International Journal of Agriculture, Environment and Biotechnology
Citation: IJAEB: 11(2): 387-395, April 2018
DOI: 10.30954/0974-1712.04.2018.21

PLANT PHYSIOLOGY

Salicylic Acid and *Trichoderma* Ameliorate Salt Stress Responses in Pea (*Pisum sativum* L.)

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Paper No. 692 Received: 18-12-2017 Accepted: 16-03-2018

ABSTRACT

Salinity Stress is one of the most important environmental stresses that cause adverse effects on crop productivity and agricultural sustainability. The present experiment was carried out as a pot-culture in the poly-house and identified various physiological and biochemical attributes which progressively reduced with increase in salinity level due to formation of reactive oxygen species i.e. hydrogen peroxide (H_2O_2) and superoxide radical (O_2^{-}). Treatment of seeds with *Trichoderma asperellum* (T42) and exogenous application of salicylic acid (SA), singly and in combination, ameliorated salt stress induced responses reflected by detoxification of both reactive oxygen species, H_2O_2 and O_2^{-} histochemically, and also changes in several growth phenotypes and physio-biochemical attributes in terms of shoot and root dry weight, chlorophyll content, protein content, sugar content and nitrate reductase activity as compared to control of respective salinity levels. Exogenous foliar application of SA (0.25 mM), singly and in combination of *Trichoderma*, ameliorated the hostile effects of salinity up to the level of 8 dSm^{-1} which showed a significant expansion of plant phenotype as compared to the untreated stressed plants.

Highlights

- Reduced plant growth and dry matter accumulation was observed under salt stress situation.
- SA is an important signaling plant molecule which overcomed salinity stress induced responses in plants.
- Bio-control agent *Trichoderma* showed significant improvement in pea under salinity stress.

Keywords: Pea, Salicylic acid, Salinity stress, *Trichoderma asperellum* T42

Pea (*Pisum sativum* L.) is one of the most important and globally known cool season legume vegetable crops belonging to fabaceae family. It has many nutritional values such as high quality protein, carbohydrates, essential amino and fatty acids, fibers, vitamin A, vitamin B6, vitamin C, vitamin K, manganese, dietary fiber, phosphorus, magnesium, cooper, iron and zinc (Nutrition Facts: Peas, 2015). It is very much sensitive to soil salinity which produces negative impacts on plant's growth and development as confirmed by decrease in plant biomass that leads to reduction in its productivity. Salinity is one of the major environmental factors which come under abiotic stress that adversely affect crop production and agricultural sustainability in many regions of the world. Salinization can be either natural or human-induced, occurs on irrigated and non-irrigated soils (Wood *et al.* 2001). Osmotic stress and ion toxicity are the two main ways identified to have the impact of salinity on plants (Munns, 2005). Osmotic stress is caused by ions Na^+ and Cl^- in the soil solution that decreases the availability of water to roots while ion toxicity occurs when plant roots take up Na^+ and/or Cl^- ions because these ions get accumulated to detrimental levels in leaves (Tejera *et al.* 2007). The oxidative stress induced by salinity has detrimental effects on certain structural and functional attributes of plants.

SA is a key endogenous signaling molecule that modulates plant responses to biotic stresses. Recent

research indicated the significant impact of SA in the regulation of diverse aspects of plant adaptive responses to many abiotic stresses (Senaratna *et al.* 2000; Shakirova *et al.* 2003; Gautam and Singh 2009; Ying *et al.* 2013). It is accumulated in the plant tissues under the impact of unfavourable environmental factors, contributing to increase of plants resistance to salinization (Ding *et al.* 2002; Kang and Saltveit 2002; Syeed *et al.* 2011; Liu *et al.* 2014). In addition, SA induces significant effect on resistance of plant growth i.e., increase in shoots and root growth, fresh weight and dry weight of shoot and roots, and plant height of salt stressed soybean (Gutierrez Coronado *et al.* 1998) and maize (Khodary 2004).

Trichoderma is a fungal bio-control agent found in many ecosystems which plays an important role in suppressing soil borne pathogens. But in recent decades, it has been reported that some *Trichoderma* strains can interact directly with roots, increasing plant growth potential, resistance to disease and tolerance to abiotic stresses (Howell 2003; Hermosa *et al.* 2012). *Trichoderma* rhizosphere-competent strains have shown to have direct effects on plants, increasing their growth potential, nutrient uptake, fertilizer use efficiency, rate of seed germination and stimulation of plant defense against biotic and abiotic damage (Shoresh *et al.* 2010). *Trichoderma* releases lots of compound that induce resistance responses to biotic and abiotic stresses (Harman *et al.* 2004, Cardona and Rodriguez 2006). The present investigation was directed towards studying effect of salicylic acid and *Trichoderma*, singly or in combination, on physio-biochemical and histochemical parameters in pea under salt stress at different stages with a view to establish best treatment of SA and *Trichoderma*.

MATERIALS AND METHODS

The present experiment was carried out as pot culture in the poly house and the Laboratory of Stress Physiology in the Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi under salinity stress situation. Disease free and healthy seeds of pea (*Pisum sativum* L.) cultivar (HUP-2) and fungal bio-control agent *Trichoderma asperellum* (T42) were procured from the Department of Genetics and Plant Breeding and Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, respectively. Healthy looking and uniform seeds of pea variety 'HUP-2' were treated with the spore suspension of *Trichoderma asperellum* (T42) for 4 to 5 h and then used for sowing in pots. After 20 days of sowing, twelve pots were imposed with 40, 80 and 120 mM NaCl treatments and maintained the Electrical conductivity (EC) in the pots at weekly intervals. SA (0.25 mM) was applied as a foliar spray after 21 days of sowing. The experiment was laid out in Complete Randomized Design (CRD) and data were obtained at different growth periods of 40, 60 and 80 days after sowing (DAS).

Physiological measurements: Plant parts (shoot and root) were well washed and the dry weight of cleaned plant samples recorded after putting them into an electric oven, first at the temperature of 105^0 C for an hour to stop the metabolic activities followed by the constant temperature of 70^0 C for a period of 72 h. Regular weighing was made on digital electronic balance till a constant dry weight of the plant material was attained.

Chlorophyll content: The chlorophyll content in the leaf samples of pea was estimated by the method given by Arnon (1949) using 80% acetone and absorbance of supernatant was recorded at 645 and 663 nm.

Estimation of Protein content: Protein content present in the plant sample was determined according to the method of Bradford (1976). Protein binding dye Coomassie Brilliant Blue G-250 was used and the blue colour developed was recorded at absorbance 660 nm using spectrophotometer.

Estimation of Total Sugar Content: Total sugar content in the plant samples was measured following method proposed by Morris (1948) with slight modification. In this method, one gm of fully expended leaf was homogenised in 10mL ethanol and centrifuged at 4000 rpm for 15 min. 2 mL of supernatant was added to 6 mL 0.2% anthrone which was prepared in 98% sulphuric acid. After that, mixed sample was placed for 3 min in boiling water bath and absorbance recorded at 620 nm.

Estimation on Nitrate Reductase (NR) Activity: NR activity in the plant was assayed *in vitro* by the method of Srivastava (1975). 0.5 g leaf samples were chopped in test tubes containing (8 mL of

phosphate buffer, 1 mL of 0.1 M KNO_3 and 1 mL of 5% propanol). Each test tube was incubated in dark for 30 min at room temperature. The reaction was stopped by placing the test tubes in boiling water bath for 2 min. After cooling, 1 mL of the extract was taken in a separate test tube and added 1 mL of the NED and 1 mL of sulphanilamide. The intensity of the pink colour developed after some time was read on a spectrophotometer at 540 nm.

Histochemical determination of H_2O_2 and O_2^- in pea leaves: The histochemical staining of H_2O_2 and O_2^- was performed as previously described (Jabs *et al.* 1996, Thordal-Christensen *et al.* 1997) with modification. In case of H_2O_2, different treated pea leaves were dipped in Diamino-benzidine (1mg ml^{-1}, pH 3) and incubated for 6-8 h in dark at 25°C. Dechlorophylization was performed by transferring leaf samples in bleaching solution [ethanol/acetic acid/glycerol (3:1:1; v/v)] and boiled on a water bath for 10-15 min at 90° C. After that leaves were briefly rinsed in distilled water twice. However, in case of O_2-, leaf samples were dipped in 0.2 mg ml^{-1} NBT in 25 mM HEPES buffer (pH 7.8) and incubated at 25°C in the dark for 3 h. Leaves were rinsed in 80% (v/v) ethanol for 15 min at 80°C and mounted in lactic acid/phenol/water (1:1:1; v/v), and developed staining on leaves were observed through microscope.

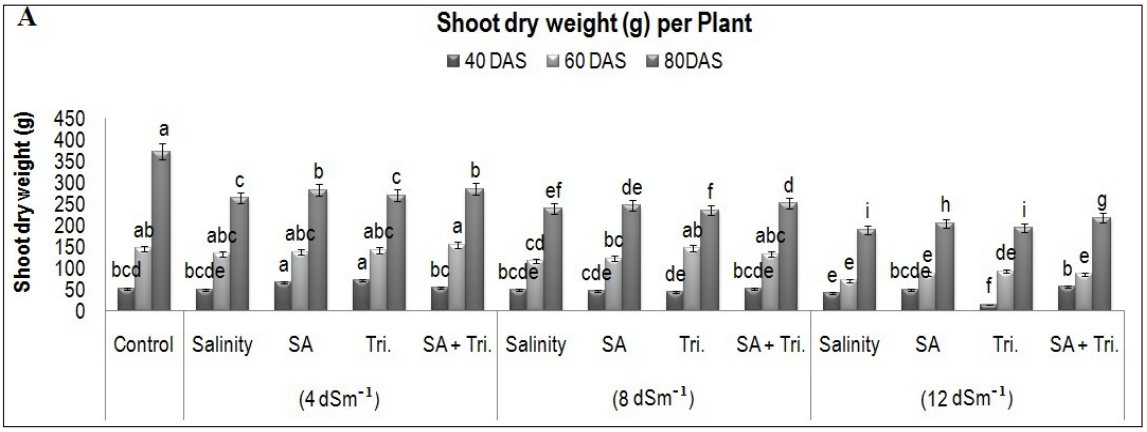
Statistical analysis: All data were presented as mean values of three replicates and analyzed using a statistical package, SPSS (Version 16.0). One-way ANOVA (analysis of variance) was employed followed by Duncan's multiple range tests to determine the significant difference among means of the treatment at $P \leq 0.05$.

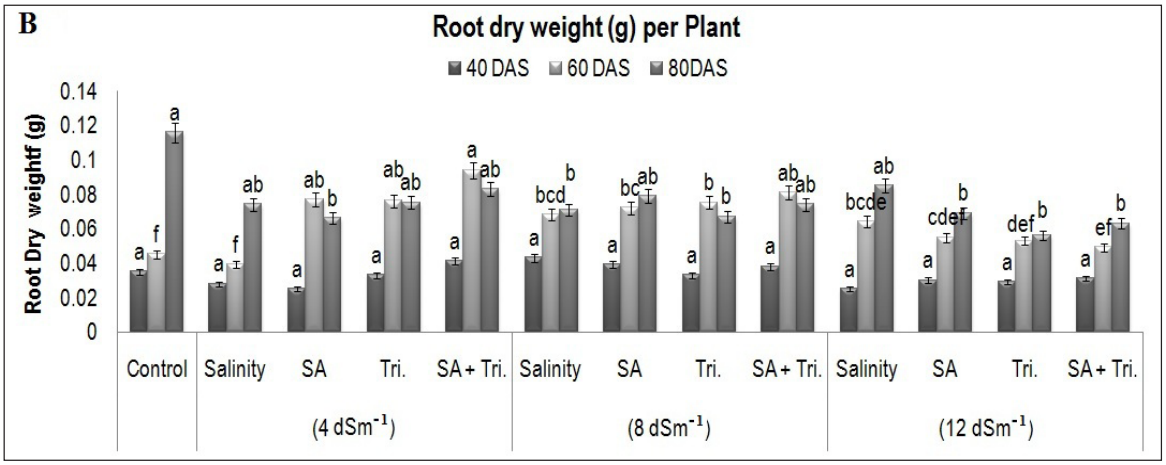
RESULTS AND DISCUSSION

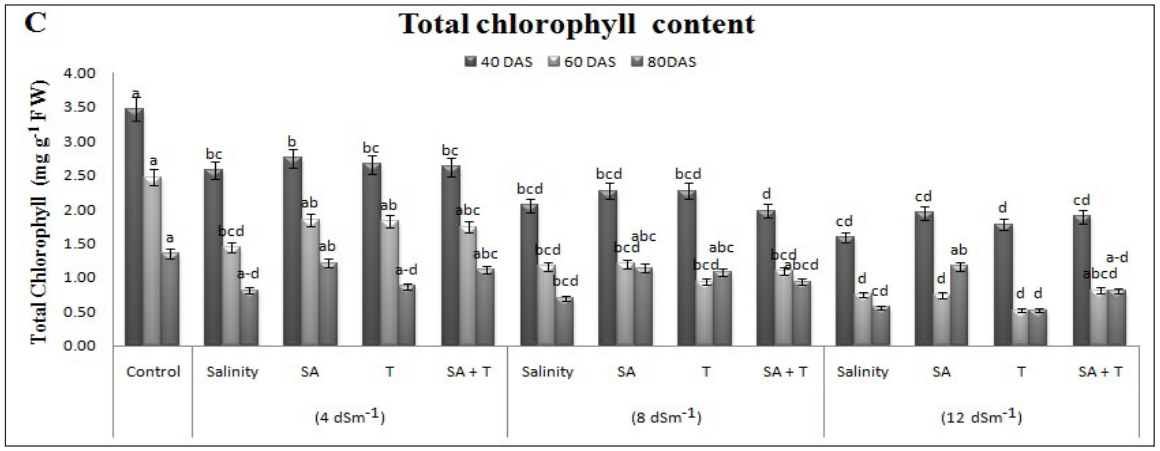
Dry weight of shoot (g) per plant: There was a significant decrease in shoot dry weight with increasing salinity levels (Fig 1A). Among the salinity levels, the maximum 60.26% dry weight reduction (0.6 g) was observed at 60 DAS in 12 dSm^{-1} salinity level. SA and *Trichoderma,* alone or in combination, showed ameliorating effects on all the salinity levels. The combination of treatments with SA and *Trichoderma* recorded maximum 70.58 % shoot dry weight (0.17 g) at 40 DAS in 4dSm^{-1} as compared to control of respective salinity level. The maximum shoot dry weight was observed in control without salinity (1.88 g) at 80 DAS. Similar result was observed by Enteshari and Sharifian (2012) where application of 0.1, 0.3 mM SA increased shoot dry weight in control plants whereas in plants treated with NaCl, shoot dry weight was significantly reduced. In plants pretreated with 0.1 mM SA and treated with NaCl, shoot dry weight significantly increased. Maximum shoot dry weight was observed in plants sprayed with 0.3 mM SA and treated with 150 mM NaCl; similar pattern of results was reported by Jamil *et al.* (2007). The colonization of cucumber roots by *T. asperellum* has been shown to enhance the availability of P and Fe to plants, with significant increases in dry weight, shoot length and leaf area (Yedidia *et al.* 2001).

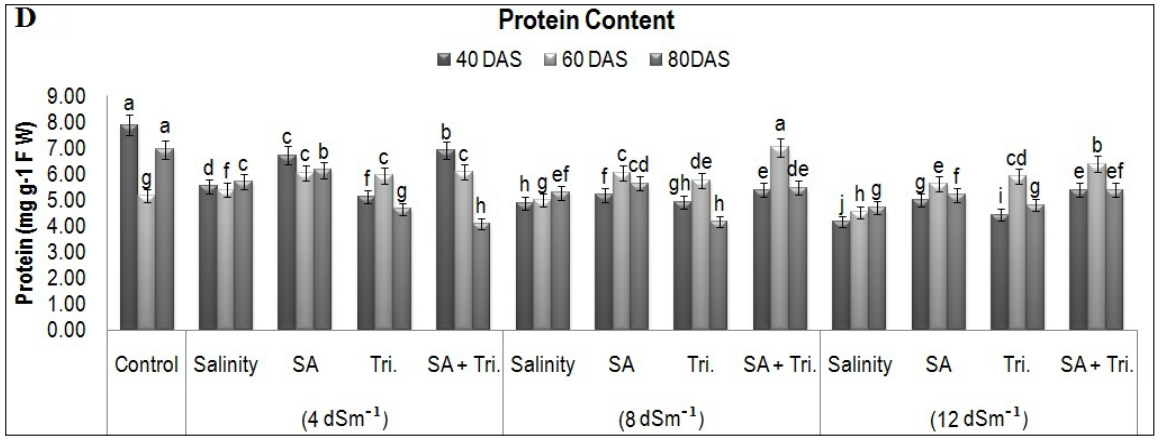
Root dry weight (g) per plant: Significant decrease in root dry weight with the increasing salinity level at different growth periods (40, 60 and 80 DAS) was observed (Fig. 1B).The maximum 28.57% decrease in root dry weight (0.025 g) was recorded at 40 DAS in 12dSm^{-1} level of salinity as compared to control without salinity (0.035 g). All the treatments i.e. SA and *Trichoderma,* alone or in combination, showed increasing root dry weight only in 4 and 8 dSm^{-1} salinity level at all growth periods i.e. 40, 60 and 80 DAS as compared to control of respective salinity levels. Combined treatment (SA and *Trichoderma*) showed reduction of root dry weight at each growth periods in 12dSm^{-1} salinity level. Among treatments, combination of SA and *Trichoderma* recorded maximum 141.0% root dry weight (0.094 g) at 60 DAS in 4dSm^{-1} as compared to control (without treatment), although the maximum root dry weight (0.116 g) was recorded in control without salinity at 80 DAS. Similar result was observed by Enteshari and Sharifian (2012); application of SA increased root dry weight in the control plants. Different concentrations of salt did not have significant effect on root dry weight. In plants pretreated with 0.1 and 0.3 mM SA and treated with NaCl, root dry weight significantly increased. Erdal *et al.* (2011) observed fresh and dry weight of root decreased significantly with exposure to salinity and reduction was severe at 120 mM of NaCl treatment without SA in root. Exogenous treatment 0.5 mM SA increased fresh and dry weights root in saline and non-saline conditions compared to control.

Total chlorophyll content (mg g^{-1} fresh weight): A critical examination of the data revealed that there


A
Shoot dry weight (g) per Plant
40 DAS
60 DAS
80DAS
Shoot dry weight (g)
Control
Salinity
SA
Tri.
SA + Tri.
(4 dSm-1)
(8 dSm-1)
(12 dSm-1)


B
Root dry weight (g) per Plant
40 DAS
60 DAS
80DAS
Root Dry weightf (g)
Control
Salinity
SA
Tri.
SA + Tri.
(4 dSm-1)
(8 dSm-1)
(12 dSm-1)


C
Total chlorophyll content
40 DAS
60 DAS
80DAS
Total Chlorophyll (mg g-1 F W)
Control
Salinity
SA
T
SA + T
(4 dSm-1)
(8 dSm-1)
(12 dSm-1)


D
Protein Content
40 DAS
60 DAS
80DAS
Protein (mg g-1 F W)
Control
Salinity
SA
Tri.
SA + Tri.
(4 dSm-1)
(8 dSm-1)
(12 dSm-1)

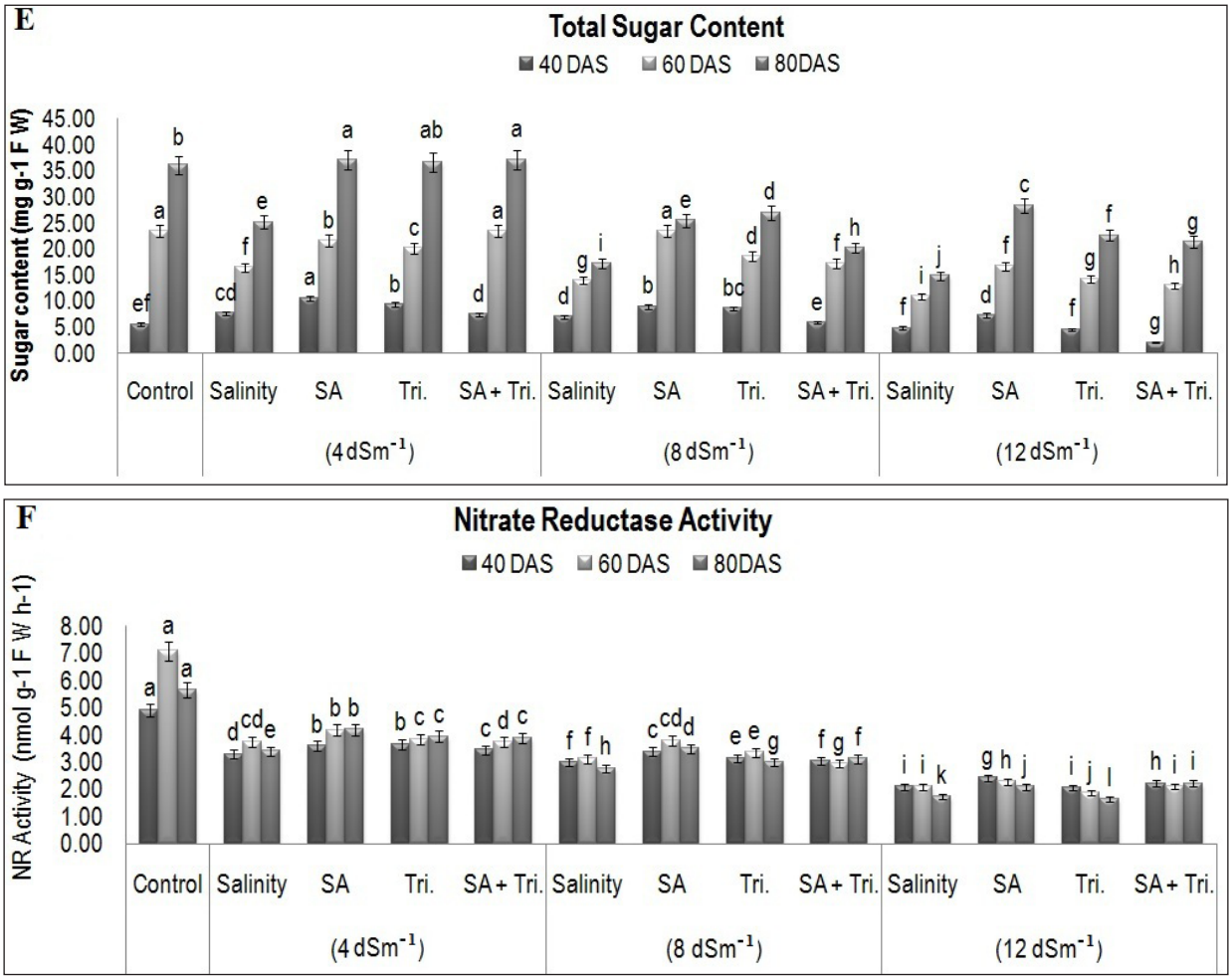


Fig. 1: Effect of Salicylic acid and *Trichorerma* on Shoot dry weight (A), Root dry weight (B), Total chlorophyll content (C), Protein content (D), Sugar content (E) and Nitrate reductase activity (F) in pea under different concentrations (4, 8 and 12 dSm^{-1}) of salinity stress

was a significant influence on the total chlorophyll content by salinity, *Trichoderma*, salicylic acid, applied singly or in combinations (Fig 1C). Among the salinity levels, the maximum 69.63% reduction of chlorophyll content (0.75 mg g^{-1} fresh weight) was observed at 60 DAS in 12 dSm^{-1} salinity as compared to control (2.40 mg g^{-1} fresh weight). Among treatments i.e., SA and *Trichoderma*, used singly or in combination, had ameliorating effects on all the salinity levels. The treatment with SA recorded maximum 49.38% chlorophyll content (1.21 mg g^{-1} fresh weight) at 80 DAS in 4 dSm^{-1} level of salinity as compared to respective salinity controls (0.81 mg g^{-1} fresh weight). In the present study, total chlorophyll content decreased with increase in the concentration of salinity and this decrement was overcomed by SA and *Trichoderma* application. Their application enhanced the capacity of plants to reduce the damage caused by ROS, which in turn increased total chlorophyll content in plants due to the protection effect of SA and *Trichoderma* on photosynthetic apparatus from salinity-induced oxidative stress (Eraslan *et al.* 2007, Azooz 2009, Shahba *et al.* 2010).

Protein content (mg g^{-1} fresh weight): There was a significant decrease in protein content with the increasing salinity level (Fig 1D). Maximum 47.33% reduction in protein content (4.15 mg g^{-1} fresh weight) was observed at 40 DAS in 12 dSm^{-1} salinity level. Among treatments, SA alone or in combination of *Trichoderma* showed ameliorating effect in each the salinity level. The combination of treatments with SA and *Trichoderma* recorded maximum 40.96% protein content (7.02 mg g^{-1} fresh weight) at 60 DAS in 8 dSm^{-1} as compared to control without salinity (5.15 mg g^{-1} fresh weight). This increased protein content induced by SA might be helpful in maintaining osmolarity in the cells during

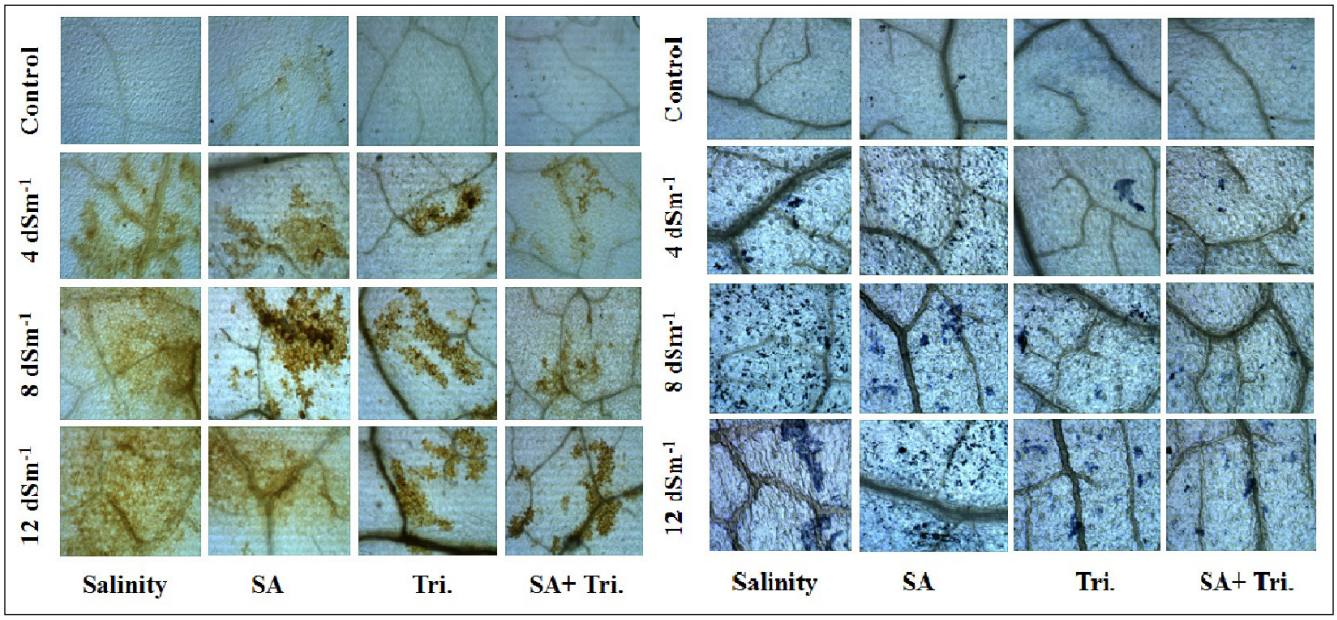


Fig. 2: Microscopic detection of H_2O_2 (Left) and Superoxide radical (Right) in treated pea leaves

where SA= Salicylic acid, Tri.= Trichoderma asperellum (T42) and SA+Tri.= combination of Salicylic acid and Trichoderma asperellum (T42).

saline stress (El-Tayeb, 2005). Agastian *et al.* (2000) also reported that soluble protein increases at low salinity and decreases at high salinity in mulberry cultivars. Ashraf and Fatima (1995) found that salt tolerant and salt sensitive accessions of safflower did not differ significantly in leaf soluble protein but decreased in response to salinity.

Total Sugar content (mg g^{-1} fresh weight): The maximum 58.84% reduction in sugar content (14.86 mg g^{-1} fresh weight) was recorded at 80 DAS in 12 dSm^{-1} level of salinity as compared to control without salinity (36.11 mg g^{-1} fresh weight) (Fig 1E). All the treatments namely SA and *Trichoderma* used singly or in combination, showed increased total sugar content with increasing salinity level i.e. 4, 8 and 12 dSm^{-1} as compared to control of respective salinity levels. Among treatments, foliar application with SA showed maximum 89.42 % total sugar content (10.57 mg g^{-1} fresh weight) as compared to control without salinity (5.58 mg g^{-1} fresh weight) at 40 DAS in 4 dSm^{-1}, although the maximum sugar content (37.21 mg g^{-1} fresh weight) was recorded in combination of treatment with SA and *Trichoderma* at 80 DAS in 4 dSm^{-1} level of salinity. Agastian *et al*. (2000) observed that in *Morus alba* plant exposed to low concentration of salinity there was an increase in soluble sugars but this decreased at high salinity level. Soluble sugar content also increased in tomato plants in relation to salt stress (Maria *et al.* 2000). Khodary (2004) pointed out that SA application increased total sugar content in maize. Our finding is also supported by Amin *et al.* (2007) who reported that salicylic acid regulates sugar contents (translocation from source to sink) and causes significant increase in total soluble sugar. Weizhen and Lei (2013) found that the application of *Trichoderma* spp. strain Q1 significantly increased the content of soluble sugar and soluble protein in cucumber leaves, whether there was salt stress or not.

Nitrate reductase activity (nM g^{-1} fresh weight h^{-1}): Data pertaining to nitrate reductase activity at different growth periods (40, 60 and 80 DAS) are presented in Fig 1 F': the treatments had significant decrement on nitrate reductase activity with increasing level of salinity. Among salinity level, the maximum 70.48% reduction in nitrate reductase activity (2.09 nM g^{-1} fresh weight h^{-1}) was recorded at 60 DAS in 12 dSm^{-1} level of salinity as compared to control without salinity (7.08 nM g^{-1} fresh weight h^{-1}). All the treatments namely SA and *Trichoderma*, alone or in combination, showed increasing nitrate reductase activity with increasing salinity level i.e. 4, 8 and 12 dSm^{-1} as compared to control of respective salinity levels except application of *Trichoderma* at each growth period in 12 dSm^{-1} salinity level. Among

treatments, application with SA showed maximum 23.46 % nitrate reductase activity (4.21 nM g^{-1} fresh weight h^{-1}) as compared to control (without treatment) at 60 DAS in 4 dSm^{-1}, although the maximum nitrate reductase activity was recorded in control without salinity at 80 DAS (5.64 nM g^{-1} fresh weight h^{-1}). These findings are supported by Hopkins (1995) where a decrease in nitrate reductase activity under salt stress may be an after effect of inhibition and/or metabolic dysfunction of this enzyme. Campbell (1999) observed that salt stress restricts the uptake of nitrate (the inducer and substrate of NR). The increased activity of NR by SA spray could be attributed to the fact that SA stabilizes the plasma membrane hence preventing damage by salt stress (Agarwal *et al.*, 2005). SA alone as well as in combination of salt significantly improved the activity of NR Hayat (2012).

H_2O_2 and O_2^- in pea leaves: The salinity stress in pea caused oxidative stress in the present experiment by the formation of reactive oxygen species (ROS: H_2O_2 and O_2^-), ROS formed maximum at the level of 12 dSm^{-1}, which was evident from the observation on DAB and NBT staining of the salt treated plant leaves; this represented qualitative analysis of increased production of H_2O_2 and O_2^- due to exposure of plants to various salt treatments (Fig. 2). However, when SA and *Trichoderma* were applied to these salt stressed plants, the H_2O_2 and O_2^- levels got significantly reduced (Fig. 2). This observation is supported by other studies where salt stress induced higher production of H_2O_2 and other ROS molecules was overcomed by SA and *Trichoderma* i.e., caused less production of ROS molecules (Overmyer *et al.* 2003, Bouchez *et al.* 2007, Steffens and Sauter 2009).

CONCLUSION

Physiological and biochemical differences were observed significantly in pea variety HUP-2 in relation to its performance under different levels of salinity stress at various growth stages; there was a significant effect of these traits on almost all the parameters evaluated under salinity stress condition, and plants treated with SA as a foliar spray and *Trichoderma* as a seed treatment or both together ameliorated the effects of salt stress. Observation showed that plants exposed to salt stress negatively affected the plant growth and development in terms of physio-biochemical and histochemical attributes such as shoot dry weight, root dry weight, sugar content, protein content, nitrate reductase activity, hydrogen peroxide and superoxide radical formation. Treatment with SA and *Trichoderma*, alone or in combination, performed best as compared to control of respective salinity levels which significantly enhanced the plant growth leading to a pronounced increase in dry matter production and overall positive impact on its productivity.

ACKNOWLEDGEMENTS

Authors are highly thankful to Department of Genetics and Plant Breeding, and Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India for providing pea seed (HUP-2) and bio-control agent, *Trichoderma asperellum* (T42), respectively.

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