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SYNERGISTIC AND INDIVIDUAL EFFECT OF *GLOMUS ETUNICATUM* ROOT COLONIZATION AND ACETYL SALICYLIC ACID ON ROOT ACTIVITY AND ARCHITECTURE OF TOMATO PLANTS UNDER MODERATE NACL STRESS

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Abstract

A pot based experiment in plastic tunnel was conducted to investigate the changes in root morphology and root activity of the tomato plants grown under moderate NaCl stress (100 mM), pretreated with arbuscular mycorrhizal fungus AMF (*Glomus etunicatum*) root colonization and acetyl salicylic acid (ASA) as salinity ameliorative agents. The results revealed that both AMF and ASA treatments significantly enhanced the fresh root weight and root morphological parameters; net length, surface area, volume, mean diameter, nodal count and number of tips to different extents as compared to those of sole salinity treatment at 90 days after transplantation. Both treatments; AMF alone and in combination with ASA significantly enhanced the root activity level in terms of triphenyl tetrazolium chloride (TTC) reduction (2.37 and 2.40 $mg^{\bullet}g^{-1\bullet}h^{-1}$ respectively) as compared to the sole salinity treatment (0.40 $mg^{\bullet}g^{-1\bullet}h^{-1}$) as well as the salt free control (1.69 $mg^{\bullet}g^{-1\bullet}h^{-1}$). On the other hand, ASA treatment alone also uplifted root activity (1.53 $mg^{\bullet}g^{-1\bullet}h^{-1}$) which was significantly higher than that of sole salt treatment. It was inferred that under moderate saline conditions (100 mM NaCl), AMF (*Glomus etunicatum*) and ASA (individually or in combination) confer protective effect on plant growth by enhanced root activity and improved root architecture. Therefore, synergistic use of AMF (*G. etunicatum*) and ASA can be eco-friendly and economically feasible option for tomato production in marginally salt affected lands and suggests further investigations.

Key words: Tomato, Salinity tolerance, *Glomus etunicatum*, Acetyl salicylic acid, Root architecture.

Introduction

Globally, soil salinity has emerged as a major stress that negatively effects crop production (Tavakkoli *et al*., 2011). Soil salinity affects around seven percent of the total and 20% of the irrigated lands of the world (Yamaguchi & Blumwald, 2005). In Pakistan, about 25% of all irrigated land is affected by varying levels of salinity and approximately 1.4 million hectares of all agricultural land has been abandoned (Anon., 2006).

Salinity reduces plant growth, imposes unwanted ionic and osmotic effects and induces oxidative stress (Khan *et al*., 2010; Hajiboland *et al*., 2010; Nadeem *et al*., 2012). Soil salinity results in reduced crop growth and slashed yields since the excessive salts in the root zone interfere with the nutrient uptake (Younis *et al*., 2014a). Yield reductions have been reported up to 50% in moderate to high saline soils (Kandel, 2011) and in the severe cases, crop production may no longer be feasible. Therefore, improvement and incorporation of salt tolerance in crops is a key global agricultural goal (Ghanem *et al*., 2011; Younis *et al*., 2015).

Tomato (*Lycopersicon esculentum* Mill.) from family *Solanaceae* is an important kitchen crop all over the world and is consumed in cooked, processed and fresh forms. However, wide spread salinity affected soils and brackish subsoil water hamper tomato cultivation with economic yields. Many studies have demonstrated that root inoculation with AM fungi improves growth of plants under a variety of stress conditions (Sharifi *et al*., 2007; Al-Khaliel, 2010; Zhu *et al*., 2012) and improves P and K uptake in inoculated tomato plants (Al-karaki *et al.,* 2001). Salicylic acid (SA) and its allied compounds are derivatives of plant phenols (Munne-Bosch & Penuelas, 2003) and their ameliorative role in salinity tolerance in wheat and maize (Gunes *et al*., 2007; Shakirova *et al*., 2003) has been documented. Acetyl salicylic acid (ASA); a derivative of salicylic acid has been reported as an ameliorative agent against heat stress in tomato (Senaratna *et al*., 2000; Khan *et al*., 2014), drought in muskmelon seedlings (Korkmaz *et al*., 2007) and low temperature in sweet pepper seeds (Korkmaz, 2005).

The combined effect of microbial and chemical ameliorative agents against salinity stress on tomato is relatively under investigated area. Rui-Hong *et al*. (2009) studied the role of AM Fungi and SA on salt tolerance of strawberry plants and observed that the leaf chlorophyll and potassium contents in both leaves and roots were significantly up regulated.

Roots may be the most vulnerable plant organs as they are directly exposed to salt stress (Ouyang *et al*., 2007; Munns & Tester, 2008; Younis *et al*., 2013). Root growth inhibition has been extensively reported as a primary response in plants under salt stress. So far, the synergistic role of ASA (chemical agent) and AMF colonization (symbiotic organisms) in root development in tomato plants under moderate saline conditions has been inadequately investigated. Though several AMF has been tested, but, *Glomus etunicatum* root colonization has rarely investigated for tomato plants regarding induction of NaCl tolerance. Most of such research work has been reported on seedling stage of tomato plants and has not been sufficiently investigated in field environmental conditions up to maturity. The use of AMF and ASA are environment friendly and relatively cost effective hence provides better chances of adaptation. Therefore, the present research project focusing entire tomato production span in plastic tunnel was exercised. The objective of this study was to investigate the role of *Glomus etunicatum* root colonization and acetyl salicylic acid, both individually and synergistically on the root growth parameters and root activity of tomato plants under salt (NaCl) stress.

Materials and Methods

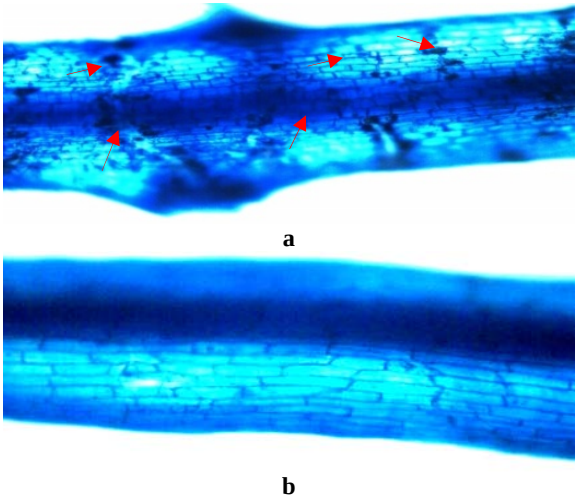
Experimental arrangements and growing seedlings: The two year experiment was conducted in a plastic tunnel at the horticultural experimental station (N 34° 16', E 108° 4') of College of Horticulture, Northwest Agriculture & Forestry University, Yangling, Shaanxi, China during Spring-2013 and 2014. For sterilization, all the materials including pots, field tools and growth medium were sprayed with formalin, wrapped in polyethylene sheet for a week and then kept in open for two weeks for fungicide evaporation. Soil was collected from open field and peat moss substrate (Pindstrup Ltd, Kekava, Latvia) was commercially procured, sterilized, thoroughly mixed (1:2 V/V) respectively and filled in the pre-sterilized 2L pots. The chemical properties of the mixture medium were as follows: pH (1:1 medium: water) 7.51; EC (1:5 medium: water) 1.2 $dS^{\bullet}m^{-1}$; available N: 121.10 $mg.kg^{-1}$; available K: 219.20 $mg^{\bullet}kg^{-1}$; available P: 178.23 $mg^{\bullet}kg^{-1}$ and organic matter: 31.92 $g^{\bullet}kg^{-1}$. Tomato seeds (cultivar: Sehao) were locally purchased, sterilized in 95% ethanol for five minutes, well rinsed and kept in hot water tub at 55°C for 15 minutes to ensure sterilization and improve germination. The seeds were then uniformly spread on wet filter paper at room temperature for initial germination and on the fourth day were transferred to the seedling trays filled with moistened peat moss and then kept in automated growth chamber operating at 12 h light period and 22/17°C day night temperatures, respectively.

Root inocculation: *G. etunicatum* inoculum was procured from AMF germplasm bank (GPB), Beijing Academy of Agriculture Science and Technology Information Institute, Beijing, China. At two leaf stage, the seedlings plugs were submerged in running tap water to wash away peat moss, keeping the roots intact. As per GPB's instructions, a suspension of AMF spores was prepared by adding 100 g inoculum in 250 mL distilled water and stirring in blender for half minute. The seedling plugs were carefully washed with tap water to expose roots and those with uniform size, vigor and root volume were chosen and five milliliter of spore suspension was drop wise poured in the root net of each seedling. This would allow the spores to trap in and adhere to the roots for subsequent colonization. The seedlings were transplanted in the 10x10x10 cm plastic pots and again kept at the same temperature and light conditions for colonization. After four weeks the plants were transplanted to the 2 L pots and placed in walk through plastic tunnel.

The AMF root colonization was evaluated using a light microscope (Olympus, Japan) after 80 days of transplantation. The sample root pieces (length ~0.5 cm) were randomly collected, cleared by submerging in 10% KOH for four days and then stained with trypan blue 0.1 g/L (w/v) in 55% acetic acid (Philips & Hayman, 1970). At least one hundred root pieces per treatment were microscopically evaluated two week before final harvest and AMF root colonization was estimated as per following equation:

$$\text{Root colonization (\%)} = \frac{\text{No. of colonized root pieces}}{\text{No. of observed root pieces}} \times 100$$

It was observed that ~68% roots per plant were well colonized by AMF *Glomus etunicatum* (Fig. 1) while no colonization was noted in none mycorrhizal treatments.



a

b

Fig. 1. AMF inoculated (A) and un-inoculated (B) root pieces.

Salinity stress and ASA treatment: Acetyl salicylic acid was purchased from Sigma-Aldrich, Beijing, China. Three days after transplantation, 0.30 mM ASA added with 1% Tween-20 (to facilitate absorption), was exogenously sprayed (20 mL/plant) wetting all leaves from both sides. Acetyl salicylic acid application was practiced biweekly until the crop was harvested. After three days of first ASA application, the plants were salinity (100 mM NaCl) stressed (Basak *et al*., 2011) in two split doses to maintain the salt stress. The practice was carried on until final harvest. Beside the salt treatment, tap water was also applied once a week to escape drought, sudden osmotic shock and ion accumulation. The experiment comprised of five treatments viz. T1: salinity free control; T2: S: sole salinity; T3: AS: ASA+NaCl; T4: FS: AMF+NaCl; T5: FAS: AMF+ASA+NaCl. The data regarding root parameters was documented at final harvesting and subsequent uprooting. The EC of NaCl free and NaCl added tap water was 0.54 $dS^{\bullet}m^{-1}$ and 7.95 $dS^{\bullet}m^{-1}$, respectively. At the time of harvest, the EC of the growth medium in none salinated pots (control) was 1.40 $dS^{\bullet}m^{-1}$ and that of salinized medium was 4.70 $dS^{\bullet}m^{-1}$ (1:5 soil: water, at room temperature) whereas, pH was 7.28 and 8.10, respectively.

Root fresh weight: At the time of harvesting (90 DAT), plants were irrigated to field capacity, carefully uprooted and were washed in running tap water until cleaned. At least five uniform plants from each treatment were selected; the roots were cut at the root-shoot juncture and blotted with filter paper. Then the fresh weight was recorded using an electronic scale (Xiangshan Ltd., Guongdong, China).

Root architecture: The data regarding various parameters of root architecture were recorded using Microtek Scan Maker *i*800 plus with software Microtec Scan wizard EZ-2.3. Owing to large root size, each root was vertically sliced in two equal halves, well washed in dH_2O, digitally scanned and then data was pooled to calculate the total of the corresponding root architectural parameter.

Root activity: Triphenyl tetrazolium chloride (TTC) reduction method was adapted for assaying the root activity (Onanuga *et al*., 2012; Li, 2000; Khan *et al*., 2014). At least three fresh root sub samples of uniform size segments were collected at the final harvesting stage (90 DAT), from rhizospheres of visually uniform plants from each treatment, well washed with dH_2O, blotted on filter paper and then stored at 4˚C to be used on the same day. A standard curve was drawn based upon spectrophotometric absorption (λ=485 nm) with varying amounts of TTC (1g/0.1 L) with $Na_2S_2O_4$ and ethyl acetate. A linear equation (Y=0.703x+0.003, R^2= 0.999) was obtained and used to calculate the root activity. Roots were then sliced to uniform sized pieces and inserted into a test tube with 5 mL 1g/0.1L (m/v) TTC and 5 mL 0.1 mol/L Na_2HPO_4–NaH_2PO_4 solution. The tubes were kept at 37 °C in the hot water tub for 1 h. Then, 2 mL of 1 mol•L^{-1} H_2SO_4 was added and the entire liquid was drained. The roots were ground in separate mortar pestles with equal quantity of ethyl acetate, the reddish extract was collected in separate test tubes and absorbance was spectrophotometrically (spectrophotometer: UV-3802, UNIC, Shanghai, China) evaluated at λ=485 nm. Root activity was calculated as per linear equation and expressed as TTC reduction intensity: mg•g^{-1}•h^{-1}.

$$\text{TTC reduction intensity} = \frac{\text{TTC reduction mass (mg)}}{\text{Root fresh mass (g) x time (h)}} \text{ x } 100$$

Statistical analysis: The pots were arranged in Randomized Complete Block Design with three replicates of each treatment and there were seven plants in each treatment. The experimental results were subjected to ANOVA using Statitix-8.1 software. Comparison of means among different treatments was performed by LSD test with 5% significance level.

Results

Root activity: The salinity stress tremendously reduced (76%) root activity in sole salinity (S) treatment as compared to the salinity free controls. However, the acetyl salicylic acid (AS), AMF (*G. etunicatum*) (FS) and the combination of both (FAS) demonstrated an ameliorative effect and significantly enhanced the root activity levels (2.88, 5.02 and 5.09 times, respectively) as compared to the sole salinity treatments. The root activity of FAS and FS treatments was even higher than the unstressed controls (Figs. 2 and 3). However, the root activity of FAS was higher than FS but was statistically at par.

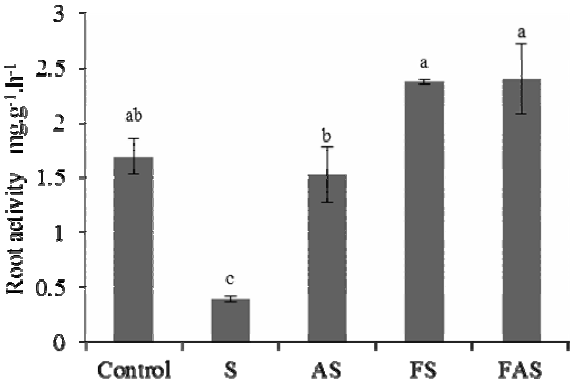


Fig. 2. The ameliorative effect of *Glomus etunicatum* colonization and exogenous acetyl salicylic acid on root activity of NaCl (100 mM) stressed tomato plants at fruiting stage. Bars headed by different alphabets represent significant difference among treatments at $p<0.05$. LSD test.

Root biomass and architecture: The prolonged salinity stress imposed deleterious impact on the growth of most of the root biomass and architectural parameters. Both AMF and ASA demonstrated varying extent of amelioration against NaCl stress. The root fresh weight was significantly (65%) reduced on account of injurious effects of NaCl in sole salinity treatment, however, the ASA and fungal treatments minimized this loss to different degrees with FS being the highest (Fig. 4.1a, b).

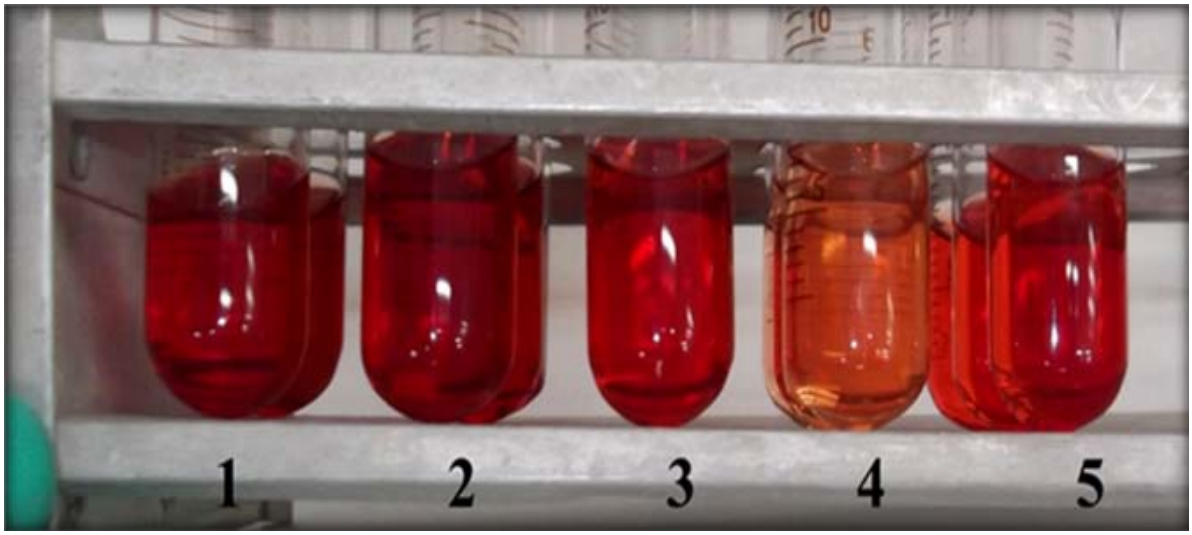


Fig. 3. Triphenyl tetrazolium chloride color development by roots of NaCl (100 mM) stressed tomato plants treated with *Glomus etunicatum* and exogenous acetyl salicylic acid. 1: (AMF+NaCl), 2: (AMF+ASA+NaCl), 3: (ASA+NaCl), 4: (sole salinity control) and 5 refers to salinity free control.

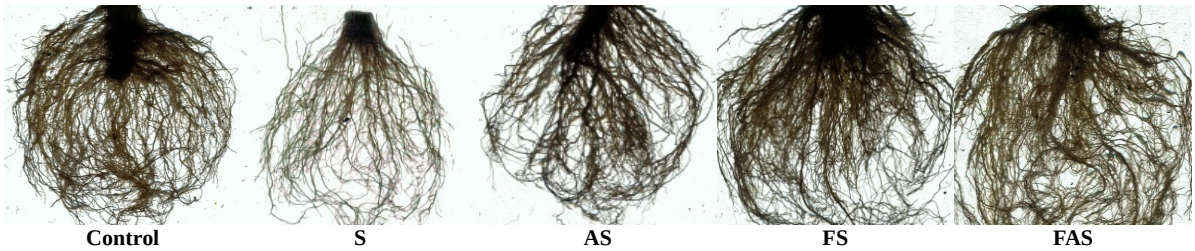


Fig. 4.1a. Combine and individual effect of *Glomus etunicatum* and ASA on tomato root architecture under moderate (100 mM) NaCl stress. S: salinity; A: acetyl SA; F: AMF *Glomus etunicatum*.

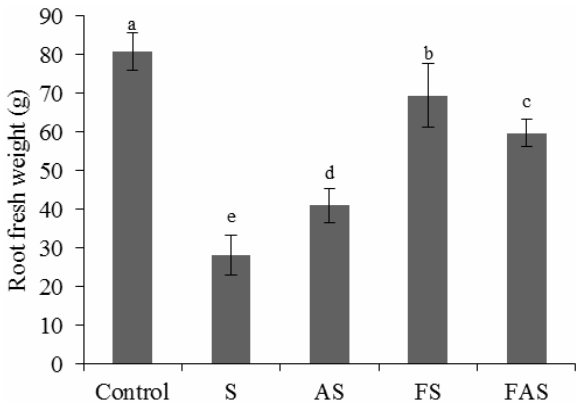


Fig. 4.1b. Tomato root fresh weight produced in saline conditions (100 mM NaCl) under five different treatments. S: salinity stress; A: exogenous acetyl salicylic acid (0.3 mM); F: AMF *Glomus etunicatum*. Bar heads containing different alphabets show significant difference among treatments at $p<0.05$, LSD.

Length of roots & volume: The total length including all root parts in both mycorrhizal treatments (FS and FAS) was significantly higher than non-mycorrhizal plants, but surprisingly it was also greater than the salinity free controls. However, application of ASA in combination with *G. etunicatum*, slightly reduced the root length (Fig. 4.2 A) but it was yet significantly higher than that of sole salinity treatments. Conclusively, both ASA and AMF treatments alone or in combination were effective to different extents to improve total root length under prolonged salinity stress.

Salinity reduced (56%) the root volume in sole salinity treatments with respect to salinity free controls. The application of ASA and *G. etunicatum* root colonization significantly minimized the loss to 47.5%, 20% and 23% in AS, FS and FAS treatments, respectively (Fig. 4.2 B).

Root diameter and surface area: The root diameter was not significantly affected by salinity stress in sole salinity treatment as compared with the unstressed controls. However, ASA (AS), mycorrhizal (FS) as well as synergistic treatments (FAS) significantly increased the mean root diameter (Fig. 4.2 C).The salinity stress posed significant reduction (37%) in root surface area as compared to non-saline controls. *G. etunicatum* colonization improved the surface area and reduced the losses to 6.3% and 1.8% in the AS and FAS treatments, respectively. However, alone AMF (FS) contributed highest root surface area and even superseded unstressed controls by 26%. Conclusively, all three treatments significantly improved root surface area as compared to the sole salinity treatment (Fig. 4.2 D).

Number of nodes and tips: The salinity stress reduced the number of nodes (38.8%) and tips (30%). Mycorrhizal treatments significantly improved both parameters. However, ASA had a minor effect on improvement in number of nodes and tips (Fig. 4.2 E, F).

Number of root connections and bifurcations: Root connections and bifurcations were reduced 40.6% and 39% respectively due to continuous salinity stress in the sole salinity treatment, however, the chemical (AS), biological (FS) and biochemical (FAS) treatments significantly improved these parameters to different extents (Fig. 4.2 G, H).

Discussion

The root architectural features (length, volume, surface area, connections, bifurcations etc.) are crucial for the plant to exploit maximum rhizosphere sources, mainly water and nutrients (Khan et al., 2014), anchorage and synthesis of growth regulators. It has been suggested that reduced shoot growth of plants whose roots have been subjected to stress is partly caused by a change in the kind and quantity of growth regulators provided by the roots (Blum *et al*., 1991; Davies & Zhang, 1991; Younis *et al*., 2014b). Thus the growth of shoot and root is interdependent. Since the root is in direct contact with the growth medium and continuous salinity stress may pose deleterious effects and negatively affect root proliferation and vitality leading to poor absorption of nutrients and water. An inadequate understanding of below ground processes is a major limitation to better predictions of crop growth and productivity (Eissenstat *et al*., 2006). In the present studies, it was observed that the salinity stress suppressed all the studied root parameters which is in agreement with the earlier findings that root growth may be affected by salt in terms of fresh weight (Khan & Panda, 2008) and root length (Kopyra & Gwóźdź, 2003). Results of our study are consistent with those of previous studies; Amjad *et al*. (2014), Oztekin and Tuzel (2011), Tantawy *et al*., (2009) and Yokas *et al*., (2008) documented reduction in root and shoot length under saline conditions. The reduction in plant growth under salinity is attributed to reduced water and nutrients availability (Kumar *et al*., 2005) to the plant due to excessive NaCl in the growth medium and subsequent ionic imbalance and specific ion toxicity (Munns *et al*., 2006) yielding down regulation of other metabolic activities.

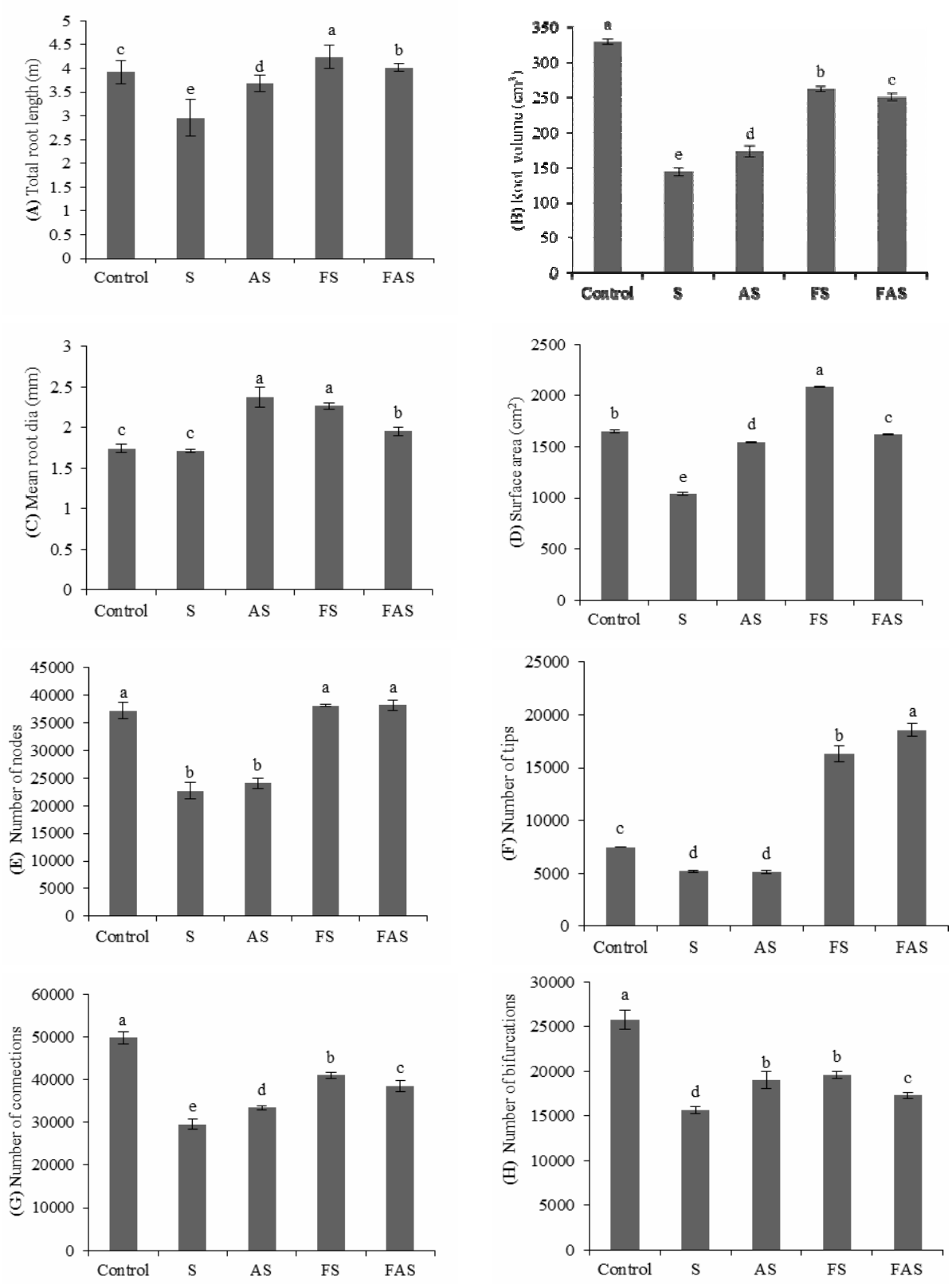


Fig. 4.2. A~H. Tomato root architectural features (per plant) in saline conditions (100 mM NaCl) under five different treatments; control: no salinity stress; S: sole salinity; AS: acetyl-SA and salinity; FS: *G. etunicatum* and salinity and FAS: *G. etunicatum*, acetyl-SA and salinity. Values represented by vertical bars are means of three replicates ± SD. Bar heads containing different alphabets show significant difference among treatments at $p<0.05$, LSD.

In the present study, we recorded significant improvement to different extents in most of the root architectural features moderated by *G. etunicatium* root colonization and exogenous application of ASA under continuous salinity stress (Fig. 4.2 A~H). Our results are supported by the findings of other workers; root colonized mycorrhizae modify the architecture and topology of root systems, generally resulting in longer or more branched roots resulting in relatively efficient absorption (Berta *et al*., 2002); AMF colonization enhanced root branching as well as root length in *Vitis vinifera* (Schellenbaum *et al*. 1991), in carob (Cruz *et al*., 2004) and improved root surface area and volume in *Plukenetia volubilis* seedlings under drought stress conditions (Tian *et al*., 2012). Our findings are also in coherence with (Turk *et al*., 2006) who documented AMF induced improvement in root proliferation, root surface area and alleviation of salinity stress by boosting water and nutrient uptake. Bryla & Roger (1998) observed that mycorrhizal (*G. etunicatum*) colonization increased tomato root length and density grown in low phosphorus soil. The results of present study are further supported by Khan *et al*. (2009) who observed that the SA and ASA treatments exhibited reduced time for 50% seedling emergence, final emergence percentage, root and shoot length, seedling fresh and dry weight and seedling vigor of hot pepper. Similarly, Khan *et al*. (2014) documented that ASA induced heat stress tolerance in tomato and improved root morphology in tomato seedlings.

In the present studies tomato plants with *G. etunicatium* colonization and exogenous ASA treatments exhibited significantly better root activity than sole salinity treatment. Triphenyl tetrazolium chloride (TTC) test is employed to asses root vitality (Ruf & Brunner, 2003; Khan *et al*., 2014). The colorless TTC is reduced by the mitochondrial dehydrogenases to red triphenyl formazan and the efficiency of this conversion provides an index of dehydrogenase activity, rate of tissue respiration (Richter *et al.*, 2006) and liveliness. The observed root activity in the present investigation were highest in the combine treatment of ASA and *G. etunicatum* colonization (FAS) which predicts more root vitality and thus better exploitation of rhizosphere resources under salt stressed environment. These findings are in close harmony to Li *et al*. (2010) who reported a significant role of combination of salicylic acid and AMF *Glomus mosseae* in alleviation of nitric oxide stress in *Avenanuda* seedlings by minimizing reduction in biomass. This led us to the conclusion that both chemical and biological agents have unidirectional ameliorative role and can be used simultaneously and confer enhanced protection against salinity stress.

In general, our results are also in line with the findings of Senaratna *et al*. (2000) who documented ameliorative role of SA and ASA against multiple stresses; heat, chilling and drought in tomato plants. Our observations are further supported by Khan *et al*. (2014) who observed a strong protective role of ASA against heat stress in tomato seedlings by improving the root activity and morphology.

In the present studies, the root activity levels are corresponding to the improved root morphological parameters of AMF and ASA treated plants. It is suggested that despite the constant NaCl stress, the enhanced root fresh weight and architectural indexes (root length, root volume, root area, bifurcations and number of tips etc.) were conferred by AMF and ASA induced improved root vitality. The findings in this study evidenced potential of the studied bioinoculant (AMF: *G. etunicatum*) and chemical (ASA) agents to reduce deleterious effects of salinity and further suggest applied research focusing their practical use for economic production of tomato in marginally salt affected lands.

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