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GLOMUS ETUNICATUM ROOT INOCULATION AND FOLIAR APPLICATION OF ACETYL SALICYLIC ACID INDUCED NACL TOLERANCE BY REGULATION OF *NAC1* & *LeNHX1* GENE EXPRESSION AND IMPROVED PHOTOSYNTHETIC PERFORMANCE IN TOMATO SEEDLINGS

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Abstract

Salinity stress hampers plant growth and cause significant yield losses thus induction of salinity stress tolerance in crop plants is one of major goals of agriculture research. Arbuscular mycorhizae fungi *Glomus etunicatum* and acetyl salicylic acid were tested for induction of NaCl stress tolerance in tomato seedlings, cultivar No. 4. The seedlings were inoculated with *Glomus etunicatum* and exogenously sprayed with acetyl salicylic acid (0.30 mM) followed by salinity stress (150 mM). It was observed that both *Glomus etunicatum* and acetyl salicylic acid (singly or in combination) were significantly effective to minimize the injurious effects of salinity by improving root morphological parameters (length, diameter, surface area, volume and number of tips, nodes, bifurcations and connections), photosynthetic parameters (net photosynthesis P_n, stomatal conductance G_s) and chlorophyll contents compared to sole salinity treatment. The bio-inoculant *Glomus etunicatum* and chemical ameliorator acetyl salicylic acid also notably improved vegetative (fresh and dry weights) and reproductive growth (percent seedlings with flower buds and opened flowers, number of flower buds and opened flowers per seedling) of the plants as compared to the sole salinity treatment. The studied salt responsive genes (LeNHX1 and NAC1) were also regulated to different extents in seedling roots and leaves which was consistent with enhanced salinity stress tolerance. From these observations it is suggested that the individual or synergetic use of the AMF (*Glomus etunicatum*) and acetyl salicylic acid can be useful for tomato cultivation in the marginally salinity effected soils and warrants further investigations.

Key words: Salt tolerance, Tomato, *Glomus etunicatum*, Arbuscular mycorhizae fungi, Acetyl salicylic acid.

Introduction

Salinity is a most common stress that affects crops all over the world causing severe yield losses. The use of new biological tools is an ecofriendly and effective way to improve salinity tolerance in agriculturally important plants. Acclimation of plants to salinized condition is linked to activation of cascade of molecular network involved in stress sensing/perception, signal transduction, and the expression of specific stress-related genes and metabolites. Salt stress affects the major plant metabolic processes, such as photosynthesis, growth, lipid metabolism, and protein synthesis (Ramoliya *et al*., 2004). Plants have evolved several adaptive mechanisms to cope with the salinity in their environments i.e regulation of genes with a role in the transport or compartmentation of Na^+ and/or K^+ such as the Na^+/H^+ antiporter SOS1, the Na^+ influx transporter family HKT and the tonoplast Na^+/H^+ antiporter family NHX. Plant tolerance to abiotic stress is mediated through complex networks of responsive genes, including TAS14 (Godoy *et al*., 1994), JERF3 (Wang *et al*., 2004) and NAC (Yang *et al*., 2011; Han *et al*., 2012). These genes regulate the response and degree of tolerance back and forth along signal transduction cascades. Such reference genes can be used to evaluate the available breeding lines and can be integrated as molecular markers in plant breeding programs. The complex network of tomato salt-responsive genes has been identified using suppression subtractive hybridization (Ouyang *et al*., 2007) and tomato micro-array analysis (Sun *et al*., 2010). Salicylic acid (SA) is a naturally occurring phenolic compound and acetyl salicylic acid is a derivative of SA with parallel properties. Role of acetyl salicylic acid has been documented for heat and drought tolerance in tomato (Khan *et al*., 2015, 2014; Senaratna *et al*., 2000). SA plays an important role in the regulation of plant growth, development, ripening and defense responses. The role of SA in the plant-pathogen relationship and induction of abiotic stress (drought, low temperature, salinity etc.) tolerance has been extensively investigated (Miura & Tada, 2014). It has been suggested that SA has considerable agronomic potential to improve the stress tolerance of agriculturally important crops.

Tomato is popularly cultivated in most parts of the world and is salinity sensitive crop. In recent years, studies have indicated that AMF can increase plant growth and uptake of nutrients, reduce yield losses of tomato under saline conditions and improve salt tolerance of tomato (Abdel-Latef & Chaoxing, 2011). Root colonization by AMF involves a series of morpho-physiological and biochemical events regulated by the interaction of plant and fungus, as well as by environmental factors (Blumwald *et al*., 2000). There are at least four LeNHX type genes (LeNHX1~4) (Rodriguez-Rosales *et al*., 2009). LeNHX1, LeNHX3 and LeNHX4 are tonoplast Na^+/H^+ antiporter (Pardo *et al*., 2006) and LeNHX2 has been shown to be a K^+/H^+ transporter (Venema *et al*., 2003). Recently, Galvez *et al*. (2012) studied expression of four tomato genes NHX (LeNHX 1~4) in wild and cultivated species by RT-PCR analysis and it was inferred that the wild species exhibited higher stress tolerant accompanied by expression of NHX that suggest an ameliorative role of the said genes.

At the cellular level, the Salt Overly Sensitive (SOS) signaling pathway that comprises SOS3, SOS2, and SOS1 has been proposed to mediate cellular signaling under salt stress, to maintain ion homeostasis (Ji *et al*., 2013). The effect of AMF and ASA on amelioration of salt stress in plants on the molecular level is less investigated area. Thus, analyzing expression of salt responsive genes viz. NAC1 and Na^+/H^+ antiporter gene LeNHX1 in relation to NaCl stress and mycorrhizal colonization is one of key objectives of the present study.

Expenditure of energy to counteract the toxic effects of NaCl negatively affects plant growth and biomass gain. However, mycorrhization was reported to increase the plant strength aiding growth and biomass (Giri *et al*., 2003; Sannazzaro *et al*., 2007; Zuccarini & Okurowska, 2008). The objective of the present study was to evaluate, the relatively less investigated synergestic and individual role of AMF *Glomus etunicatum* and acetyl salicylic acid towards induction of NaCl stress tolerance in tomato seedlings.

Material and Method

Sterilization of materials: Before initiation of the experiment, all the materials to be used were sterilized with fungicide. Briefly, the growth medium (peat moss mixed with sand 1:1 v/v) and the pots were sprayed with formalin solution, wrapped in polythene sheet for five days and were then kept in open for the next week to allow evaporation of the fungicide.

Growing seedlings: The tomato seeds (Cv. No.4) were locally purchased, sterilized and then germinated on wet filter paper in Petri plates at room temperature. The germinated seeds (about 2 cm roots each) were then transferred to formalin sterilized germination trays filled with peat moss (Pindstrub, Latvia). The trays were placed in growth chamber with 12 h photoperiod and 22°C/18°C day/night temperature regimes.

Root inoculation: Inoculum of *Glomus etunicatum* was obtained from Beijing Academy of Agriculture Science and Technology Information Institute, Beijing, China. At two leaf stage, the seedling plugs were washed to get the roots free of peat moss. Seedlings with uniform shoot and root vigor were transplanted in small pots (8×8×10 cm) containing equal quantity of sterilized growth medium and 5 g inoculum was placed beneath the roots. After one month, the AMF root colonization was confirmed following trypan blue staining method (Philips & Hayman, 1970).

Acetyl salicylic acid treatment and salinity stress: Acetyl salicylic acid (ASA) was procured from Sigma-Aldrich, Beijing. After one month of AMF inoculation, the plants were sprayed to run off with 0.30 mM ASA solution added with 1% Tween-20 (to aid absorption). On third day of first ASA application, the plants were salinity stressed i.e. saline (150 mM NaCl) tap water (50 mL/ plant with EC: 13.08 $dS^{\bullet}m^{-1}$ at 27°C) was applied on alternate days. The application of acetyl salicylic acid was repeated after five days of first salinity treatment. The study comprised of five treatments viz. **T1**: Control; **T2**: S: sole salinity; **T3**: AS: ASA+NaCl; **T4**: FS: AMF+NaCl; **T5**: FAS: AMF+ASA+NaCl. Each treatment had ten replicates and the experiment was repeated thrice. The EC of salinity free growth medium (control) was 2.15 $dS^{\bullet}m^{-1}$ and that of salinity medium was 6.01 $dS^{\bullet}m^{-1}$ at 27°C (growth medium: distilled water: 1:5), whereas pH was 7.35 and 8.21, respectively.

Vegetative & reproductive growth of the plants: The data regarding plant growth parameters and initiation of reproductive phase was recorded after ten days of continuous salinity stress.

Root development: The roots were washed in the running tap water and architectural features were recorded after 15 days of salinity stress. The roots were submerged in water tray and growth parameters (total length, volume, surface area, number of connections, bifurcations, nodes and tips) were computed using digital scanner Microtek ScanMaker *i*800plus with software Microtec Scan wizard EZ-2.3.

Photosynthesis and chlorophyll contents: Total chlorophyll content was estimated on 5th and 10th day of salinity treatment using SPAD-502 chlorophyll meter (Minolta, Japan). In all plants, readings were taken on the third fully expanded leaves from the plant apex as previously adapted by Khan *et al*. (2015) and Ling *et al*. (2011). After ten days of salinity stress period, the plants were taken out of the growth chamber and kept in open early in the morning for Sun-adaptation. Next day photosynthetic performance of second fully developed leaf from apex was measured at 10:00 am using portable photosynthesis system LI-6400 (LI-COR Inc., Lincoln, NE, USA). Net photosynthetic rate (P_N, μmol CO_2 $m^{-2}s^{-1}$) of leaves was measured at a leaf temperature of 25.5±2°C. The ambient CO_2 concentration was ~400 μL of $CO_2{}^{\bullet}L^{-1}$ air. Stomatal conductance gs (mol$^{\bullet}$$m^{-2\bullet}s^{-1}$), intercellular CO_2 concentration C_i (mmol$^{\bullet}$$m^{-2\bullet}s^{-1}$), transpiration rate E (μmol$^{\bullet}$$mol^{-1}$) were also measured. Water use efficiency WUE (μmol CO_2.$mmol^{-1}$) was computed as $WUE = P_n/E$ where P_n is net photosynthesis and E refers to transpiration rate. Stomatal limitation (Ls) was calculated as: Ls = 1 (Ci/Ca) where C_a is concentration of CO_2 in the air and Ci is intercellular CO_2. At least five seedlings per treatment were tested for photosynthetic performance.

Gene expression: On third day of salinity treatment, the samples from root tips and leaf apex of uniform seedlings were excised for RNA extraction. The samples were wrapped in aluminum foils and flash frozen in liquid nitrogen immediate after excision. The samples were powdered in liquid nitrogen and RNA was extracted using RNA out Kit (Tian En Ze, Beijing) according to manufacturer's instructions. The cDNA from the extracted RNA was developed using All in One RT Master Mix (www.abmgood.com) following manufacturer's protocols. Actin was selected as reference gene (Zhou *et al*., 2007) and the primers for salt responsive genes; NAC1 and LeNHX1 were used (Sangon Biotech. Co. Shanghai, China, Table 1). The fluorescence of amplified samples was detected using Takara SYBR® Premix Ex Taq II RNAs H-plus PCR master mix (www.takara-bio.com) after PCR thermal program: (95°C 3 min, 94°C 30 sec, 60/56°C 20 sec, 70°C 20 sec, 72°C 10 min). Real time RT-PCR was performed using Bio-Rad *i*Q5 multicolor real time PCR detection system (*i*Q™5) optical module and analyzed by Bio-Rad *i*Q5 2.1 optical system software.

Table 1. Primers for salinity responsive genes studied for their expression in NaCl stressed tomato seedlings with AMF root inoculation and /or Acetyl-SA treatment.

Gene	Forward 5'-3'	Reverse 5'-3'
NAC1	CAAATTGGATTATGCACGAGTACCGC	AAGTAGTCGTTTGCTGGTGTCGATCG
LeNHX1	ATGTTGTTGGTGCCGTCG	AGGCTGCTCGTCCTGATT
Actin	GGACTCTGGTGATGGTGTTAG	CCGTTCAGCAGTAGTGGTG

Results

Vegetative growth: On account of salt injury, the fresh and dry weights were many times reduced in the sole salinity treatment as compared to the salinity free controls. However, the AS, FS and FAS treatments markedly improved these parameters (Table 2). It was observed that the percent dry weight in sole salinity treatment (S) was highest (17.98%) which shows less water uptake whereas lowest percent dry weight observed in the salinity free controls suggests better water uptake. Our results are in agreement with (Sheng *et al*., 2008) who observed that, under salt stress, mycorrhizal maize plants had a higher dry weight accumulation in shoot and root, higher relative chlorophyll content and better water status compared to non-mycorrhizal maize plants. The salinity imposed about 50% and 21.6% reduction in the stem length and thickness in the sole salinity controls as compared to the salinity free plants. Similarly, the stem thickness, leaf count and number of leaflets per leaf were also significantly reduced and subsequently rescued by the AS, FS and FAS treatments to varying extents. Among the salinity stressed treatments, the highest stem length, stem thickness, leaf and leaflet count were induced by FS followed by AS, FS and FAS, respectively (Table 3).

Table 2. Effect of AMF and ASA on biomass production in salinity stressed tomato seedlings.

Treatment	Fresh weight per plant (g)	Dry weight per plant (g)	Dry weight (%) per plant
Control	31.21 ± 2.67^{a}	3.43 ± 0.40^{a}	11.36 ± 0.47^{c}
S	8.68 ± 0.58^{c}	1.48 ± 0.06^{c}	17.98 ± 0.69^{a}
AS	14.09 ± 1.09^{b}	2.10 ± 0.18^{b}	15.80 ± 0.23^{b}
FS	14.71 ± 0.70^{b}	2.10 ± 0.17^{b}	14.55 ± 0.76^{b}
FAS	14.49 ± 1.07^{b}	2.21 ± 0.14^{b}	15.55 ± 0.60^{b}

Data are the mean of at least five uniform seedlings ± SD. CL: no salinity; S: sole salinity; AS: acetyl salicylic acid and salinity; FS: *G. etunicatum* root inoculation and salinity; FAS: *G. etunicatum* root inoculation along with acetyl salicylic acid and salinity. Means followed by different alphabets in a column depict significant difference among treatments at $p<0.05$. LSD test

Reproductive growth: The salinity stress remarkably delayed the appearance of flower buds and only 20.83% plants produced reproductive buds in sole salinity treatment contrary to 100% in the salinity free controls. Among salinated treatments, highest number of plants (100%) produced flower buds in ASA treatment followed by FAS and FS. Similarly, among the salinated treatments, opened flowers were only observed in the AMF treatments (FS and FAS) however there was no difference between FS and FAS treatments regarding percent plants bearing opened flowers and number of opened flowers per plant (Table 4) which suggests a potential role of AMF in induction of flowering in salt stress conditions. It is suggested that both AMF and ASA played role in establishment and or maintenance of hormonal balance necessary for flowering.

Chlorophyll contents: Chlorophyll is vital component of chloroplasts and its quantity is directly correlated to photosynthetic performance (Anjum *et al*., 2011). In the present studies, chlorophyll contents were noted in terms of SPAD units at 5th and 10th day of first salinity treatment. It was observed that there was significant reduction in chlorophyll pigments in sole salinity treatment as compared to the salinity free controls. Previous studies demonstrate that salt stress cause reduction in chlorophyll content in plants such as tomato (Sudhir & Murthy, 2004) and less photosynthetic pigment may lead to declined photosynthesis. In the present study, *Glomus etunicatum* root colonization alone and combined with ASA significantly reduced losses in chlorophyll contents and notably there were minute reduction but no significant difference with the salinity free controls at both dates (5th and 10th day of salinity treatment). However, exogenous ASA application was best to induce higher chlorophyll contents on these dates. It was observed that in the given conditions AS was the only treatment which remarkably increased chlorophyll contents on the 5th and 10th day of salinity stress treatment (Fig. 1). These findings are also consistent with the results of photosynthetic performance.

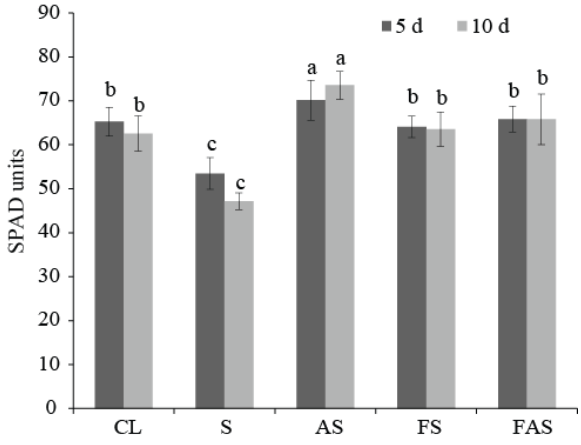

Fig. 1. Effect of acetyl salicylic acid and *G. etunicatum* root colonization on chlorophyll contents of salinity (150 mM) stressed tomato seedlings. CL: no salinity; S: sole salinity; AS: acetyl salicylic acid and salinity; FS: *G. etunicatum* root inoculation and salinity; FAS: *G. etunicatum* root inoculation along with acetyl salicylic acid and salinity. Different alphabets show statistically significant difference among treatments at $p<0.5$, LSD.

Photosynthetic performance: The salinity stress had deleterious effect upon the photosynthetic machinery and net photosynthesis (Pn) was significantly reduced to 6.16 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the sole salinity treatment as compared to the salinity free control (12.32 $\mu mol \cdot m^{-2} \cdot s^{-1}$). However,

other three treatments viz. AS, FS and FAS rescued the photosynthetic system and significantly improved net photosynthesis (Pn) as compared to the sole salinity treatment (Table 5). Similar trend was observed in the stomatal conductance (Gs) and transpiration rate (E). However, water use efficiency (WUE) was higher (1.13 μmol CO_2·$mmol^{-1}$) in sole salinity treatment due to reduced stomatal conductance and increased stomatal limit.

Root architecture: The continuous salinity stress reduced over all root vigor (Fig. 2).The total root length in sole salinity treatment was slashed up to 52.9% as compared to salinity free controls (Fig. 3A). However, treatment with AMF (*Glomus etunicatum*) significantly reduced the root length losses to 22.8% whereas ASA and ASA+AMF completely eliminated the injurious effects of salinity. Moreover, total root length in these treatments was statistically at par with that of salinity free controls (Fig. 3A). A similar trend was observed regarding root total surface area and volume where the AS, FS and FAS treatments were observed to contribute to reduce the losses caused by salinity to different extents (Fig. 3 B&C). The root mean diameter was not significantly affected by salinity as compared to the salinity free controls. The mean root diameter in sole salinity (S) and ASA treatments was at par with the salinity free controls, however, treatments involving AMF viz. FS and FAS yielded significantly improved mean root diameter (Fig. 3 D). The total number of nodes, tips, bifurcations and connections were significantly reduced in sole salinity treatments and subsequently rescued by all three treatments viz. ASA, AMF and ASA+AMF. Total number of nodes and tips were particularly up regulated and were significantly higher than the salinity free controls in the ASA and AMF treatments (Fig. 3 E, F, G&H) which suggest a root growth regulatory role of AMF and notably that of ASA.

Induced gene expression

Expression of NaC1: The amount of NAC1 transcripts was significantly regulated by both acetyl salicylic acid and *Glomus etunicatum*. The expression of NAC1 in roots was generally higher than in the leaves. In leaves, highest expression of NAC1 was noted in FS treatment followed by hybrid (AMF+ASA) treatment AFS that lead us to conclude that *Glomus etunicatum* has up-regulatory effect for NAC1 expression in salt stressed tomato leaves. The lowest level of expression was observed in AS treatment that mentions a down regulatory effect of acetyl salicylic acid in leaves (Fig. 4 A). On the other hand, highest NAC1 expression was observed in AS roots followed by sole salinity roots. From this data it is apprehended that contrary to down regulatory effect on NAC1 expression in leaves, acetyl salicylic acid up-regulated it in roots whereas *Glomus etunicatum* had slight down-regulatory effect here in roots. From the Fig. 4 A, it is further evident that the salinity also increased the transcript level of NAC1 both in leaves and roots compared to the salinity free controls.

Expression of LeNHX1: In leaves the expression level of sole salinity was highest in sole salinity treatment followed by FS. However, treatments with acetyl salicylic acid appear to induce a limiting effect on the transcript levels in the leaves. This result is reciprocal in case of roots where salinity had a little effect on LeNHX1 expression whereas FS, AS and AFS induced highest transcript levels in roots, respectively revealing a significant regulatory role of both acetyl salicylic acid and AMF (*Glomus etunicatum*) towards LeNHX1 expression (Fig. 4 B).

Table 3. Effect of AMF and ASA on vegetative growth of salinity stressed tomato seedlings.

Treatment	Stem length (cm)	Stem thickness (mm)	No. of leaves/plant	No of leaflets/ leaf
Control	51.35 ± 2.07^{a}	5.32 ± 0.23^{a}	8.80 ± 0.84^{a}	10.50 ± 0.55^{a}
S	26.85 ± 0.62^{c}	4.17 ± 0.04^{c}	5.71 ± 0.76^{c}	6.57 ± 0.79^{c}
AS	31.68 ± 0.91^{b}	4.61 ± 0.08^{b}	7.00 ± 0.82^{b}	8.50 ± 0.55^{bc}
FS	35.10 ± 1.02^{b}	4.50 ± 0.06^{bc}	8.80 ± 0.84^{a}	9.60 ± 1.14^{b}
FAS	29.65 ± 1.02^{c}	4.51 ± 0.03^{bc}	8.33 ± 0.82^{a}	9.67 ± 0.52^{b}

Data are the mean of at least five uniform seedlings ± SD. Means followed by different alphabets in a column depict significant difference among treatments at $p<0.05$. LSD test

Table 4. Effect of AMF and ASA on reproductive growth of salinity stressed tomato seedlings

Treatment	Plants bearing flower buds (%)	No. of flower buds/plant	Plants bearing open flowers (%)	No. of open flowers/plant
Control	100	3.33 ± 1.03^{a}	25^{a}	2 ± 0.81^{a}
S	20.83	1.2 ± 0.44^{d}	-	-
AS	100	1.86 ± 0.69^{c}	-	-
FS	54.16	1.66 ± 0.51^{c}	20.83^{b}	1.4 ± 0.54^{b}
FAS	62.5	2.5 ± 0.83^{b}	20.83^{b}	1.4 ± 0.54^{b}

Data are means of at least five uniform seedlings ± SD. Means followed by different alphabets in a column depict significant difference among treatments at $p<0.05$. LSD test

Table 5. The photosynthetic performance of salinity stressed tomato plants exogenously sprayed with acetyl-SA and/or root colonized by *Glomus etunicatum*.

Treatment	Photo synthetic rate ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	stomatal conductance ($mol \cdot m^{-2} \cdot s^{-1}$)	intercellular CO_2 concentration ($\mu mol \cdot mol^{-1}$)	transpiration rate ($mmol \cdot m^{-2} \cdot s^{-1}$)	water use efficiency ($\mu mol\ CO_2 \cdot mmol^{-1}$)	Stomatal limit (Ls)
Control	12.32 ± 0.53^{a}	0.38 ± 0.03^{a}	281.74 ± 5.55^{d}	11.57 ± 0.97^{a}	1.07 ± 0.10^{ab}	0.20 ± 0.02^{a}
S	6.16 ± 0.15^{c}	0.16 ± 0.02^{c}	284.33 ± 10.94^{cd}	5.54 ± 0.65^{d}	1.13 ± 0.14^{a}	0.21 ± 0.03^{a}
AS	8.29 ± 0.82^{b}	0.25 ± 0.03^{b}	289.18 ± 5.09^{ab}	7.60 ± 0.68^{c}	1.09 ± 0.08^{a}	0.17 ± 0.01^{cd}
FS	8.64 ± 0.31^{b}	0.25 ± 0.01^{b}	285.84 ± 2.94^{bc}	8.77 ± 0.27^{b}	0.99 ± 0.05^{c}	0.18 ± 0.01^{bc}
FAS	8.50 ± 0.66^{b}	0.26 ± 0.02^{b}	290.66 ± 1.92^{a}	8.44 ± 0.46^{b}	1.01 ± 0.08^{bc}	0.17 ± 0.01^{d}

Data are the means of at least 10 replicates ±SD. Means followed by different alphabets in a column depict significant difference among treatments for a certain parameter at $p<0.05$. LSD test

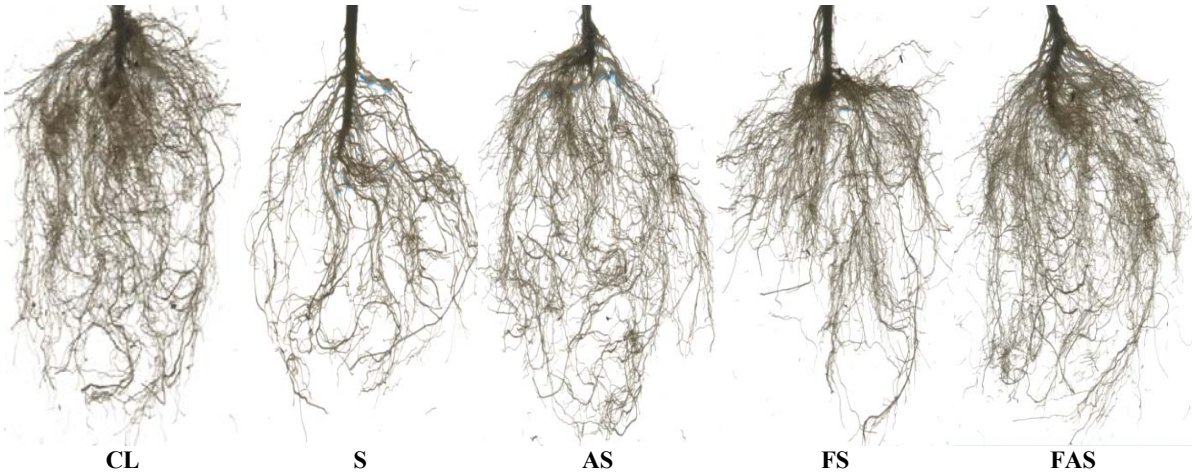

Fig. 2. Effect of acetyl salicylic acid and *Glomus etunicatum* on root morphology of salinity (150 mM) stressed tomato seedlings.

Discussion

In the present study it was observed that the salinity stress reduced fresh and dry plant biomass whereas *Glomus etunicatum* and acetyl salicylic acid either alone or in combination, minimized the losses to considerable extents which is aligned with the findings of Giri *et al*. (2007) who reported higher root and shoot dry weight in the mycorrhizal than non-mycorrhizal *Acacia nilotica* seedlings. Similarly Al-karaki (2000) documented a higher shoot and root dry weight, fresh fruit yield, fruit weight and fruit number in mycorrhizal tomato plants than in a nonmycorrhizal tomato plant. Our findings are further supported by Colla *et al*. (2008) who reported improved growth, yield, water status, nutrient content and quality of fruits of salinity stressed *Cucurbita pepo* plants colonized by *Glomus intraradices*. Similarly, Wasti *et al*. (2012) reported that SA (0.01mM) treatment through drenching significantly improved shoot growth and leaf area of salinity stressed tomato plants compared to the sole salinity controls.

In the present study, salt deleteriously reduced root physical features including total length, volume, surface area, number of connections, nodes and tips. However, AMF increased these parameters, which is consistent with previous studies that document improvement in root architecture with increased branching in AMF colonized root systems (Norman *et al.,* 1996; Locatelli *et al*., 2002; Aguin *et al*., 2004; Fan *et al.*, 2011). Previously, Khan *et al*. (2014) attributed better root development in heat stressed tomato seedlings to the exogenous acetyl salicylic acid induced root activity and vitality. Auge (2001) and Yao *et al*. (2005) interpreted that AMF induced modified nutritional status and altered phytohormone levels might be responsible for improved root-system. The relationship between phytohormones and root-system morphology has been established since phytohormones are major signaling factors conferring the effects of environmental cues on root-system morphology (Yao *et al*., 2005). Moreover, the extended root system itself facilitates better moisture and nutrients uptake especially under stress conditions.

In our study, it was found that there was significant reduction in chlorophyll pigments in sole salinity treatment as compared to the salinity free controls which is in agreement with previous studies; (Sudhir & Murthy, 2004) noted that salt stress reduced chlorophyll content in plants such as tomato which may lead to declined photosynthesis process. We further observed that *Glomus etunicatum* root colonization alone and in combination with ASA significantly minimized losses in chlorophyll contents and there was no significant difference between stressed and salinity free controls at both dates (5^{th} and 10^{th} day of salinity treatment). This suggests that regarding chlorophyll synthesis, salt stress interfered to lesser extent in mycorrhizal plants than in non-mycorrhizal plants (Giri & Mukerji, 2004). Our findings are in agreement with the results of many workers (Sannazzaro *et al*., 2007; Colla *et al*., 2008; Sheng *et al*., 2008) who reported higher chlorophyll contents in leaves of mycorrhizal plants under saline conditions. We

observed that in spite of salt stress to the *Glomus etunicatum* colonized seedlings, the chlorophyll contents were sustained to the level of the salinity free controls. Giri *et al*. (2003) observed that the antagonistic effect of Na^+ on Mg^{2+} uptake is counterbalanced and suppressed in mycorrhizal plants. Thus AMF inoculated plants under salt stress reach levels of photosynthetic capacity (estimated by chlorophyll content) even superior to those of non-stressed plants, which mentions that at least in this aspect, AMF are capable of fully counterbalancing salt stress (Zuccarini., 2007). We further observed that the improvement in the chlorophyll contents was significantly higher in the seedlings with acetyl-SA foliar application that superseded rest of treatments both at 5^{th} and 10^{th} day of salinity stress. Other workers have infrequently studied acetyl-SA in salinity stress scenario in tomato. Previously, Khan *et al*. (2014, 2015) have reported heat stress ameliorative role of acetyl-SA in tomato seedlings and attributed same to better root vitality and development, and improved photosynthetic pigment contents. Our results are also supported by Wasti *et al*. (2012) who reported that NaCl (100 mM) caused around 50% reduction in chlorophyll contents in tomato leaves compared to control plants but application of 0.01 mM salicylic acid to NaCl stressed plants increased chlorophyll accumulation in the leaves.

In this study, reduced stomatal conductance in sole salinity treatment (S) accompanied by low net photosynthesis (Pn) suggested poor functioning of photosynthetic apparatus which is attributed to NaCl induced damage. Whereas acetyl salicylic acid (AS), AMF (FS) and combination of both (FAS) treatments played an ameliorative role and protected the photosynthetic apparatus by improved net photosynthesis (Pn), better stomatal conductance (Gs), improved transpiration (E) and reduced stomatal limitation (Ls) (Table 5). These results are supported by Szepsi *et al*. (2005) who showed that physiological parameters of salicylic acid (10^{-4} M) pre-treated tomato plants stressed with 100 mM NaCl, exhibited better values than those of salt stressed controls. The primary photochemical processes of photosynthesis (Fv/Fm, ΦPSII, qP) functioned equally or better than that of the control plants. Improvements in photosynthetic activity or water-use efficiency have been also reported in mycorrhizal plants growing under salt-stress conditions (Sheng *et al*., 2008; Zuccarini & Okurowska, 2008). Previously it was observed that AM symbiosis improved the photosynthetic capacity of maize (Sheng *et al*., 2008) and sweet basil leaves (Zuccarini & Okurowska., 2008) by uplifting gas exchange capacity, improving efficiency of PSII and by regulating the energy bifurcation between photochemical and non-photochemical events.

In our experiment, we investigated how the salt responsive genes (LeNHX1 and NAC1) respond to the salt stress (150 mM), AMF *Glomus etunicatum* and acetyl salicylic acid (0.3 mM) application. It was observed that salinity stress, *Glomus etunicatum* colonization as well as acetyl-SA affected the gene expression levels to varying extents. *Glomus etunicatum* had up regulatory effect for NAC1 expression in salt stressed tomato leaves whereas acetyl-SA had down regulatory effect. Contrary to leaves, the effect of *G. etunicatum* and acetyl-SA in roots was reverse i.e. down regulation of NAC1 by *G. etunicatum* and up regulation by acetyl-SA in roots. The salinity itself had up regulatory effect on the NAC1 transcript volumes both in leaves and roots compared to the salinity free controls. Though the *G. etunicatum* and acetyl-SA induced opposite effects in the leaves and roots on the NAC1 expression levels, however the overall effect was establishment of better salt tolerance in both treatments. This led us to suggest that the *G. etunicatum* and acetyl-SA might have different modes of salt amelioration in both the plant parts.

There are at least four LeNHX type genes (LeNHX1~4) in tomato (Rodriguez-Rosales *et al*., 2009). LeNHX1, 3 and 4 are tonoplast Na^+/H^+ antiporters (Pardo *et al*., 2006). Galvez *et al*. (2012) concluded that the LeNHX 1~4 expression is higher in wild species accompanied by better salt tolerance. In the present studies, we observed that the Na^+/H^+ antiporter LeNHX1 gene expression was higher in (FS; AMF+salinity) and (FAS; AMF+acetyl-SA+salinity) as well as in the (AS; acetyl-SA+AMF) treatments than the sole salinity in the seedling roots which was consistent with the enhanced salt tolerance. However, our results are not in agreement with Ouziad *et al*. (2006) and He & Huang (2013) who evaluated expression of two Na^+/H^+ antiporters LeNHX1 and LeNHX2 in dependence on salt and mycorrhizal (*Glomus geosporum* and *Glomus intraradices*) colonization in roots and leaves of tomato plants and observed no significant transcript alterations under given conditions. However, in our studies we noted that the *Glomus etunicatum* or acetyl-SA had either no or inhibitory role in the expression of LeNHX1 in the seedling leaves compared to the sole salinity treatments. It is suggested that Na^+/H^+ exclusion is more crucial in the root zone thus the activation of the LeNHX1 induced by *G. etunicatum* and acetyl-SA is of prime importance. Previously, Zhang *et al*. (2001) and Waditee *et al*. (2002) reported that over expression of Na^+/H^+ antiporters in plants or microorganisms induced salt stress tolerance. Thus we suggest that the regulation of the NAC1 and LeNHX1 genes by *G. etunicatum* and acetyl-SA might have major role and be one of principal sources of defense against NaCl stress in the tomato plants. Root colonization by AMF involves a series of morpho-physiological and biochemical events regulated by the interaction of plants and fungus, as well as by environmental factors (Blumwald *et al*., 2000). From the results of this study we attribute the improved vegetative and reproductive performance of salinity stressed tomato seedlings to the AMF (*G. etunicatum*) and ASA (0.3 mM) regulated NAC1 and LeNHX1 gene expression and sustained photosynthetic performance due to protected photosynthetic apparatus and up regulated chlorophyll contents. Arbuscular mycorrhizal fungi (AMF) have the potential to improve the profitability and sustainability of salt tolerance (Aggarwal *et al*., 2012) in plants. The present study suggests feasibility of practical use of eco-friendly bio-inoculant; *Glomus etunicatum* and chemical ameliorator; acetyl salicylic acid, individually and in combination and warrants further large scale investigations to explore their ameliorative potential under natural salt stressed conditions.

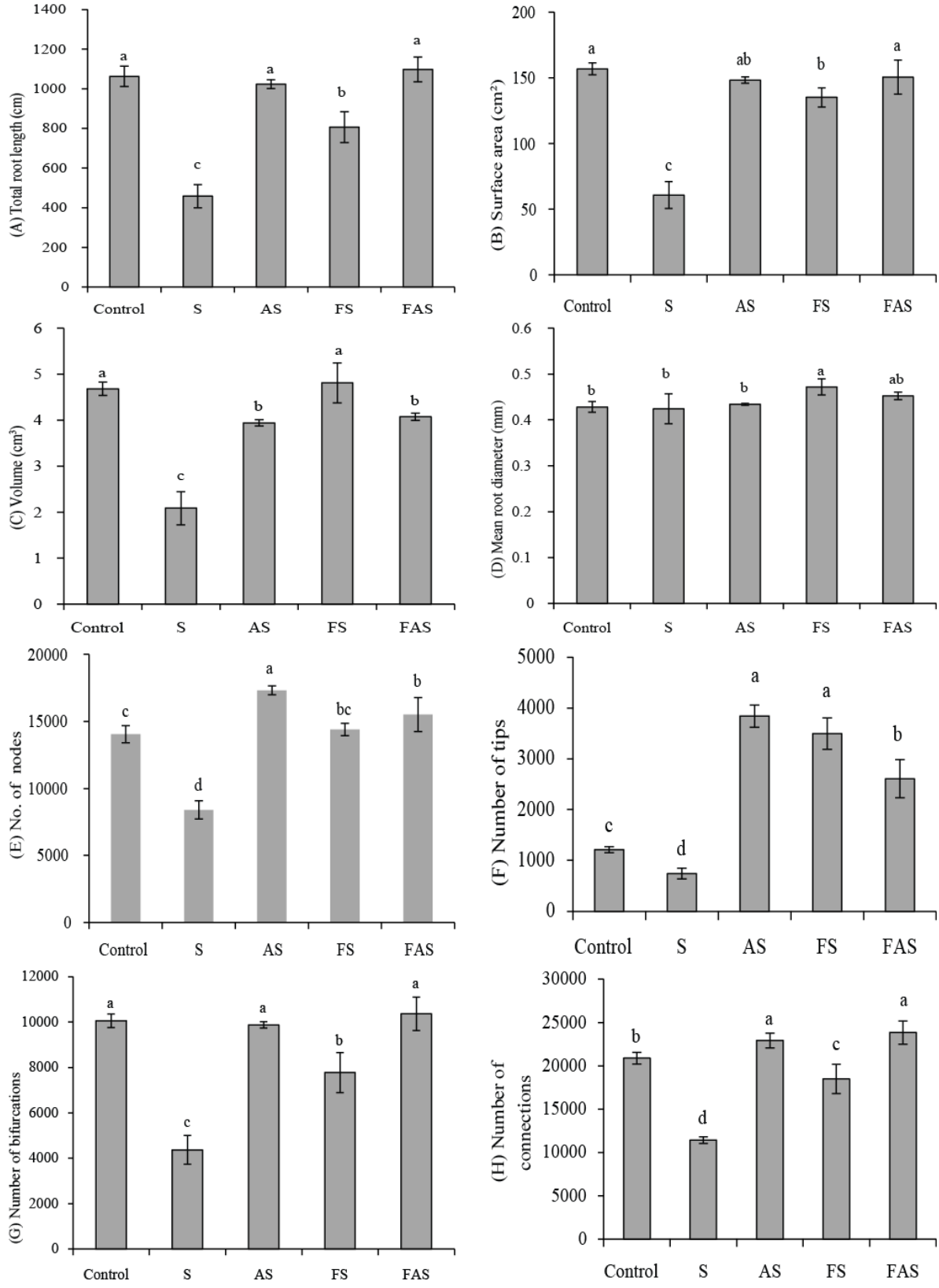

Fig. 3. Effect of acetyl salicylic acid and *Glomus etunicatum* root colonization on root morphology of salinity (150 mM) stressed tomato seedlings. Error bars show treatment means ±standard deviation (n=3) and different alphabets being bar heads show significant difference among treatments at $p<0.5$, LSD.

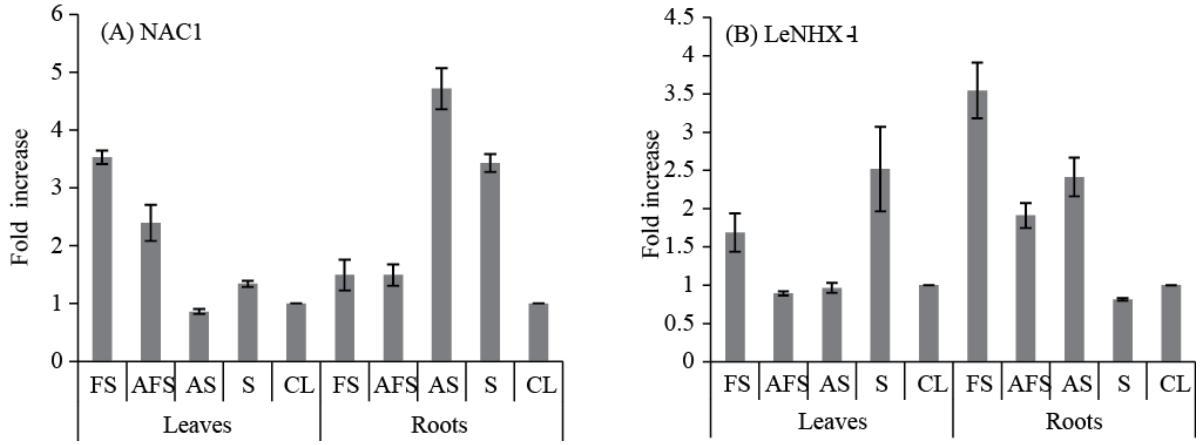

Fig. 4. Regulatory effect of AMF *Glomus etunicatum* root colonization and acetyl salicylic acid on expression of salt responsive genes (NAC1 and LeNHX-1) in leaves and roots of salinity stressed tomato seedlings. FS: Salinity stressed *G. etunicatum* colonized seedlings, AFS: Salinity stressed *G. etunicatum* colonized seedlings with exogenous application of acetyl-SA; AS: acetyl salicylic acid and salinity; S refers to sole salinity control and CL refers to salinity free control. Error bars show treatment means ± SD, n=3.

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