

INFLUENCE OF EXOGENOUSLY APPLIED SALICYLIC ACID AND PLANT GROWTH PROMOTING RHIZOBACTERIA INOCULATION ON THE GROWTH AND PHYSIOLOGY OF SUNFLOWER (*HELIANTHUS ANNUUS* L.) UNDER SALT STRESS

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Abstract

Present attempt is to evaluate the effect of Salicylic acid (SA) alone and in combination with plant growth promoting rhizobacteria (*Azospirillum* and *Pseudomonas*) on sunflower plant under salt stress. Two hybrids of sunflower were inoculated with *Azospirillum* spp. and *Pseudomonas* spp. applied as seed soaking treatment prior to sowing. Salt stress (20dSm^{-1}) was induced 28d after sowing. Foliar application of Salicylic acid (10^{-4} M) was made 4h after induction of salt stress. The osmotic potential, antioxidants (SOD, POD) analyses were made from the plant leaves. Salicylic acid application alone and in combination with *Azospirillum* and *Pseudomonas* minimized the inhibitory effects of salt stress. The survival efficiency of *Azospirillum* and *Pseudomonas* under salt stress in the presence of Salicylic acid was higher as compared to salt treatment made alone. The salt tolerance in these treatments was mediated by increase in the superoxide dismutase and peroxidase activities in leaves of sunflower and increase in growth of sunflower hybrids. The adverse effects of salt stress could be alleviated by foliar application of Salicylic acid used alone and more effectively in combination with *Azospirillum* and *Pseudomonas* inoculations.

Introduction

Amongst the abiotic stresses salinity, is the major environmental constraints to crop productivity worldwide (Taiz & Zeiger, 2006). In Pakistan, saline area is about 6.67 million hectares out of the total 20 million hectares of agricultural land (Khan *et al.*, 2006). Soil salinity is common in arid and semiarid regions, where rainfall is insufficient to leach salts and excess sodium ions out of the rhizosphere (Aslam *et al.*, 2000). Pakistan agriculture is facing the salinity and sodicity problem affecting the crop yield badly by disturbing the water and nutritional balance of the plant due to deterioration of physical and chemical properties of soil.

Salinity is an environmental-stress factor for crop plants and is included in chemical stress group (Idikut *et al.*, 2012). Salinity induced osmotic stress triggers the formation of reactive oxygen species (ROS) which can damage mitochondria and chloroplast by disturbing cellular structures (Mittler, 2002). Salicylic acid (SA), a plant Phenolic is now considered as a hormone-like endogenous regulator and its role in the defense mechanisms against biotic and a biotic stress has been well documented (Yalpani *et al.*, 1994; Szalai *et al.*, 2000). Application of exogenous SA enhanced the drought and salt stress resistance of plants (Senaratna *et al.*, 2000; Tari *et al.*, 2002).

SA application may alleviate the adverse effects of abiotic stress due to its important role in nutrient uptake (Glass, 1974), stomatal regulation (Arfan *et al.*, 2007), photosynthesis and growth (Khan *et al.*, 2003; Arfan *et al.*, 2007), besides its identical role in inducing systemic resistance in plants. The exogenous application of SA mitigated the adverse effects of salinity on maize plants by osmoregulation which is possibly mediated by increased production of sugar as well as proline (Fahad & Bano 2012).

Azospirillum, is the most researched associative bacterium (Barassi *et al.*, 2006), stress conditions appear to emphasize its growth-promoting effects on plants (Barassi *et al.*, 2000). The beneficial effects of *Azospirillum* spp. on the inoculated plants however are not only restricted to direct growth promotion, its effect in alleviation of water and salt stress has also been reported (Mayak *et al.*, 2004).

Soil salinity significantly reduces absorption of mineral nutrients, especially phosphorus (P) because phosphate ions precipitate with Ca^{+2} ions in salt stressed soil and become unavailable to plants (Grattan & Grieve 1999). Phosphorus solubilizing bacteria are known to play a major role in the solubilization of unavailable forms of soil phosphorus and the uptake of its applied forms (Khan *et al.*, 2006).

Therefore, our rational was that exogenous application of SA and PGPR inoculants could improve the resistance against salt stress in plants. The aim of this work was to study the ability of SA and two PGPR strains to improve the growth and to induce resistance in sunflower plant against salt stress.

Materials and Methods

An experiment was conducted in the net house of Quaid-i-Azam University, Islamabad. Seeds of two sunflower hybrids (Hysun & Parsun) were obtained from Oil Seed Program, National Agriculture Research Centre (NARC) Islamabad. The seeds were sown in earthen pots (20 cm length \times diameter) with drainage hole, containing soil and sand 3:1 under natural conditions, using a Completely Randomized Design.

Sterilization of seeds was done with 95% ethanol followed by shaking in 10% chlorox for 2-3 min, thereafter, the seeds were thoroughly rinsed three times with sterilized water.

Azospirillum was isolated from arid field (14% soil moisture) and have been identified as *Azospirillum brasiliense* (Accession number GQ 255949) (Ilyas and

Bano 2010). Phosphate solubilizing bacteria *Pseudomonas* isolated from rhizosphere soil of Khewra salt range (ECe; 4300 dS/m) was identified on the basis of colony morphology, QTS test and phosphate solubilization (Yasmin & Bano, 2010). Fresh culture of *Azospirillum* was prepared by using LB media (Miller, 1972). Broth culture of *Pseudomonas* was prepared in Pikovskaya's media (Pikovskaya, 1948). The seeds were soaked overnight in cultures of *Azospirillum* and *Pseudomonas* prior to sowing. Five plants pot⁻¹ were allowed to grow.

The application of salt started four weeks after inoculation. Aqueous solution equal to ECe= 20dSm⁻¹ of NaCl was applied to the rhizosphere soil of potted plants till saturation; the required salt concentration was maintained by measuring electrical conductivity and pH of soil (Radojevic & Bashkin, 1999). Watering was made to the control plants as and when required. Salicylic acid (10⁻⁴M) was foliar applied to plants 4h after the salt treatment. The electrical conductivity of the soil from representative pots was monitored regularly to ascertain actual NaCl concentrations in the rooting medium.

Treatments made

S.No.	Treatments	Symbols
1.	Control	C
2.	NaCl (20dsm ⁻¹)	S
3.	<i>Pseudomonas</i> + NaCl	P+S
4.	Salicylic acid (10 ⁻⁴ M)	SA+S
5.	<i>Azospirillum</i> + NaCl	A+S
6.	<i>Pseudomonas</i> + Salicylic acid + NaCl	P+SA+S
7.	<i>Azospirillum</i> + Salicylic acid + NaCl	A+SA+S

Sampling procedure: Young and fully expanded leaves in three replications were collected to analyze the Shoot length, Shoot fresh weight and dry weight, osmotic potential and activity of antioxidant enzymes 7d after the induction of salt treatment.

Colony forming unit (CFU g⁻¹ Soil): Colony forming unit (CFU) count of *Azospirillum* in 1g soil were determined by plating 0.1 ml of decimal dilution on LB medium. For phosphate solubilizing bacteria, Pikovskaya's media was used (Pikovskaya & Hoben, 1985). Number of bacteria/gm soil was calculated from the colony forming units obtained on plates by formula given by James (1978).

$$\text{Colony forming unit} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of inoculum}}$$

Soil moisture content: At the time of sampling, soil moisture was determined gravimetrically. Soil (20g) was taken from uniform depth i.e. 6 inches from the upper soil surface of pots. The soil was dried in the oven for 72 h at 70°C till constant weight the percent moisture content of soil was determined as follow;

$$\text{Soil moisture content (\%)} = \frac{\text{Fresh weight of soil} - \text{dry weight of soil}}{\text{Fresh weight of soil}} \times 100$$

Osmotic potential: The osmotic potential of the cell sap was measured from the flag leaves with a freezing point osmometer according the method of Capell & Doerffling (1993).

Readings were taken from freezing point osmometer (Gonotec GmbH model OSMOMAT 010) as mosmol/Kg and was converted to MPa. Values using following formula:

$$\text{Osmotic potential (MPa)} = - \text{osmolarity (mosmol Kg}^{-1}) \times$$

$$0.831 \times 10^{-5} \times T(K).$$

$$0.831 \times 10^{-5}(R) = \text{Gas constant}$$

$$T(K) = \text{Thermodynamic temperature}$$

Extraction of antioxidant enzymes

Superoxide dismutase: SOD was determined following the method of Beauchamp & Frodovich (1971).

Peroxidase activity (POD): The POD activity was measured by the method of Vetter *et al.*, (1958) as modified by Gorin & Heidema (1976). The assay mixture contained 0.1ml enzyme extract, 1.35ml 100mM MES buffer (pH 5.5), 0.05% H₂O₂ and 0.1% phenylenediamine. Changes in absorbance were recorded at 485nm for 3 min with the spectrophotometer. The activity of POD was presented as change in OD485 nm /min /mg protein.

Statistical analysis of data: The data were subjected to factorial ANOVA and the mean values were compared with Duncan's Multiple Range Test (DMRT) using MSTAT-C version 1.4.2

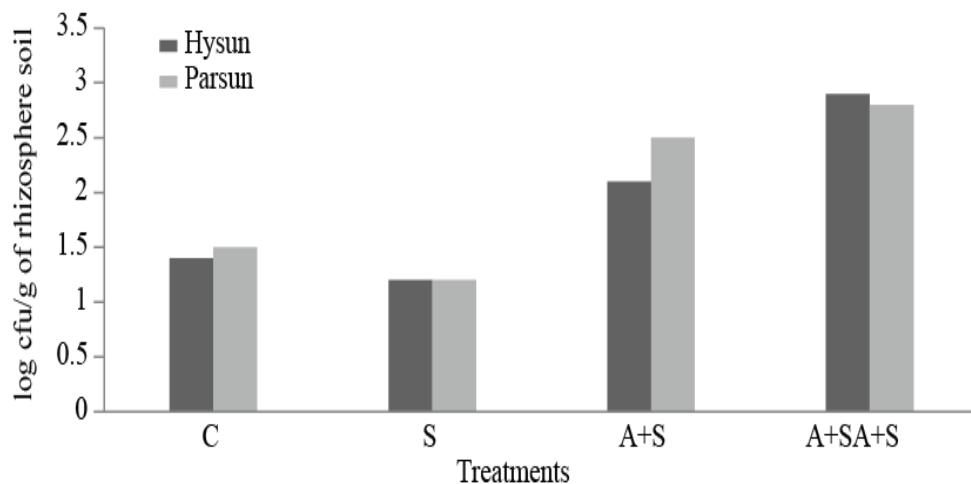
Results

Soil used for cultivation was analyzed prior to sowing and was found sandy loam having pH 8.5 and ECe varied between 7.00-8.56 dS/m.

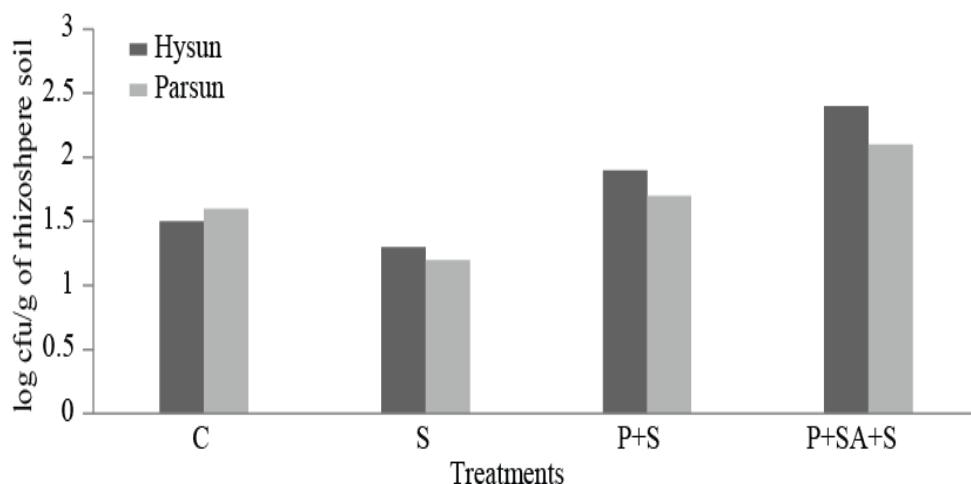
Survival efficiency of *Azospirillum* (log cfu/g): Colony forming units g⁻¹ of rhizospheric soil was observed after 7d of salt stress. Both the hybrids inoculated with *Azospirillum* and treated with 20dSm⁻¹ NaCl showed higher cfu of *Azospirillum* spp. /g of rhizospheric soil as compared to that of uninoculated salt treated and uninoculated unstressed treatments (Fig. 1).

Survival efficiency of *Pseudomonas* (log cfu/g): The cfu of *Pseudomonas* was almost equal to uninoculated control but higher than uninoculated NaCl treatment made alone in rhizosphere of Hysun and Parsun but foliar application of SA augmented the cfu of *Pseudomonas* (Fig. 2).

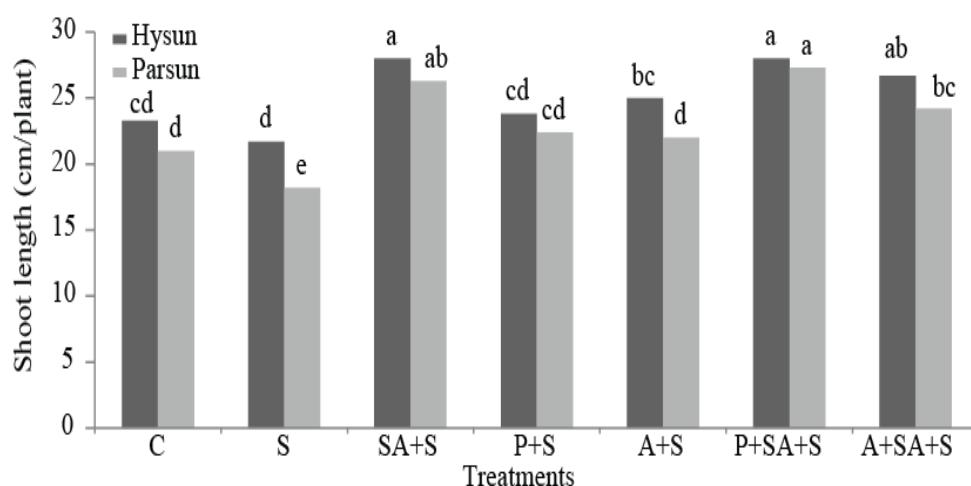
Growth parameters: Shoot length, shoot fresh weight and dry weight of both the sunflower hybrids were significantly reduced due to imposition of salt stress (Figs. 3-5) as compared to unstressed control. However, the foliar application of salicylic acid alone and in combination with *Azospirillum* and *Pseudomonas* significantly ameliorated the adverse effects of salt on the shoot length, shoot fresh and dry weight of both the sunflower hybrids as compared to salt stressed plants.

Fig. 1. Effect of salt stress (20dsm^{-1}) and Salicylic acid on colony counts of *Azospirillum*.

C= un-inoculated control, S= un-inoculated exposed to salt stress, A+S= inoculated with *Azospirillum* and exposed to NaCl stress, A+SA+S= inoculated with *Azospirillum* and foliar application of Salicylic acid exposed to NaCl stress

Fig. 2. Effect of salt stress (20dSm^{-1}) and Salicylic acid on colony counts of *Pseudomonas*.

C= un-inoculated control, S= un-inoculated exposed to salt stress, P+S= inoculated with *Pseudomonas* and exposed to NaCl stress, P+SA+S= inoculated with *Pseudomonas* and foliar application of Salicylic acid exposed to NaCl stress

Fig. 3. Effect of *Azospirillum*, *Pseudomonas* and Salicylic acid on Shoot length (cm/plant) of two sunflower hybrids under salt stress.

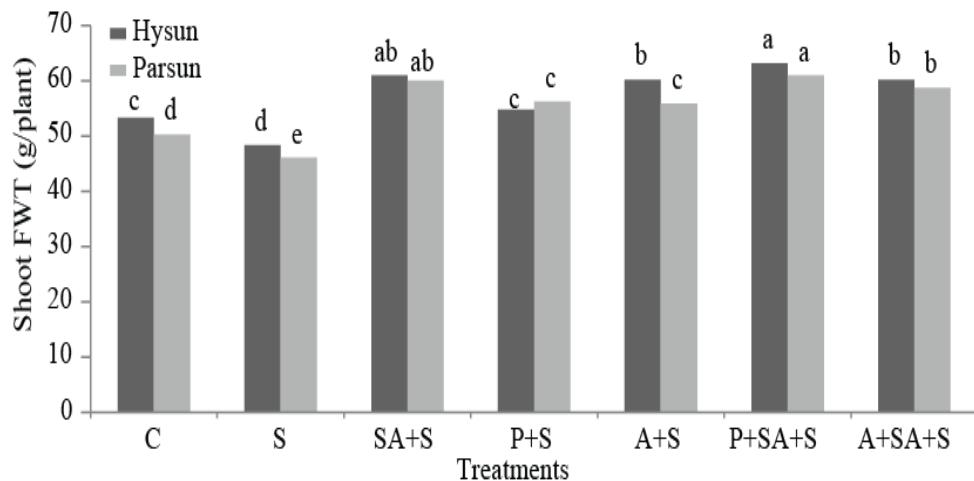


Fig. 4. Effect of *Azospirillum*, *Pseudomonas* and Salicylic acid on Shoot fresh weight (g/plant) of two sunflower hybrids under salt stress.

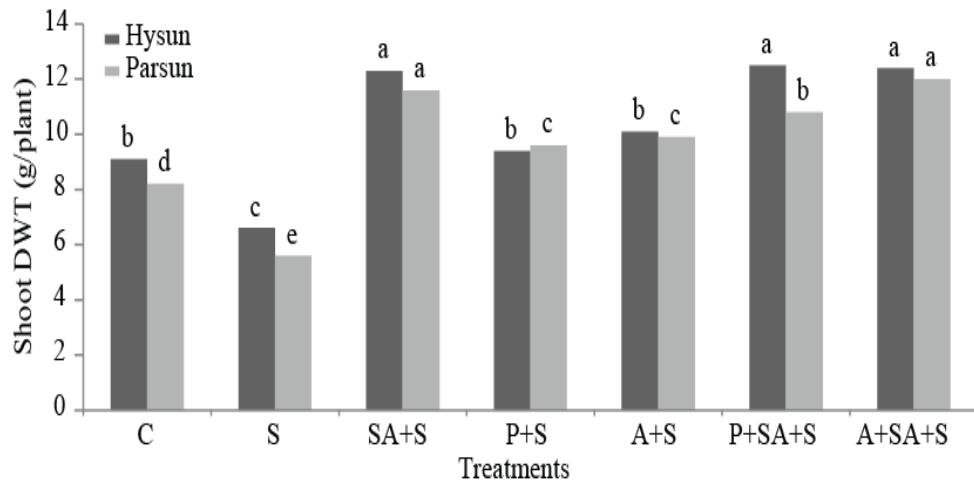


Fig. 5. Effect of *Azospirillum*, *Pseudomonas* and Salicylic acid on Shoot dry weight (g/plant) of two sunflower hybrids under salt stress.

There was no significant effect of salt on the shoot length of Hysun, whereas the shoot length of Parsun was significantly (13%) decreased. All the treatments significantly alleviated the effect of salt. The SA application to salt treated plants ameliorated the salt induced inhibition in shoot length of both the hybrids whereas, the *Pseudomonas* has no significant effect as compared to control. The maximum stimulation was observed with the combined treatment of *Azospirillum* and *Pseudomonas* under salt stress.

Salt stress significantly decreased the fresh weight and dry weight of shoot, the pronounced decrease in shoot dry weight was 27% and 32% in hybrids Hysun and Parsun respectively. Among all the treatments *Azospirillum* alone was more effective than *Pseudomonas* in stimulating both shoot fresh and shoot dry weight.

Soil moisture content: Results showed that salt treatment

with 20dSm⁻¹ resulted in less uptake of water from soil hence the soil moisture content of both the hybrids were higher than control (Table 1). Under salt stress Hysun and Parsun rhizospheric soil samples had 57% and 31% higher soil moisture content respectively than unstressed uninoculated control. Under salt stress, *Azospirillum* + SA resulted in significant decrease in soil moisture content as compared to salt stress in both the hybrids. All the treatments (except *Pseudomonas* in Hysun) in both the hybrids showed significant decrease in percent soil moisture as compared to salt stress.

Osmotic potential: Results presented in Table 2 showed that in Hysun NaCl treatment significantly decreased the osmotic potential by 65% as compared to control. The treatments significantly ameliorated the adverse effects of NaCl stress on osmotic potential and the osmotic potential was higher as compared to un-inoculated salt stressed plants.

Table 1. Effect of *Azospirillum*, *Pseudomonas* and Salicylic acid on soil moisture content of two hybrids (Hysun & Parsun) under salt stress.

Treatments	Soil moisture content (%)	
	Hysun	Parsun
C	14.00 ^b	24.50 ^b
S	32.47 ^a	37.37 ^a
P+C	28.00 ^a	19.90 ^b
SA+S	18.97 ^b	19.67 ^b
A+S	19.03 ^b	20.20 ^b
P+SA+S	17.10 ^b	18.63 ^b
A+SA+S	16.80 ^b	18.53 ^b

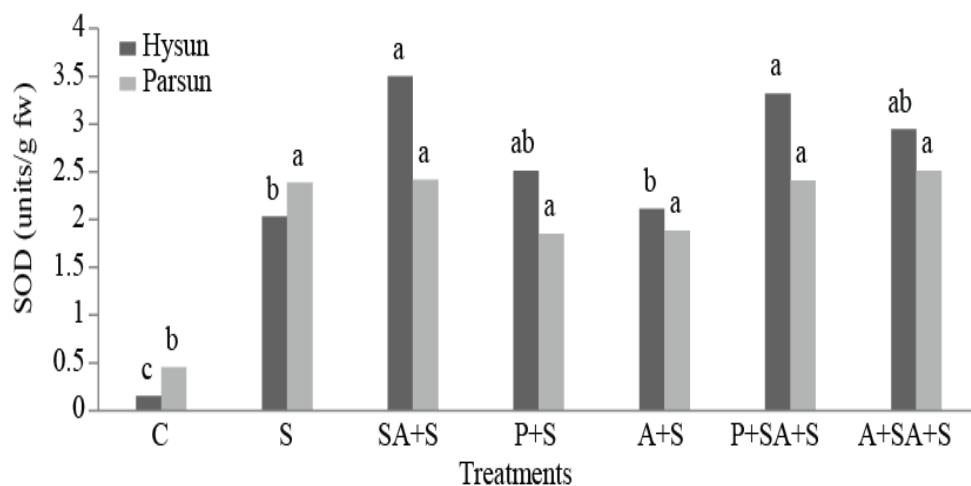
C = un-inoculated control, S = un-inoculated exposed to salt stress (20dSm^{-1}) P+S = inoculated with *Pseudomonas* and exposed to NaCl stress, SA+S = foliar application of Salicylic acid and exposed to NaCl stress, A+S = inoculated with *Azospirillum* and exposed to NaCl stress, P+SA+SA = inoculated with *Pseudomonas* and foliar application of Salicylic acid exposed to NaCl stress, A+SA+S = inoculated with *Azospirillum* and foliar application of Salicylic acid exposed to NaCl stress

All means which share different letters are significantly different at 5% level of significance

Table 2. Effect of *Azospirillum*, *Pseudomonas* and Salicylic acid on Osmotic Potential (-MPa) of two sunflower hybrids (Hysun & Parsun) under salt stress.

Treatments	Hysun OP(-MPa)	Parsun OP(-MPa)
C	1.113 ^a	1.175 ^a
S	0.674 ^b	0.722 ^c
P+S	1.023 ^a	1.052 ^{ab}
SA+S	1.086 ^a	1.023 ^{ab}
A+S	0.988 ^a	0.893 ^{bc}
P+SA+S	1.080 ^a	1.042 ^{ab}
A+SA+S	1.106 ^a	1.007 ^{ab}

All means which share different letters are significantly different at 5% level of significance

Fig. 6. Effect of *Azospirillum*, *Pseudomonas* and Salicylic acid on SOD (units/g fw) of two sunflower hybrids under salt stress.

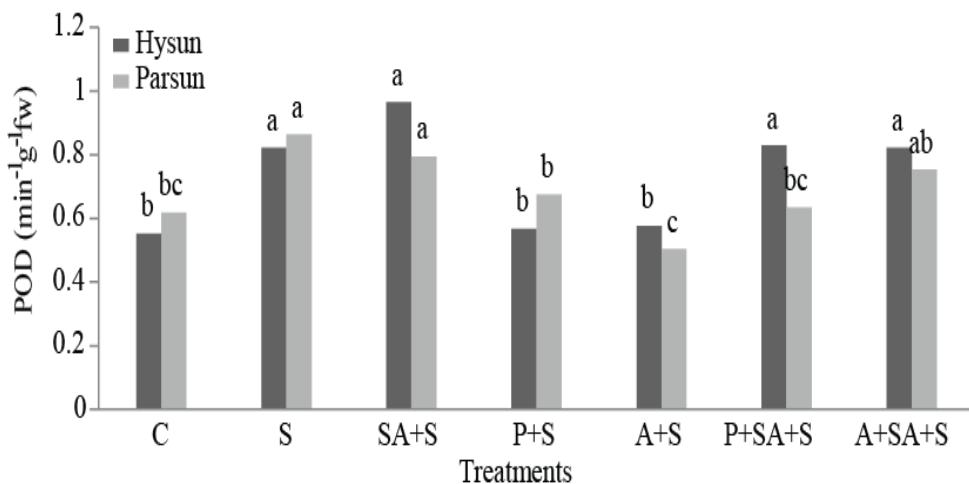


Fig. 7. Effect of *Azospirillum*, *Pseudomonas* and Salicylic acid on POD (min⁻¹ g⁻¹ fw) activity of two sunflower hybrids under salt stress.

In Parsun NaCl also had significant inhibitory effect on osmotic potential. All the treatments showed significant increase over NaCl treatment except *Azospirillum* inoculated plants that was unable to overcome salt induced inhibition.

Superoxide dismutase activity (SOD) of leaves: Results presented in Fig. 6 revealed that SOD activity was increased under salt stress in both the hybrids as compared to un-stressed control. In Hysun, the magnitude of stimulation of SOD activity was greater (92%) as compared to un-stressed control. The stimulatory effect of salt was further augmented by SA alone and *Pseudomonas*+SA.

In Parsun, salt treatment significantly (81%) increased the SOD activity over control. The treatments differed non-significantly over that of salt treatment.

Peroxidase activity (POD) of leaves: The POD activity was significantly increased (33%) under salt stress in Hysun as compared to respective un-inoculated unstressed control (Fig. 7). Under salt stress the *Pseudomonas* and *Azospirillum* inoculated plants both in combination with SA as well as SA applied alone exhibited significant increase at P>0.05 over control, although they differ non-significantly from the salt stressed plants.

In Parsun salt treatment significantly (9%) increased the POD activity over control. The treatments differed non-significantly over that of salt treatment.

Discussion

Results revealed that salt stress caused reduction in the growth of both sunflower hybrids. Inhibitory effect of salt stress was more pronounced in Parsun. The cfu of rhizospheric soil indicated that both *Azospirillum* and *Pseudomonas* successfully survive and proliferate in the presence of 20dSm⁻¹ NaCl. Though the value was less than that of control but *Azospirillum* and SA treatment further augmented their survival efficiency significantly (Figs. 1&2).

Nabti *et al.*, (2007) reported that *Azospirillum* can tolerate 300 mM/L NaCl in the absence of osmoprotectants and upto 600 mM/L NaCl in the

presence of osmoprotectant. *Pseudomonas* was reported to alleviate NaCl stress and significantly promote the seedling growth of annual ryegrass under NaCl stress in gnotobiotic growth pouch assay (Ji & Huang, 2008). *Pseudomonas* induced growth under saline conditions is possibly mediated by solubilization of soil P in available form to plant. The results in Table 1 indicated that soil under saline conditions retained high moisture content but the relative water content of the leaves of salt treated plants were less than that of control, this is because of the osmotic imbalance under salt stress conditions roots may fail to absorb water from the soil and even loss of water from the roots may occur (Waisel *et al.*, 1991; Blum & Johnson, 1992). The less available moisture in pot soil having *Azospirillum* + SA treatment possibly indicate the treatment induced increase in hydraulic conductivity of the root, resulting in better water uptake by the root.

Azospirillum and *Pseudomonas* did not ameliorate the salt induced inhibition in shoot length but partially inhibit the adverse effects of salt stress on sunflower hybrids. Foliar application of salicylic acid alone and in combination with *Azospirillum* and *Pseudomonas* ameliorated the salt induced inhibition in growth. These results were similar to the earlier studies which showed that exogenous application of SA promotes growth and counteracts the stress-induced growth inhibition in some crop species (Tari *et al.*, 2002; Singh & Usha, 2003). *Azospirillum* have been catching researcher's interest on account of remarkable ability to improve plant growth and productivity under various environmental stresses (Bano *et al.*, 2013). Cantrell & Linderman (2001) reported better growth of inoculated plants over non-inoculated plants under salt stress conditions.

A decrease in water availability under soil salinity causes osmotic stress, which leads to decreased turgor. The salt induced decrease in osmotic potential of Parsun was higher than Hysun over that of control (Table 2).

Osmotic potential was increased by SA application alone and in association with *Pseudomonas* and *Azospirillum*. Szepesi *et al.*, (2005) reported that SA treatment overcome the salinity induced inhibition in osmotic potential. Bano & Fatima (2009) have reported that inoculation with *Pseudomonas* under unstressed conditions maintains the osmotic potential of leaves in maize. Chinnusamy & Zhu (2003) have suggested that plant survival depends on maintaining a positive turgor, which is indispensable for expansion growth of cells and stomatal opening. *Azospirillum* applications also results in increase of osmotically active components of the cell sap of maize plants under salinity (Hamdia & EL-Komy, 1998).

Salt stress disturbs the ion homeostasis resulting in osmotic stress and ion toxicity both of which cause generation of reactive oxygen species (ROS), which trigger phytotoxic reactions such as lipid peroxidation, protein degradation (McCord & Fridovich, 2000; Mittler, 2002). To overcome salt mediated oxidative stress, plants detoxify ROS by upregulating antioxidant enzyme, like superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and catalase. Mittova *et al.*, (2002) demonstrated that salt tolerance is often correlated with the efficient oxidative system.

The SOD plays a key role in the antioxidative defense system and it is most effective antioxidant enzyme in preventing cellular damage. Salt induced activity of the SOD was greater in Parsun than that of Hysun (Fig. 6). Previous findings reported increased SOD activity in plant exposed to different environmental stresses, including salinity (Benavides *et al.*, 2000). The H₂O₂ generated as a result of scavenging action of SOD is detoxified by POD. The observed marked increase in POD activity in Hysun following SA, *Pseudomonas* and *Azospirillum* inoculation alone and in combination may possibly indicate the better adaptability of Hysun for the antioxidant system (Fig. 7).

Conclusion

The exogenous application of SA as foliar spray alone as well as with *Azospirillum* and *Pseudomonas* mitigated the adverse effects of salt stress on the growth and physiology of sunflower. SA appears to assist *Pseudomonas* and *Azospirillum* for mitigating the adverse effects of salt stress. Superoxide dismutase activity may be used as physiological markers as they appear to correlate with the salt tolerance e.g. they were higher in hybrid Hysun than that of Parsun. Further studies related to SA activation of gene expression need to be elucidated.

Inocula of studied microbes *Azospirillum* and *Pseudomonas* are economically feasible and sustainable, can be implicated in fields along with SA for better growth of plant.

Acknowledgement

The authors express their deep senses of gratitude to Higher Education Commission of Pakistan for financial support in this research work.

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(Received for publication 30 December 2011)