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Modulating effect of Salicylic acid and *Trichoderma* in pea (*Pisum sativum* L.) under salt stress

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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 morpho-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 ($O_2^{\cdot-}$). Treatment of seeds in *Trichoderma asperellum* (T42) and exogenous application of salicylic acid, singly and in combination, ameliorated salt stress induced responses reflected by detoxification of both reactive oxygen species, H_2O_2 and $O_2^{\cdot-}$ histochemically, and also changes in several growth phenotypes and physio-biochemical attributes in terms of length of shoot, length of root, number of leaves per plant, leaf area and plant dry weight, chlorophyll a and b, and carotenoid content 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.

Keywords: hydrogen peroxide, *Pisum sativum*, salicylic acid, salinity stress, superoxide radical, *Trichoderma asperellum* (T42)

Introduction
Pulse production in India has reduced very much due to biotic and abiotic stresses because of its sensitivity. Pea (*Pisum sativum* L.) is one of the most important and globally known cool season leguminous vegetable crops. 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 comes under abiotic stress that adversely affect crop production and agricultural sustainability in many regions of the world. It also reduces the value and productivity of affected land. Salinization can be either natural or human-induced, occurs on irrigated and non-irrigated soils (Wood *et. al.*, 2001) [33]. There are two main ways of identifying the impact of salinity on plants that is osmotic stress and ion toxicity (Munns, 2005) [19]. Osmotic stress is caused by ions (mainly Na^+ and Cl) in the soil solution that decreases the availability of water to roots because of high osmotic potential as compared to root cell sap. On the other hand, 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) [30]. The oxidative stress induced by salinity has detrimental effects on certain structural and functional attributes of plants.

Salicylic acid (SA) is a key endogenous signalling molecule that modulates plant responses to pathogen infection. Recent research indicated the significant role of SA in the regulation of diverse aspects of plant adaptive responses to many abiotic stresses (Senaratna *et al.* 2000; Shakirova *et al.* 2003) [21, 26]. It is accumulated in the plant tissues under the impact of unfavourable abiotic factors, contributing to the increase of plants resistance to salinization (Ding *et al.* 2002; Kang and Saltveit, 2002) [3, 16]. In addition, SA-induced 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) [7] and maize (Khodary, 2004) [18].

Trichoderma is a fungal genus found in many ecosystems which plays an important role in biological control of soil borne pathogens and has been discussed over a period of time by several workers. 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) [13, 12]. *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, increasing their growth potential, nutrient uptake, fertilizer use efficiency,

rate of seed germination and stimulation of plant defence against biotic and abiotic damage (Shoresh *et al.*, 2010) [27]. Biopriming is a process of biological seed treatment that refers to a combination of seed hydration and seed inoculation with beneficial organisms to protect seed. The technique helps seeds to evenly germinate even under adverse soil conditions (Singh *et al*., 2003) [29]. Biocontrol agent *Trichoderma*, releases lots of compound that induce resistance responses to biotic and abiotic stresses (Harman *et al*., 2004; Cardona and Rodriguez, 2006) [8, 2]. The present investigation was directed towards studying effect of salicylic acid and *Trichoderma*, alone or in combination, on morpho-physiological, 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

Experimental Details: 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, Uttar Pradesh, India. Disease free and healthy seeds of pea (*Pisum sativum* L.) cultivar (HUP-2) and fungal bio-control agent *Trichoderma asperellum* (T42) were obtained from the Department of Genetics and Plant Breeding and Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, respectively. The experiment was laid out in Complete Randomized Design (CRD) which consisted of 13 treatments, 3 replications for each treatment. The data were obtained at different growth periods of 40, 60 and 80 days after sowing (DAS).

Seed Treatment: Good, 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 germination, a population of five plants per pot was maintained. After 20 days of sowing, twelve pots were imposed with 40, 80 and 120 mM NaCl treatment, which produced 4, 8 and 12 dSm^{-1}, respectively as measured by Electrical conductivity (EC) in order to maintain the required salinity levels in the pots at weekly intervals. In each salinity level, three pots were treated with SA, three with *Trichoderma* and three with both SA and *Trichoderma* combination, and similar number of pots were not given any salinity treatment and they served as control.

Morphological measurement: Shoot length of three tagged plants was measured in centimetre from the base of the plant to the growing tips of the main shoot with the help of a meter scale and expressed in cm. Root length of plant was measured from the root tip to the base of the root. Root and Shoot length of three plants was averaged to obtain respective root and shoot length per plant. Number of leaves per plant was recorded by counting the leaves from top to bottom of the plant and mean value of the three tagged plants was selected from each treatment and expressed as number per plant. In case of leaf area, three tagged fully mature leaf of plant was measured with the help of leaf area meter and expressed in cm^2. The leaf area of three plants was averaged to obtain the leaf area per plant.

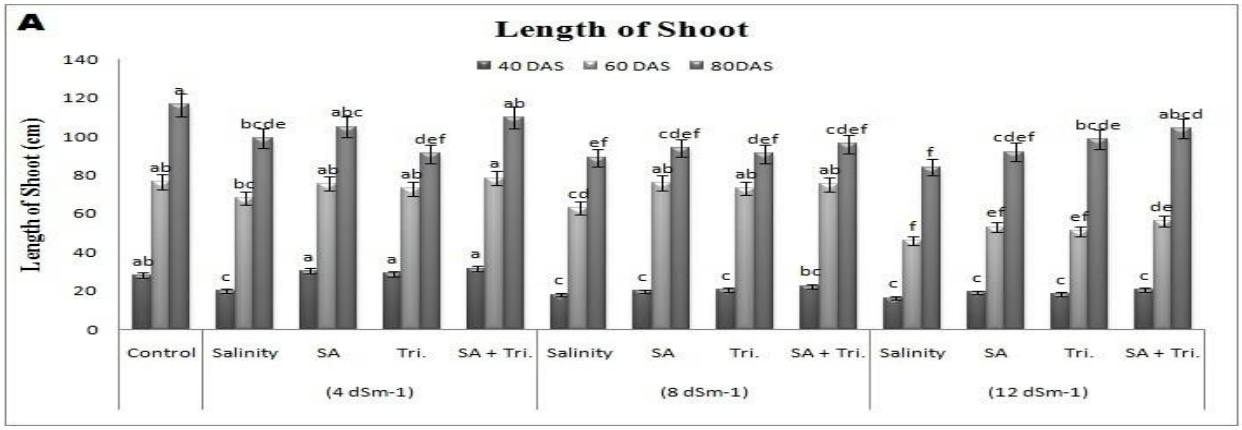
Physiological measurements: Plant parts (shoot and root) were well washed and the dry weight of cleaned plant samples recorded after putting them into an electric oven, first at the temperature of 105^0 C for an hour to stop the metabolic activities followed by the constant temperature of 70^0 C for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

Biochemical measurements: Chlorophyll and carotenoid content in the leaf samples was estimated by the method of Arnon (1949) [1] using 80% acetone and absorbance was recorded at 440, 645 and 663 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) [14, 31] with modification. In case of H_2O_2, different treated pea leaves were deeped in Diamino-benzidine (1mg ml^{-1}, pH 3) and incubate 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.

Data analysis: All data were presented as mean values of three replicates and analyzed by 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



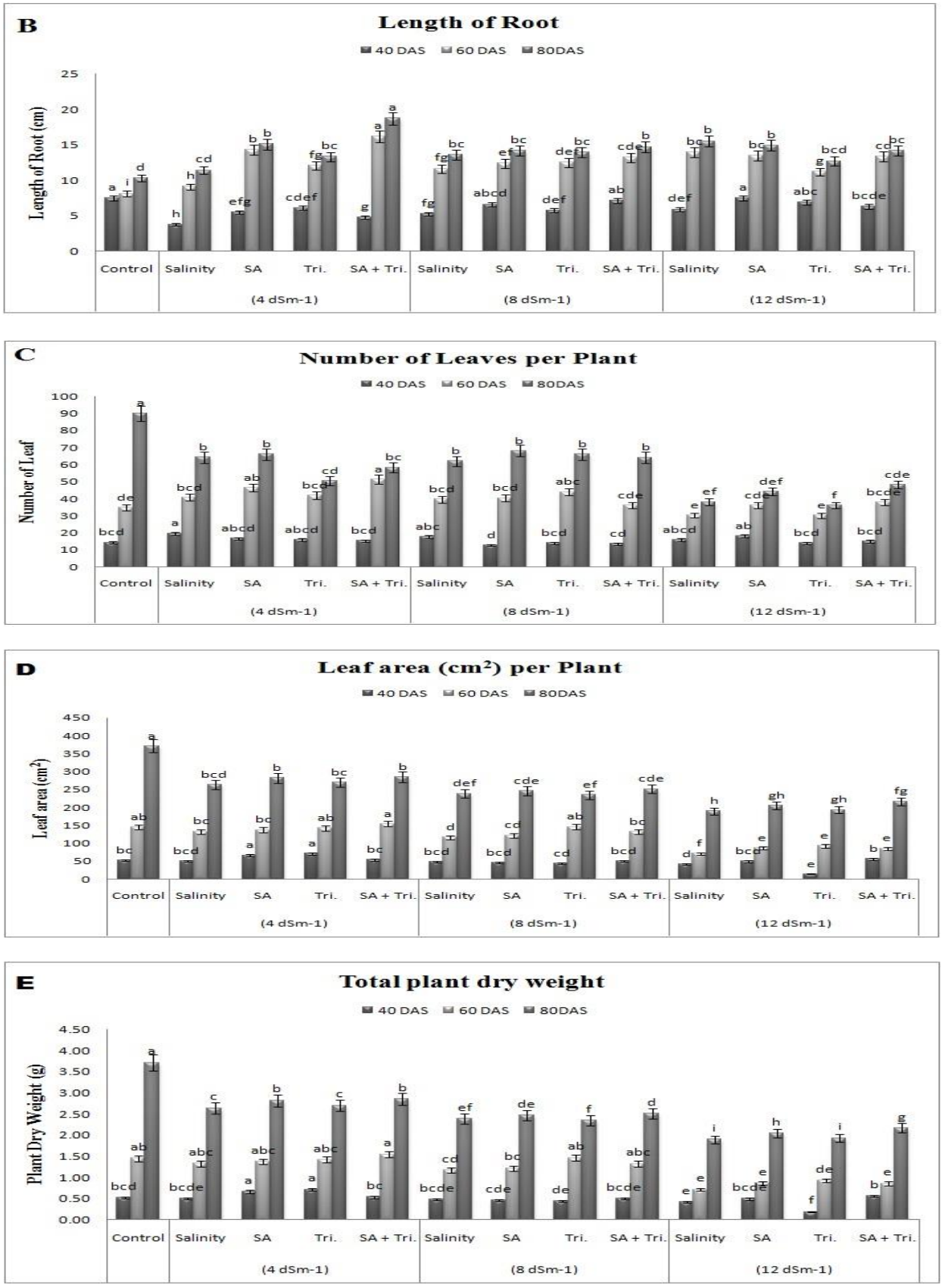


Fig 1: Effect of Salicylic acid and *Trichorerma* on Length of Shoot (Fig 1A), Length of Root (Fig 1B), Number of leaves per plant (Fig 1C), Leaf area per plant (Fig 1D) and Total Plant dry weight (Fig 1E) in pea under different concentrations (4, 8 and 12 dSm^{-1}) of salinity stress

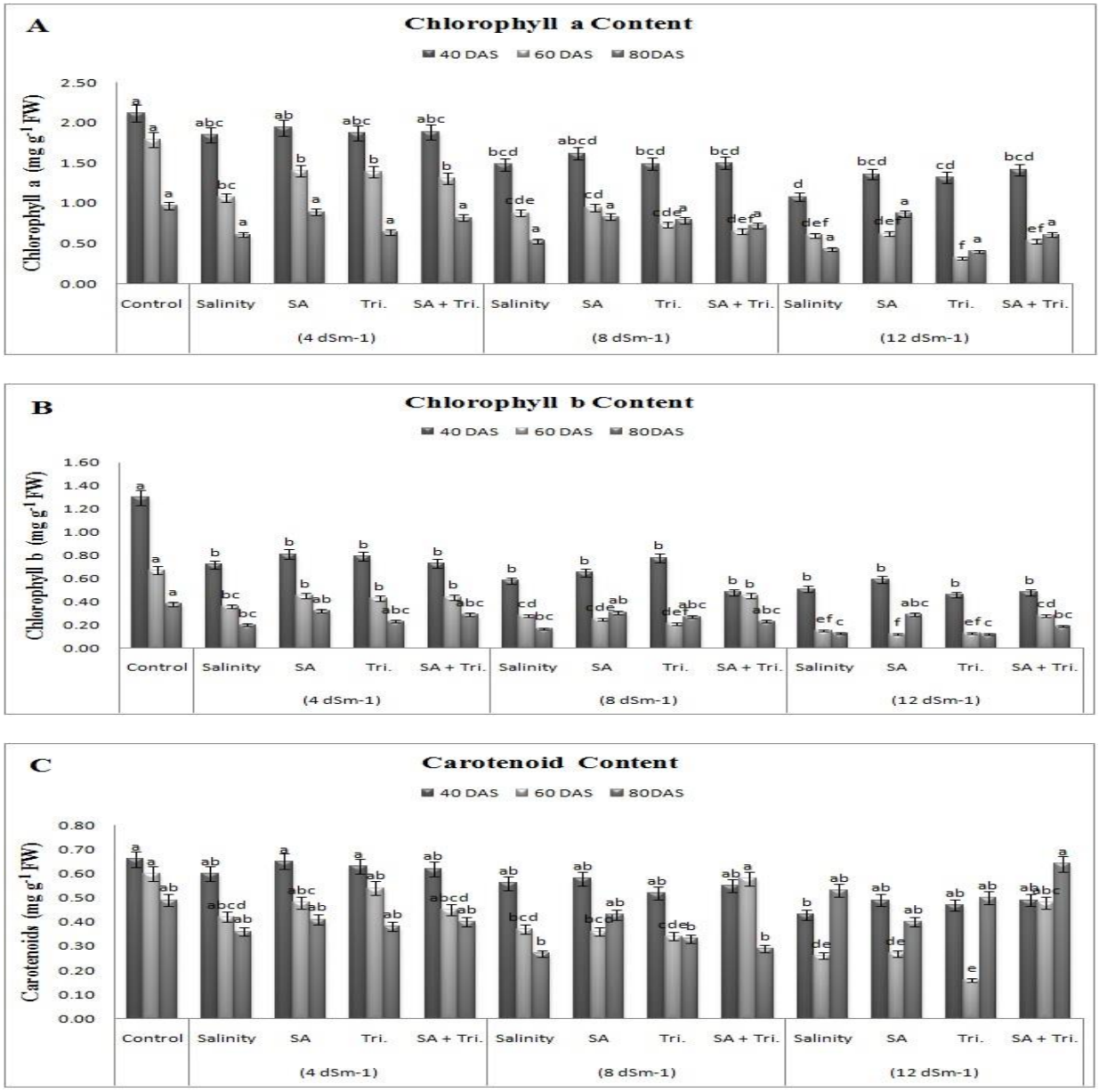


Fig 2: Effect of Salicylic acid and *Trichorerma* on Chlorophyll a content (Fig 2A), Chlorophyll b content (Fig 2B) and Carotenoid content (Fig 2C) in pea under different concentrations (4, 8 and 12 dSm^{-1}) of salinity stress

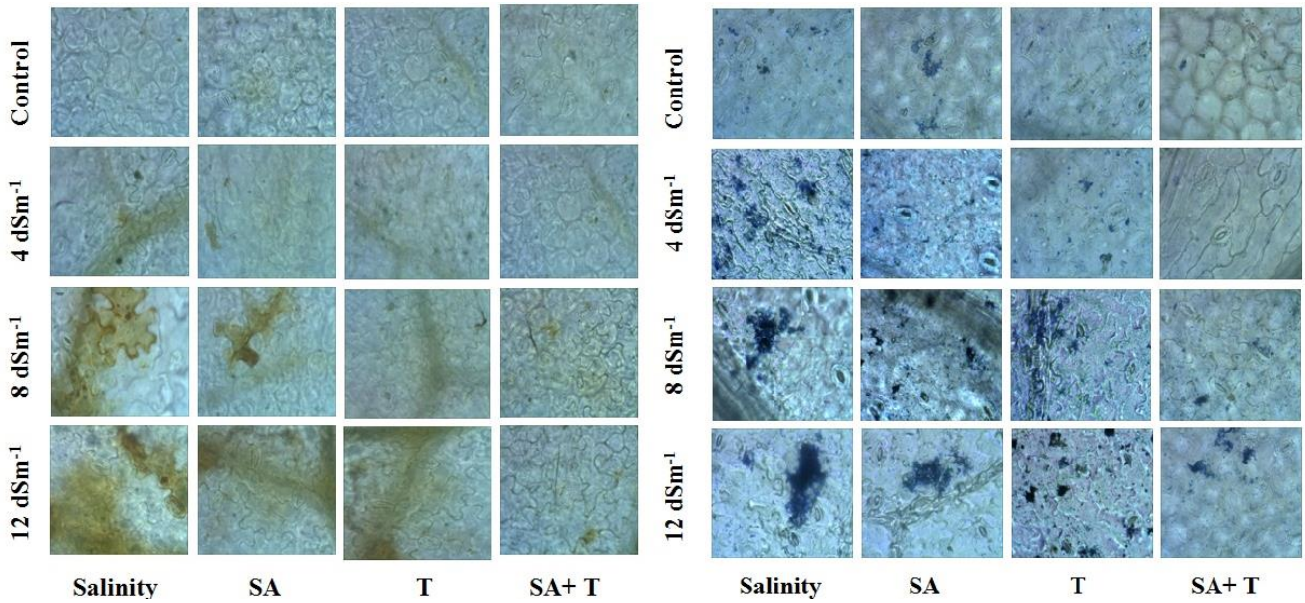


Fig 3: Microscopic detection of H_2O_2 (Left) and Superoxide radical (Right) in treated pea leaves. Where SA= Salicylic acid, T= *Trichoderma asperellum* (T42) and SA+T= combination of Salicylic acid and *Trichoderma asperellum* (T42)

Length of shoot (cm): Length of shoot significantly decreased with increase in the salinity level (Fig 1A). Among the salinity level, maximum 48.88% length reduction (16.25 cm) was observed at 40 DAS under 12 dSm^{-1} level of salinity as compared to control (27.96 cm). The treatments of SA and *Trichoderma*, alone or in combination, showed ameliorating effect at all the salinity levels but combination of treatments with salicylic acid and *Trichoderma* recorded maximum 57.73% shoot length (31.5 cm) at 4 dSm^{-1} at 40 DAS compared to control (27.96 cm) at same growth period. The maximum shoot length was observed in control without salinity at 80 DAS. Similar results has been reported by Gutierrez-Coronado *et al.* (1998) [7] and Hayat *et al.* (2012) [11] who observed that shoot length per plant increased by the treatment of SA in stressed plant as compared to control without SA treatment. It has also been mentioned that SA enhanced growth of wheat plants under water stress (Singh and Usha, 2003) [28]. Shoot length significantly decreased in all levels of salinity as compared to the plants under control. The increase in the shoot length was significant when plants were treated with 0.1 and 0.3 mM SA under salt stress (Enteshari and Sharifian, 2012) [5].

Length of root (cm): Data indicated significant increase in root length with increasing salinity level at different growth periods 40, 60 and 80 DAS (Fig 1B). Among salinity level, maximum 72.22% increase in root length (13.95 cm) was recorded at 60 DAS under 12 dSm^{-1} level of salinity as compared to control with (8.10 cm). SA and *Trichoderma*, alone or in combination, showed increase in root length with increasing salinity level i.e. 4, 8 and 12 dSm^{-1} as compared to control of respective salinity levels. Among treatments, combination of SA and *Trichoderma* showed maximum 78.28% increase in root length (16.17cm) as compared to control (without treatment) at 60 DAS under 4 dSm^{-1}, although the maximum root length (18.70 cm) was recorded in combination of treatment with SA and *Trichoderma* at 80 DAS under 4 dSm^{-1} level of salinity. These findings are supported by Hayat *et al.* (2012) [11] who observed that the treatment of stressed plant with SA also increased the root length as compared to those grown without SA. Similar increase by SA treatment has been reported by Gutierrez-Coronado *et al.* (1998) [7]. SA enhanced growth of maize plants under salt stress (Khodary, 2004) [18].

Number of Leaves per plant: Data pertaining to number of leaves per plant at different growth periods (40, 60 and 80 DAS) are given in (Fig 1C) which elucidate that there was a significant decrease in number of leaves per plant with increasing level of salinity. Maximum 57.65% decrease in number of leaves (38.17) per plant was observed at 80 DAS at 12 dsm^{-1} level of salinity as compared to control without salinity (90.13). SA, alone or in combination of *Trichoderma*, showed ameliorating effects at all the growth periods except 40 DAS in each level of salinity viz. 4, 8 and 12 dsm^{-1} but performance of *Trichoderma* was reduced at 12 dSm^{-1} level of salinity. The treatment of SA in combination with *Trichoderma* recorded maximum 26.46% increase in number of leaves (48.27) per plant at 80 DAS under 12 dSm^{-1} level of salinity. Shahid *et al*. (2012) [24] observed analogous results where data regarding the plant biomass revealed that salt tolerant cultivars offered maximum number of leaves per plant as compared to the sensitive ones under salinity stress. 'Climax' salt tolerant genotype of pea exhibited the highest salt tolerance potential by maintaining maximum number of leaves, branches, internodal distance, while 'Euro', highly salt sensitive genotype of pea showed maximum susceptibility in this regard. Reduced number of leaves per plant is a common phenomenon under salinity stress in various plant species (Zhu, 2001) [34]. Further Harman (2000) [9] publicized that *Trichoderma* also increases crop yield, seedling fresh weight and foliar area.

Leaf area (cm^2) per plant: Data presented in Fig 1D are related to leaf area per plant at different growth periods (40, 60 and 80 DAS) and indicate significant influence by salinity, *Trichoderma*, SA when used singly or in combination. There was a significant reduction in the leaf area per plant with the increasing salinity stress. Among salinity level, maximum 50.96% reduction in leaf area (71.17 cm^2) was recorded at 60 DAS under 12 dSm^{-1} salinity level as compared to control without salinity (145.15 cm^2). SA and *Trichoderma*, alone or in combination, showed enhanced leaf area with increased salinity level i.e. 4, 8 and 12 dSm^{-1} as compared to control of respective salinity level. Among treatments, combination of SA and *Trichoderma* showed maximum 36.41% increase in leaf area (56.16 cm^2) as compared to control (without treatment) 40 DAS at 12 dSm^{-1} salinity level, although maximum leaf area (371.36 cm^2) was recorded in control without salinity at 80 DAS. The treatment of stressed plants with SA also increased leaf area as compared to control plants grown without SA treatment. It has also been mentioned that SA enhanced leaf area and growth of barley plant under salt stress (El Tayeb, 2005) [4] and wheat plants under water stress condition (Singh and Usha, 2003) [28].

Plant dry weight (g): The salinity treatment significantly decreased plant dry weight (Fig 1E). Maximum 57.42% plant dry weight reduction (0.668 g) was observed at 60 DAS at 12 dSm^{-1} salinity level. Among treatments viz. 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 72.16% plant dry weight (0.334 g) at 40 DAS in 4 dSm^{-1} as compared to control of respective salinity level. The maximum plant dry weight (1.943 g) was observed with treatment SA at 80 DAS in 4 dSm^{-1} salinity level as compared to control without salinity (1.897 g) at same growth period. Similar result was observed by Shahid *et al*. (2012) [24] where data regarding the plant biomass revealed that salt tolerant cultivars exhibited maximum fresh and dry plant weight per plant as compared to the sensitive ones under salt stressed conditions. Saeidnejad *et al.* (2012) [20] reported that the total dry matter (TDM) was significantly affected by different salinity levels, which was highly decreased at 200 mM salinity treatment compared to control. SA alleviated harmful effects of both 100 mM and 200 mM salinity levels. Salinity induced retardation of growth in wheat was extremely alleviated by salicylic acid application (Shakirova, 2007) [26]. Khan *et al*. (2003) [17] was also reported that SA could enhance the dry mass production in corn and soybean. Another study by Fariduddin *et al.* (2003) [6] showed that the dry matter accumulation was significantly increased in *Brassica juncea* with SA application.

Chlorophyll a content: There was a significant decrease in chlorophyll a content with increasing salinity level (Fig 2A). Among the salinity level, the maximum 66.48% reduction in chlorophyll a (0.6 mg g^{-1} fresh weight) 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 while the individual performance of *Trichoderma* declined at 12 dSm^{-1} treatment. SA treatment recorded

maximum 102.3% chlorophyll a content (0.87 mg g^{-1} fresh weight) at 80 DAS in 12 dSm^{-1} as compared to control of respective salinity level. The maximum chlorophyll a content (2.12 mg g^{-1} fresh weight) was observed in control without salinity at 40 DAS. Shahba *et al.* (2010) [23] observed the amount of chlorophyll a to decrease with the increase in the concentration of salinity in tomato. Exogenously applied *Trichoderma* strain Q1 on cucumber increased the content of chlorophyll a as compared to those of the non-inoculated controls (Weizhen and Lei, 2013) [32].

Chlorophyll b content: Data elucidate that the treatments significantly decreased chlorophyll b content with increasing level of salinity (Fig 2B). Among the salinity level, the maximum reduction of 77.61% chlorophyll b content (0.15 mg g^{-1} fresh weight) 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 except in 12 dSm^{-1}. The treatment with SA recorded maximum 123.0% chlorophyll b content (0.29 mg g^{-1} fresh weight) at 80 DAS in 12 dSm^{-1} as compared to control of respective salinity level. The maximum content of chlorophyll b (1.30 mg g^{-1} fresh weight) was observed in control without salinity at 40 DAS. Hassanein *et al*. (2009) [10] reported reduction in chlorophyll b content in wheat crop under high salinity stress. Senthil *et al*. (2003) [22] noticed that there was an increase in chlorophyll content in the green gram plants when sprayed with 100 ppm salicylic acid and NAA at 40 ppm at flowering stage.

Carotenoid Content: There was a significant decrease in carotenoid content with the increasing salinity level showed in (Fig 2C). Among the salinity level, maximum 56.6% reduction in the carotenoid content (0.26 mg g^{-1} fresh weight) was revealed at 60 DAS in 12 dSm^{-1} salinity level. SA and *Trichoderma*, singly or in combination, showed ameliorating effects at all the salinity levels. The combination of treatments with SA and *Trichoderma* recorded maximum 84.61% carotenoid content (0.48 mg g^{-1} fresh weight) at 60 DAS in 12 dSm^{-1} as compared to control of respective salinity levels. The maximum carotenoid content (0.66 mg g^{-1} fresh weight) was observed in control without salinity at 40 DAS. These findings are supported by Kalarani *et al*. (2002) [15] who reported that SA caused an increased carotenoid content at each level of salinity. Foliar application SA (50, 100, 150 and 200 ppm) showed its distinct role in increasing carotenoid content in tomato crop. Hassanein *et al*. (2009) [10] also reported that high salinity caused reduction in carotenoid content in wheat crop.

Histochemical determination of H_2O_2 and O_2^- in leaves: The salinity stress in pea caused oxidative stress, 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 3). However, when SA and *Trichoderma* were applied to these salt stressed plants, the H_2O_2 and O_2^- levels got significantly reduced (Fig 3). This observation is supported by other studies where salt stress induced higher production of H_2O_2 and other ROS molecules and SA and *Trichoderma* mitigated the oxidative stress i.e., caused less production of ROS molecules.

Conclusion

Salinity is considered a significant factor affecting crop production and agricultural sustainability. Data showed that salinity had deleterious effects in pea plants. Plants could sustain up to 8 dSm^{-1} level of salinity with application of SA and *Trichoderma*, alone or in combination, but beyond 8 dSm^{-1} salinity level it reduced most of the morpho-physiological and biochemical attributes. Based on the above results, it is concluded that SA (0.25 mM) and *Trichoderma asperellum* (T42), alone or in combination, showed better response up to 8 dsm^{-1} salinity level in comparison to respective salinity levels.

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