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MOLECULAR AND PHYSIOLOGICAL RESPONSES OF SUNFLOWER (*HELIANTHUS ANNUUS* L.) TO PGPR AND SA UNDER SALT STRESS

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Abstract

This paper presents the efficacy of PGPR (*Azospirillum* and *Pseudomonas*) and its modulation by salicylic acid. Two hybrids of sunflower (Hysun and Parsun) were inoculated with *Azospirillum* spp. and *Pseudomonas* spp. prior to sowing. Salt stress (20 dSm^{-1}) was applied 28 d after sowing followed by foliar spray of salicylic acid (100 µM) after 4 h of salt treatment. *Azospirillum* and *Pseudomonas* inoculation alone and in combination with salicylic acid alleviated the effects of salt stress on both the sunflower hybrids. The salt tolerance in these treatments was mediated by an increase in relative water content, carotenoids, proline, ABA, induction of new polypeptide bands and yield of sunflower hybrids. In response to salt stress four new polypeptide bands were synthesized in both Hysun, whereas, a group of six polypeptide bands were observed in Parsun. Application of salicylic acid alone and in combination with *Azospirillum* found to induce four new polypeptide bands in Hysun and Parsun. It is inferred that synthesis of new proteins in response to the combined application of salicylic acid and *Azospirillum* under salt stress, may play an important role as stress proteins in tolerance of sunflower hybrids to salt stress.

Key words: *Azospirillum*, Phytohormones, *Pseudomonas*, SDS-PAGE.

Introduction

The most important abiotic stress is salinity, which is increasing day by day and reducing the agricultural productivity in large areas of the world (Hasanuzzaman *et al*., 2013). The destructive effects of accumulation of salt in agricultural soils have influenced both modern and ancient civilizations (Ahmad *et al*., 2012). The reduction in plant growth and yield due to salinity is especially severe in the arid and semi-arid regions of the world because soil salinity is a common feature of arid and semi-arid regions of the world (Ashraf, 2004). The agriculture of Pakistan is also facing the salinity problems affecting crop production by disturbing nutrient and water balance of plants due to high concentrations of salts in the soil (Munns & Tester 2008. Salinity stress inhibits the plant growth severely mainly in two ways; first by the water deficit or osmotic effects of salt stress and second by ion excess or salt-specific effects (Sobhanian *et al*., 2010). The enhancement of salt tolerance in crops is highly challenging as it involves participation of multiple physiological and biochemical pathways (Babu *et al*., 2012). Adaptation to salinity stress requires modifications in the gene expression and protein profile of the plant. It is a complex process at the cellular and whole plant levels. The proteome or protein profiling is not static but somewhat responsive to many external and internal factors (Zhu *et al*., 1995). According to Zörb *et al*. (2004), for the verification of molecular mechanism of salt tolerance, the identification of the differentially-regulated proteins are quite helpful in the analysis of changes in gene expression under salt stress.

Many efforts have been made to minimize the severe effects of salt stress on the crop growth and productivity. Biological approaches such as inoculation of seeds/plants with plant growth promoting bacteria (PGPR) and application of growth regulators to induce resistance against stress have already been attempted (Hayat *et al*., 2010). The most suitable solution or method/approach in this regard is to use the salt tolerant bacterial isolates that may induce salt tolerance thus being useful in facilitating plant growth and yield under salt stress (Bacilio *et al*., 2004). PGPR may prevent the harmful effects of one or more types of environmental stresses (Woitke *et al*., 2004). *Azospirillum* is the most researched associative bacterium (Barassi *et al*., 2006). Different type of stress conditions may be helpful in emphasizing its growth promoting effects on plants (Barassi *et al.,* 2000). Mayak *et al*. (2004) has reported the beneficial effects of *Azospirillum* in alleviation of salt and drought stress conditions. Phosphorus solubilizing bacteria are known to play an important role in the solubilization of unavailable types of soil phosphorus and maintenance of nutrient status of soil (Khan *et al*., 2006). Phosphorus solubilizing bacteria such as *Pseudomonas* in combination with growth regulator (salicylic acid) have been reported for its positive effects on plant growth under salt stress (Naz & Bano, 2013).

Salicylic acid is a hormone-like endogenous growth regulator playing defensive role against biotic and abiotic stresses (Szalai *et al*., 2000). Salicylic acid application may alleviate the toxic effects of salt stress due to its significant role in stomatal regulation, growth, yield and photosynthesis (Arfan *et al*., 2007). It has also role in inducing systemic type of resistance in plants (Delaney, 2004).

The aim of the present study was to determine the effects of PGPR isolates (*Azospirillum* and *Pseudomonas*) and growth regulator (Salicylic acid) under salt stress conditions on the physiological properties, hormonal status and yield of sunflower. Another objective was to investigate the effectiveness of *Azospirillum*, *Pseudomonas* and salicylic acid on the protein profile SDS-PAGE in mitigating the adverse effects of salt stress on the growth of sunflower plants.

Materials and Methods

An experiment was conducted in the net house of Quaid-i-Azam University, Islamabad. Seeds of two sunflower hybrids (Hysun and Parsun) were selected and obtained from Oil Seed Program, National Agriculture Research Centre (NARC), Islamabad.

Inoculum preparation and application: *Azospirillum* was isolated from arid field (14% soil moisture) and has been identified as *Azospirillum brasilience* (Accession number GQ 255949) (Ilyas & Bano 2010). Phosphate solubilizing bacteria *Pseudomona*s isolated from rhizosphere soil of Khewra salt range (ECe; 4300 dS/m) was identified on the basis of colony morphology, Quick Test Strip (QTS) test and phosphate solubilization (Yasmin & Bano, 2011. Fresh culture of *Azospirillum* (Ilyas & Bano, 2010) was prepared by using LB medium (Miller, 1972). Similarly culture of *Pseudomonas* was prepared in Pikovskaya's media (Pikovskaya, 1948).

The seeds (10 seeds/pot) were sown in earthen pots (25x40 cm^2) with drainage hole, containing soil and sand 3:1 under natural conditions, using a Completely Randomized Design (CRD). The seeds were soaked overnight in cultures of *Azospirillum* and *Pseudomonas* prior to sowing. A week after germination the plants were thinned to five per pot. The plants were watered daily as required. All the treatments including control were triplicated

Induction of salt stress: The application of salt started four weeks after sowing. Aqueous solution equal to ECe= 20 dSm^{-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. The control plants watered as and when required. Salicylic acid (100 µM) was sprayed to plants 4 h 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

Treatments	Symbols
Control	C
NaCl (20 dsm^{-1})	S
Pseudomonas + NaCl	P+S
Salicylic acid (100 µM) + NaCl	SA+S
Azospirillum + NaCl	A+S
Pseudomonas + Salicylic acid + NaCl	P+SA+S
Azospirillum + Salicylic acid + NaCl	A+SA+S

Sampling: Young and fully expanded leaves in three replications were collected during 1000 h–1200 h to analyze the relative water content, carotenoids, proline content, protein content and phytohormone production 7 d after the induction of salt treatment.

Relative water content (RWC): Relative water content of leaves was determined following the method given by Gupta (1995). The leaves (number 2) of plants were harvested from plant and weighed. Than soaked in distilled water for 24 h. The fully turgid leaves were again weighed. There after leaves were dried in oven for 72 h at 70ºC.

Relative water content was calculated by applying the formula:

$$RWC\ (\%) = (FW - DW) / (FTW - DW) \times 100$$

where:
RWC = Relative water contents
FW = Fresh weight
DW = Dry weight
FTW = Fully turgid weight

Carotenoid content: Carotenoid content was estimated according to the method of Lichtenthaler & Wellburn (1983). The leaf material (0.05 g) in 10 mL dimethylsulfoxide (DMSO) was heated at 65° C for 4 h and then the absorbance of extract was recorded at 665 and 645 nm.

$$\text{Carotenoid content} = A_{OD\ (470\ nm)} \times 4$$

Protein content (µg/g): Protein content of leaves harvested at vegetative stage was determined following the method of Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) as standard.

Fresh leaves 0.1 g were ground with the help of mortar and pestle in 1 mL of phosphate buffer pH 7.5 with the help of mortar and pestle and was centrifuged for 10 min at 3000 rpm. The supernatant (0.1 mL) was poured in the test tubes. Distilled water was added to make the total volume of 1 mL was made with distilled water.1 mL of reagent C was added. After shaking for 10 min 0.1 mL of reagent D was added. The absorbance of each sample was recorded at 650 nm after 30 min incubation. The concentration of protein content was determined with reference to standard curve made by using standard BSA (Bovine Serum Albumen). The BSA of different concentrations viz., 20, 40, 60, 80, 160, 320 and 640 mg were prepared. Finally the absorbance of protein extract and BSA was recorded at 650 nm.

Proline content of leaves (µg/g): Proline content of leaves was estimated following the method of Bates *et al*. (1973). Fresh plant material (0.1 g) was homogenized with 4 mL sulfosalicylic acid (3.0%) in mortar and placed overnight at 5ºC. Suspension was centrifuged at room temperature at 3000 rpm for 5min. Supernatant was mixed with 4 mL acedic ninhydrin reagent. Reaction mixture was shaken; the contents in the tubes were heated in boiling water bath for 1 h. Thereafter content in the tubes were cooled and the mixture was extracted with 4 mL of toluene in a separating funnel. The absorbance of toluene layer was recorded at 520 nm.The concentration of the unknown sample was calculated with reference to the standard curve.

Determination of protein profile: The determination and identification of different protein fractions from achenes of sunflower hybrids was done by using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). Gels were stained by Coomassie brilliant blue R-250 and destained with 5% methanol/acetic acid mixture (Laemmili, 1970). The destained gels were photographed and protein banding patterns were analyzed quanlitatively using a Gel Documentation System (Bio-Rad).

Hormone analysis

Extraction and purification of ABA: The extraction and purification was made following the method of Kettner & Doerffling, (1995).

The plant leaves (1 g) were ground in 80% methanol, at 4°C with an antioxidant butylated hydroxy toluene (BHT). The leaf was extracted at 4°C for 72 h with subsequent change of solvent. The extracted sample was centrifuged and the supernatant was reduced to aqueous phase using rotary film evaporator .The pH of aqueous phase was adjusted to 2.5-3.0 and partitioned four times with ½ volume of ethyl acetate. The ethyl acetate was dried down completely using rotary thin film evaporator (RFE). The dried sample was re-dissolved in 1 mL of methanol (100%).

Samples ware analyzed on HPLC (Shimadzu, C-R4A Chromatopac; SCL-6B system controller) using U.V. detector and C-18 column. For identification of hormones, samples filtered through 0.45-millipore filters were injected into column. Pure ABA was used as standard for identification and quantification of plant hormone. This growth hormone is identified on the basis of retention time and peak area of the standards. Methanol, acetic acid and water (30:1:70) were used as a mobile phase. For ABA the injected sample was eluted with 0.1% acetic acid and methanol (30-70 % methanole, linear gradient over 30 min) at 254 nm wavelength.

Determination of salicylic acid: Salicylic acid was extracted and purified according to the method of Enyedi *et al*. (1992) and Seskar *et al*. (1998) with some modifications.

Seed phosphorus contents: The total seed phosphrus was determined by wet digestion procedure.

Statistical analysis of data: The effect of salt stress on host plants was observed on daily basis and samples were collected in three replications per treatment to analyze the relative water content, carotenoids, proline / protein content and phytohormone production after 7 d of salt stress. The data were subjected to factorial Analysis of Variance (ANOVA) and the mean values were compared with Duncan's Multiple Range Test (DMRT) using MSTAT-C version 1.4.2.

Results and Discussion

Soil used for cultivation was analyzed prior to sowing and was found sandy loam having pH 8.5 and ECe varied between 700–856 dS/m.

Relative water content: Results shown in Table 1 indicated that salt stress has no significant decrease in the relative water content in Hysun. Similar non-significant responses were observed after the inoculation with *Pseudomonas, Azospirillum* and *Azospirillum*+SA under NaCl stress. In contrast, *Pseudomonas* inoculation + SA treatment exhibited significant increase of 17% in relative water content of leaves as compared to NaCl treatment made alone. In Parsun relative water content was significantly decreased by 14% under NaCl treatment as compared to un-stressed control. Maximum significant increase of 28% in relative water content was observed in *Pseudomonas* + SA as compared to un-inoculated NaCl stressed plants. The lack of any significant effect of salt on relative water content of leaves of Hysun as compared to Parsun also suggest the better water conservation mechanism of the former hybrid to sustain the adverse effects of salt on water economy of plants. The SA was significantly more effective in combination with *Pseudomonas* to ameliorate the adverse effect of salt. Agarwal *et al.* (2005) reported that SA decreased the transpiration and increased the relative water content.

Table 1. Effect of *Azospirillum, Pseudomonas* and Salicylic acid on Relative Water content (%age) and Osmotic Potential (-MPa) of two Sunflower hybrids (Hysun & Parsun) under salt stress. *Azospirillum* and *Pseudomonas* were applied as seed soaking treatment prior to sowing. Salt stress was induced after 28 d of sowing and salicylic acid was applied after 4 h of induction of salt stress. Plants were harvested 7 d after application of 20 dSm^{-1}NaCl.

Treatments	Hysun (RWC (%))	Parsun (RWC (%))
C	70.03^{ab}	70.47^{b}
S	66.07^{b}	60.60^{c}
P+S	65.73^{b}	67.57^{b}
SA+S	66.40^{b}	70.07^{b}
A+S	61.13^{c}	75.53^{a}
P+SA+S	77.57^{a}	77.57^{a}
A+SA+S	61.97^{c}	68.37^{b}

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

Table 2. Effect of *Azospirillum*, *Pseudomonas* and Salicylic acid on Carotenoid contents of two Sunflower hybrids (Hysun & Parsun) under salt stress. *Azospirillum* and *Pseudomonas* were applied as seed soaking treatment prior to sowing. Salt stress was induced after 28 d of sowing and salicylic acid was applied after 4 h of induction of salt stress. Plants were harvested for the determination of carotenoid content 7 d after application of 20 dsm^{-1}NaCl.

Treatments	Carotenoids content (mg/g) Hysun	Parsun
C	2.26[a]	2.150[a]
S	0.60[d]	1.440c
P+S	1.81[ab]	1.743[b]
SA+S	2.17[a]	1.960[a]
A+S	1.15[c]	1.423[c]
P+SA+S	2.15[a]	1.700[b]
A+SA+S	1.60[b]	1.843[ab]

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

Carotenoid content: Carotenoids serve as accessory pigments and also assist in scavenging free radicals (Paiva & Russell, 1999). In Hysun and Parsun, the carotenoid contents were significantly reduced under NaCl stress as compared to control that was also reported by El-Tayyab, 2005. Foliar application of SA under salt stress resulted in significant increase in carotenoid content of leaves compared to stress control (Table 2). The marked decrease in carotenoid content of the sensitive hybrid Parsun as compared to Hysun suggest the better defense strategy in Hysun mediated by free radical scavenging activity of carotenoids.

Leaf proline content: The NaCl stress caused increase in accumulation of leaf proline in both the hybrids of sunflower as compared to control (Fig. 1). The higher accumulation of proline in leaves of inoculated plants under salt stress may contribute to cellular adaptation to salt stress as reported by Jain *et al*. (2001). According to Stewart & Lee (1994) and Maiti *et al*. (2000) proline accumulation is a mechanism for plant adaptation to abiotic stress conditions and also act as source of energy, carbon and nitrogen for recovery. In Hysun, NaCl exhibited significant (73%) increase in leaf proline as compared to control. In Parsun there was non-significant increase (12%) in leaf proline under NaCl stress as compared to control. The *Pseudomonas* inoculation in conjunction with foliar application of SA as well as SA treatment made alone caused significant increase in leaf proline accumulation as compared to un-inoculated NaCl stressed plants. Shakirova *et al*. (2003) also reported enhanced accumulation of proline in wheat seedlings treated with SA. *Pseudomonas* inoculation under unstressed conditions did not affect the leaf proline content of maize as reported by Bano & Fatima (2009).

Leaf protein content and changes in protein profile: The NaCl treatment significantly decreased leaf protein content of both the hybrids as compared to un-stressed control. The percent decrease (64%) was significantly greater in Parsun. All the treatments under salt stress significantly increased the leaf protein content as compared to salt treatment alone at $p<0.05$. Foliar application of SA induced 126% and 274% increase in protein content in Hysun and Parsun respectively as compared to salt treatment made alone. Plants produce proteins in response to abiotic and biotic stress and many of these proteins are induced by phytohormones including SA (Jin *et al*., 2000). The *Pseudomonas* + SA treatment showed 117% and 260% increase in leaf proteins in Hysun and Parsun respectively as compared to NaCl treatment made alone (Fig. 2).

The electrophoretic pattern of achene's protein of both the hybrids (Fig. 3) differed in different treatments. Seed protein, DNA marker, morphological and biochemical properties have been used for analyzing the genetic diversity within and between different species (Miernyk & Hajduch, 2011; Tavaud-Pirra *et al*., 2009).

Salt stress induced four new polypeptide bands of molecular weight 20, 50, 65 and 85 KDa in Hysun, whereas, six polypeptide bands of 23, 30, 40, 45, 85 and 100 KDa were observed in Parsun. Under salt treatment there was a disappearance of two bands of 45 KDa and 58 KDa and four polypeptide bands of 20, 25, 57 and 60 KDa molecular weight in Hysun and Parsun, respectively. It has been reported that salinity causes a decrease in intensity of several protein bands in *Brassica parviflora* (Parida *et al*., 2004) and the degree of decrease of these protein bands seems to be proportional to the external NaCl concentration. The polypeptide bands of 70 KDa and 23 KDa was appeared in all the treatments in Hysun except salt-stressed control, while in Parsun there was a disappearance of 70 KDa in all the treatments and appearance of 23 KDa in *Azospirillum* + SA treatment. The polypeptide band of 85 KDa was present in plants exposed to salt stress and in the SA and *Azospirillum* + SA treatment in Hysun, while in Parsun it was disappeared in all the treatments except salt stressed treatment. In Hysun the band of 100 KDa of high intensity was appeared in SA treatment and *Azospirillum* + SA under salt stress. In the foliar application of SA there was four new polypeptide bands of 23, 45, 70 and 100 KDa was appeared in Hysun and four polypeptide bands of 20, 26, 57 and 60 KDa in Parsun as compared to salt treatment alone.

ABA accumulation in leaves: The NaCl treatment exhibited 138% and 89% increase in ABA accumulation in Hysun and Parsun respectively as compared to unstressed control (Fig. 4). Taylor *et al.* (2000) reported that ABA level could be elevated in response to various environmental stresses. The *Azospirillum* inoculated plants receiving foliar application of SA significantly increased the endogenous ABA level in leaves which was 113% of un-inoculated salt stressed plants. The growth promotion of plants treated with *Azospirillum* lies in its ability to produce and metabolize various phytohormones

(Cassan *et al*., 2001) that improves root growth, absorption of water and minerals subsequently resulting in higher yield and more productive plants (Dobbelaere *et al*., 2001). In Parsun, the *Azospirillum* inoculation receiving foliar application of SA significantly ($p<0.05$) increased (55%) the endogenous ABA accumulation in leaves as compared to *Azospirillum* and *Pseudomonas* inoculation made alone. Application of SA alone and in combination with *Azospirillum* to plants increased ABA level has previously been reported by Shakirova *et al*. (2003). The production of ABA was also reported by *Azospirillum brasilense* sp 245 (Cohen *et al*., 2008).

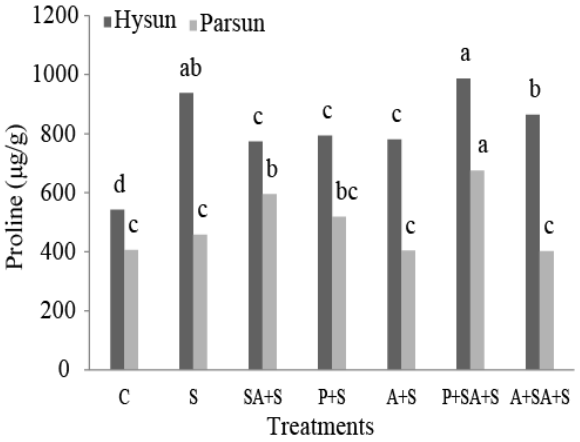

Fig. 1. Effect of *Azospirillum, Pseudomonas* and salicylic acid on proline contents (µg/g) of two sunflower hybrids under salt stress.

Values with different letters are significantly different at $p<0.05$. C= un-inoculated control, S= un-inoculated exposed to salt stress (20 dSm^{-1}), P+S= inoculated with *Pseudomonas* and exposed to NaCl stress (20 dSm^{-1}), SA+S= foliar application of Salicylic acid and exposed to NaCl stress (20 dSm^{-1}), A+S= inoculated with *Azospirillum* and exposed to NaCl stress (20 dSm^{-1}), P+SA+S= inoculated with *Pseudomonas* and foliary applied Salicylic acid exposed to NaCl stress (20 dSm^{-1}), A+SA+S= inoculated with *Azospirillum* and foliary applied Salicylic acid exposed to NaCl stress (20 dSm^{-1}).

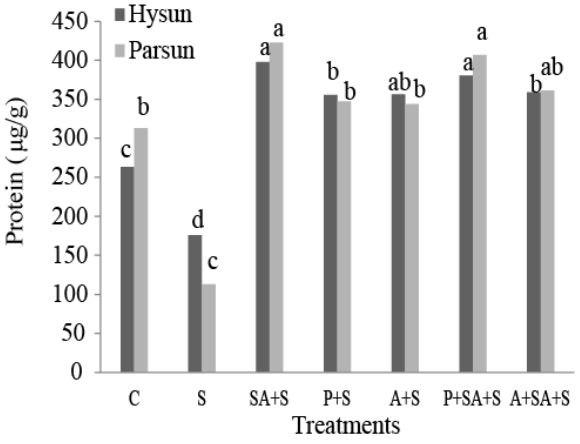

Fig. 2. Effect of *Azospirillum, Pseudomonas* and salicylic acid on protein contents (µg/g) of two sunflower hybrids under salt stress.

Values with different letters are significantly different at $p<0.05$. Labelling is explained in Figure 1.

SA contents in leaves: Similar to ABA, basal level of endogenous SA concentration was relatively lower in Parsun as compared to Hysun (Fig. 5). The NaCl treatment non-significantly increased the total endogenous SA concentration in Hysun. The SA application was unable to have any significant effect on endogenous SA production. The inoculation with *Pseudomonas, Azospirillum* alone and in combination with SA significantly increased the total SA content. The maximum increase (244%) was due to the combined application of *Pseudomonas* and SA under salt stress. In Parsun, the NaCl treatment resulted in significant increase in SA content. The maximum increase of 113% in SA was due to the combined application of *Pseudomonas* + SA under salt stress. SA appears to participate in the maintenance of water budget, osmoregulation as well as antioxidant responses.

Number and weight of achene's / capitulum: Results showed that NaCl treatment significantly decreased number of achene per capitulum by 35% and 39% as compared to control in Hysun and Parsun, respectively (Table 3). All the treatments significantly increased the number of achene per capitulum as compared to NaCl treatment. Foliar application of salicylic acid in Hysun was found to be significantly (75%) more effective to increase the number of achene per capitulum and this increase followed by *Pseudomonas* inoculation (47%) as compared to NaCl treatment. In Hysun, NaCl treatment significantly decreased achene's weight by 50% and 21% as compared to control (Table 3). In Parsun, salt treatment decreased the achene's weight by 21% of the control. All the treatments significantly increased the achene's weight as compared to salt stress. Zahir *et al*. (2009) reported that *Pseudomonas* under salt stress increase the yield of wheat plant. Yield increases with SA application has been reported in different crop species (Singh & Usha, 2003; Khodary, 2004; El-Tayeb, 2005), which is attributed to the observed increase in the number of spikelets and number of grains, SA is known to upregulate flower induction in maize (Khan *et al*., 2003) and wheat (Sairam *et al*., 2002).

Seed phosphorus content: In Hysun and Parsun, NaCl treatment significantly decreased the seed phosphorus contents by 62% and 61% respectively over that of control (Table 4). The *Pseudomonas* + SA treatment exhibited maximum significant increase (247% and 187%) in seed phosphorus contents as compared to salt treatment in both the varieties. Foliar application of SA in Hysun and *Azospirillum* inoculation in Parsun had no significant effect on seed phosphorus content, while all other treatments significantly increased the seed phosphorus content. This may be due to the ability of *Pseudomonas* to solubilize and mobilize the phosphorus (Rajendra *et al*., 1998). Many researchers reported increased seed P content by phosphate solubilizing microorganisms (Zaida *et al*., 2003).

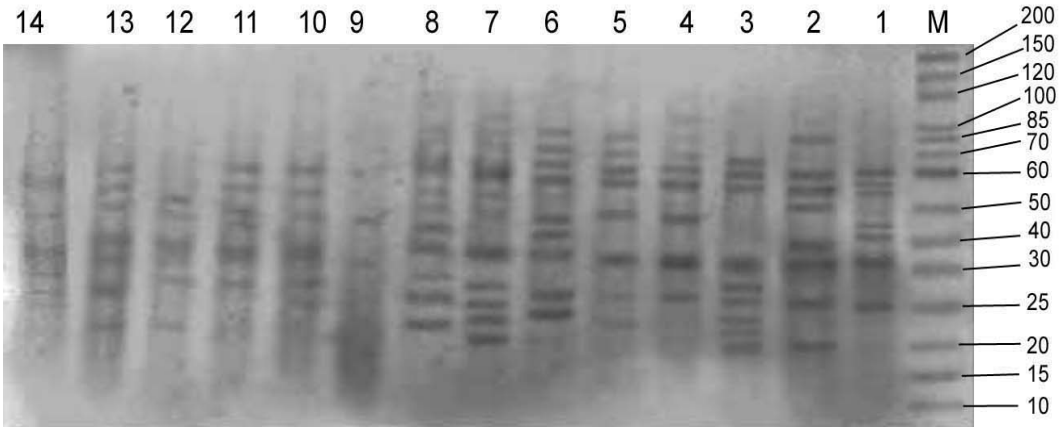

Fig. 3. SDS-PAGE protein profile extracted from sunflower seeds of two hybrids (Hysun and Parsun) under salt stress. *Azospirillum* and *Pseudomonas* were applied as seed soaking treatment prior to sowing. Salt stress (20 dSm^{-1}) was induced after 28 d of sowing and salicylic acid was applied after 4 h of induction of salt stress. Seeds were collected for the determination of protein profiling at harvesting stage.

HySun:

M: Marker, Lane 1: Control, Lane 2: NaCl (20 dSm^{-1}), Lane 3: *Pseudomonas* + Salicylic acid (100 µM) + NaCl (20 dSm^{-1}), Lane 4: *Pseudomonas* + NaCl (20 dSm^{-1}), Lane 5: *Azospirillum* + NaCl (20 dSm^{-1}), Lane 6: Salicylic acid (100 µM) +NaCl (20 dSm^{-1}), Lane 7: *Azospirillum* + Salicylic acid+NaCl (20 dSm^{-1})

Parsun:

Lane 8: NaCl (20 dSm^{-1}), Lane 9: Control, Lane10: *Azospirillum* + Salicylic acid (100 µM) +NaCl (20 dSm^{-1}), Lane11: *Pseudomonas*+ Salicylic acid (100 µM) +NaCl (20 dSm^{-1}), Lane12: *Azospirillum* + NaCl (20 dSm^{-1}), Lane13: Salicylic acid (100 µM) +NaCl (20 dSm^{-1}), Lane14: *Pseudomonas* + NaCl (20 dSm^{-1})

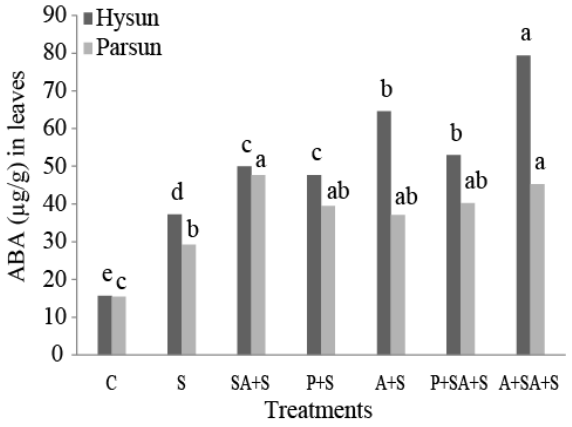

Fig. 4. Effect of *Azospirillum, Pseudomonas* and salicylic acid on ABA accumulation in leaves of sunflower hybrids under salt stress. Values with different letters are significantly different at $p<0.05$. Labelling is explained in Figure 1.

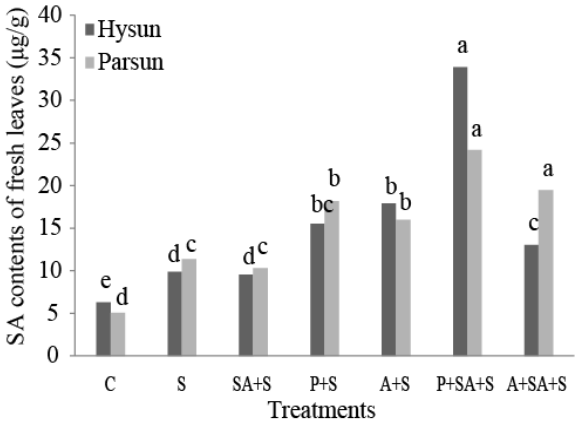

Fig. 5. Effect of *Azospirillum, Pseudomonas* and salicylic acid on endogenous Salicylic acid contents of leaves under salt stress. Values with different letters are significantly different at $p<0.05$. Labelling is explained in Figure 1.

Table 3. Effect of *Azospirillum, Pseudomonas* and Salicylic acid on No. of Achene/capitulum and 100 Achene's weight of two Sunflower hybrids (Hysun & Parsun) under salt stress. *Azospirillum* and *Pseudomonas* were applied as seed soaking treatment prior to sowing. Salt stress 20 dSm^{-1} was induced after 28 d of sowing and salicylic acid (10^{-4}) was applied after 4 h of induction of salt stress. Plants were grown till yield stage to determine the effect of 20 dSm^{-1}NaCl on yield of sunflower plant.

Treatments	Hysun		Parsun	
	No. of Achene/capitulum	100 Achene's weight (g)	No. of Achene/capitulum	100 Achene's weight (g)
C	53.68^{b}	2.667^{ab}	47.67^{a}	2.4^{ab}
S	35.00^{e}	1.333^{d}	29.00^{c}	1.9^{b}
P+S	51.33^{bc}	2.167^{c}	42.33^{b}	2.9^{a}
SA+S	61.33^{a}	3.100^{a}	47.33^{a}	2.8^{a}
A+S	45.68^{cd}	2.133^{c}	44.00^{b}	2.7^{a}
P+SA+S	44.00^{cd}	2.267^{bc}	50.00^{a}	2.9^{a}
A+SA+S	42.68^{d}	2.267^{bc}	47.67^{a}	2.6^{a}

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

Table 4. Effect of *Azospirillum, Pseudomonas* and Salicylic acid on seed Phosphorus content (ppm) of hybrids (Hysun and Parsun) under salt stress. *Azospirillum* and *Pseudomonas* were applied as seed soaking treatment prior to sowing. Salt stress 20 dSm^{-1} was induced after 28 d of sowing and salicylic acid (10^{-4}M) was applied after 4 h of induction of salt stress. Rhizospheric soil samples for determination of P content were collected 7 d after application of 20 dSm^{-1}NaCl.

Treatments	Seed P content (ppm)	
	Hysun	Parsun
C	151.1[b]	222.3[abc]
S	57.6[c]	86.03[d]
P+S	157.5[b]	174.6[abc]
SA+S	132.9[b]	164.03[cd]
A+S	158.4[b]	171.9[bc]
P+SA+S	199.9[a]	247.2[ab]
A+SA+S	161.9[b]	253.4[a]

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

Conclusion

The exogenous application of SA as foliar spray alone as well as with *Azospirillum* and *Pseudomonas* enhanced the mitigating effects of salt stress on the physiology and yield of sunflower. Proline accumulation, phytohormone production and synthesis of more polypeptide bands may be used as physiological and molecular markers as they appears to correlate with the salt tolerance e.g. they were higher in hybrid Hysun than that of Parsun. Further studies on the role of SA in the activation of gene expression are therefore needed to in order to get more information. Soil/seed inoculation of studied microbes *Azospirillum* and *Pseudomonas* are economically feasible and sustainable, can be implicated in fields along with SA for better growth of plants and higher yield.

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