saline conditions. Shoot length, shoot fresh and dry weight was determined. Leaf area was determined by leaf area meter (CID, CI-202).

Estimation of Physiological Attributes

Chlorophyll contents of flag leaf were quantified by using a acetone maceration procedure (Bruinsma, 1963) Extract were collected in separate test tubes absorbance were recorded at 645, 663 and 672 by using UV spectrophotometer. The membrane stability index was examined according to Sairam (1994). Method of Bates *et al.* (1973) was followed for estimation of proline contents. Method of Dubois (1951) was followed for estimation of total soluble sugars. To determine osmotic potential of each fresh leaf method of (Capell and Doerffling, 1993) was followed by using freezing point osmometer (Model: Wescor Vapro 5520). For determination of water potential of flag leaf Scholander pressure chamber (SAPS 11, Model 3115) was used (Scholander *et al*., 1965).

Super Oxide Dismutase Assay

SOD was determined by the method of Giannopolistis and Ries (1977). 0.5 g fresh leaves were homogenized in 10ml of phosphate buffer (pH 7) containing (2.17 g of K_2HPO_4, 1.70 g of KH_2PO_4 along with 2.5 g of PVP and 9.25 g of EDTA). Homogenate was centrifuged at 1500 ramps for 15 min. Supernatant was used for SOD assay. 0.1 mL supernatant was taken and 3 mL of the buffer was added and shaken well. Two sets of test tubes were made one set was placed in florescent light (40W) and another set was placed in a dark. The reaction was carried out for 8 min until the yellow color turns to dark. Absorbance was recorded at 560nm on UV spectrophotometer.

Statistical Analysis

The software used for statistical analysis was statistics 9.0. Two way ANOVA with the factorial block design was carried out for all treatments.

Results

A. lipoferum strain had shown positive antifungal activity against *A. niger* and produced a clear inhibition zone of 25 mM. A comparatively larger inhibition zone of 30 mM diameter (Table 1) was produced by *A. lipoferum* against *Pseudomonass spp*. Optimum optical density was recorded up to the concentration of 150 mM NaCl (Table 2). Results have shown a reasonable production of different osmolytes in culture broth. In supernatant 0.697 µmol/mL extracellular proline was recorded at 150 mM. In the same way significant amount of 13.96 µmol/mL soluble protein at 150 mM NaCl was produced by *A. lipoferum*. Concentration of total soluble sugar was 1.17 µmol/mL at similar salinity level. The antioxidant enzyme activity of super oxide dismutase was 1.15 unit proteins per minute (Table 3).

Table 1: Antifungal activity of *A. lipoferum*

Fungal strains	Inhibition zone (mM)	Antifungal activity
Aspergillus niger	25 mM	+
Aspergillus terus	Nil	_
Bacterial strains	Inhibition zone (mM)	Antibacterial activity
Pseudomonass spp.	30 mM	+
Escherischia.coli	Nil	_

Table 2: Production of osmolytes by *A. lipoferum*

STRAIN	Protein µ mol/ml	Sugar µ mol/ml	SOD (unit protein per minute)	Proline µ mol/ml
Azospirillum lipoferum	1.39	1.17	0.15	0.697

Table 3: Growth conditions of *A. lipoferum* at different salinity levels

Treatments	OD at 600 nm
Control	0.576
25 mM NaCl	0.825
50 mM NaCl	0.742
100 mM NaCl	0.665
150 mM NaCl	0.714
200 mM NaCl	0.313

Germination Attribues

Significant reduction in germination percentage was observed in plants with salt stress as compared to control. Inoculation under stress condition had shown an increase of 3% at 50 mM, 9% at 100m M and 20% at 150 mM NaCl as compared to un-inoculated plants growing at a same salinity level. A similar trend was observed in germination index, seeds inoculation remained effective at all salinity levels with an increase of 7% at 50 mM, 10% at 100 mM, 11% at 150 mM NaCl. Results for promptness index remained significant ($p \leq 0.05$) at all salinity levels. The percentage decrease in promptness index was 20% at 50 mM NaCl, 31% at 100 mM NaCl, 42% at 150 mM NaCl. Results for *A. lipoferum* inoculation remained parallel with control in normal conditions. The inoculation has increased the promptness index of seedlings up to 8.8% at 50 mM NaCl, 31% at 100 mM and 10% at 150 mM. Highly significant results of seedling vigor index were observed for all treatments. With the concomitant increase in salinity, seedling vigor index has shown more reduced values. In stress condition inoculation has produced a significant increase in seedling vigor index of 52.5% at 150 mm NaCl, 36% at 100 mM and 23% at 50 mM as compared to un-inoculated seeds with similar salinity conditions.

Plant growth in Stress Conditions

Considerable decline in morphological parameters was observed under different salinity conditions. In case of vegetative parameters 12.9%, 16% and 22.5% decrease in shoot length (Fig. 1A) at 50 mM, 100 mM and 150 mM salt concentration was recorded. A similar decline was observed in shoot fresh and dry weight (Fig. 1B, C) of plants at different salt concentrations. In stress exposed plants, inoculation resulted in 8.2%, 9.8% and 11.6% increase in shoot length with respect to un-inoculated plants at 50,100 and 150 mM NaCl. The similar effect of inoculation on shoot fresh weight was observed in stressed exposed plants with a 7.7% increase at 50 mm NaCl, 10.9% at 100 mM NaCl and 12% at 150 mM salt concentration. While in case of shoot dry weight, inoculation cause maximum increase of 12.8% at 150 mM salt level. Significant difference in leaf area was recorded between control plants and salt stressed plants. 150 mM salt concentrations cause 7.7% reduction in leaf area. Inoculation with microbial strain resulted in 8% and 8.2% increase in leaf area at control condition and 150 mM salt level, respectively. (Fig. 1D).

Physiological Attributes

In salt stress a significant reduction in chlorophyll contents of flag leaf was recorded (Fig. 2A). Increase in salinity levels from 50 mM to 150 mM produced 15% to 37.9% reduction in chlorophyll contents. Results of inoculation were effective in both control and salt stress conditions and mitigate the salt effect up to 12%. Significant variation was encountered between plants grown in normal condition and those with salt stress with respect to membrane stability index. There was a decrease of 27.4% at 150 mm salt concentration in membrane stability from control plants (Fig. 2B). The percent increase of 15.5% at 50 mM, 20.4% at 100 mM and 15.3% at 150 mM was observed in inoculated plants compared to their respective control ones. Leaf water potential of plants has shown more negative values with increase in salt level as compared to control plants. Significant increase of 10 % in water potential was observed in *A. lipoferum* inoculated plants compared to control. Decrease of 32%in leaf water potential was measured in 150 mM salinity level over control. Inoculation proved to be effective in plants which were exposed to different salinity levels with increase of 16.5% at 150 mM

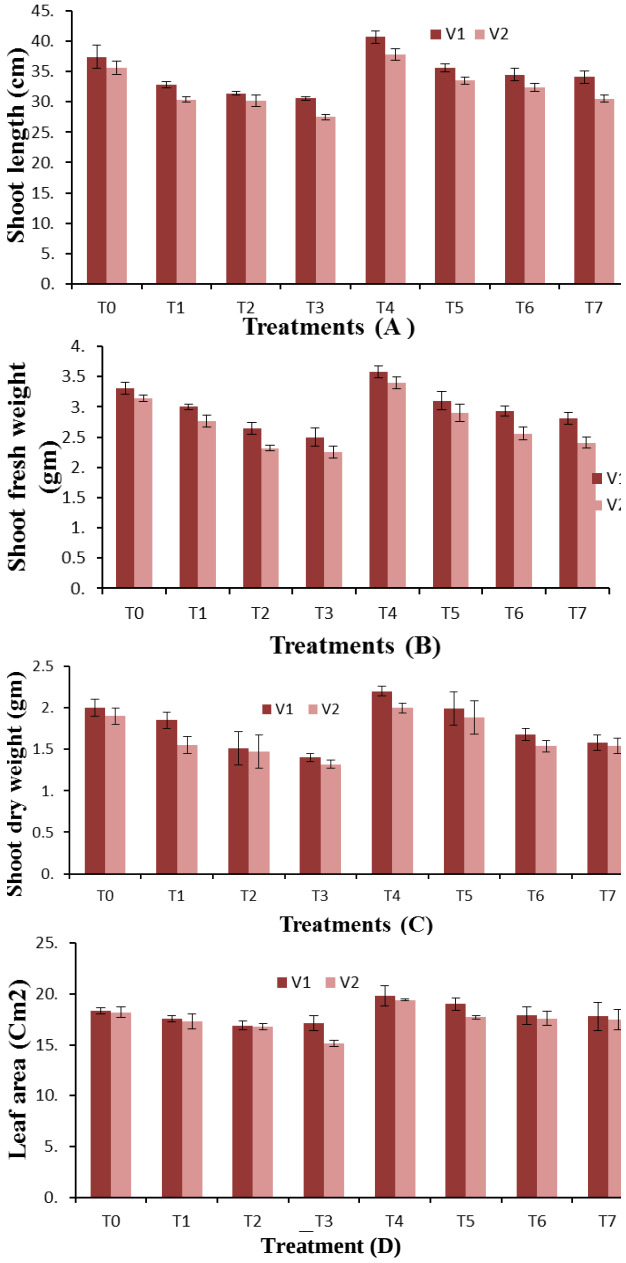

Fig. 1: Inoculation effect of *Azospirillum lipoferum* on shoot length (A), Shoot fresh and dry weight (B, C), Leaf Area (D) of two wheat varieties under different salinity levels of NaCl. Error bars shows the standard error of deviation at significant level of ($p \leq 0.05$)

NaCl, 13.5% at 100 mM and 13% at 50 mM NaCl respectively (Fig. 3C). Data regarding to osmotic potential of the leaf showed decrease in osmotic potential of Plants grown in salt stress as compared to control plants (Fig. 3D). Results of inoculation were effective in both control and salt stress conditions. Results have shown an increase of 11% at 150 mM NaCl and percent increase was same at 50 and 100 mM NaCl as compared to un-inoculated stress plants.

Effect on Compatible Solutes

Proline acts as a compatible solute under stress conditions. Significant increase in proline content was observed in plants under salt stress. At 150 mM salt concentration maximum increase of 23% was recorded in proline content followed by 21% at 100 mM NaCl and 9% at 50 mM NaCl as compared to control (Fig. 3A). The similar effect of salt stress was seen in sugar content of plants with a maximum increase of 30.4% at 150 mM salt concentration. *A. lipoferum* inoculation facilitated the plants to maintain high level of proline and soluble sugars in comparison to un-inoculated plants. Maximum amount of proline 35.6% and soluble sugar 28% was recorded in inoculated plants under 150 mM salt concentration (Fig. 3A, B).

Super Oxide Dismutase Assay

Results have shown a reasonable increase in activity of antioxidant enzyme superoxide dis mutase under different salinity levels. Significant difference was observed at 50 mM NaCl, 100 mM NaCl and 150 mM NaCl with increase of 11.6%, 22% and 33% as compared to control plants. The inoculation has mitigated the saline stress by decreasing superoxide dismutase activity up to 10% as compared to un-inoculated stress imposed plants (Fig. 4).

Discussion

In present study *A. lipoferum* has shown strong potential towards biotic components of rhizosphere including fungal pathogenic strain *A. niger* as well as against *Pseudomonas* strain (Table 1). *A. lipoferum* has the ability to modify the residential status of microbial communities (Baudoin *et al.*, 2010). Pathogenic inhibition may be exhibited due to several disease resistance mechanisms such as production of secondary metabolites e.g. iron chelating siderophores, fungal wall degrading enzyme Chitinase, ammonia and cyanide (Lovic *et al.*, 1993; Weller, 2007; Tortora *et al.*, 2011).

A. lipoferum has shown a threshold potential uptill 150 mM of NaCl. Similar findings for salt tolerance of bacteria were documented by Ramados *et al.* (2013). Adaptation of current environmental conditions and synthesis of protective substances is responsible for stabilizing in stress conditions (Finkel and Kolter, 1999).

Accumulation of osmoprotectants such as proline, protein and soluble sugars was recorded in bacterial strain which enables the bacteria to survive under stress conditions. In extreme osmotic stress compatible solutes could be synthesized by bacteria that act as osmoprotectants. These compatible solutes includes soluble sugars, proline and their derivatives, that helps the organisms to survive in osmotic stress (Da-Costa *et al.*, 1998; Bacilio *et al.*, 2004; Parida and Das, 2005).

During present study it was evaluated that salt stress

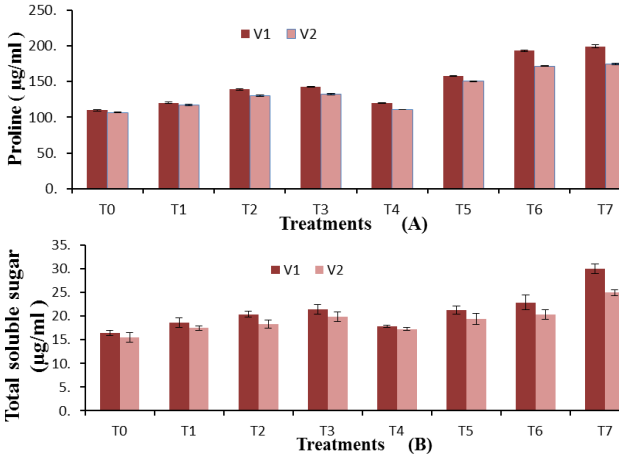

Fig. 3: Inoculation effect of *A. lipoferum* on proline (A) and Soluble sugars (B) of two wheat varieties under different salinity levels of NaCl. Error bars shows the standard error of deviation at significant level of ($p \leq 0.05$)

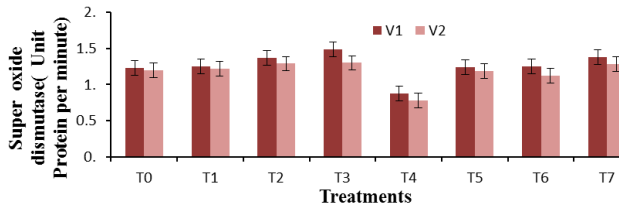

Fig. 4: Inoculation effect of *A. lipoferum* on super oxide dismutase of two wheat varieties under different salinity levels of NaCl

greatly affects the germination as well as all germination parameters of wheat seedlings. Germination of uninoculated and inoculated seedlings was significantly affected at different salt concentration but the effect was more pronounced at 150 mM NaCl and results are in accordance with Duan *et al.* (2007). Decrease in germination percentage may be due to ion accumulation in plant tissues, which hindered several cellular processes (Sidari *et al.*, 2008). *A. lipoferum* inoculation has shown an increase in germination percentage, germination index and promptness at different salt levels. Several studies have been reported for beneficial germination due to plant growth promoting bacteria (Barassi *et al.*, 1997; Nadeem *et al.*, 2013). Increase in germination percentage may confer due to production of growth promoting substances under salt stress conditions (Rueda-Puente *et al.*, 2007).

A significant reduction was observed in vegetative features of the wheat plant under prevailing salinity levels. Salinity resulted in successive reduction in shoot length, shoot fresh and dry weight of Wheat. Disturbance in ionic equilibrium reduces the uptake of nutrients such as Magnesium, Phosphorus, Potassium and Nitrogen which reduces the shoot length and biomass (Gunes *et al.*, 1996;

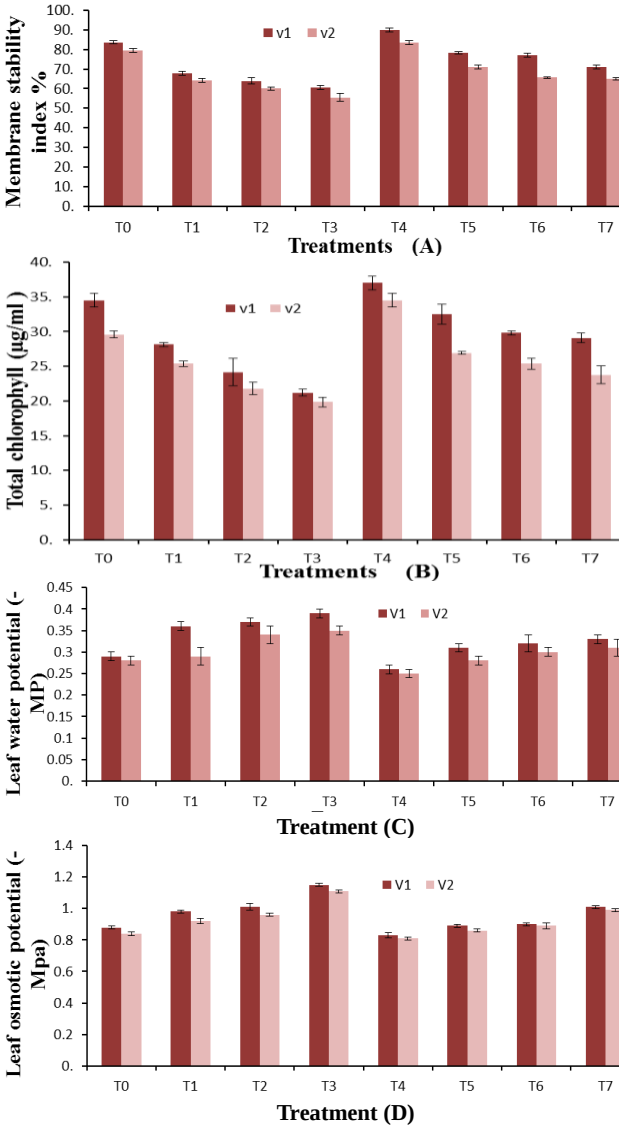

Fig. 2: Inoculation effect of *A. lipoferum* on membrane stability index (A) and Chlorophyll contents (B) Leaf water potential (c) Leaf Osmotic Potential (d) of two wheat varieties under different salinity levels of NaCl. Error bars shows the standard error of deviation at significant level of ($p \leq 0.05$)

Abdel-Ghaffar *et al.*, 1998). Reduction is also result of osmotic and ionic stress (Hagemann and Erdmann, 1997). Inoculation significantly increases shoot length, shoot fresh and dry weight of wheat at all salinity levels.

In our study both wheat cultivars have shown a considerable decrease in chlorophyll contents and yellowing of leaves was more prominent at higher salinity levels. Tuna *et al.* (2008) reported a significant decrease of chlorophyll *a* and *b* in response to saline conditions in maize. Biosynthesis of several proteolytic enzymes was reported in saline conditions such as chlorophyllase which

may be responsible for the disintegration of chlorophyll (Parida and Das, 2005). *A. lipoferum* inoculation leads towards better stabilization of membranes in wheat plants at different salinity levels. Inhibitory effect of salt was more pronounced on osmotic potential and water potential. At higher salinity levels more negative values of water and osmotic potential were observed and same effect was reported by (Munns, 2002). Results have shown that severity of effect depends upon the concentration of salt applied it may be due to Na^+ ion buildup in soil and plants which expels water from tissues and results in decreased water contents (Yeo *et al.*, 1991). Effect of salt stress on water and osmotic potential mitigated by inoculation with *A. lipoferum* and this increase in water content may be due to altered Na^+/K^+ ratio as well as due to accumulation of several compatible solutes (Afrasayab and Husnain, 2000).

Proline acts as potent compatible solute and proline concentration has a direct correlation with salinity. The inoculation has greatly increased the concentration of proline and soluble sugar in wheat after exposure to salt stress. Accumulation of proline may lead to higher leaf water potential as well as it protects the plants from oxidative stress imposed by salt (Zarea *et al.*, 2012) and proline accumulation maintain redox balance in stress conditions (Ashraf and Foolad, 2007). Soluble sugars act as osmolytes and maintain osmotic balance in cytoplasm (Gadhallah *et al.*, 1999) and concentration of soluble sugars increase in wheat plant at higher salinity levels (Sairam *et al.*, 2002).

According to our findings, at the higher salinity level activity of superoxide dismutase increases to scavenge reactive oxygen species as compared to control plants and these results are in accordance with (Yu and Rangel, 1999; Joseph and Jini, 2011). *A. lipoferum* inoculation reverted the injurious effect of stress by decreasing the activity of super oxide dismutase (Upadhyay *et al.*, 2012).

Results of present research *A. lipoferum* (Accession no. GQ255949) has shown strong potential to enhance the growth and development of wheat under saline conditions. *Azospirillum* has the ability to mitigate salt stress and this may confers due to its adaptability to survive in such prevailing conditions. Strain under consideration was previously isolated from Arid region and is well adapted to grow in stress conditions such as drought and salt stress. Results of present research work are also in accordance with previous studies which reported that salinity tolerance can be conferred by *A. lipoferum* strain when isolated from saline area and they performed better in saline conditions (Kerepesi and Galiba, 2000). There is needed to perform similar experiment in field conditions and different types of inoculums should be produced in future for better crop production in natural conditions.

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