Studies on Salinity Tolerance in Mycorrhizal Vegetable Crops

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(数種野菜でのアーバスキュラー菌根菌による耐塩性誘導機構に関する研究)

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CHAPTER 1-1

Estimation of salinity tolerance in mycorrhizal vegetable crops and antioxidant changes to ion stress under salinity condition

Introduction

Salinity is one of the most important agricultural and environmental problems nowadays, which is increasing steadily in many parts of the world especially in Asian region (Evelin et al., 2009; Porcel et al., 2012). Saline soils have been estimated to occupy more than 7% of the Earth's land surface and it is expected to be increased by up to 50% by the middle of the twenty-first century (Ruiz-Lozano et al., 2012). Typhoon, tsunami, sea water influx and inappropriate cultivation practices have exacerbated the concentration of salts in soil and water. Excess salt in soil is a major constraint to food production because it limits agricultural yield as it negatively affects plant growth and development, decreasing crop production over 20% (Porcel et al., 2012). In green house cultivation system high evaporation, low precipitation and fertilizer use induce gradual accumulation of salt in the soil. Salinity stress impacts many aspects of a plant's physiology such as osmotic stress, nutrient imbalance through ion stress, damage cell organelles, hamper energy and lipid metabolism, and disrupt photosynthesis, protein synthesis and respiration (Evelin et al., 2009, 2012). However, the effect of salinity stress on plants depends on the several conditions such as, severity and time of exposure of salinity stress, plant genotypes and environmental factors.

To deal with salinity and minimize crop loss, scientists have searched for new salt-tolerant crop plants and developed through breeding. Leaching of excessive salts or desalinizing seawater for use in irrigation are other methods employed to combat salt stress. Though successful, these approaches are costly and beyond the economic means of developing nations (Cantrell and Linderman, 2001).

Plants, in their natural environment are colonized by both external and internal microorganisms. Some microorganisms, particularly beneficial bacteria and fungi can improve plant performance under stress environments consequently, enhance yield (Creus et al., 1998). Arbuscular mycorrhizal fungi (AMF) are associated with the roots of over 80%

terrestrial plant species including halophytes, hydrophytes and xerophytes (Smith and Read, 2008). Utilization of this natural relationship to alleviate salinity stress may be beneficial for crop production.

Horticulture is the most promising area for practical use of AMF for nursery and green house plants. There are two main benefits from introducing mycorrhizal fungi to horticultural crops: stronger growth in the nursery or green house and improved performance after planting in the field (Beltrano et al., 2013). On the other hand, in protective cultivation systems, utilizing fertilizers and when irrigation is made just to meet the plant needs, salts gradually build up in the root zone such as strawberry cultivation (Jamalian et al., 2013). Insufficient leaching of irrigation water in the soil is a major cause of salt accumulation in the root zone.

Moreover, horticultural crops especially vegetable with high salinity tolerance are very limited and no exist in major vegetable crops. From these facts, establishing the methods which increase salt tolerance independent from vegetable species has been very important subject in horticultural production.

During salt stress, different metabolic pathways are uncoupled and electrons are transferred to molecular oxygen, leading to the formation of reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl radicals (OH^-). These ROS are toxic molecules capable of disrupting normal metabolism through denaturation of proteins, mutagenesis of DNA and lipid peroxidation (Estrada et al., 2013). To detoxify and eliminate the generated ROS, plants possess a well-developed antioxidant defence system, which comprises non-enzymatic as well as enzymatic antioxidants. The salt stress tolerance in plants has been associated with the induction of antioxidants and reduction of oxidative damages (Ruiz-Lozano et al., 2012).

AMF can help plants to cope with abiotic stress conditions (Barea et al., 2013) and they found to be present even under severe saline conditions (Wilde et al., 2009). Until now, salt

stress tolerance studies have recommended that AMF inoculated plants grow better due to improved mineral nutrition, physiological processes like photosynthesis or water use efficiency, higher K^+/Na^+ ratios and higher antioxidative activities (Evelin et al., 2009, 2012). Several studies suggested that mycorrhizal symbiosis helps plants to alleviate salt stress by enhancing the activities of antioxidant enzymes (Evelin et al., 2009; Hajiboland et al., 2010). Antioxidant activities vary with the plant tissue, salinity level and duration of stress (Abogadallah, 2010; Evelin and Kapoor, 2014). However, about the influence of AMF symbiosis on the accumulation of non-enzymatic antioxidants, such as ascorbic acid, glutathione and polyphenol contents in host plants, has been still unclear (Evelin et al., 2009; Ruiz-Lozano et al., 2012).

Therefore, the present study was aim to check the salt tolerance and changes in antioxidative ability through AMF application on asparagus, tomato and strawberry plants under salinity stress.

Materials and Methods

Experiment 1:

Plant materials and AMF inoculation: In this experiment, asparagus (*Asparagus officinalis* L., cv. Welcome), tomato (*Solanum lycopersicum* L., cv. Momotaro 8) and strawberry (*Fragaria* × *ananassa* Duch., cv. Tochiotome) plants were used. Seeds of asparagus and tomato plants were sowed and two months old strawberry runner plants were planted in plastic pots (13.5×27.0×15.5 cm for asparagus, 10.5 cm in diameter with depth 9.0 cm for tomato and 10.5 in diameter for strawberry) containing autoclaved (121^{o}C, 1.2 kg/cm^2, 30 min) commercial potting media SM-2 (Ibigawa Industry Co. Ltd., Japan) (Fig. 1). The potting media consist of Canadian sphagnum peat moss 85%, perlite, vermiculite, dolomitic and calcitic limestone and wetting agent. At the time of sowing and transplanting, half of the pots were inoculated with AMF inocula 5 g/plant (*Glomus fasciculatum* for asparagus and

strawberry and *Gigaspora margarita* for tomato) at a depth of 3 cm and mixed with potting media. The non-inoculated/non-mycorrhizal plants received the same amount of sterilized inocula. The mycorrhizal inocula of unknown spore density were obtained (exclusively for research purposes) from Centralgrass Co. Ltd., Tokyo, Japan (*Gigaspora margarita*) and Idemitsu Agri. Co. Ltd. Tokyo, Japan (*Glomus fasciculatum*). Plants were fertilized with slow release granular fertilizer (Long total 70 type, JCAM AGRI Co. Ltd., Japan) at the rates of N:P:K = 13:11:13, 1g/plant, after seedling emergence for asparagus and tomato and immediately after transplanting for strawberry. Plants were grown in a greenhouse at 25 ± 3/19 ± 3^{o}C day/night temperature with a 12-13 h photoperiod (750-1000 µmol/m^{2}/s) and 60%-70% relative humidity.

Treatment with NaCl: The plants were subjected to salinity stress with 200 mM NaCl solution after nine (strawberry) and fourteen (asparagus and tomato) weeks of AMF inoculation. Plants were irrigated with NaCl solution (40 ml/plant) for twelve days (4 times/week). The no salt-treated/control plants received an equal amount of distilled water. The electrical conductivity (EC) of saturated soil extract {1:5 soil: water (w/v) suspension} were measured weekly from 10 randomly selected pots for every treatment and replication. The average EC of saturated soil extract increased from 0.0013 mS/cm to 4.1, 8.5 and 14 mS/cm after 1st, 2nd and 3rd weeks of salt treatment, respectively.

Experimental setup: The experiment contains two factors: AMF and salt stress, each containing two treatments, non-mycorrhizal and mycorrhizal plants and no salt/control and 200 mM NaCl solution, respectively. Each treatment contains twenty plants with three replications arranged in completely randomized design.

Experiment 2: To check the AMF effect on seawater salinity, another experiment was simultaneously carried out with strawberry plants. Two months old strawberry (Tochiotome) runner plants with uniform sized were planted in plastic pots (13.5 × 100 × 18.5 cm)

containing autoclaved (121^{o}C, 1.2 kg/cm^{2}, 30 min) commercial potting media SM-2. Each pot contains three plants. At the time of transplanting half of the plants were inoculated with 5 g of AMF inocula at a depth of 3 cm of each plant and mixed with soil. Two types of AMF inocula were use, GM, *Gigaspora margarita* and Gf, *Glomus fasciculatum*. Plants were also fertilized with slow release granular fertilizer (Long total 70 type, JCAM AGRI Co. Ltd., Japan) at the rate of N:P:K = 13:11:13, 1 g/plant and grown in a greenhouse. Nine weeks after AMF inoculation plants were watered with seawater. Seawater was collected from Pacific Ocean near the Aichi prefecture of Japan and its EC was 62 mS/cm. Plants were watered with 80 ml sea water contained EC 3 and 6 mS/cm four times in a week. The seawater was diluted with distilled water. To maintain the required soil medium salt levels, the EC of the soil medium was measured periodically by portable EC meter and the required amount of treated water was added. So, the experiment contains two factors: AMF and salinity stress, AMF factors contained two treatment, non-mycorrhizal and mycorrhizal plants and salinity factors contained three treatments control, EC 3.0 and 6.0 mS/cm. Each treatment contains twenty plants with three replications arranged in completely randomized design.

Mycorrhizal colonization: After three weeks salt treatment, all the plants were uprooted. The lateral roots of the fresh non-mycorrhizal and mycorrhizal asparagus, tomato and strawberry plants (including seawater treatment) were preserved with 70% ethanol and stained with trypan blue according to the method of Phillips and Hayman (1970). In this process, after removing the 70% ethanol with washing tap water, roots were autoclaved at 121ºC, 1.2 kg/m^{2}, 15 min. After that the roots were immersed in 10% potassium hydroxide (KOH) solution. Thereafter, washing with distilled water and the roots were stained with trypan blue solution (glycerine 50 ml, lactic acid 50 ml, distilled water 50 ml and trypan blue 1 g). Then the stained roots were cut into a length of 0.5 to 1.0 cm section and placed on a glass slide for observing the colonization using an optical microscope. The rate of AMF colonization in 1 cm

segments of the lateral roots (abbreviated RFCSL) was calculated. Hence, RFCSL express the percentage of 1 cm AMF colonized segments to the total 1 cm segments of all lateral roots. The number of segments was approximately 50 per plant, used for 10 plants with three replications.

Plant growth: The dry weight of shoots and roots of ten plants per treatment (experiment 1 and 2) were measured after oven drying at 60-70ºC for 24 h while the remaining plants were frozen in liquid nitrogen to preserve for further analysis.

Na^+ content determination: The Na^+ content of shoots/leaves and main roots of asparagus, tomato and strawberry plants (including experiment 2) were measured using the Compact Na^+ (B-722) meter (Horiba Ltd., Tokyo, Japan). Frozen sample (0.2 g) was extracted using 2.5 ml of distilled water and then centrifuged at 13,000 rpm, 4°C, 10 min. The supernatant was subsequently used as a sample solution for measurement of Na^+.

PO_4^{3-}, Ca^{2+}, Mg^{2+} contents measurement:

The PO_4^{3-}, Ca^{2+}, Mg^{2+} contents were measured using RQflex plus 10 reflectometer and Reflectoquant test strip (Merck KGaA, Darmstadt, Germany) and analyzed by the following procedures. The Frozen samples of leaves and roots (0.1 g) were extracted using 10 ml of distilled water and the extract was centrifuged at 4,000 rpm, 4ºC, 10 min. The supernatant was used as sample solution for the measurement of PO_4^{3-}, Ca^{2+}, Mg^{2+} contents.

PO_4^{3-}content: Ten drops of reagent were shaken with 5 ml of sample solution and a test-strip dipped in the solution for 2 seconds and then removed. After 85 seconds the test-strip was inserted into the reflectometer and the concentration in mg PO_4^{3-}/L was displayed 5 seconds later. The measuring range of the system was quoted as 5-120 mg PO_4^{3-}/L.

Ca^{2+} content: Ten drops of reagent were shaken with 5 ml of sample solution and a test-strip dipped in the solution for 2 seconds and then removed. After 15 seconds the test-strip was inserted into the reflectometer and the concentration was displayed. The measuring range of

the system was quoted as 5-125 mg Ca^{2+}/L.

Mg^{2+} content: Ten drops of reagent were shaken with 5 ml of sample solution and a test-strip dipped in the solution for 2 seconds and then removed. After 15 seconds the test-strip was inserted into the reflectometer and the concentration was displayed. The measuring range of the system was quoted as 5-100 mg Mg^{2+}/L.

Chlorophyll content determination: Chlorophyll (a+b) content in leaves was determined according to Porra et al. (1989). The Frozen sample of leaves (0.1 g) was extracted using 4 ml of 100% methanol and the extract was centrifuged at 13,000 rpm, 5°C, 5 min. Then the supernatant was diluted 10 times with 100% methanol and the absorbance at wavelengths of 652 nm, 665.2 nm, and 750 nm was measured with a spectrophotometer (U-1900, Hitachi Co. Ltd., Tokyo, Japan). From the obtained absorbance, the chlorophyll content was determined by the following formula:

Chlorophyll (a + b) content (μg/ml) = 2.71 (A 665.2 - A 750) +22.12 (A 652 - A 750)

MDA (malondialdehyde) content determination: Measurement of MDA content was carried out according to the method of Heath and Packer (1968) (Fig. 2). Frozen sample (0.1 g) was extracted using 5 ml of 0.1% (w/v) TCA (trichloroacetic acid) solution and the extract was centrifuged at 13,000 rpm, 4°C, 5 min. Then the supernatant (1 ml) was mixed with 4 ml of 0.5% (w/v) TBA (2-thiobarbituric acid) and 20% (w/v) TCA solution in a test tube and incubated for 30 minutes in a boiling water bath. After cooling in ice the mixture was centrifuged at 4,000 rpm, 4°C, 10 min. The absorbance of the supernatant was measured at 532 nm and 600 nm by a spectrophotometer. Based on the obtained absorbance, the MDA content was determined by the following formula. The molar extinction coefficient was set to 155/mM/cm.

MDA content (nmol/g FW) = (A 532 - A 600) × 1000/ε × V/M

ε: Molar extinction coefficient, V: 0.1% (w/v) TCA addition amount (ml), M: Frozen sample

weight (g).

Hydrogen peroxide (H_2O_2) content determination: The H_2O_2 content was measured according to the method of Velicova et al. (2000) (Fig. 3). Frozen samples (0.5 g) were extracted using 5 ml of 0.1% (w/v) TCA and centrifuged at 13,000 rpm, 4°C, 15 min. Then the supernatant (1 ml) was mixed with 1 ml of 10 mM potassium phosphate buffer solution (pH 7.0) and 2 ml of 1 M potassium iodide and allowed to stand at room temperature for 1 hour in the dark. Then the absorbance of the reaction mixture was measured at a wavelength of 390 nm using a spectrophotometer. To make calibration curve 0.3 to 30 µg/ml H_2O_2 solutions was used and the H_2O_2 content was calculated from the obtained calibration curve.

Measurement of Superoxide dismutase (SOD) activity: Measurement of SOD activity was carried out according to the method of Beauchamp and Fridovich (1971) (Fig. 4). Frozen sample (0.1 g) was extracted using 3 ml of 50 mM phosphate buffer (pH 7.0) and the extract was centrifuged at 13,000 rpm, 4°C, 10 min. Then the supernatant (0.1 ml) was mixed with 2.3 ml of 50 mM sodium carbonate buffer (pH 10.2), 0.1 ml of 1.0 mM NBT (nitro blue tetrazolium), 0.1 ml of 4.0 mM xanthine, 0.1 ml of 3.0 mM EDTA (ethylenediaminetetraacetic acid), 0.1 ml of 0.15% (w/v) BSA (bovine serum albumin), 0.1 ml of 12.5 units/ml xanthine oxidase (100-fold diluted solution). The mixture was then allowed to stand at room temperature for 30 minutes in dark condition. Thereafter, 0.2 ml of 14 mM $CuCl_2$ [copper (II) chloride] solution was added and the absorbance of the reaction mixture was measured at a wavelength of 560 nm with a spectrophotometer. The one unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25ºC.

Measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability: The DPPH radical scavenging ability was measured according to the method of Burtis and Bucar (2000) (Fig. 5). Frozen sample (0.1 g) was extracted using 3 ml of 80% ethanol and the extract was centrifuged at 13000 rpm, 4°C, 10 min. Then the supernatant (0.15 ml) was mixed

with 0.9 ml of 400 µM DPPH solution, 0.9 ml of 0.2 M MES buffer solution (pH 6.0), 0.9 ml of 20% ethanol and 0.75 ml of 80% ethanol. Then the mixture was allowed to stand at room temperature for 30 min in dark condition. At that time, a blank was prepared by adding 0.15 ml of 80% ethanol. After completion of the reaction, the absorbance at 520 nm was measured with a spectrophotometer. Trolox (10 to 100 µg/ml) was used to create a calibration curve, and the blank measurement value was subtracted from the measurement value of the analysis sample, and the value calculated on the calibration curve was taken as the DPPH radical scavenging ability.

Glutathione content determination: The glutathione content was measured according to the method of Wu et al. (2006) (Fig. 8). Frozen sample (0.5 g) was extracted using 5 ml of 5% (w/v) trichloroacetic acid and centrifuged at13,000 rpm, 4°C, 15 min and the supernatant was used as an analytical sample. 1 ml of the analytical sample and 2.7 ml of 0.6 mM 5,5'-dithiobis (2-nitrobenzoic acid)-100 mM phosphate buffer (pH 7.7) were mixed and allowed to stand at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 412 nm with a spectrophotometer. To prepare a calibration curve, reduced glutathione (1 to100 µg/ml) was used and glutathione content was calculated from the obtained calibration curve.

Polyphenol content determination: The method of MacDonald et al. (2001) using Folin-Denis reagent was adopted for the determination of the polyphenol contents in the prepared extracts, and absorbance was measured at 700 nm (Fig. 7). Frozen sample (0.1 g) was extracted by using 4 ml of 80% methanol and centrifuged at 13,000 rpm, 4°C, 10 min. Then the supernatant (0.2 ml) was mixed with 2.4 ml of distilled water, 0.2 ml of a Follin Denis reagent [distilled water 70 ml, sodium tungstate dihydrate 10 g, phosphomolybdic acid [12 molar (IV) phosphoric acid n-hydrate 2 g], 5 ml phosphoric acid and 0.4 ml saturated sodium carbonate. Then the mixture was allowed to stand in dark condition at 30ºC for 30 minutes. At

that time, a blank was prepared by adding 0.2 ml of distilled water instead of Folin-Denis reagent. After completion of the reaction, the absorbance of the reaction mixture was measured at a wavelength of 700 nm using a spectrophotometer. Quercetin (1 to100 µg/ml) was used for the calibration curve preparation, and the blank value was subtracted from the measurement value of the sample solution, and the value calculated from the calibration curve was taken as the polyphenol content.

Ascorbic acid content: For ascorbic acid, samples were extracted using 5% metaphosphoric acid at a ratio of 0.15 g/5 ml and analyzed as described by Mukherjee and Choudhuri (1983). Then the supernatant (0.5 ml) was mixed with 0.5 ml of 0.03% DCIP (sodium 2,6-dichloroindophenol solution), 0.5 ml of a 2% thiourea-5% metaphosphoric acid solution and 0.25 ml of 2% DNP (2,4-dinitrophenylhydrazine) solution. Then the mixture was kept in water bath at 50ºC for 70 min. After completion of the reaction, 2.0 ml of 85% sulfuric acid was slowly added while cooling in ice and the mixture was allowed to stand in dark condition at room temperature for 30 minutes. At the same time, the test tube which was used as blank (2% DNP solution) was also kept in dark condition but without adding the sulfuric acid solution. Then the absorbance of the reaction mixture was measured at a wavelength of 520 nm using a spectrophotometer. For the calibration curve creation, L-ascorbic acid (1 to100 µg/ml) was used, and the value obtained by subtracting the blank value from the measurement value of the sample solution was applied to the calibration curve to calculate the ascorbic acid content.

Statistical analysis: Mean values were tested separated by a *t*-test for colonization level at $P \leq 0.05$. Dry weight, antioxidants analysis and others were analyzed by Tukey's test at $P \leq 0.05$. All analyses were performed using XLSTAT 2012 pro statistical analysis software (Addinsoft, New York).

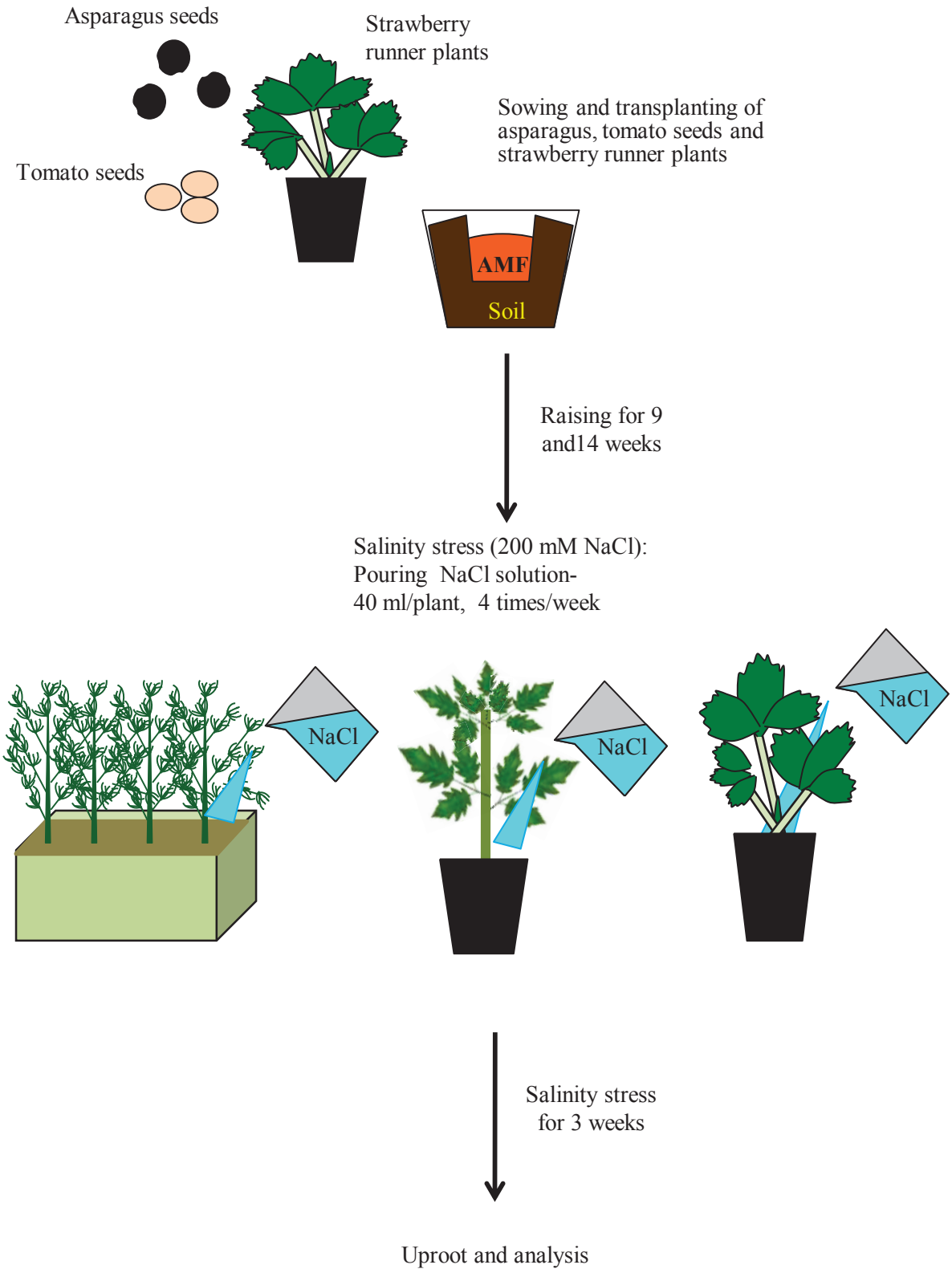


Fig. 1. AMF application and NaCl treatment in asparagus, tomato and strawberry plants. AMF, arbuscular mycorrhizal fungi; NaCl, NaCl 200 mM.

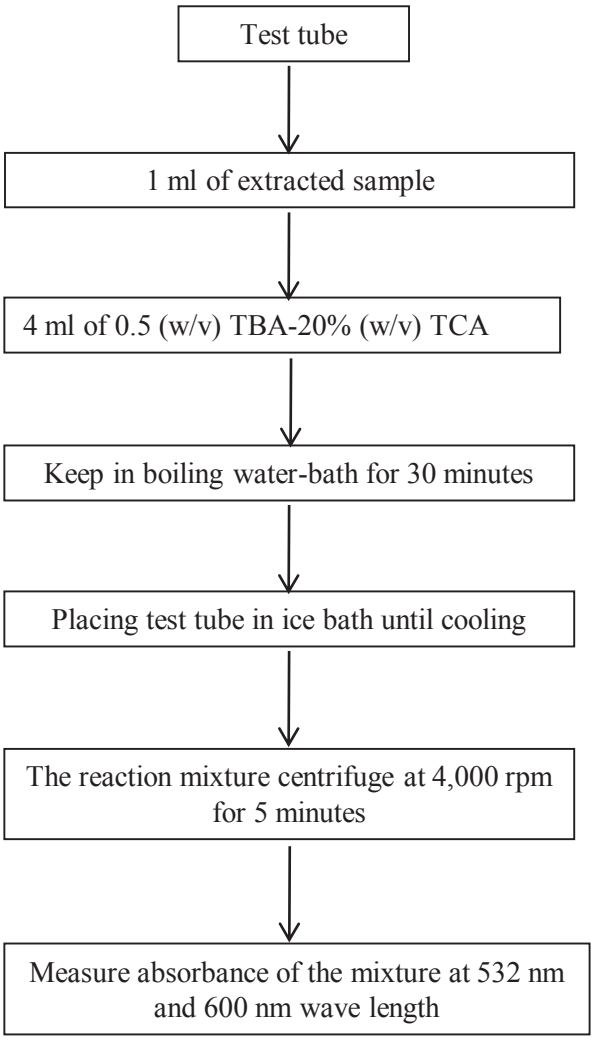


Fig. 2. Flow diagram of the procedures in MDA content assay.

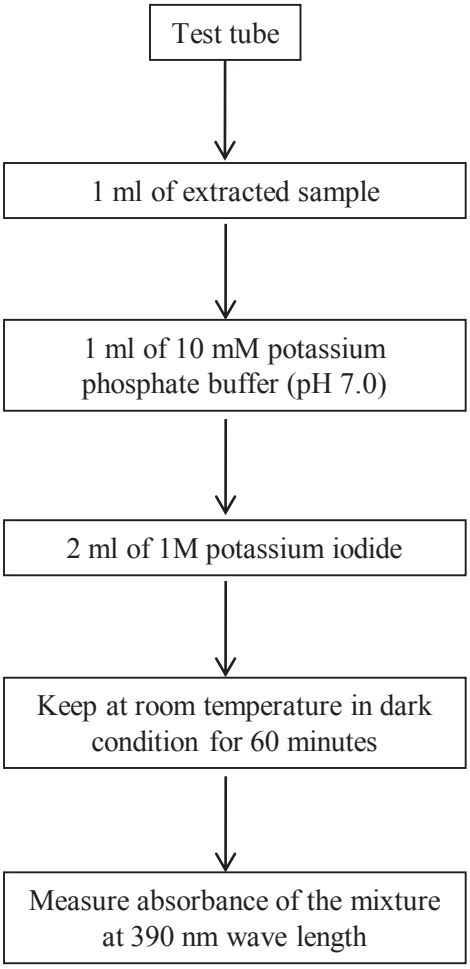


Fig. 3. Flow diagram of the procedures in hydrogen peroxide content assay.

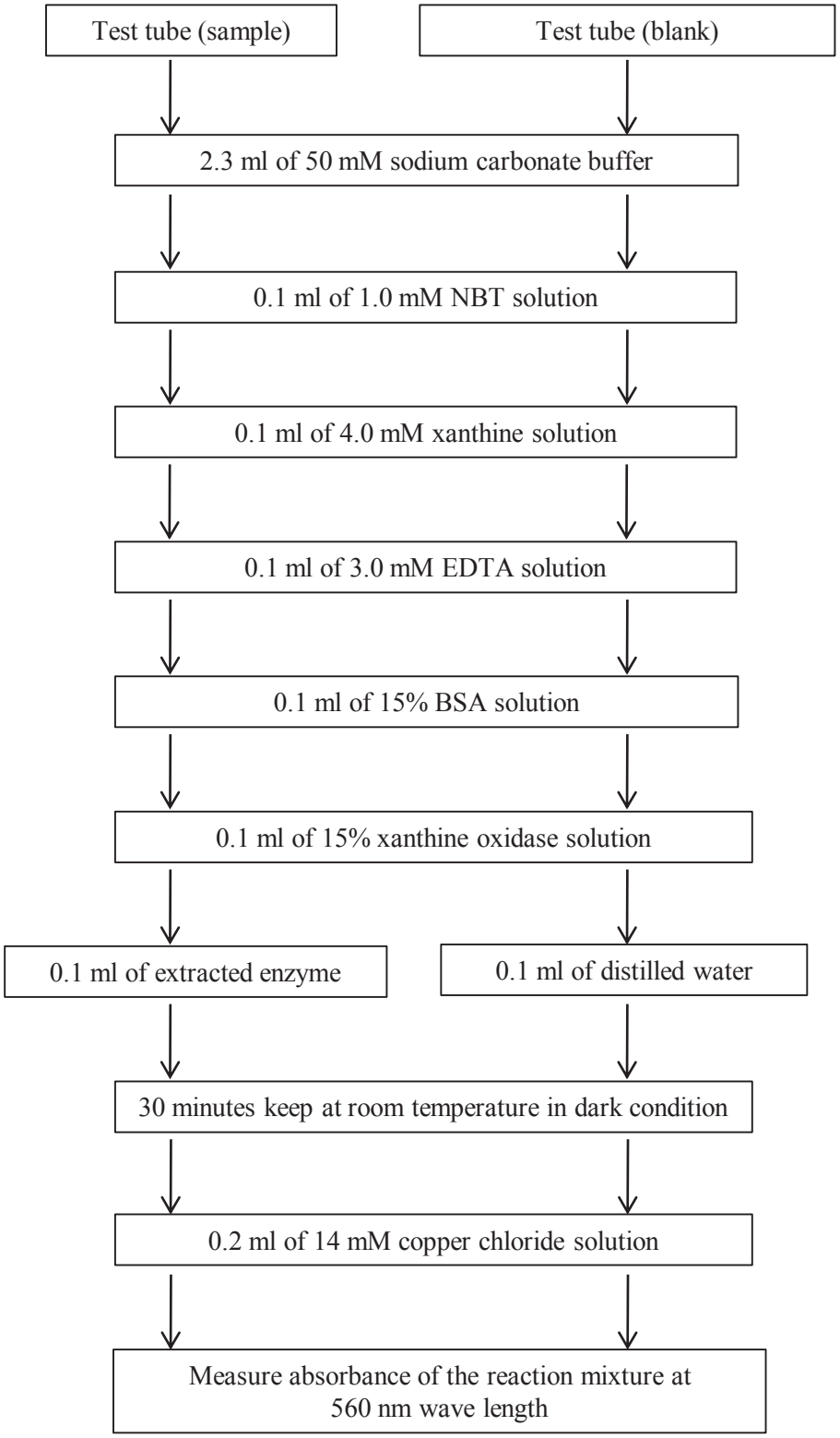


Fig. 4. Flow diagram of the procedures in SOD analysis.

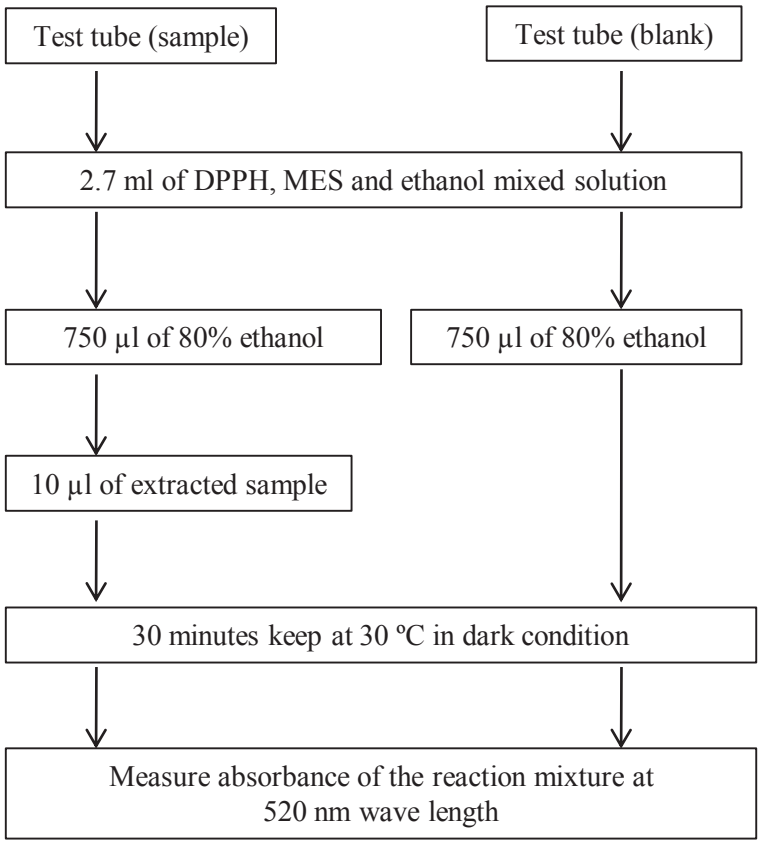


Fig. 5. Flow diagram of the procedures in DPPH radical scavenging activity analysis.

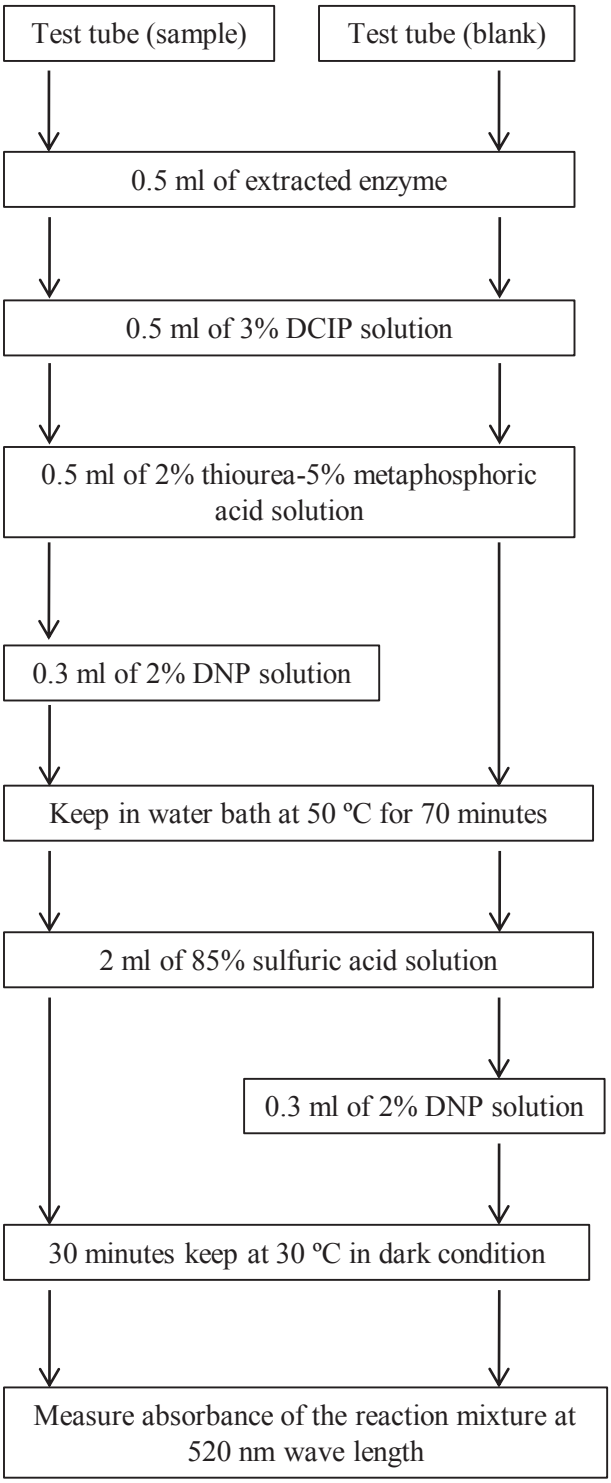


Fig. 6. Flow diagram of the procedures in ascorbic acid assay.

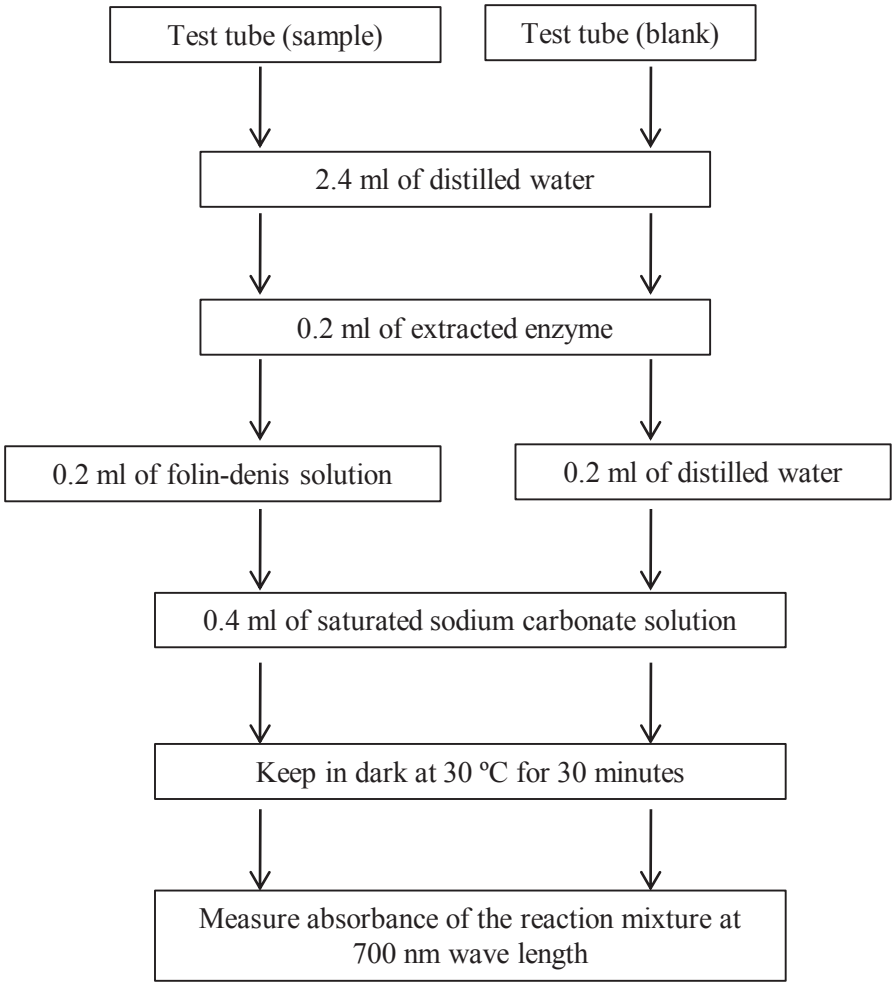


Fig. 7. Flow diagram of the procedures in polyphenol content assay.

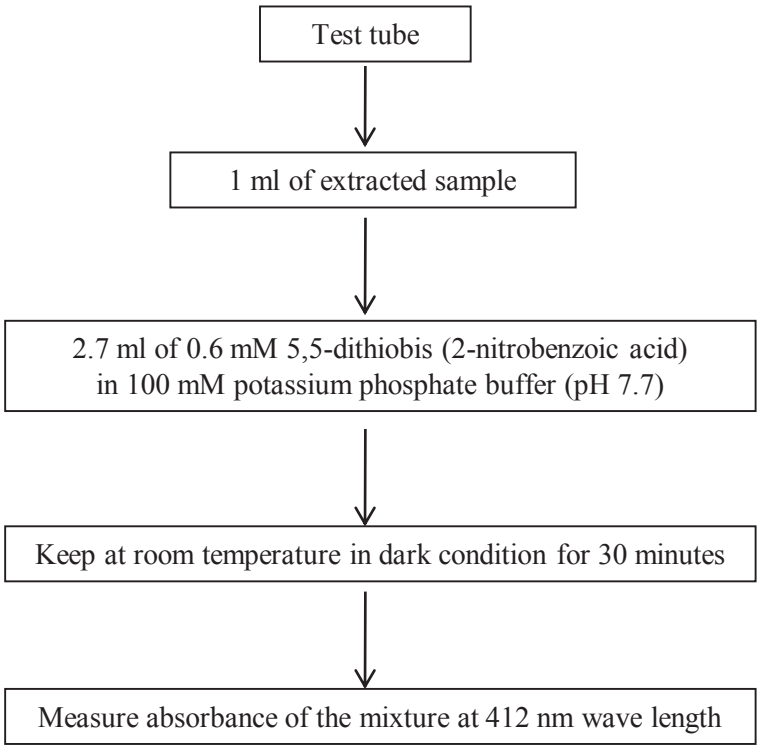


Fig. 8. Flow diagram of the procedures in glutathione content assay.

Results

Plant growth response: After three weeks of salinity stress, dry weights of shoots and roots significantly decreased in non-mycorrhizal plants compared to control on three vegetables (Fig. 9). In mycorrhizal plants, there were no decreases observed compare to control except asparagus shoots. However, significant difference observed between mycorrhizal and non-mycorrhizal plants under salinity condition (Fig. 9 and 10). Here, mycorrhizal plants had higher dry weight than non-mycorrhizal plants in all three vegetables.

AMF colonization: The microscope assessment confirmed AMF colonization successfully occurred in all inoculated plants and non-inoculated plants had no colonization (Fig. 11 and 12). Among the three plants, asparagus showed the highest colonization about 82% and lowest was found in tomato 43% (Fig. 11). After salinity stress, the colonization level decreased in all three plants. However, the reduction was not statistically significant for asparagus and strawberry but significantly decreased for tomato.

Chlorophyll content: In case of chlorophyll content, under salinity stress, non-mycorrhizal plants drastically reduced chlorophyll in all three vegetables (Fig. 13). On the other hand, mycorrhizal plants maintained chlorophyll content under salinity except asparagus.

Na^+ content: Salinity stress increased Na^+ content in leaves and roots of non-mycorrhizal and mycorrhizal plants in all three vegetables (Fig. 14). However, mycorrhizal asparagus, tomato and strawberry plants accumulated less Na^+ compared to non-mycorrhizal plants under salt stress.

PO_4^{3-}, Ca^{2+}, Mg^{2+} contents: In asparagus and tomato plants, mycorrhizal application increased PO_4^{3-}, contents than non-mycorrhizal plants under both salinity and control conditions (Fig. 15). In strawberry plants, there was no significant difference observed between AMF inoculated and non-inoculated plants under with and without salinity stress. The Ca^{2+} content was decreased in AMF inoculated asparagus shoots under salt stress

however in tomato and strawberry leaves it were not significantly differ between mycorrhizal and non-mycorrhizal plants (Fig. 16). In case of Mg^{2+} content, there is no significant difference present between AMF inoculated and non-inoculated plants under salinity stress in all three vegetables (Fig. 17).

MDA and H_2O_2 contents: MDA content was increased in leaves and roots of all three vegetables under salinity stress (Fig. 18). The mycorrhizal plants had lower MDA content than non-mycorrhizal plants under salt stress in three vegetables. Subsequently, H_2O_2 content increased in leaves and roots of asparagus, tomato and strawberry plants under salinity stress (Fig. 19). However, AMF inoculated plants had lower H_2O_2 content than non-inoculated plants under salinity except tomato leaves, where it was same.

Antioxidant activities: Both salinity and AMF inoculation altered the antioxidants in asparagus, tomato and strawberry plants and the responses were varied among leaves, roots and species. The enzymatic antioxidant superoxide dismutase (SOD) activity was higher in mycorrhizal asparagus, tomato and strawberry plants compared to non-mycorrhizal plants under salinity stress, except in tomato and strawberry roots (Fig. 20). As for the DPPH radical scavenging activity, all three plants had higher DPPH activity in both shoots/leaves and roots of asparagus, tomato and strawberry plants inoculated with AMF under salinity stress (Fig. 21). The non-enzymatic antioxidant ascorbic acid contents were greater in mycorrhizal compared to non-mycorrhizal tomato and strawberry plants under salt stress (Fig. 22). In asparagus shoots and roots there were no changed in ascorbic acid contents between AMF inoculation and salinity treatment plants. Polyphenol contents were higher in roots of all three AMF inoculated plants under salinity stress compare to non-inoculated plants (Fig. 23). In shoots and leaves of asparagus and tomato mycorrhizal plants had higher polyphenol content than non-mycorrhizal plants. In strawberry leaves, it was same between mycorrhizal and non-mycorrhizal plants. Another non-enzymatic antioxidant, glutathione contents were increased

in all three plants leaves and roots (except strawberry leaves) when inoculated with AMF compared to non-inoculated plants under salinity stress (Fig. 24).

In experiment 2, under seawater salinity (EC 3.0 and 6.0 mS/cm), the dry weight of shoots of non-mycorrhizal strawberry plants were no changed but, roots dry weight were significantly decreased (Fig. 25 and 26). On the other hand, there was no decrease observed in AMF (GM, Gf) inoculated plants. In AMF colonization level, there was no significant difference found between the no salt and salt treatments however, colonization was different between the AMF species (Fig. 27). In case of Na^+ content in shoots and roots, there were no changed of Na^+ between AMF inoculated and non inoculated plants under without salinity stress condition (Fig. 28). After salinity stress it was slightly increase under EC 3.0 and in EC 6.0 mS/cm it increased drastically in non-inoculated plants. Conversely, AMF inoculated plants showed lower amount of Na^+ compared to non-inoculated plants under salinity stress conditions.

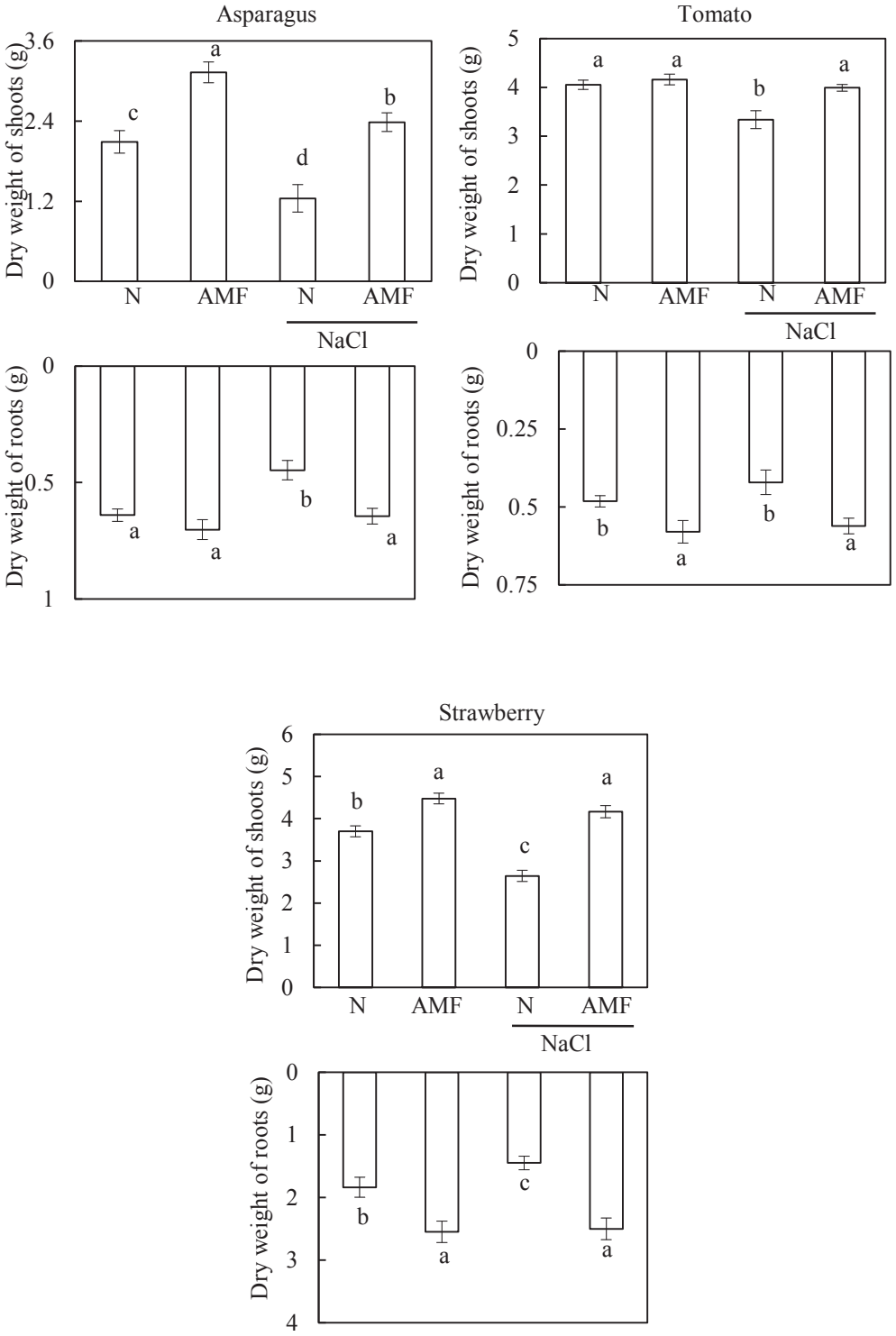
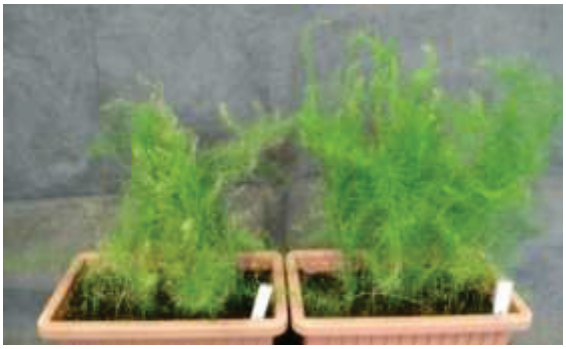


Fig. 9. Dry weight of shoots and roots in asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

Asparagus

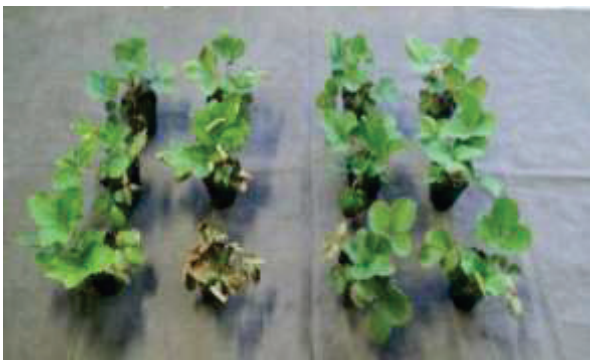


N + NaCl AMF + NaCl

Tomato

N + NaCl AMF + NaCl

Strawberry



N + NaCl AMF + NaCl

Fig. 10. Effect of salinity stress on growth of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM.

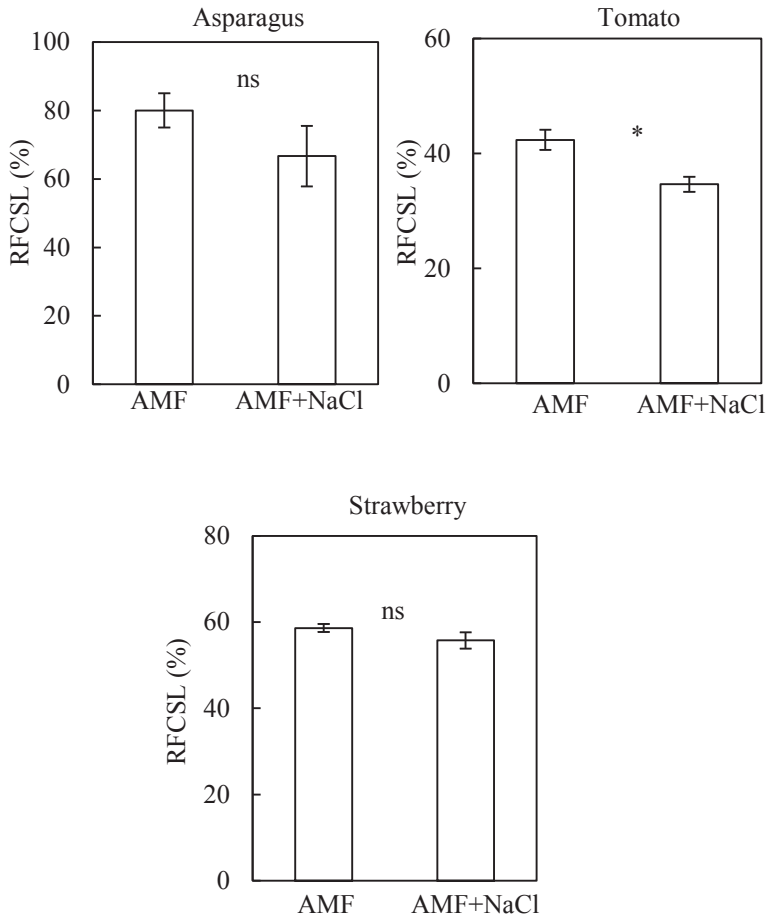


Fig. 11. AMF colonization level (RFCSL) in asparagus, tomato and strawberry plants. AMF, mycorrhizal plants, NaCl 200 mM. Bars represent standard errors (n=10). *, significant difference present; ns, no significant difference (*t*-test, $P \leq 0.05$).

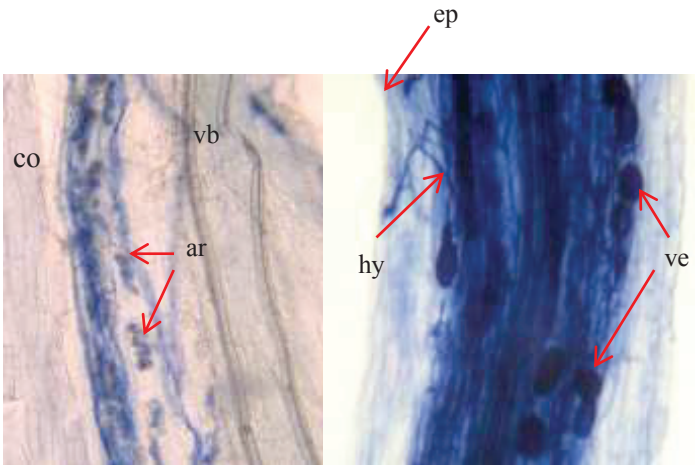


Fig. 12. Colonized strawberry roots showing arbuscules and vesicles (*Glomus fasciculatum*). ar, arbuscules; co, cortex; ep, epidermis; hy, hypha; vb, vascular bundle; ve, vesicles.

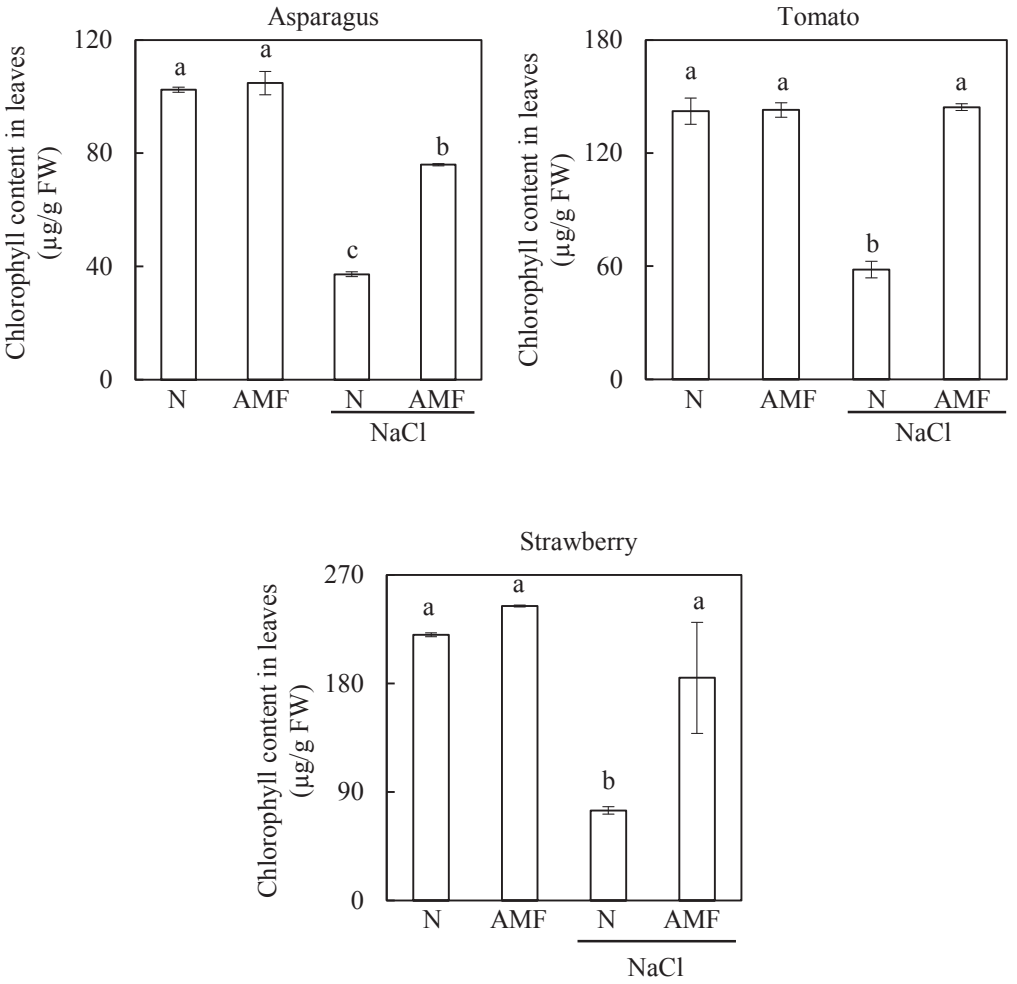


Fig. 13. Chlorophyll content in asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

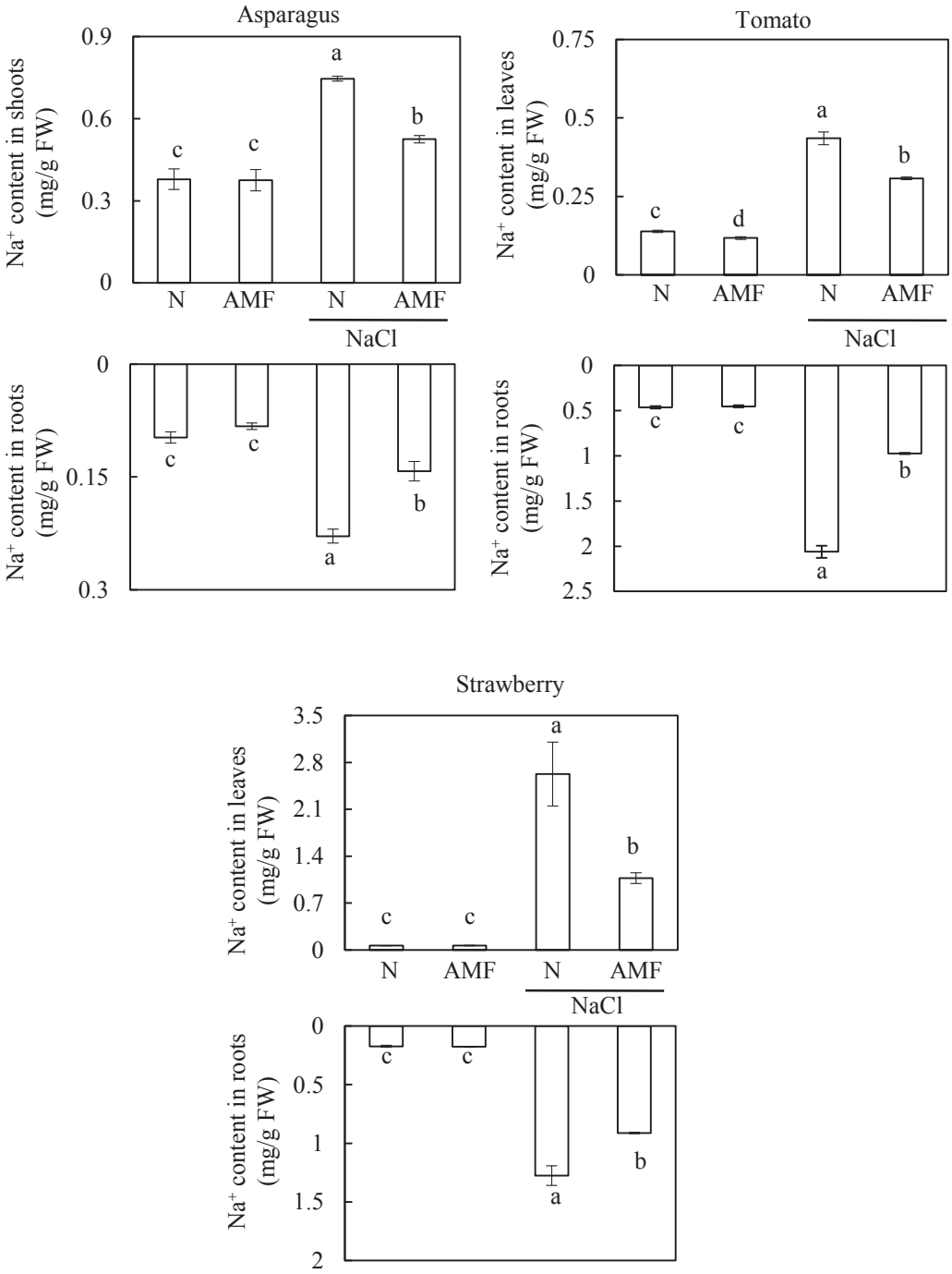


Fig. 14. Na^+ content in shoots/leaves and roots of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

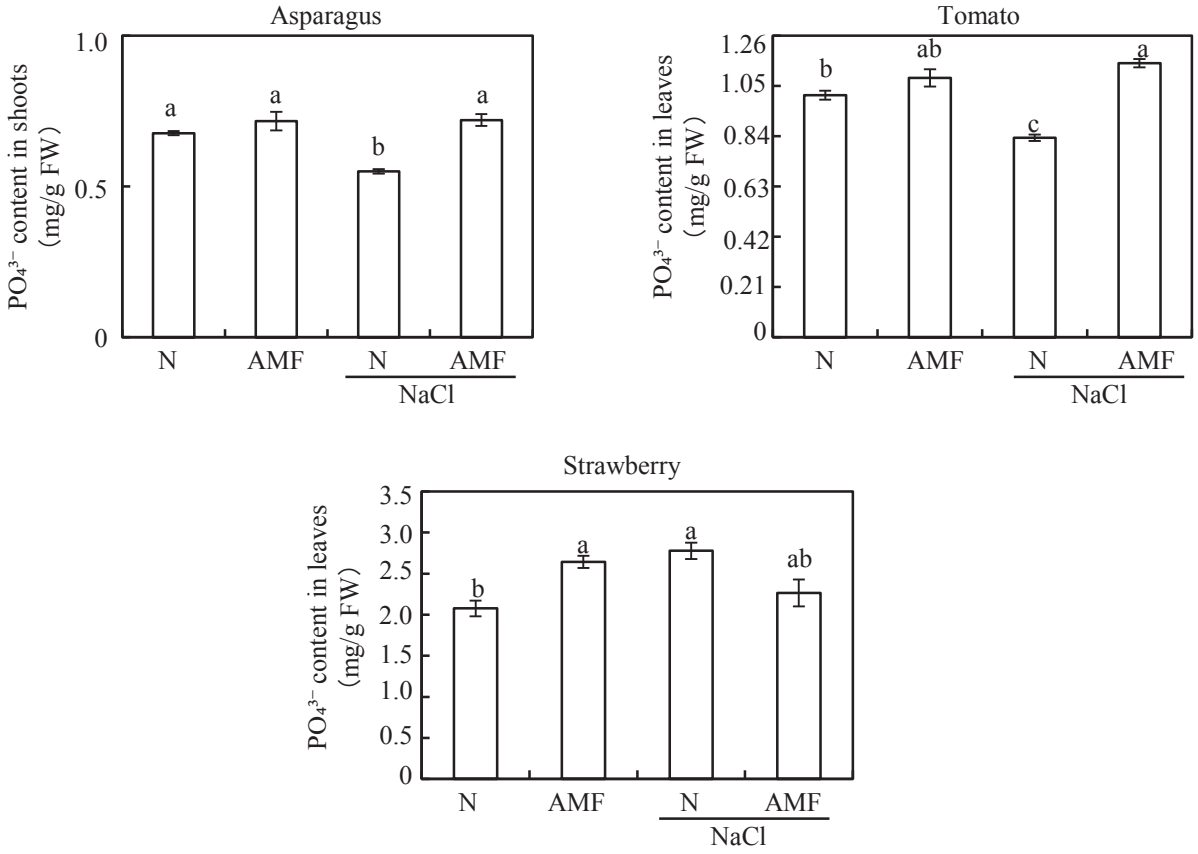


Fig. 15. PO_4^{3-} content in shoots and leaves of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

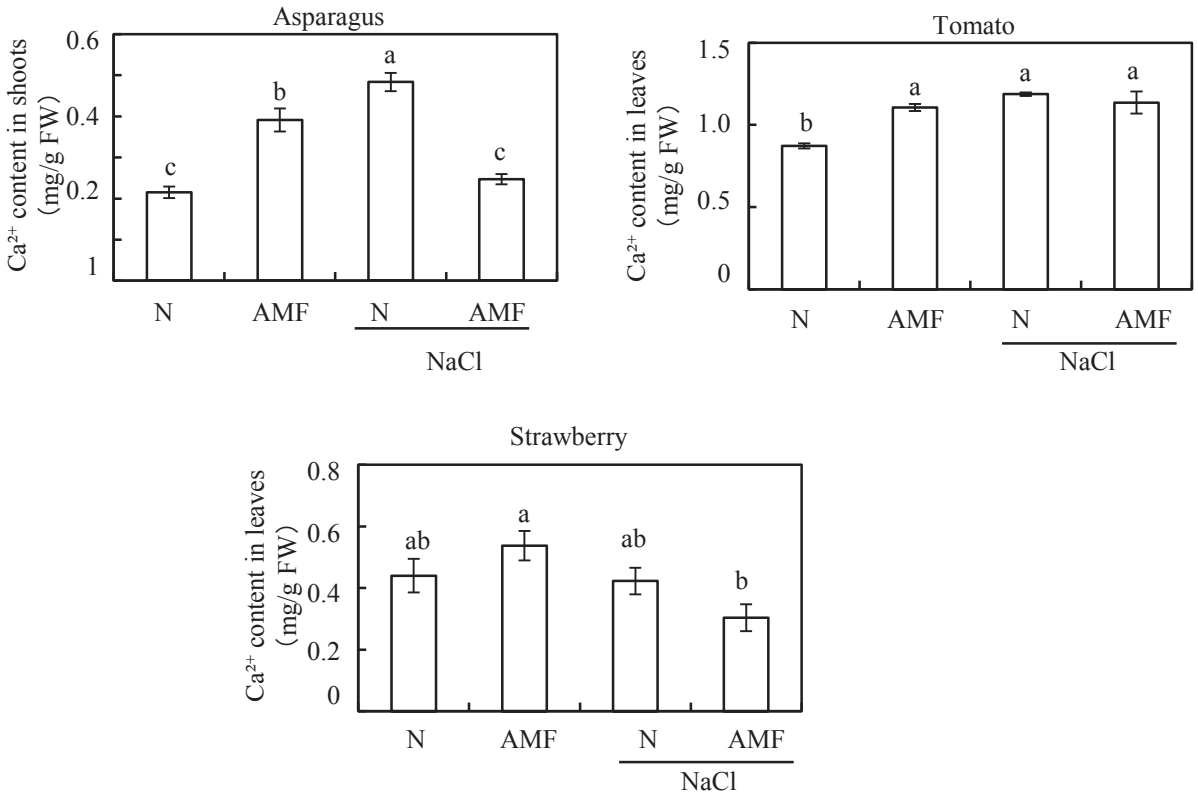


Fig. 16. Ca^{2+} content in shoots and leaves of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

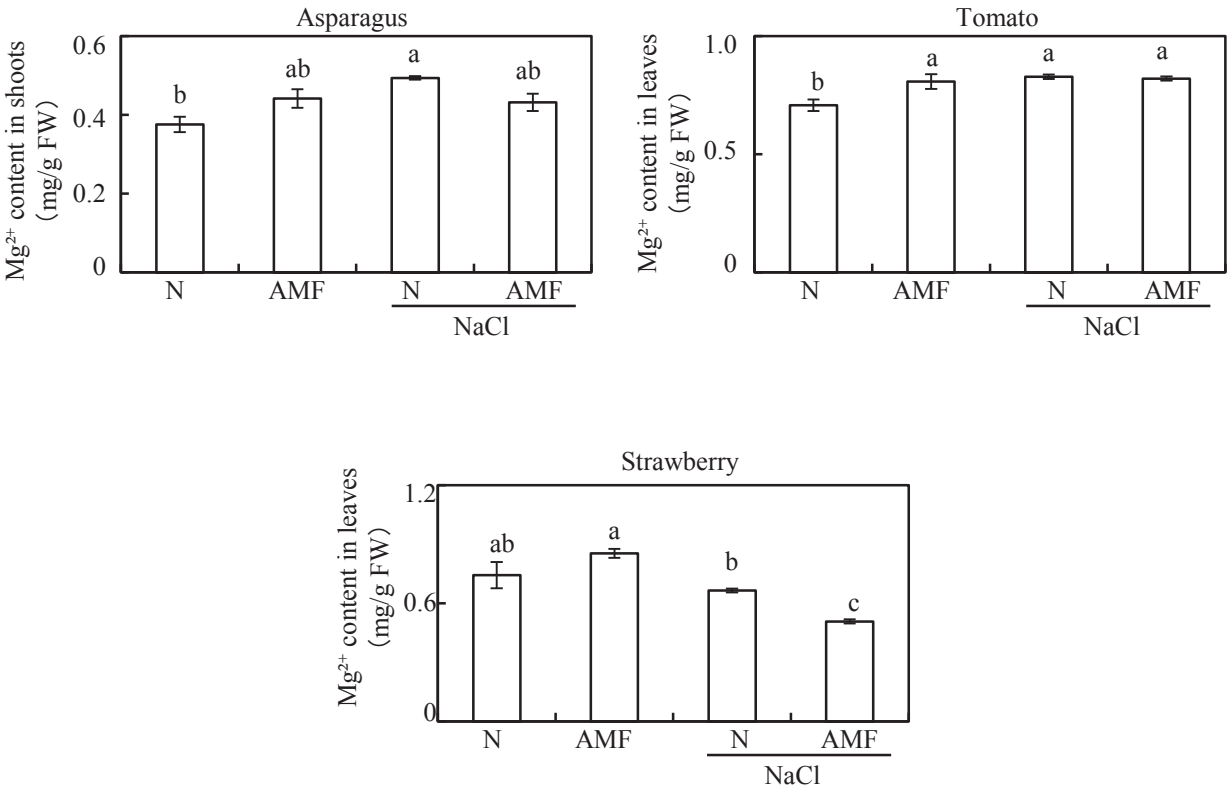


Fig. 17. Mg^{2+} content in shoots and leaves of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

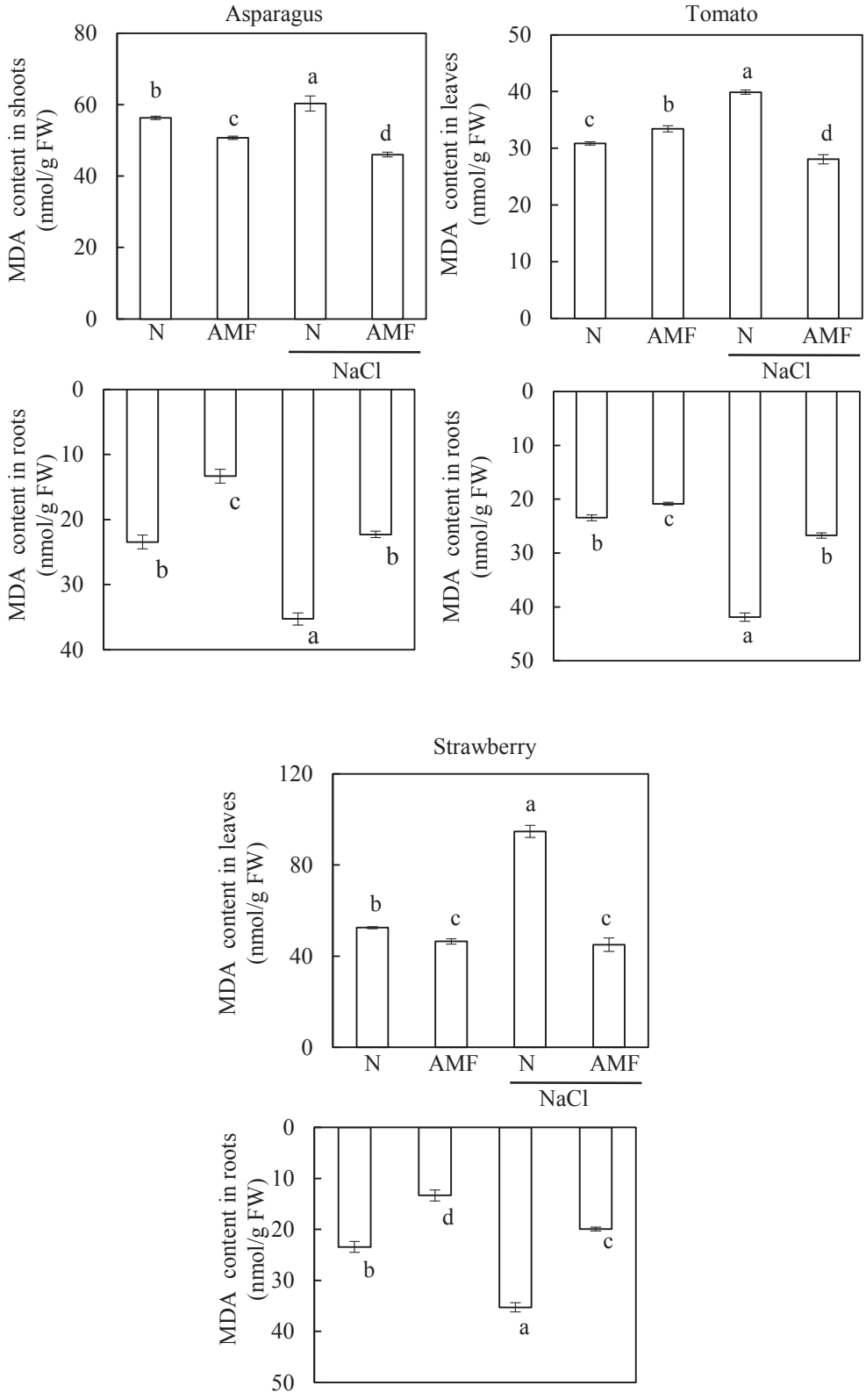


Fig. 18. Malondialdehyde (MDA) content in shoots/leaves and roots of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

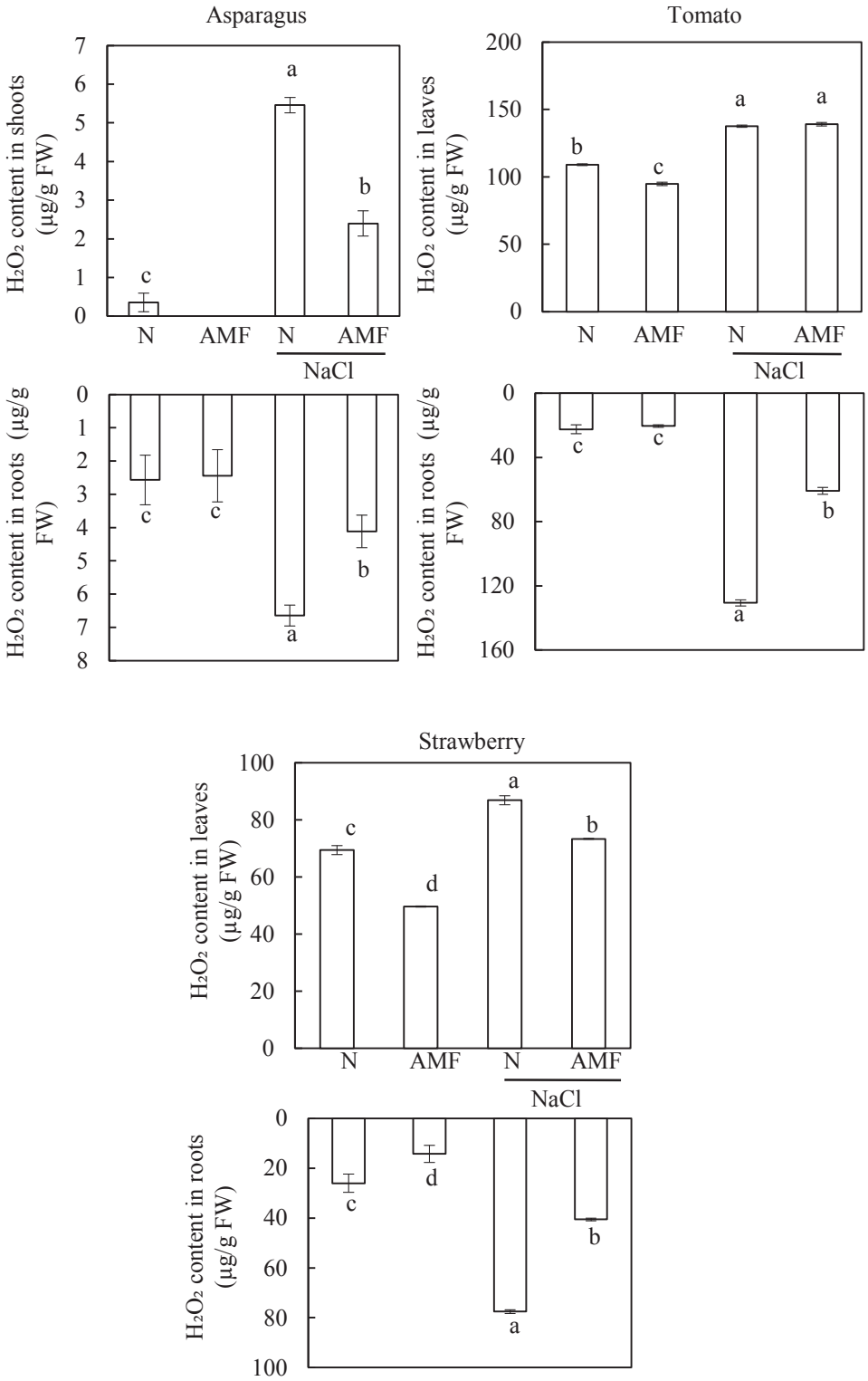


Fig. 19. H_2O_2 content in shoots/leaves and roots of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

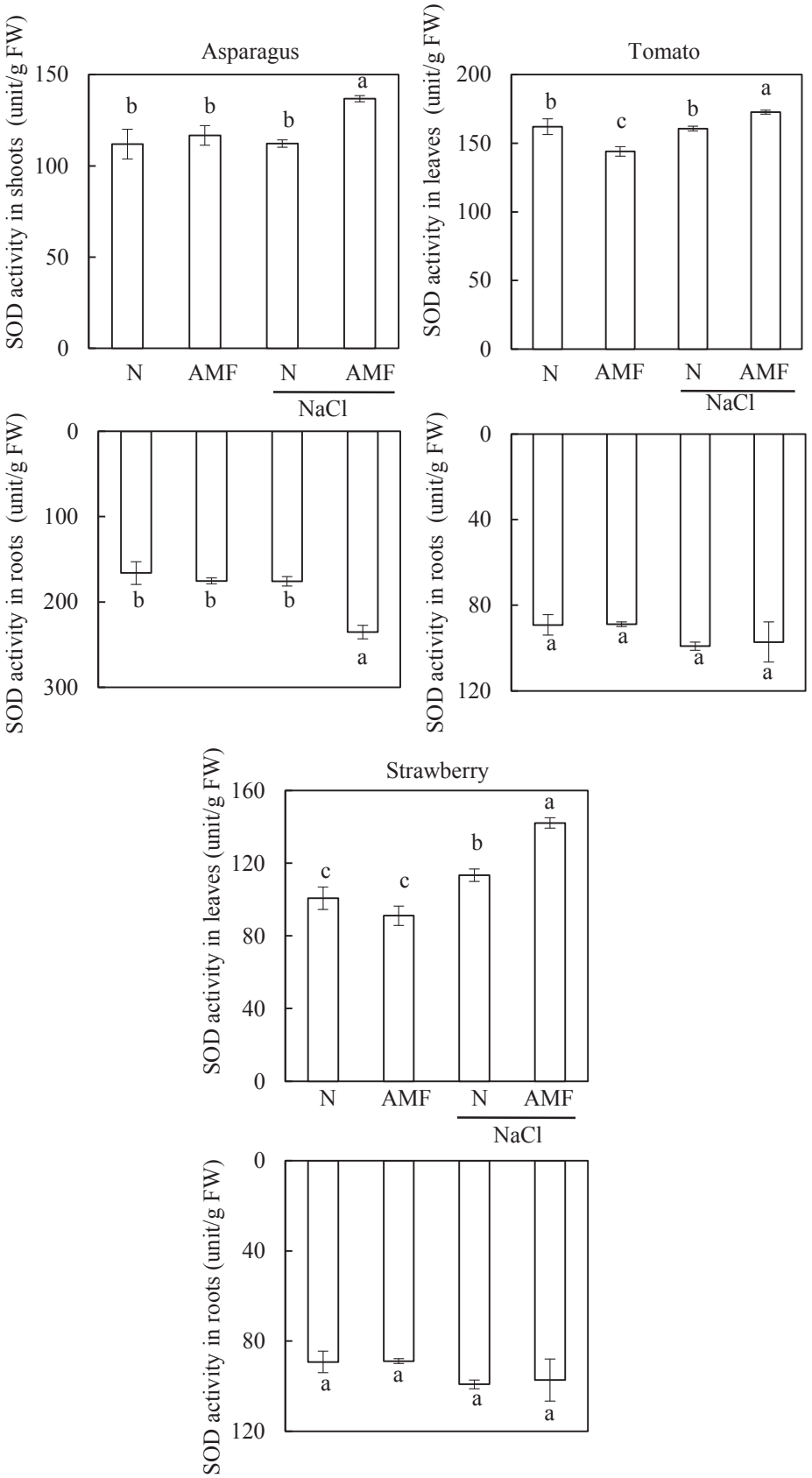


Fig. 20. Superoxide dismutase (SOD) activity in shoots/leaves and roots of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

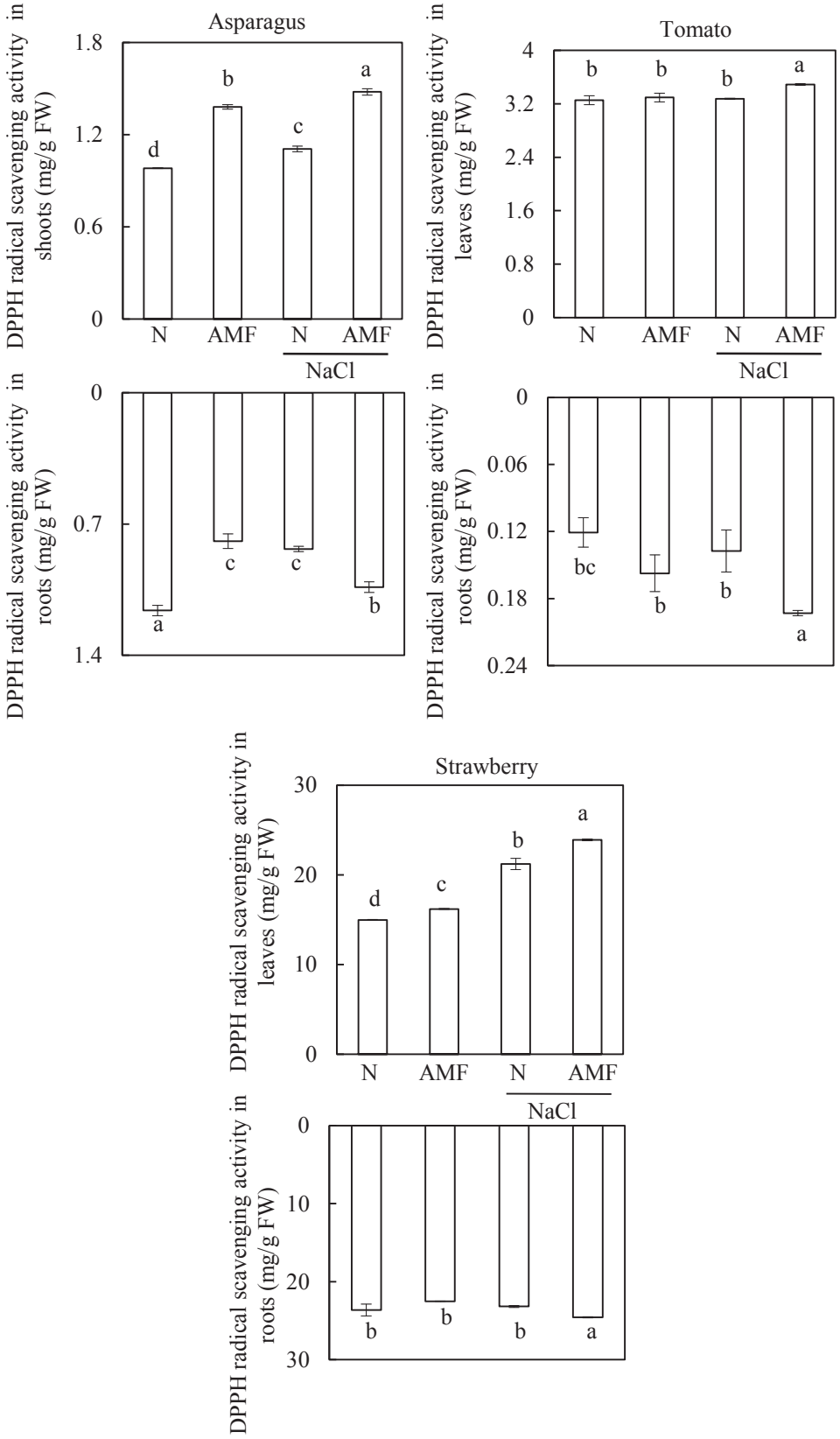


Fig. 21. DPPH radical scavenging activity in shoots/leaves and roots of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

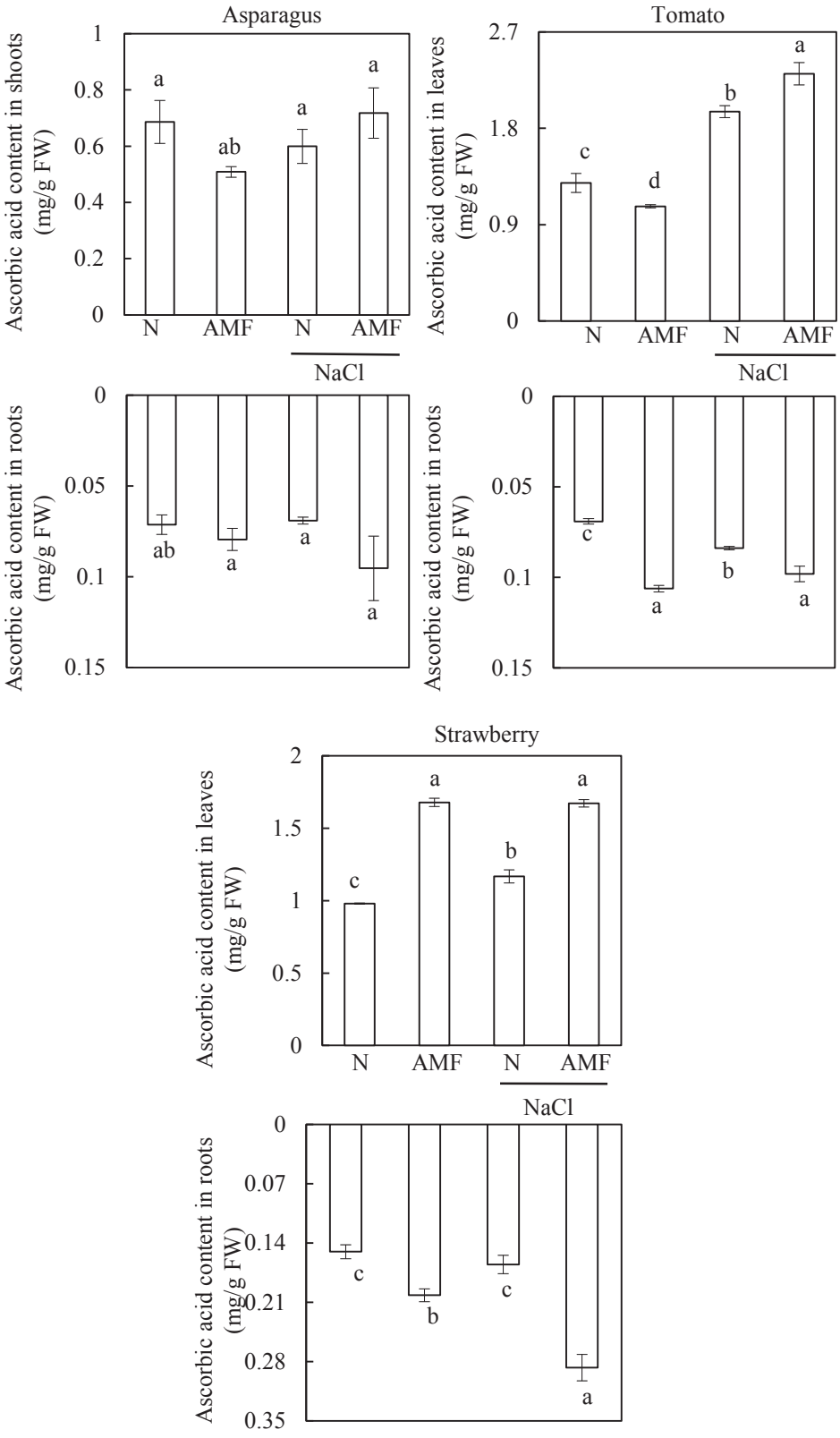


Fig. 22. Ascorbic acid content in shoots/leaves and roots of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

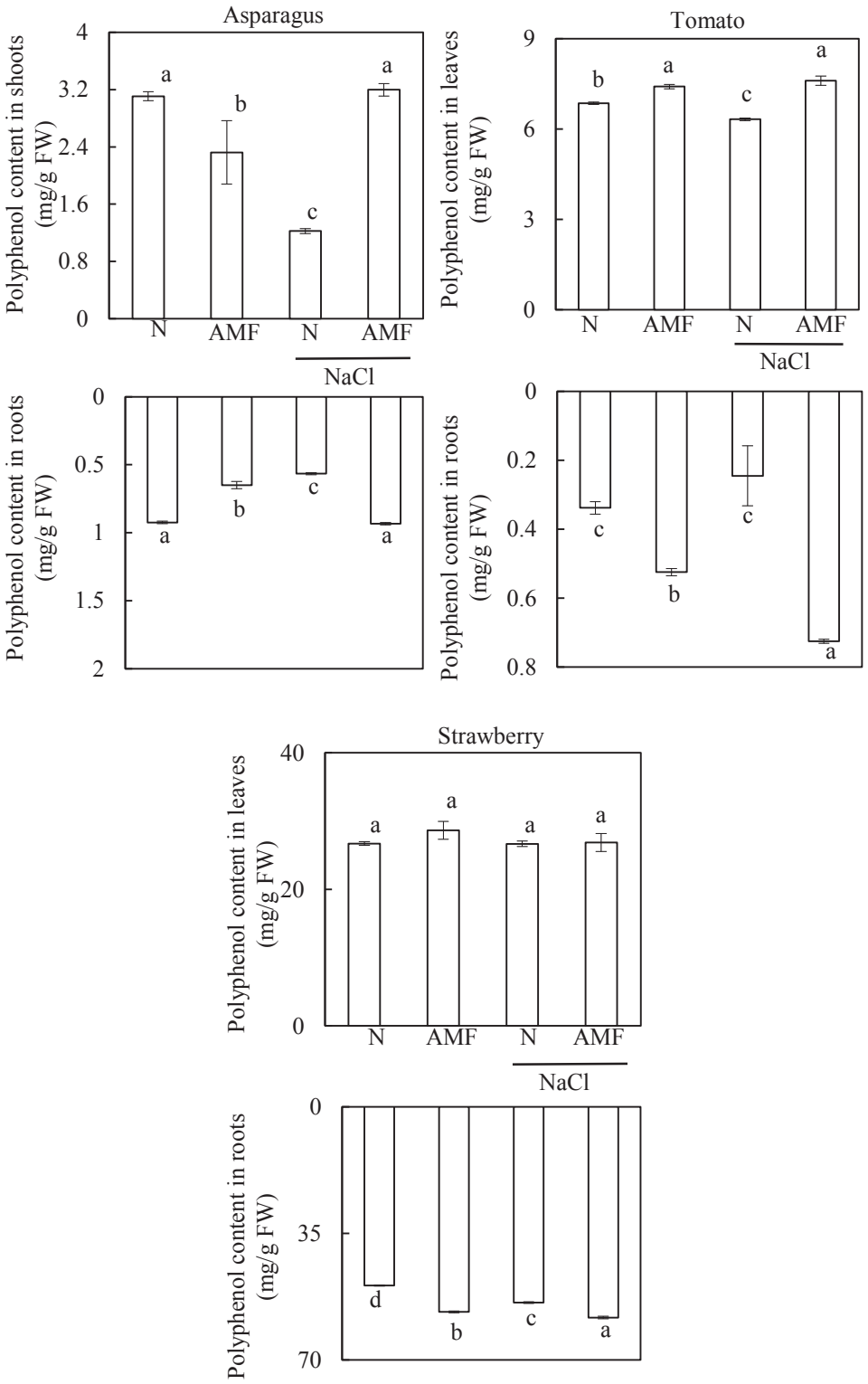


Fig. 23. Polyphenol content in shoots/leaves and roots of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

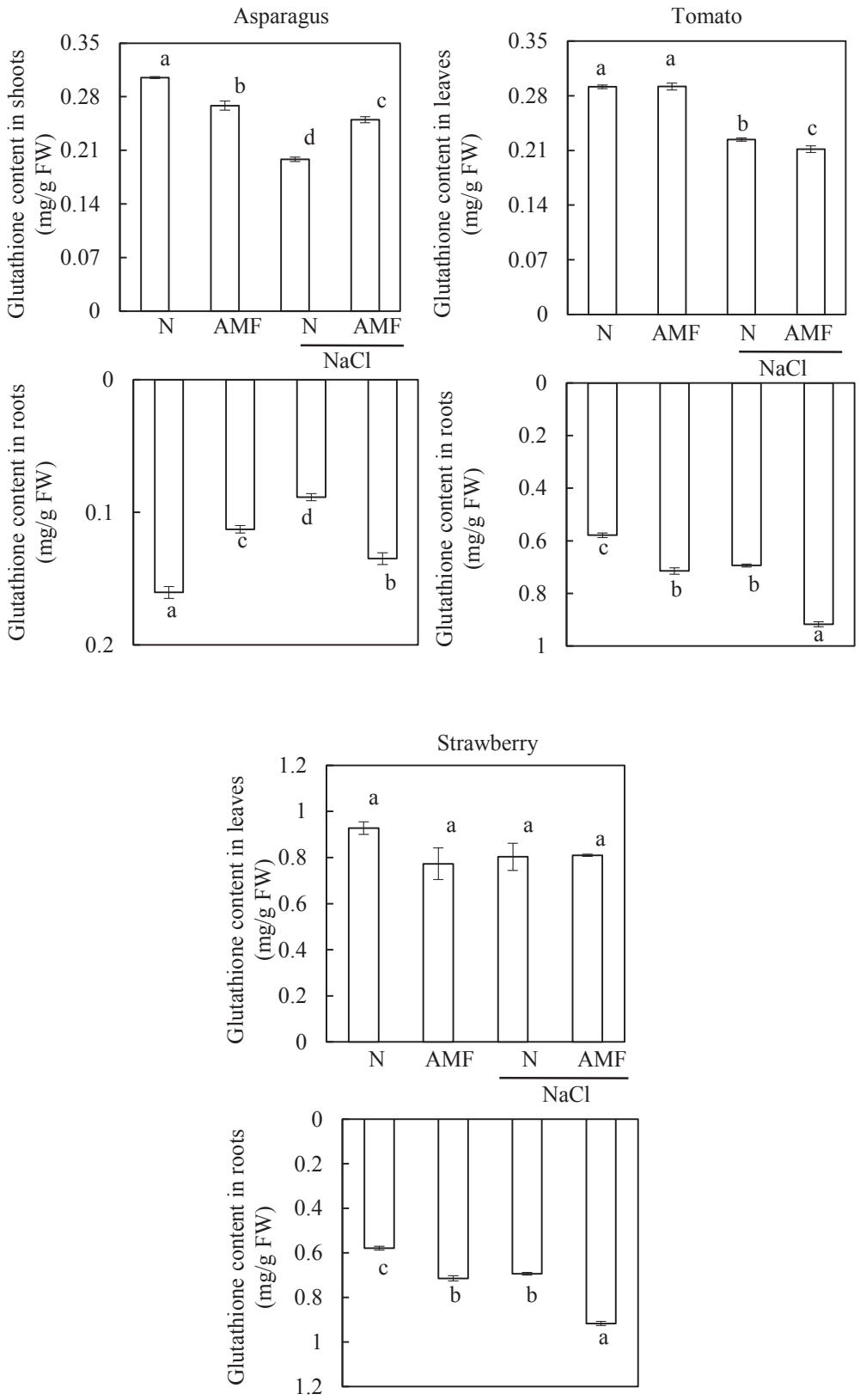


Fig. 24. Glutathione content in shoots/leaves and roots of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

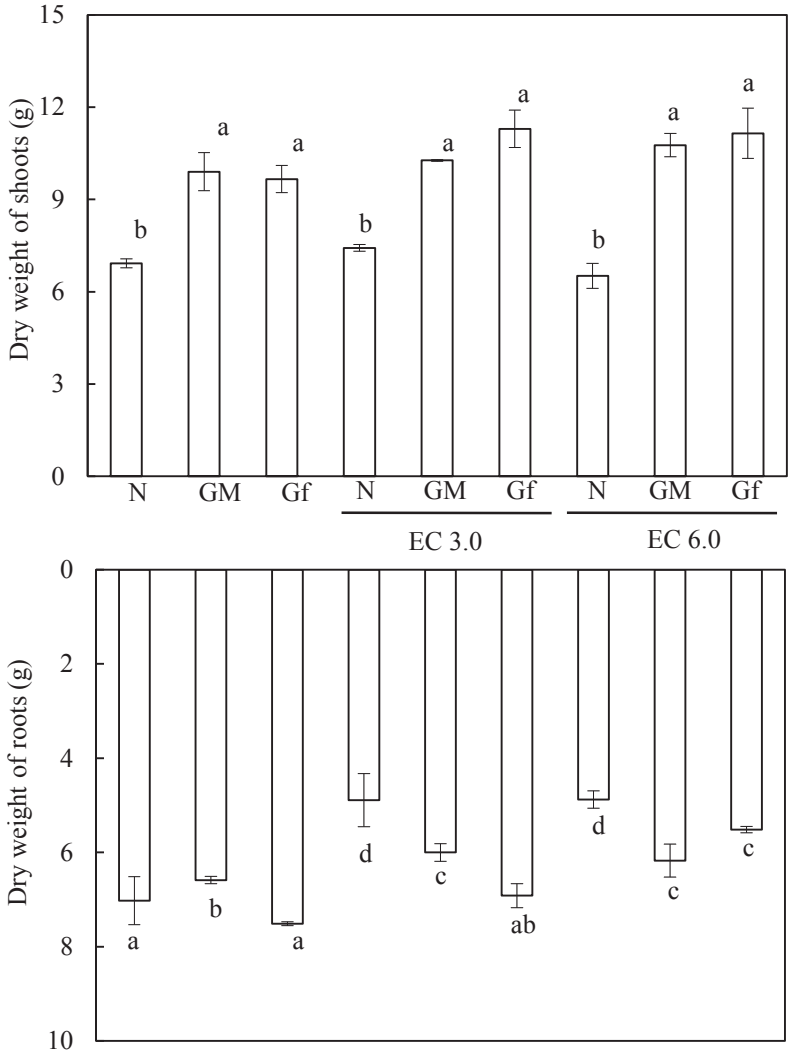


Fig. 25. Dry weight of shoots and roots in strawberry plants. N, non-mycorrhizal plants; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; EC 3.0, 6.0, electrical conductivity of seawater 3.0 and 6.0 mS/cm. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

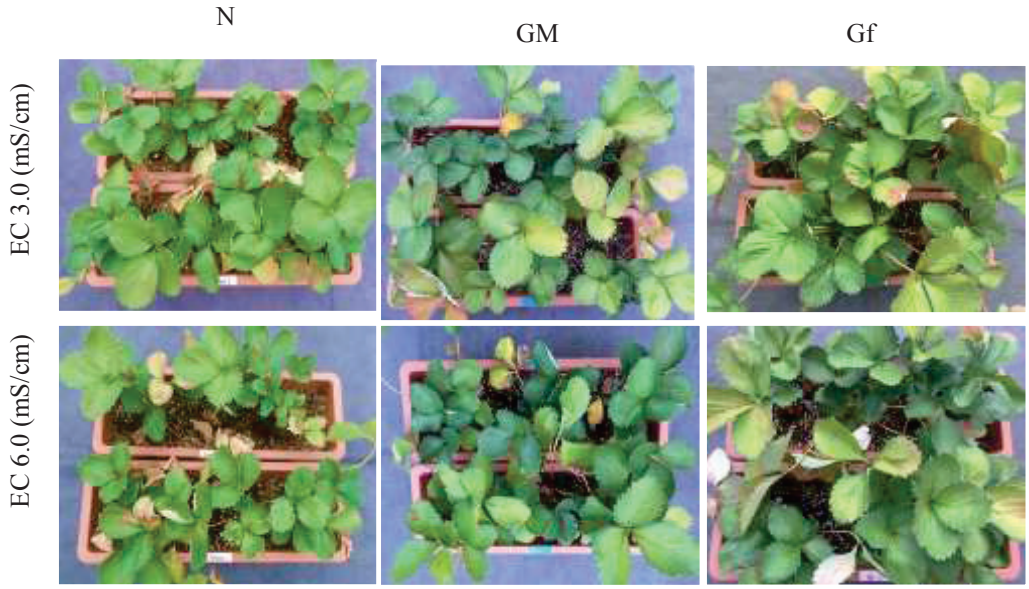


Fig. 26. Growth of strawberry plants under salinity stress. N, non-mycorrhizal plants; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*. EC 3.0, 6.0 mS/cm, electrical conductivity of seawater.

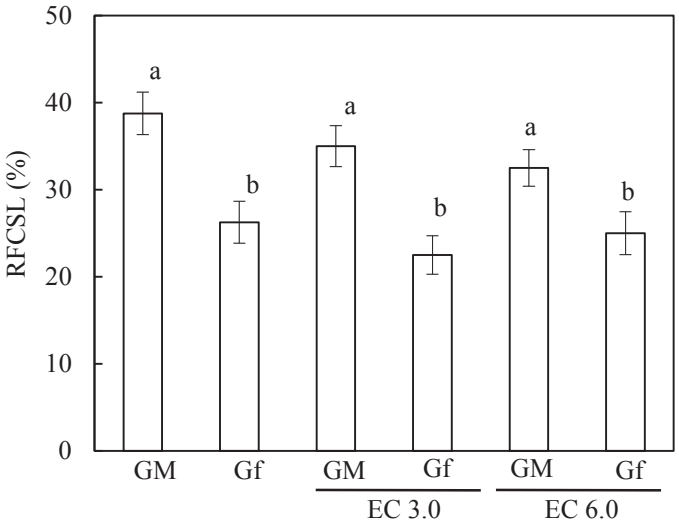


Fig. 27. AMF colonization level (RFCSL) in strawberry plants. GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*. EC 3.0, 6.0, electrical conductivity of seawater 3.0 and 6.0 mS/cm. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

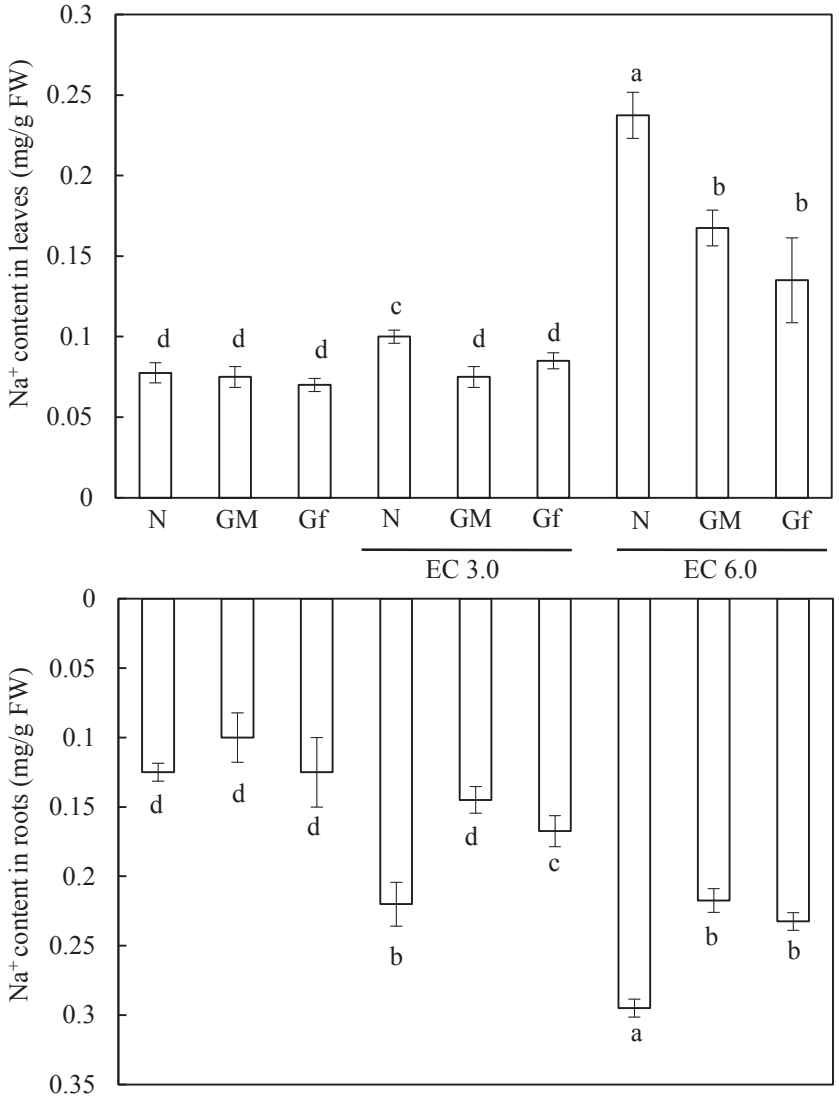


Fig. 28. Na^+ content in leaves and roots of strawberry plants. N, non-mycorrhizal plants; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* EC 3.0, 6.0, electrical conductivity of seawater 3.0 and 6.0 mS/cm. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

Discussion

In this experiment, to obtain a method for improving salinity tolerance in vegetables and elucidating the mechanism of salt stress alleviation, we studied the effect of AMF symbiosis on asparagus, tomato and strawberry plants. We also investigate the AMF effect on seawater salinity in strawberry plants. The dry weight, chlorophyll, Na^+ contents and antioxidants analysis checked in AMF inoculated and non-inoculated plants.

In the present study, AMF colonization occurred in all the inoculated plants. However, salinity stress did not influence the colonization level (*Glomus fasciculatum*) in asparagus and strawberry but reduced in tomato plants (*Gigaspora margarita*). Presence of excess salt had a suppression effect on mycorrhizal hyphal growth or hyphal spreading after initial infection and spore germination (Talaat and Shawky, 2014). The decrease of colonization level in tomato might be due to such suppression effect of salt on fungus. Fan et al. (2011) also reported that AMF root colonization decreased significantly under salt stress in strawberry plants. In addition, colonization was also not changed in seawater salinity where both species were used in strawberry. From the above facts we can concluded that, *Glomus fasciculatum* species might be more salt tolerant compared to *Gigaspora margarita*. And, the colonization is also depending with the host plant interaction. Moreover, the possible reason for not reduction of colonization might be selection of newly developed lateral roots for the assessment. Further study will be needed on this aspect including the difference between AMF and host species.

As a result of the plants growth, dry weights in the shoots and roots of the non-inoculated asparagus, tomato and strawberry plants were decreased by salinity stress. On the other hand, in the inoculated plants, particularly no decrease observed in dry weight by salinity stress in both NaCl and seawater salinity. In our experiment, we select three plants based on their salinity tolerance (Brouwer et al., 1985) however, all three pants showed mostly same growth

reduction pattern under 200 mM salinity stress. Although, the growth condition of three plants were different. In chlorophyll content we also found the same reduction in all three plants. So, salt tolerance and sensitivity also depends on plant growth condition not only the species. We also confirmed, AMF application improved growth in all the growth stage of tested plants. Several researchers have reported that AMF inoculated tomato, pepper and strawberry plants had higher shoots and roots dry weight than non-inoculated plants under salt stress (Al Karaki, 2000; Latef and Chaoxing, 2014; Sinclair et al., 2014). Our results of higher dry weights were also concomitant with their findings. In horticultural aspects, growth improvement of plants is very much important for better yield. Therefore, AMF inoculation alleviates salinity stress and confirmed better plants growth under salinity condition.

AMF applications have a positive influence on the composition of mineral nutrients (especially poor mobility nutrients such as phosphorus) of plants grown in salt-stress conditions (Al-Karaki, 2000). In the present study, mycorrhizal asparagus and tomato plants had higher phosphate (PO_4^{3-}) content than non-mycorrhizal plants under salinity stress. The higher uptake of phosphate is facilitating by the extensive hyphae of the fungus which allows them to explore more soil volume than the non-mycorrhizal plants (Ruiz-Lozano and Azcon, 2000). On the other hand, the Ca^{2+} content was reduced and no changed in mycorrhizal plants under salinity stress. Similar result was also found by Giri et al. (2003) in *Acacia auriculiformis* plants. This suggests that AMF may not be so important to the nutrients moving to plant roots by mass flow as compared with nutrients moving by diffusion. Moreover, mycorrhizal inoculation depressed the Ca:P ratio by increased production of oxalate in the mycorrhizosphere, which is able to scavenge Ca^{2+} from the soil solution (Azcon and Barea, 1992). The low Ca^{2+} content in this study might be regulated by these factors.

As a result of investigating chlorophyll content as an indicator of degree of plants yellowing, reduction of chlorophyll content by salinity was suppressed in mycorrhizal plants.

As well as, lower accumulation of toxic Na^+ in this study on both NaCl and seawater salinity stress indicates alleviation of salt stress. Previously, Zuccarini and Okurowska (2008) and Balliu et al. (2015) suggested that mycorrhizal sweet basil and tomato plants have lower concentrations of Na^+ than non-mycorrhizal plants under salinity conditions. In addition, a positive relationship between photosynthetic attributes and growth has already been reported for various plants grown under salt conditions e.g., strawberry (Husaini and Abdin, 2008) and pepper (Beltrano et al., 2013). Formerly, higher chlorophyll content in mycorrhizal peanut and tomato plants under saline conditions were also confirmed by Al-Khaliel (2010) and Latef and Chaoxing (2011). Therefore, better growth of mycorrhizal asparagus, tomato and strawberry plants under salinity stress might have relation with greater chlorophyll and lower Na^+ contents. In addition, in this study, we choose three plants based on their salinity stress mitigation ability. And, we found the salt sensitivity of all three plants was same under 200 mM salinity though, the growth stages of three plants were different. Moreover, under field conditions, salinity level is not an adjustable variable. So, the procedure used in this study of pre-inoculation with AM fungi can be of practical importance in the cultivation of many horticultural crops grown under saline conditions, especially high-value crops such as fruit and vegetables.

The presence of excess salt has been found to reduce plant growth and biomass by inducing oxidative stress (Hajiboland et al., 2010; Porcel et al., 2012). Lower oxidative stress in mycorrhizal plants is due to their improved growth and physiological amelioration of salt stress through ionic and osmotic homeostasis, and a better enzymatic antioxidative capacity (Evelin et al., 2012, 2013). In addition, salt stress causes peroxidation of membrane lipids, there by disrupting membrane integrity (Juan et al., 2005). Increased lipid peroxidation with increasing concentration of NaCl was also reported by Rasool et al. (2013a) in different genotypes of chickpea. In this study, under salt conditions, lower MDA contents in leaves and

roots of all three mycorrhizal plants helped in decreasing the oxidative damage in lipids. On the other hand, high concentration of H_2O_2 leads to oxidative stress and increases lipid peroxidation and electrolyte leakage (Imlay, 2003). In present study, mycorrhizal asparagus, tomato and strawberry plants had lower H_2O_2 and MDA levels than the non-mycorrhizal plants under salinity stress, suggesting mycorrhizal symbiosis alleviated the oxidative stress.

In our present work, SOD activity increased in the upper parts of the most of the vegetables whereas, in roots it was no changed. SOD is considered to be the most important key enzyme in antioxidative abilities in plants (Fridovich, 1986). The higher SOD activity in mycorrhizal leaves in this study consistent with previous observations on leaves of tomato plants colonized by *Glomus intraradices* subjected to NaCl salinity (Hajiboland et al., 2010). So, SOD activity might be related to detoxify oxidative stress in the place where the salinity stress is not in a severe manner. Although, SOD activity was measured only once after salt stress, so that it is difficult to estimate whether SOD activity in mycorrhizal root was related to further ROS detoxification compared to non-mycorrhizal plants. Further investigation will be needed to clarify this aspect.

The DPPH radical scavenging activity is commonly used a rapid, simple and inexpensive method to measure the free radical scavenging capacity of total antioxidants in biological compounds, and involves the use of the free radical DPPH (Marxen et al., 2007). In this study, the DPPH radical scavenging activity was greater in all three mycorrhizal plants than non-mycorrhizal plants in both leaves and roots when treated with salt. In addition, mycorrhizal plants increased several non-enzymatic antioxidants, such as ascorbic acid, glutathione, and polyphenol contents in leaves and roots under saline conditions. The increased DPPH radical scavenging activity induced salt tolerance in maize plants (Hichem et al., 2009). Ascorbic acid and glutathione are involved in the neutralization of the secondary products of ROS reactions. The higher ascorbic acid and glutathione contents enable plants to directly scavenge the $^1O_2$

and H_2O_2, as well as other ROS-like hydroxyl radicals (Noctor and Foyer, 1998). In addition, ascorbic acid is considered to be an effective antioxidant in plants which can detoxify free radicals as well as oxidants (Li et al., 2012). On the other hand, polyphenols are also excellent oxygen radical scavengers, because the electron reduction potential of the phenolic radical is lower than that of oxygen radicals (Grace, 2005). Subsequently, less H_2O_2 in leaves and roots and a higher amount of ascorbic acid, glutathione and polyphenol contents in mycorrhizal plants greatly accelerated detoxification of ROS under salt stress.

In this study, non-enzymatic antioxidants (ascorbic acid, glutathione and polyphenol) mostly increased in mycorrhizal roots rather than in shoots, and the root is the organ directly exposed to salinity stress (Hajibagheri et al., 1987; Hasegawa et al., 2000). Moreover, plant tolerance to salinity largely depends on the salinity tolerance of the roots (Hajibagheri et al., 1987; Jeschke and Wolf, 1988). An improved root system mediated by AMF that alleviates salt stress in strawberry plants were also mentioned by Fan et al. (2011) and Sinclair et al. (2014). From this work, it is supposed that oxidative stress alleviation under salinity stress in mycorrhizal plants might be closely related to the response of non-enzymatic antioxidants, where the salt stress is greater.

In all of the asparagus, tomato and strawberries tested in this experiment, growth improvement, suppression of Na^+ adsorption and increase of SOD activity with other antioxidant substance was confirmed under salinity stress with AMF application. The antioxidant activities were changed in plant species and plant parts under salt stress and also with AMF association. Therefore, AMF association can maintain growth and also effectively manage oxidative stress in plants under salinity stress. This findings suggested that AMF as an effective biocontrol tool for the salinity stress management in vegetable plants. However, Na^+ reduction method by AMF inoculation was not clearly understood. Further study will be needed on this aspect including the difference between AMF and host species.

CHAPTER 1-2

Salinity tolerance and SEM-EDX analysis of Na localization with histological factors in mycorrhizal plants

Introduction

Salinity stress occurs when soluble salts (usually NaCl) are elevated in soil and water (Bruning and Rozema, 2013). The stress is globally present in about 20% of cultivated land and 50% of irrigated systems (Latef and Chaoxing, 2014). Salinity stress involves changes in various physiological and metabolic processes in plants, depending on severity and duration of the stress (Munns, 2005; James et al., 2011). Increased soil salt concentrations decrease the ability of a plant to take up water and, once Na^+ and Cl^- are taken up in large amounts by roots, both excess Na^+ and Cl^- negatively affect growth by impairing metabolic processes and decreasing photosynthetic efficiency (Deinlein et al., 2014). Excess Na^+ disrupt the structure of enzymes and other macromolecules, damage to the cell organelles and plasma membrane, disruption also occurs in respiration and protein synthesis and nutrient imbalance by ion deficiency (Evelin et al., 2009; Hasegawa, 2013).

Several studies have found that diverse types of soil microorganisms improve plant growth, especially when plants are under stressful conditions. Most important are the arbuscular mycorrhizal fungi (AMF) which form mycorrhizal colonization with approximately 80% of plant species and are present in almost all terrestrial ecosystems (Smith and Read, 2008). The association between the AMF and plants makes the host plant more tolerant to abiotic stresses like salt, drought and heat etc. (Dodd and Ruiz-Lozano, 2012). AMF also found under extreme salt conditions, and they can be adapted to these conditions (Wilde et al., 2009). Previously, AMF symbiosis has been demonstrated to increase salt tolerance in a variety of host plants such as tomato, maize etc. (Al-Karaki, 2000; Sheng et al., 2011).

In case of salt sensitivity of a plant, excess Na^+ uptake and distribution within the plants are considered the major determinants. Prevention of excess Na^+ entry into the roots, transport to, and allocation within the leaves, and sequestration into the vacuoles are strategies by which plants cope with salt stress (Ruiz-Lozano et al., 2012). Moreover, Xue et al. (2014)

reported, salt tolerance of wild soybean compared to cultivated soybean is depend on the accumulation of excess Na^+ in roots and suppression of migration of Na^+ to leaves. In mycorrhizal plants, AMF may act as the first barrier for ion selection during nutrient uptake from the saline soil or during transfer to the plant host, suggesting that they induce a buffering effect on the Na^+ uptake (Hammer et al., 2011). Mycorrhizal colonization has also been shown to enhance K^+ absorption under salt conditions while preventing excess Na^+ translocation to shoot tissues, resulting in a higher K^+/Na^+ ratio in mycorrhizal plants (Giri et al., 2007; Estrada et al., 2013). On the other hand, there is a little understanding of the molecular mechanism of excess Na^+ suppression in mycorrhizal plants (Ruiz-Lozano et al., 2012). However, He et al. (2013) reported that the mechanism that reduced the Na^+ damage to tomato induced by AMF has little relation to vacuolar Na^+/K^+ antiporter gene (LeNHX1). So, changes in the Na^+ permeability in the cell wall outside the cell membrane may be considered as a factor of the control of the Na^+ inclusion other than the ion transporter. In this regards, Krishnamurthy et al. (2011) stated that apoplast barrier was deposited in rice under weak salt stress, reduced the apoplast Na^+ absorption and increased survival under salt stress. Therefore, it can hypothesize that under salt stress mycorrhizal plants might have some mechanism to reduce excess Na^+ uptake or localized it inside plant organs and tissues.

In this study, excess Na localization was analyzed in different plant parts of asparagus, tomato and strawberry plants. For check Na localization in plant tissues through scanning electron microscope and energy dispersive x-ray spectroscopy (SEM-EDX) with nano-suit technique strawberry plants were investigated. In addition, the histological components were also analysed to elicit the mechanism of Na reduction in mycorrhizal plants.

Materials and Methods

Plant materials and AMF inoculation: In this experiment asparagus (*Asparagus officinalis* L., cv. Welcome), tomato (*Solanum lycopersicum* L., cv. Momotaro 8) and strawberry

(*Fragaria* × *ananassa* Duch., cv. Tochiotome) plants were used. Seeds of asparagus and tomato plants wee sowed and two months old strawberry runner plants were planted in plastic pots (13.5×27.0×15.5 cm for asparagus, 10.5 cm in diameter with depth 9.0 cm for tomato, 10.5 in diameter for strawberry) containing autoclaved (121^{o}C, 1.2 kg/cm^{2}, 30 min) commercial potting media SM-2 (Ibigawa Industry Co. Ltd., Japan) (Fig. 1). At the time of asparagus and tomato seed sowing and strawberry transplanting, half of the pots were inoculated with AMF inocula 5 g/plant (*Glomus fasciculatum* for asparagus and strawberry and *Gigaspora margarita* for tomato) at a depth of 3 cm and mixed with potting media. The non-mycorrhizal plants received the same amount of sterilized inocula. The mycorrhizal inocula of unknown spore density were obtained (exclusively for research purposes) from Centralgrass Co. Ltd., Tokyo, Japan (*Gigaspora margarita*) and Idemitsu Agri. Co. Ltd. Tokyo, Japan (*Golmus fasciculatum*). Plants were fertilized with slow release granular fertilizer (Long total 70 type, JCAM AGRI. Ltd., Japan) at the rates of N:P:K = 13:11:13, 1g/plant, after seedling emergence for asparagus and tomato and in strawberry immediately after transplanting. Plants were grown in a greenhouse at 25 ± 3/19 ± 3^{o}C day/night temperature with a 12-13 h photoperiod (750-1000 µmol/m^{2}/s) and 60%-70% relative humidity.

Treatment with NaCl: The plants were subjected to salinity stress with 200 mM NaCl solution after nine (strawberry) and fourteen (asparagus and tomato) weeks of AMF inoculation. Plants were irrigated with NaCl solution (40 ml/plant) for twelve days (4 times/week). The no salt-treated/control plants received an equal amount of distilled water. The electrical conductivity (EC) of saturated soil extract {1:5 soil: water (w/v) suspension} were measured weekly from 10 randomly selected pots for every treatment and replication. The average EC of saturated soil extract increased from 0.0015 mS/cm to 5.1, 10.2 and 15 mS/cm after 1st, 2nd and 3rd weeks of salt treatment, respectively.

Experimental setup: This experiment contains two factors: AMF and salt stress, each containing two treatments, mycorrhizal and non-mycorrhizal plants and no salt/distilled water and 200 mM NaCl respectively. Each treatment contains twenty plants with three replications arranged in completely randomized design.

Plant uproot: After three weeks salt treatment, all the plants were uprooted. Ten plants from every treatment were used for recording shoot and root dry weights while the remaining plants were frozen in liquid nitrogen to preserve for further analysis.

Mycorrhizal colonization: The lateral roots of the mycorrhizal asparagus, tomato and strawberry plants were preserved with 70% ethanol and stained with trypan blue according to the method of Phillips and Hayman (1970). In this process, after removing the 70% ethanol with washing tap water, roots were autoclaved at 121ºC, 1.2 kg/m^2, 15 min. After that the roots were immersed in 10% potassium hydroxide (KOH) solution. Thereafter, the potassium hydroxide was removed by washing with distilled water and the roots were stained with trypan blue solution (glycerine 50 ml, lactic acid 50 ml, distilled water 50 ml and trypan blue 1g). Then the stained roots were cut into a length of 0.5 to 1.0 cm section and placed on a glass slide for observing the colonization using an optical microscope. The rate of AMF colonization in 1 cm segments of the lateral roots (abbreviated RFCSL) was calculated. Hence, RFCSL express the percentage of 1 cm AMF colonized segments to the total 1 cm segments of all lateral roots. The number of segments was approximately 50 per plant, used for 10 plants with three replications.

Determination of Na^+ and K^+ contents: Na^+ and K^+ contents from young leaflets and petioles (3-4 weeks age), old leaflets and petioles (7-9 weeks age) and main roots and lateral roots of strawberry plants, young leaves, old leaves, stems, main roots and lateral roots of tomato and young shoots, old shoots, storage roots and feeder roots of asparagus plants were measured using Compact Na^+ (B-722) and K^+ (B-731) meter, (Horiba Ltd., Tokyo, Japan).

Frozen sample (0.2 g) was extracted using 2.5 ml of distilled water and then centrifuged at 13,000 rpm, 4°C, 10 min. The supernatant was subsequently used as a sample solution and used drop wise in the measuring section of a Compact Na^+ (B-722) and K^+ (B-731) meter, (Horiba Ltd., Tokyo, Japan).

Scanning electron microscope and energy dispersive x-ray spectroscopy (SEM-EDX) with nano-suit analysis: A cross-section with a thickness of about 2 mm was made from the old petiole and main root of strawberry plants (Fig. 29) A nano-suit formation treatment was done in the cross-section according to the method of Takaku et al. (2013). Plant raw slices were immersed in a 1% Tween 20 solution and then subjected to plasma irradiation for 20 seconds with a Neoc-Neo Osmium Coater (Meiwafosis Co. Ltd., Tokyo, Japan) to form nano-suit. The observation was performed with a scanning electron microscope (S-4300, Hitachi Co. Ltd., Tokyo, Japan). A line scan of energy dispersive x-ray spectroscopy (EDX) was performed on the petiole and root cross-section, and element distribution of Na was analyzed. Acceleration voltage was 20 kV and operation mode was Analysis.

Determination of cellulose, hemicelluloses, and lignin in main roots of strawberry: Cellulose, hemicellulose, and lignin contents were measured according to Detergent Fiber method (Van Soest and Wine, 1967) by the following procedure.

Determination of neutral detergent fiber (NDF, cellulose + hemicellulose + lignin): Frozen roots (0.5 g) wrapped in standard filter paper and placed in a test tube containing 10 ml of a neutral detergent solution (Table 1), 0.5 ml of decalin and 0.05 g of anhydrous sodium sulphite and immersed in a 100°C water bath for 1 hour. After that, the filter paper was washed with hot water (80 to 90°C), and when foams were disappeared, it was washed twice more with acetone. Subsequently, the sample taken out from the filter paper was air dried at 100°C for 1 day and weighed. Next, the sample was incinerated for 2 hours with a muffle at

550°C, and then weighed again, and the difference in mass before and after ashing was taken as the NDF amount.

Determination of acidic detergent fiber (ADF, cellulose + lignin): Frozen roots (0.5 g) of sample were wrapped in standard filter paper. It was placed in a test tube containing 10 ml of an acidic detergent solution (Table 2) and 0.5 ml of decalin and immersed in a 100°C water bath for 1 h. Thereafter, the filter paper was washed with hot water and acetone in the same manner as the NDF procedure. Then air dried at 100°C for 1 day and night, then incinerated for 2 h in a muffle at 550°C, and the difference in mass before and after ashing was taken as the ADF amount.

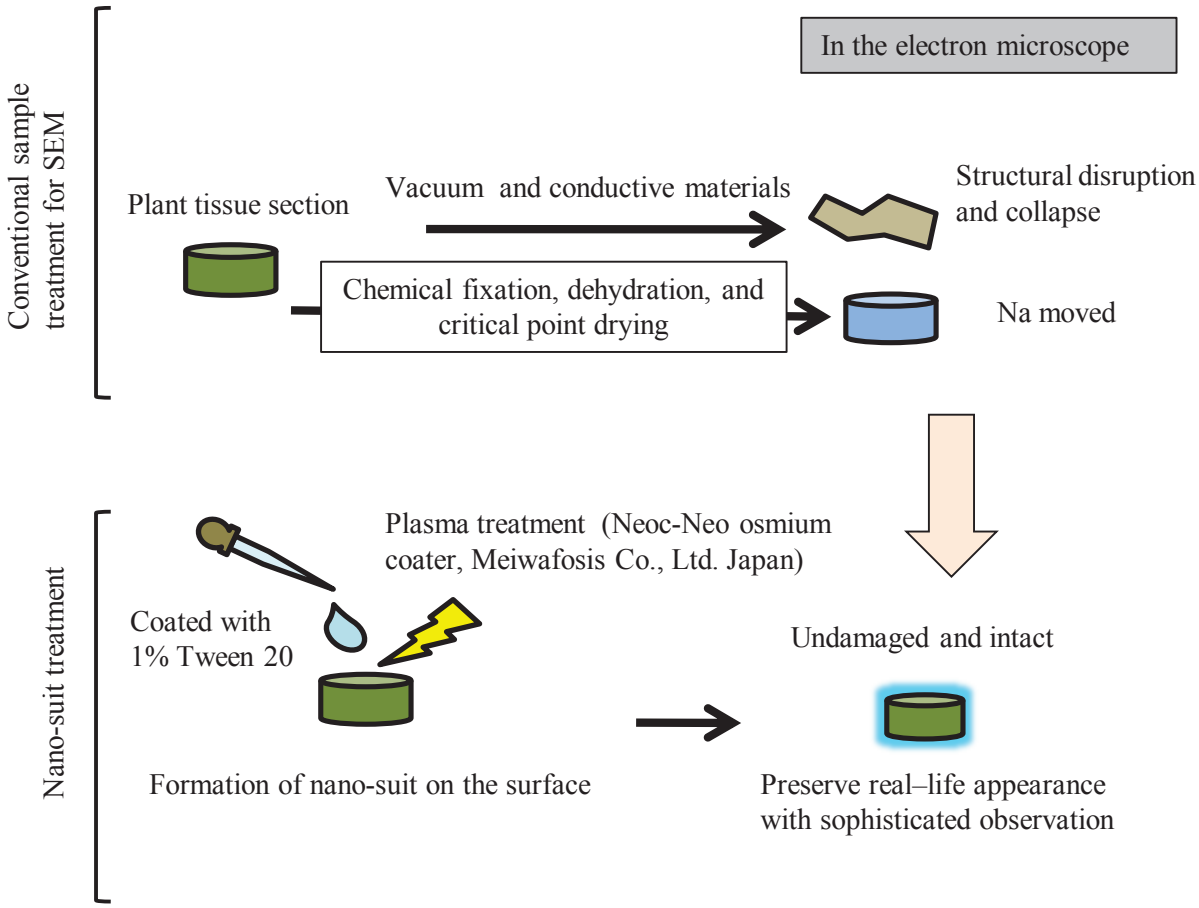
Determination of lignin: The sample obtained after ADF analysis was transferred to a test tube and 1 ml of 72% H_2SO_4 was added three times at intervals of 30 min to wet the whole. Thereafter, it was washed with hot water, dried and incinerated like NDF and ADF, and the difference in mass before and after ashing was defined as the amount of lignin. The cellulose and hemicellulose contents were measured by the following formula:

cellulose = ADF – lignin, hemicellulose = NDF – ADF.

Determination of pectic substance: The root samples were extracted separately with 80% ethanol. The alcohol-insoluble solid (AIS) was then extracted successfully with water (water-soluble pectin, WP), hexametaphosphate (hexametaphospahte-soluble pectin, PP) and hydrochloric acid (HCL-soluble pectin, HP) by the following procedure. AIS were extracted with water at 20ºC for 15min for 3times. Then the filtrates were used as WP and the residue were again extracted with 0.4% hexametaphosphate at 20ºC for 15 min for 3times. The filtrates were used as PP and the residue were again extracted with 0.05N HCl at 100ºC for 1 hour for 3 times. The filtrates of HCl extraction were used as HP. The pectic substances in the three fractions were determined by carbazole - sulfuric acid method (Bitter and Muir, 1962). Extracted sample solution (0.5 ml) was taken in a water-cooled test tube and 2.5 ml of reagent

(A) (Table 3) was gently mixed, and heated to 100°C for 10 min. Thereafter, it was cooled in room temperature and 0.1 ml of reagent (B) (Table 4) was mixed and heated to 100°C for 15 min. Then, it was cooled in room temperature and the absorbance was measured at 530 nm wave length. Distilled water was used for the blank and galacturonic acid was used for preparing the calibration curve.

Statistical analysis: Mean values were separated using a *t*-test for colonization level at $P \leq 0.05$. Dry weights, Na^+ and K^+ content, cellulose, hemicelluloses, lignin content and pectic substance were analyzed by Tukey's test at $P \leq 0.05$. All analyses were performed using XLSTAT 2012 pro statistical analysis software (Addinsoft, New York).



Electronic microscope: SEM S-4300 with EDX (Hitachi Co. Ltd., Tokyo, Japan)

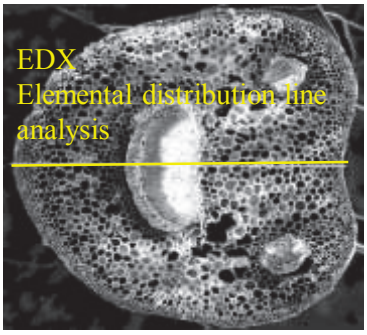


Fig. 29. Difference between conventional sample preparation and nano-suit treatment for scanning electron microscope (SEM) observation with EDX analysis.

Table 1. Composition of neutral detergent (1L) solution.

Sodium lauryl sulfate	30 g
Ethylenediaminetetraacetic acid disodium salt 2-hydrate	18.6 g
Sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$)	6.8 g
di-Sodium hydrogen phosphate, anhydrous (Na_2HPO_4)	4.5 g
2-Methoxyethanol	10 ml
Distilled water	1 L

Table 2. Composition of acid detergent (1L) solution.

n-Hexadecyltrimethylammonium bromide (CTAB)	20 g
1N sulfuric acid	1 L

Table 3. Composition of reagent (A).

Sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$)	0.95 g
Concentrated sulfuric acid	100 ml

Table 4. Composition of reagent (B).

Carbazole	0.12 g
Methanol	100 ml

Results

After three weeks of salinity stress, mycorrhizal plants had higher dry weight compare to non-mycorrhizal plants (data no shown). The pictorial presentation (Fig. 30) confirmed the growth improvement of mycorrhizal plants under salinity condition. Microscopic observation confirmed the colonization in all three mycorrhizal vegetable plants (data not shown). Salinity stresses did not significantly decrease the colonization in all three plants.

Salt stress increased Na^+ content in all the investigated site of both non-mycorrhizal and mycorrhizal plants in all three vegetables (Fig. 31). However, mycorrhizal plants significantly decreased Na^+ compared to non-mycorrhizal plants. In asparagus, mostly excess Na^+ was accumulated in old shoots then in young shoots. This pattern was same in both non-mycorrhizal and mycorrhizal plants. In case of tomato, excess Na^+ was accumulated in lateral roots and main roots. This phenomenon was not change for AMF application. In strawberry plants, the old petioles had the higher Na^+ content compared to other parts such as, young leaflets and petioles and old leaflets in both non-mycorrhizal and mycorrhizal plants. On the other hand, in roots, the highest Na^+ content observed in main roots (no colonization occurred) than lateral roots (colonization occurred) in both non-mycorrhizal and mycorrhizal. plants. In case of Na^+/K^+ ratio, after salt application, all the mycorrhizal plants showed relatively lower Na^+/K^+ ratio than non-mycorrhizal plants in all the tested site of three vegetables except lateral roots of tomato and young petioles of strawberry (Fig. 32). However, the pattern of site specific Na^+/K^+ ratio was same between mycorrhizal and non-mycorrhizal plants for all three vegetables.

The SEM and EDX analysis of strawberry old petiole showed, Na present in all the tissues and highest localized occurred in vascular bundle margin (inner cortex, endodermis and pericycle) (Fig. 33). However, no change observed in this Na localization procedure in the presence or absence of AMF. In the line scan of strawberry main root tissues, a higher

accumulation of Na found in vascular bundle margin like petiole, compared to the other tissues. There was no large change in this accumulation due to the presence of AMF. However, in both petiole and root tissues, mycorrhizal plants accumulated less Na than the control plants.

The cell wall component like pectin contents of roots were no changed under salt stress, except hexametaphosphate soluble pectin increased in mycorrhizal plants under stress condition (Fig. 34). The cellulose and lignin contents in roots significantly increased in mycorrhizal plants compared to non-mycorrhizal plants under with and without salt stress conditions (Fig. 35). On the other hand, higher hemicellulose content found in mycorrhizal plants than non-mycorrhizal plants under control condition. However, there was no difference in hemicellulose content between non-mycorrhizal and mycorrhizal plants in salinity stress.

Asparagus

N+ NaCl AMF + NaCl

Tomato

N + NaCl AMF + NaCl

Strawberry

N + NaCl AMF + NaCl

Fig. 30. Effect of salinity stress on growth of asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM.

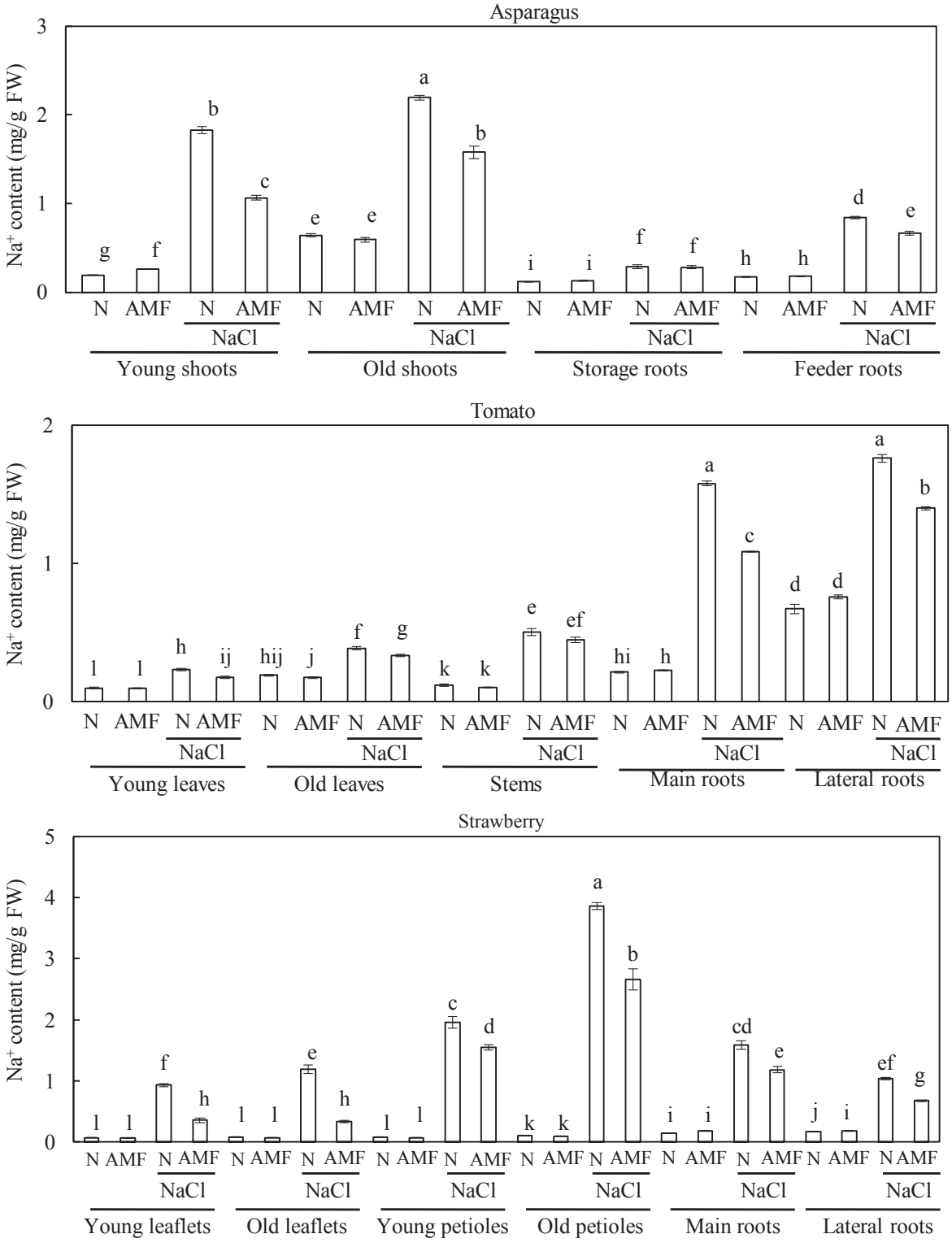


Fig. 31. Na^+ content in asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

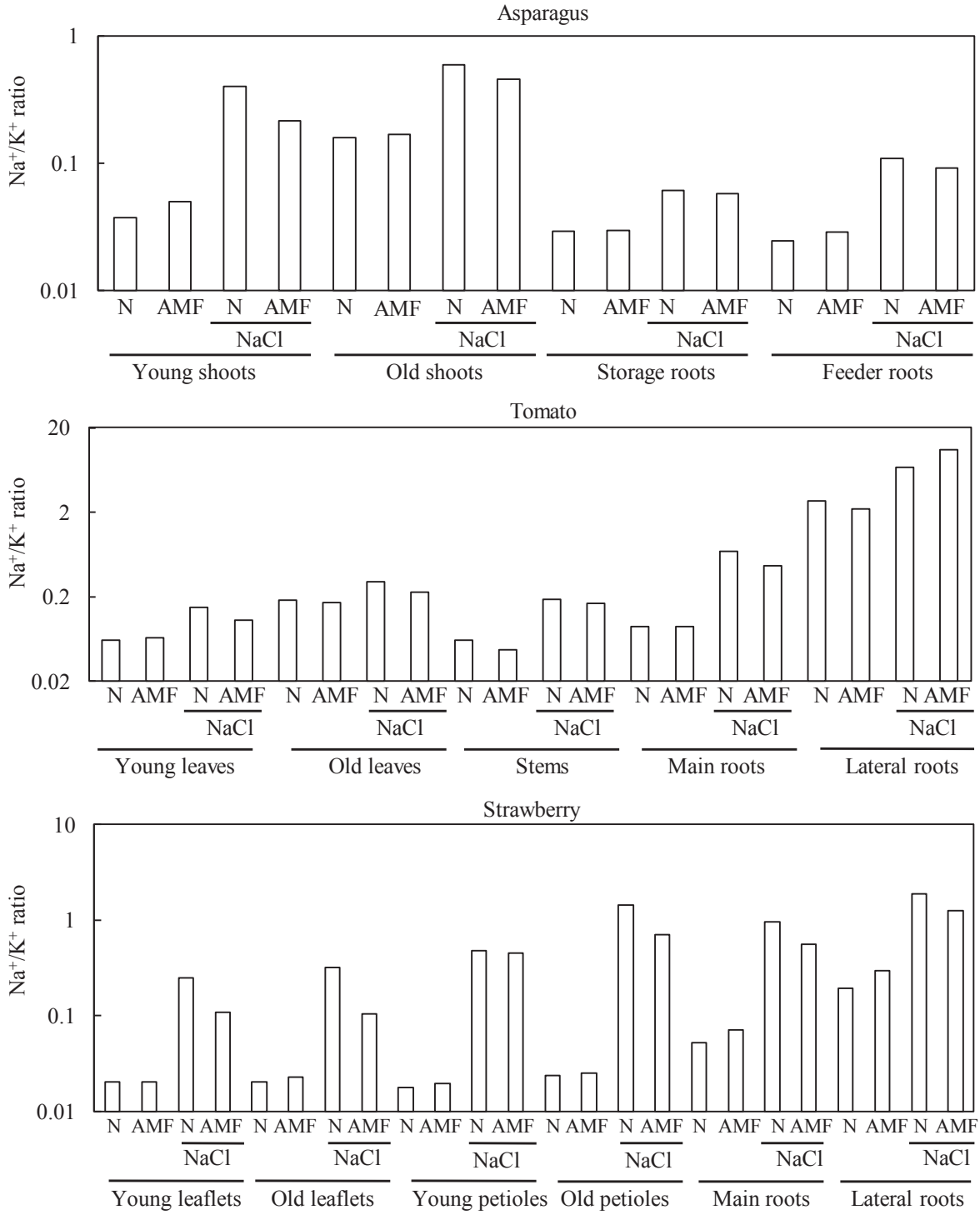


Fig. 32. Na^+/K^+ ratio in asparagus, tomato and strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM.

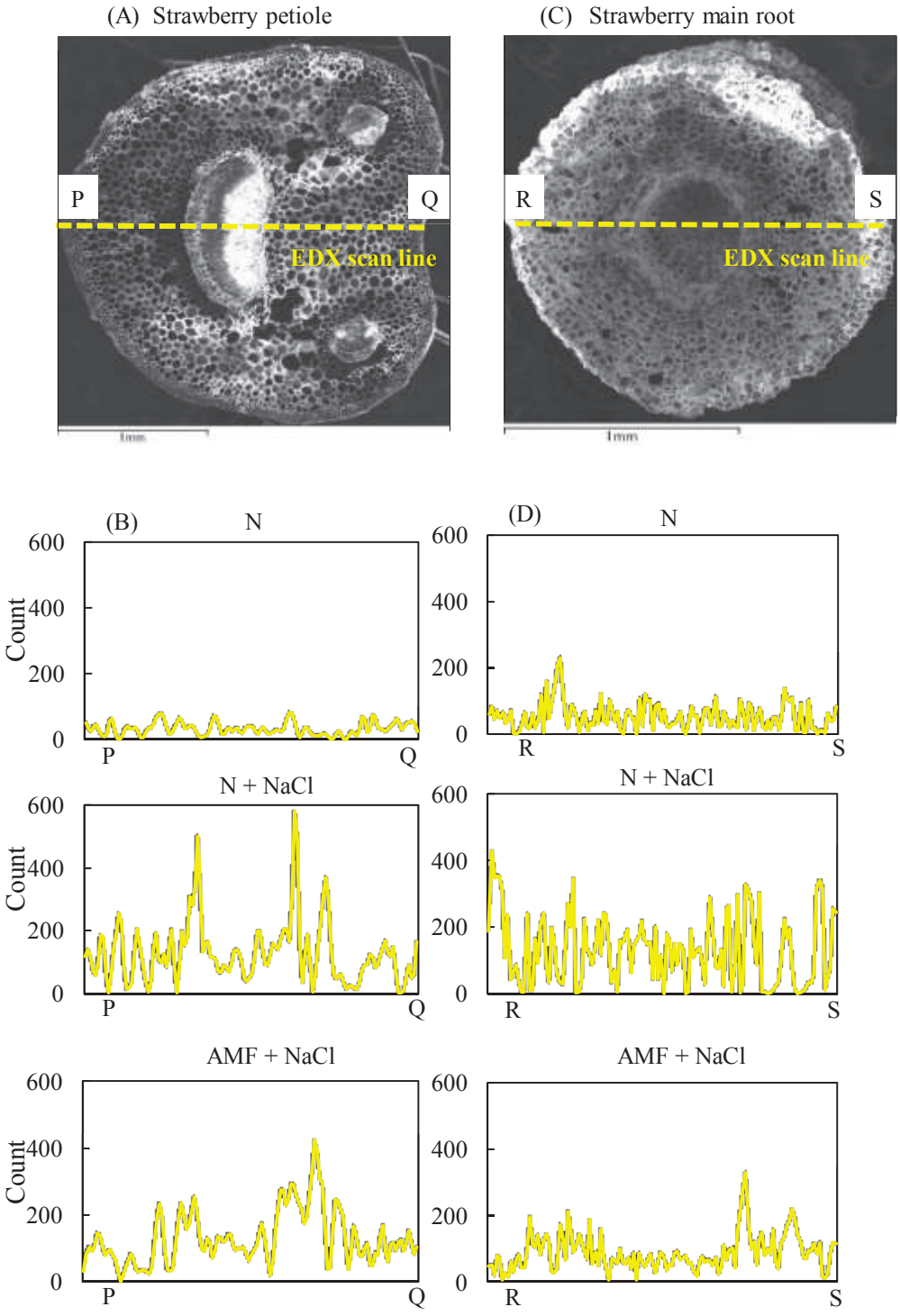


Fig. 33. SEM observation (A, petiole; C, main root) and SEM-EDX analysis of Na localization (B, petiole; D, main root) in strawberry plants with nano-suit. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, NaCl 200 mM. PQ and RS showed EDX scan line in petiole and main root section, respectively.

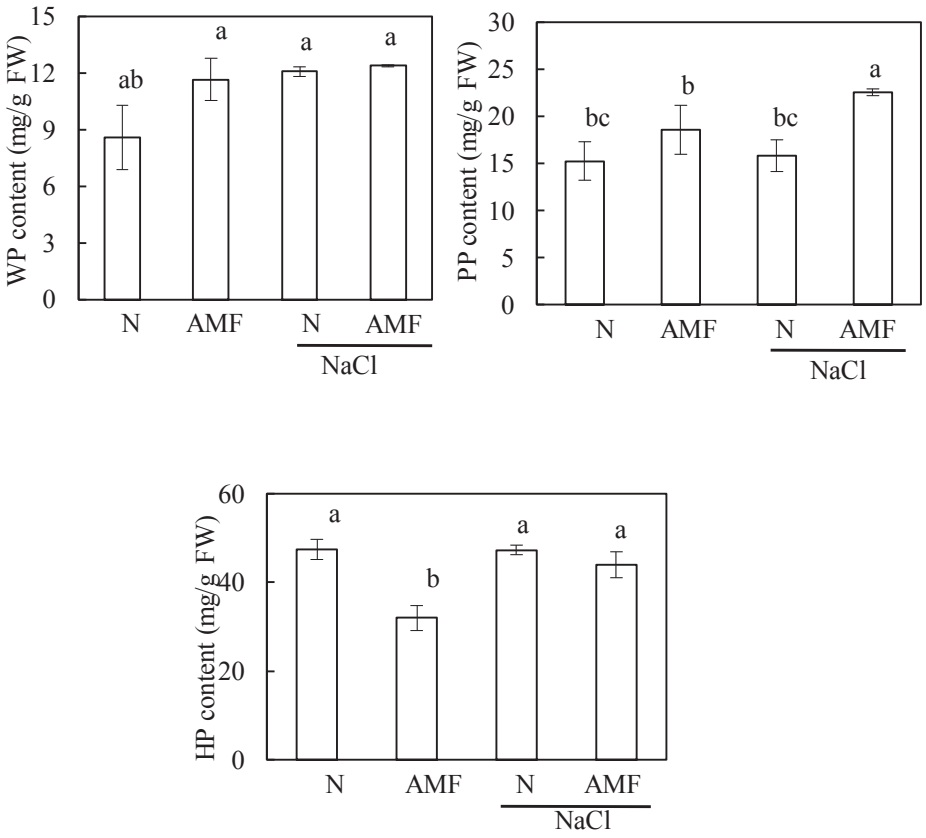


Fig. 34. Water-soluble pectin (WP) , hexametaphosphate-soluble pectin (PP) and HCl-soluble pectin (HP) contents in main roots of strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl. Columns denoted by different letters indicate significant difference according to Tukey's test ($P \leq 0.05$).

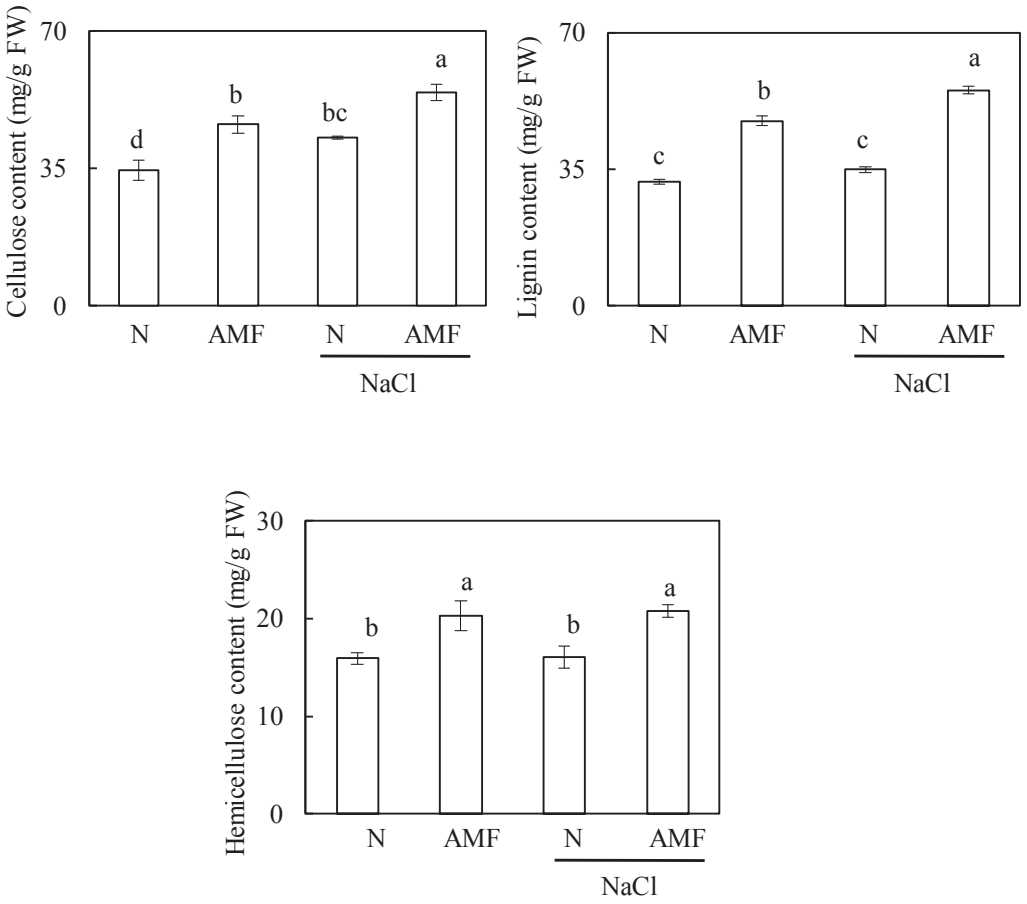


Fig. 35. Cellulose, lignin and hemicellulose contents in main roots of strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl. Columns denoted by different letters indicate significant difference according to Tukey's test ($P \leq 0.05$).

Discussion

In the present study, AMF colonization occurred successfully in all the inoculated plants and not significantly decreased under salt stress. The dry weight of all the three vegetables of mycorrhizal plants were also increased compared to non-mycorrhizal plants under salinity stress. Previously, Latef and Chaoxing (2014) and Sinclair et al. (2014) indicated that mycorrhizal symbiosis improved pepper and strawberry plant growth under salt stress. Additionally, lower Na^+ content and Na^+/K^+ ratio in mycorrhizal plants for all three vegetables suggested the alleviation of salinity stress. Low Na^+ content implies less interference of salt with chlorophyll biosynthesis and this is due to the inhibition of Na^+ transport in leaves, which leads to better functioning of photosynthetic machinery under salinity stress (Alenazi et al. 2015). So, in this experiment AMF induced salinity tolerance was confirmed.

In salt condition, plants increasingly accumulate Na^+ and excess Na^+ in plant cells directly damages membrane system and organelles (Porcel et al., 2012). In the present study, Na^+ content increased after salt stress in all organs of three plants and Na^+ mostly distributed in the older organ than the younger parts. However, this distribution was not changed through AMF application. Previously, Parida and Das (2005) reported in their review that plants protect young leaves from ion harm via the large accumulation of toxic Na^+ in old leaves during adaptation to salt stress. Our results showed similar patterns to those findings. On the other hand, in tomato most of the Na^+ accumulated in lateral roots and main roots. In asparagus and strawberry it was in shoot part. Such as in strawberry, Na^+ mostly accumulated in petioles (old) of both non-mycorrhizal and mycorrhizal plants. So, excess Na^+ distribution inside plant body to protect its toxicity, varied on plant species and every species have their own strategy for Na^+ toxicity reduction. AMF application has no influence on the excess Na^+ distribution on different plant organs during adaptation to salt stress.

For SEM observation, a nano-suit technique was used to protect the surface of plant tissue section from rapid dehydration and maintain stable physiological condition. The use of nano-suit provides good integrity of the organism's surface without interfering with high resolution imaging in the SEM (Takaku et al., 2015). So, the SEM observation including nano-suit provides more precise and correct data than the conventional SEM. The SEM-EDX analysis of strawberry petiole tissue confirmed localization of Na occurred mainly in the cells of vascular bundle margin and no change observed in this localization through AMF application. In case of roots Na present in all the tissues, and highest localization occurred around vascular bundle but the amount of Na was lower than petioles. Moreover, the same pattern of localization found in mycorrhizal plants. So, the cells of vascular bundle margin of the strawberry petioles may have the function of accumulate or absorbed excess Na and restrict their translocation to the leaflets under salt stress. The AMF application only reduced the Na content inside plant body, not changed the translocation in the plant organ and inside tissues. However, the low Na^+ content and decrease in Na^+/K^+ ratio in mycorrhizal plants was mentioned by Evelin et al. (2009) and Porcel et al. (2012) in their reviews. Our results showed consistence with previous findings. From above facts, it will be hypothesized that the main factor of decrease Na^+ content in mycorrhizal plants may be Na^+ absorption suppression rather than the control of Na^+ translocation in plant organs and tissues.

It has recently been suggested that the mycorrhizal fungal mycelium may act as first barrier for ion selection by selectively uptaking K^+ and Ca^{2+} and alleviate salt stress in plants by pre-selecting nutrients and preventing toxic ion like Na^+ from entering the plant (Hammer et al. 2011). The low Na^+ content and low Na^+/K^+ ratio in this study might be related with this selective ion uptake mechanism of AMF. Moreover, Sinclair et al. (2014) stated AMF have a strong relation with the strawberry root system. So, it can be expected that suppression of Na^+ absorption was happened in the vicinity of the root surface of strawberry plants. Scheriber et

al. (1999) stated that the cell wall forming the root apoplast barrier. In this regards, analysis of the cell wall component of strawberry roots showed, cellulose and lignin content increased in mycorrhizal roots than non-mycorrhizal plants under salt stress. The hemicellulose content was increased only in mycorrhizal roots in no salt treatment. However, roots pectin content no changed in mycorrhizal plants than non-mycorrhizal under salt stress. These results suggested that increased cell wall component (cellulose and lignin) on the mycorrhizal roots act as apoplast barrier for Na inflow in to the plant body. In this case, it is also expected that material deposition on cell wall simultaneously decrease the permeability of other ion such as K^+. However, in this examination, lower Na^+/K^+ ratio confirmed accumulation of K^+ inside plants. Recently, Estrada et al. (2013) correlated higher K^+/Na^+ ratios with regulation of ZmAKT2, ZmSOS1 and ZmSKOR gene expression in mycorrhizal maize roots, contributing to K^+ and Na^+ homeostasis in plants. So, under salt stress, K^+ can be absorbed more than the apoplast path through more specific and better regulated mechanism. Therefore, excess deposition of cellulose and lignin in mycorrhizal roots make apopalst barrier for the Na inclusion into the plant body.

In conclusion, AMF application induced salt tolerance in asparagus, tomato and strawberry plants by increased dry matter, low Na^+ content and Na^+/K^+ ratio. The excess Na^+ accumulation in plants organ were not influenced by AMF. It depends on plant species. Subsequently, the excess Na localized around the vascular bundle tissues of particularly older organ than younger parts. Then, the main factor for the reduction of Na^+ in mycorrhizal plants was suppression of Na^+ absorption inside plant body and the higher cellulose and lignin content in cell wall of roots may have contributed to this suppression. So, AMF only suppress the Na absorption inside plant body, it has no effect on localization of Na in regard to salt tolerance.

CHAPTER 1-3

Relationship between salinity tolerance and free amino acid contents as compatible solute in mycorrhizal plants

Introduction

Soil salinity is a frequently occurring abiotic stress, which can limit agricultural production worldwide. Typhoon, tsunami, sea water influx and inappropriate cultivation practices have exacerbated the concentration of salts in soil and water (Ruiz-Lozano et al., 2012). It has been estimated that 7% of all land globally is in high in salt content, which results in perturbations in plant growth and development (Evelin et al., 2009). Salinity induces changes in various physiological and metabolic processes in plants, depending on the severity and duration of the stress (Munns, 2005; James et al., 2011). High levels of salt in soil or irrigation water affect the metabolism of the plant, causing hyperionic and hyperosmotic stresses. However, plants have evolved several biochemical and molecular mechanisms for coping with the negative effects of salinity (Porcel et al., 2012).

Salt accumulation in soil results in, excessive amount of Na^+ and Cl^- in the soil causes osmotic stress that reduces the osmotic potential of the soil solution and water absorption by plant roots (Rasool et al., 2013b). In response to salt-induced lowered water potential, plants accumulate K^+ ions and compatible solutes in the cytoplasm which include sugars (fructose, glucose, etc.), onium compounds (glycine betaine, dimethylsulfoniopropionate), and amino acids (Nuccio et al., 1999). An increase in free amino acids acts as an indicator of tolerance to salinity. Among the amino acids, an increase in proline is one of the most frequently reported alterations that are induced by salt stress in plants. Under saline conditions, several plants accumulate proline as a non-toxic and protective osmolyte for maintaining osmotic balance under low water potentials (Evelin et al., 2009).

Numerous beneficial soil microorganisms, particularly arbuscular mycorrhizal fungi (AMF), can help plants cope with different abiotic stress conditions (Barea et al., 2013). AMF symbiosis can be defined as a specialized system for nutrient uptake and transfer that is

more efficient than plant roots alone. AMF can alleviate the detrimental effects of salinity and enhance plant growth through several means (Evelin et al., 2009 and Ruiz-Lozano et al., 2012). In addition, AMF can be found under extremely saline conditions, and can further be adapted to these conditions (Wilde et al., 2009). However, numerous details remain unclear concerning the mechanism by which AMF alleviates salt stress in plants.

Previous studies have described the changes in free amino acid levels in plants resulting from mycorrhizal colonization. Such as, Fattah and Mohamedin (2000) and Sood (2003) stated that free amino acid content increases in both mycorrhizal tomato and sorghum plants. In contrast, Rolin et al. (2001) described, a decrease in total amino acid content in the roots of mycorrhizal leek plants (*Glomus etunicatum*), and relative proportions of different amino acids are not affected by mycorrhizal colonization. Talaat and Shawky (2014) reported that free proline increased in mycorrhizal wheat under salt stress condition. Contradictorily, Latef and Chaoxing (2014) suggested that AMF increased free amino acid concentrations and decreased proline in pepper plants under saline conditions. However, the effect of AMF symbiosis on individual free amino acid contents (other than proline) under salt stress remains unclear. Thus, the present study aimed to investigate the role of AMF symbiosis in amino acid and sugar contents as an osmoprotectant under salt stress in strawberry and asparagus plants.

Materials and Methods

Plant materials and AMF inoculation: In this experiment asparagus (*Asparagus officinalis* L., cv. Welcome) and strawberry (*Fragaria* × *ananassa* Duch., cv. Tochiotome) plants were used. Seeds of asparagus plants were sowed and two months old strawberry runner plants were planted in plastic pots (13.5×27.0×15.5 cm for asparagus, 10.5 in diameter for strawberry) containing autoclaved (121^{o}C, 1.2 kg/cm^{2}, 30 min) commercial potting media SM-2 (Ibigawa Industry Co. Ltd., Japan). At the time of sowing and transplanting, half of the

pots were inoculated with 5 g of AMF/plant (*Gigaspora margarita*) inocula at a depth of 3 cm and mixed with potting media. The non-mycorrhizal plants received the same amount of sterilized inocula. The mycorrhizal inocula of unknown spore density were obtained (exclusively for research purposes) from Centralgrass Co. Ltd., Tokyo, Japan. Plants were fertilized with slow release granular fertilizer (Long total 70 type, JCAM AGRI Co. Ltd., Japan) at the rates of N:P:K = 13:11:13, 1g/plant, after seedling emergence for asparagus and immediately after transplanting for strawberry. Plants were grown in a greenhouse at 25 ± 3/19 ± 3^{o}C day/night temperature with a 12-13 h photoperiod (750-1000 µmol/m^{2}/s) and 60%-70% relative humidity.

Treatment with NaCl: The plants were subjected to salinity stress with 200 mM NaCl solution after nine (strawberry) and fourteen (asparagus) weeks of AMF inoculation. Plants were irrigated with NaCl solution (40 ml/plant) for twelve days (4 times/week). The no salt-treated/control plants received an equal amount of distilled water. The electrical conductivity (EC) of saturated soil extract {1:5 soil: water (w/v) suspension} were measured weekly from 10 randomly selected pots for every treatment and replication. The average EC of saturated soil extract increased from 0.0013 mS/cm to 4.1, 8.5 and 14.2 mS/cm after 1st, 2nd and 3rd weeks of salt treatment, respectively.

Experimental setup: The experiment contains two factors, AMF and salt stress, each containing two treatments, non-mycorrhizal and mycorrhizal plants and no salt/control and 200 mM NaCl solution, respectively. Each treatment contains twenty plants with three replications arranged in completely randomized design.

Plant uproot: After three weeks salt treatment, all the plants were uprooted. Ten plants from every treatment were used for recording shoot and root dry weights while the remaining plants were frozen in liquid nitrogen to preserve for further analysis.

Mycorrhizal colonization: The lateral roots of the mycorrhizal asparagus and strawberry

plants were preserved with 70% ethanol and stained with trypan blue according to the method of Phillips and Hayman (1970). In this process, after removing the 70% ethanol with washing tap water, roots were autoclaved at 121ºC, 1.2 kg/m^2, 15 min. After that the roots were immersed in 10% potassium hydroxide solution. Thereafter, the potassium hydroxide was removed by washing with distilled water and the roots were stained with trypan blue solution (glycerine 50 ml, lactic acid 50 ml, distilled water 50 ml and trypan blue 1g). Then the stained roots were cut into a length of 0.5 to 1.0 cm section and placed on a glass slide for observing the colonization using an optical microscope. The rate of AMF colonization in 1 cm segments of the lateral roots (abbreviated RFCSL) was calculated. Hence, RFCSL express the percentage of 1 cm AMF colonized segments to the total 1 cm segments of all lateral roots. The number of segments was approximately 50 per plant, used for 10 plants with three replications.

Free amino acid analysis using liquid chromatography-mass spectrometry (LC-MS): Sample preparation: LC-MS was carried out using the Ultra-Performance Liquid Chromatography (UPLC) - Mass Spectrometry (MS) system (Waters Corporation, Milford, USA). Frozen samples (0.2 g, leaves and roots, excluding lateral roots) were extracted using 3 ml of 0.2 N perchloric acid solution and then centrifuged at 4,000 rpm at 4ºC for 10 min. After adjustment to pH 4, the supernatant was again centrifuged at 13,000 rpm at 4ºC for 5 min. The supernatant was subsequently filtered through a syringe filter for use as an analysis sample (Nimbalkar et al., 2012).

Analytical samples were derivatized using the AccQ·Tag Ultra Derivatization Kit (Waters Corporation, Milford, USA) (Fig. 36). Analytical samples (30 µl) were mixed with 210 µl of borate buffer and 60 µl of derivatization reagent. The reaction mixture was immediately mixed and left for 1 min at room temperature. Subsequently, the solution was incubated at 55ºC for 10 min in a water bath. After cooling, the reaction mixture was used for UPLC

injection.

Instrumentation and chromatographic condition for UPLC-MS: The ACQUITY UPLC BEH C18 (1.7 μm, 2.1 × 100 mm, Waters Corporation, Milford, USA) reversed phase column was used under 25°C. The chromatographic condition and gradient used in the experiment showed in Table 5. The mass spectrometer (Xevo Q Tof MS, Waters Corporation, Milford, USA) measured the analysis mass range electro spray ionization in positive mode at 100-1000 m/z. A mass chromatogram (Abs Window 0.05 Da) of the m/z value of each amino acid was prepared from the measurement results, and the amino acid content was measured according to the peak integration value (Fig. 37 and 38) (Table 6). Data analysis was executed using the Waters Masslynx software, USA. Amino acid mixed standard solution H-type (Wako Pure Chemical Industries Ltd., Japan) was used for making a standard curve. Twenty kinds of free amino acids were analyzed, all of which were included in the standard solution.

Free sugar analysis: Sugars were extracted in 62.5% (v/v) methanol for 15 min at 55ºC and concentrations were determined enzymatically with a Boehringer Mannheim Sucrose/D-Glucose/D-Fructose Test Kit (catalogue number-716 260, R-Biopharm AG, Darmstadt, Germany) following the method of Irving and Hurst (1993).

Statistical analysis: Mean values were separated using a *t*-test for colonization level at $P \leq 0.05$. Dry weights and free amino acid contents were analyzed using Tukey's test at $P \leq 0.05$. All analyses were performed using XLSTAT 2012 pro statistical analysis software (Addinsoft, New York).

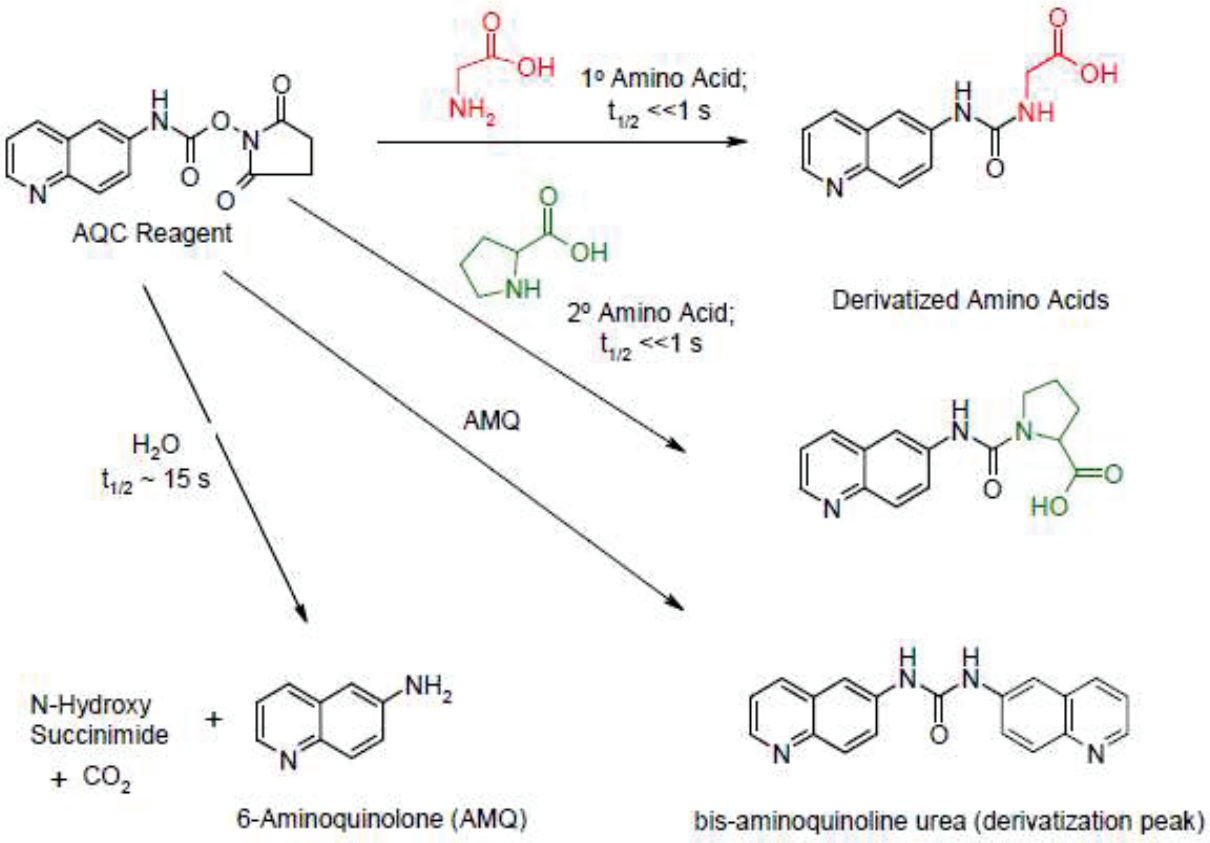


Fig. 36. Amino acid derivatization by AccQ-Tag Ultra Derivatization Kit (Waters Corporation, Milford, USA).

Table 5. Chromatographic conditions

Column	ACQUITY UPLC BEH C 18 (1.7 µm, 2.1 × 100 mm, reversed phase column, Waters).			
Mobile phase A	0.1% formic acid			
Mobile phase B	acetonitrile			
Injection volume	5 µl			
Gradient	Time (min)	Flow (ml/min)	Mobile phase A (%)	Mobile phase B (%)
	0.0	0.4	99.9	0.1
	12.0	0.4	50.0	50.0
	13.0	0.4	99.9	0.1
	15.0	0.4	99.9	0.1

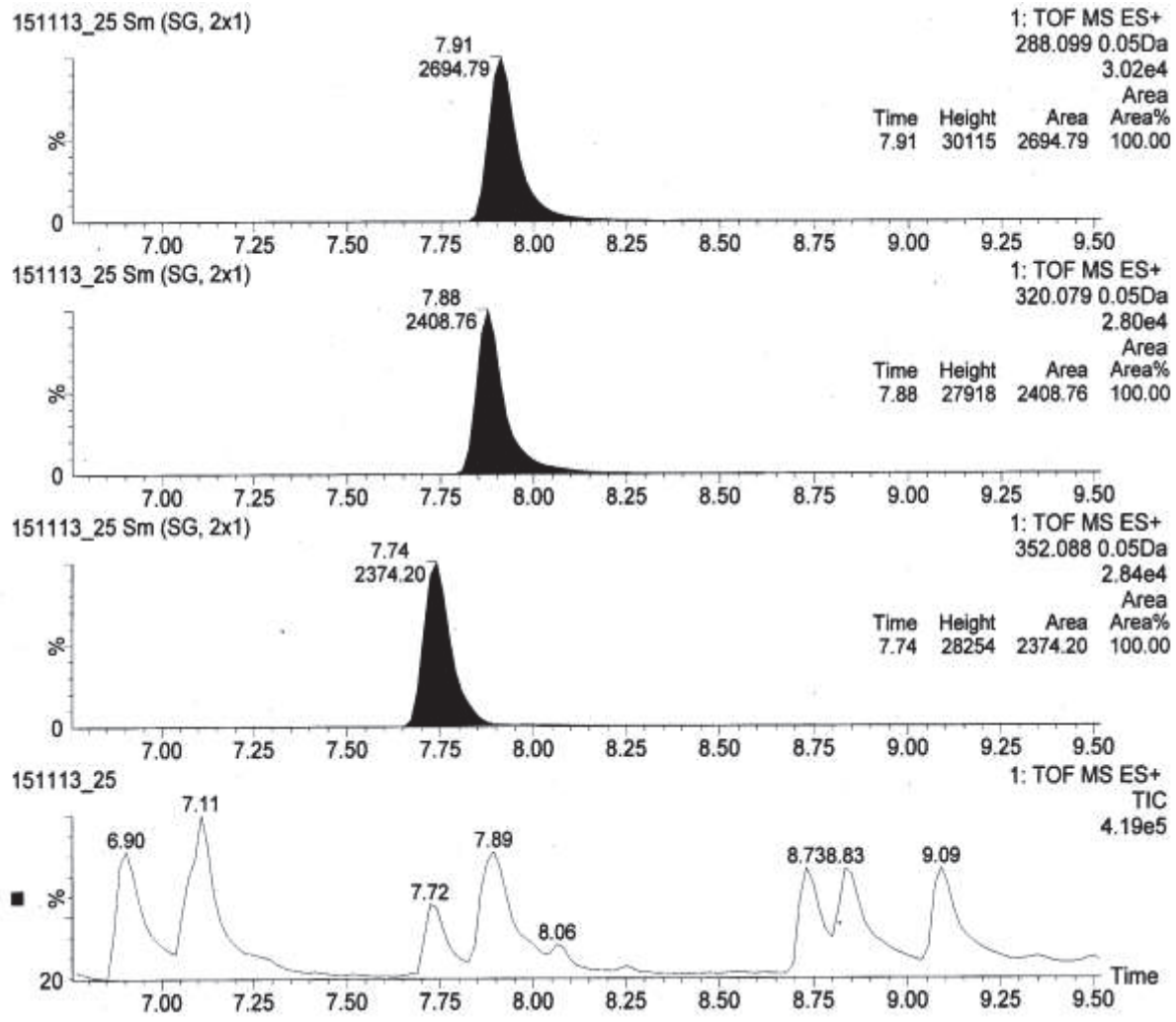


Fig. 37. Peak integration on mass chromatogram.

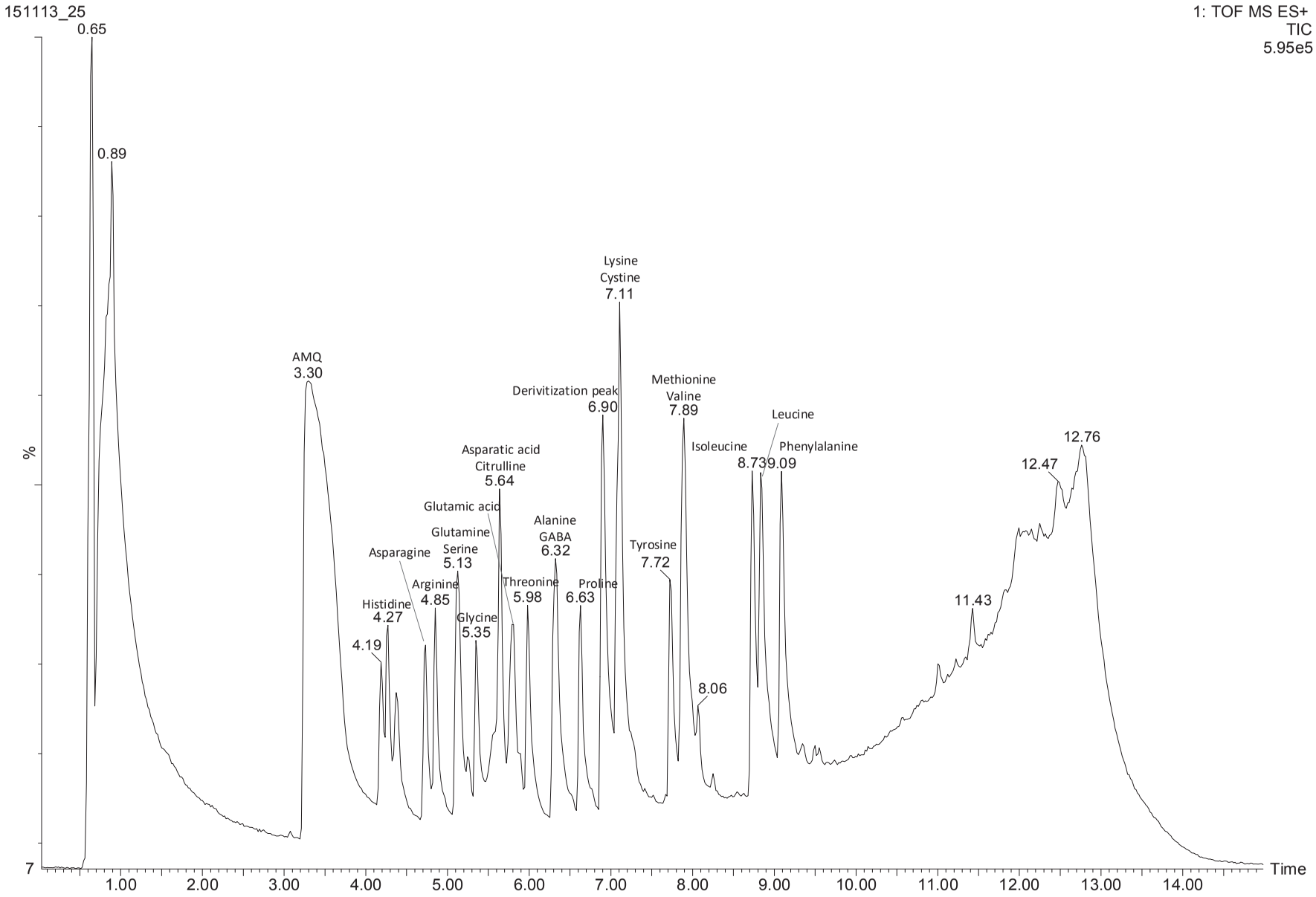


Fig. 38. UPLC/MS chromatogram of standard amino acids solution.

Table 6. Detected retention time (RT) and m/z of derivatized amino acids.

Name	RT (min)	m/z (positive)
6-aminoquinoline (AMQ)	3.30	145.04
Histidine	4.27	326.14
Asparagine	4.23	303.10
Arginine	4.85	345.17
Glutamine	5.11	317.12
Serine	5.13	276.08
Glycine	5.35	246.07
Aspartic acid	5.62	304.08
Citrulline	5.64	346.15
Glutamic acid	5.81	318.09
Threonine	5.98	290.09
Alanine	6.30	260.08
γ-aminobutyric acid (GABA)	6.34	274.01
Proline	6.63	286.10
derivatization peak (derivatized AMQ)	6.90	315.11
Lysine	7.09	487.20
Cystine	7.11	581.11
Tyrosine	7.72	352.09
Methionine	7.88	320.08
Valine	7.89	288.10
Isoleucine	8.73	302.12
Leucine	8.85	302.12
Phenylalanine	9.09	336.10

Results

Microscopic observation confirmed the colonization in mycorrhizal strawberry and asparagus plants (data not shown). Salinity stresses significantly decrease the colonization in strawberry plants. In case of asparagus, there was no significant deceased observed. As plant growth, mycorrhizal plants showed greater dry weight in both strawberry and asparagus plants under salt stress (data not shown).

Total free amino acid content in the leaves and roots of mycorrhizal strawberry and asparagus plants increased under salt stress conditions compared to non-mycorrhizal plants (Fig. 39). In the case of free amino acids in leaves of strawberry plants, aspartic acid, GABA, histidine, and glutamine had higher contents in mycorrhizal plants than non-mycorrhizal plants under no-salt conditions (Fig. 40). Subsequently, under salt stress, aspartic acid, GABA, glutamine, and proline contents significantly increased in mycorrhizal plants relative to non-mycorrhizal plants. Conversely, in non-mycorrhizal plants under salt stress, histidine and lysine content increased while serine content remained unchanged when compared with mycorrhizal plants. In addition, we detected that the increase in aspartic acid, GABA, glutamine, and proline was much greater in mycorrhizal plants when compared with non-mycorrhizal plants. Moreover, the concentrations of aspartic acid and GABA were higher than the other amino acids, however, after salt stress a significant increase was observed in mycorrhizal plants than non-mycorrhizal plants.

When observing the roots of strawberry plants (Fig. 41), alanine, GABA, asparagine, serine, proline, tyrosine and leucine showed an increased concentration in mycorrhizal plants when compared with non-mycorrhizal plants under conditions without salt treatment. Under salt stress, several amino acids including alanine, GABA, asparagine, glutamine, serine, aspartic acid, citrulline, proline, tyrosine, valine, isoleucine, and leucine contents increased when measured in mycorrhizal plants as compared with non-mycorrhizal plants. However,

glutamic acid content was higher in non-mycorrhizal plants compared with mycorrhizal plants under salt stress treatment. Alanine, GABA, and asparagine concentrations showed no significant difference under salt and no salt conditions. However, they were significantly lower than mycorrhizal plants under both salt and no-salt conditions. The amount of GABA and alanine (0.8-2 μmol/g FW) was higher (two-fold) in mycorrhizal plants when compared with non-mycorrhizal plants under both no-salt and salt conditions.

In case of asparagus shoots (Fig. 42), under no salt stress conditions free amino acids GABA, glutamine, serine, arginine, threonine, lysine and valine contents increased in mycorrhizal plants. Under salinity stress condition alanine, GABA, proline, arginine, glycine and valine contents increased in AMF inoculated plants.

In asparagus roots (Fig. 43), no significant difference observed in most of the free amino acid content between mycorrhizal and non-mycorrhizal plants. On the other hand, after salinity stress several amino free amino acids content were changed in both non-mycorrhizal and mycorrhizal plants. Free amino acids asparagine, arginine, glutamine, proline, histidine, aspartic acid, citruline, glutamic acid, GABA, lysine, tyrosine, methionine, valine and leucine contents increased in mycorrhizal plants compared to non-mycorrhizal plants.

The free sugar content of leaves and roots of strawberry plants showed, glucose, fructose and sucrose contents increased in AMF inoculated plants under salt condition compared with non-inoculated plants (data not shown).

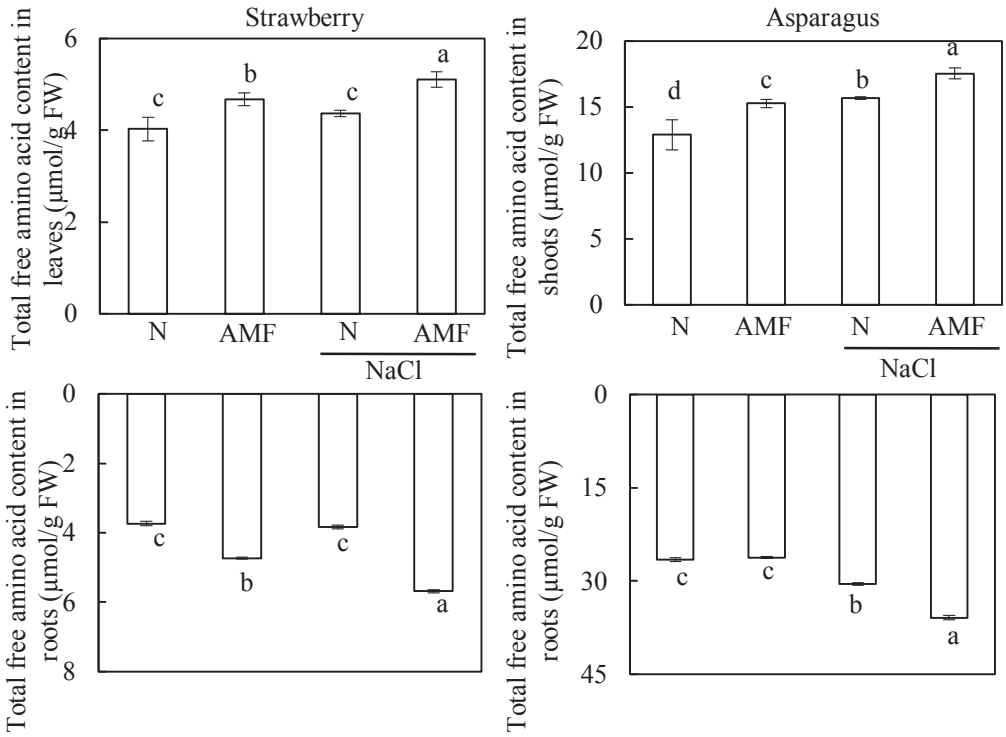


Fig. 39. Total free amino acid contents in leaves/shoots and roots of strawberry and asparagus plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; 200 mM NaCl. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

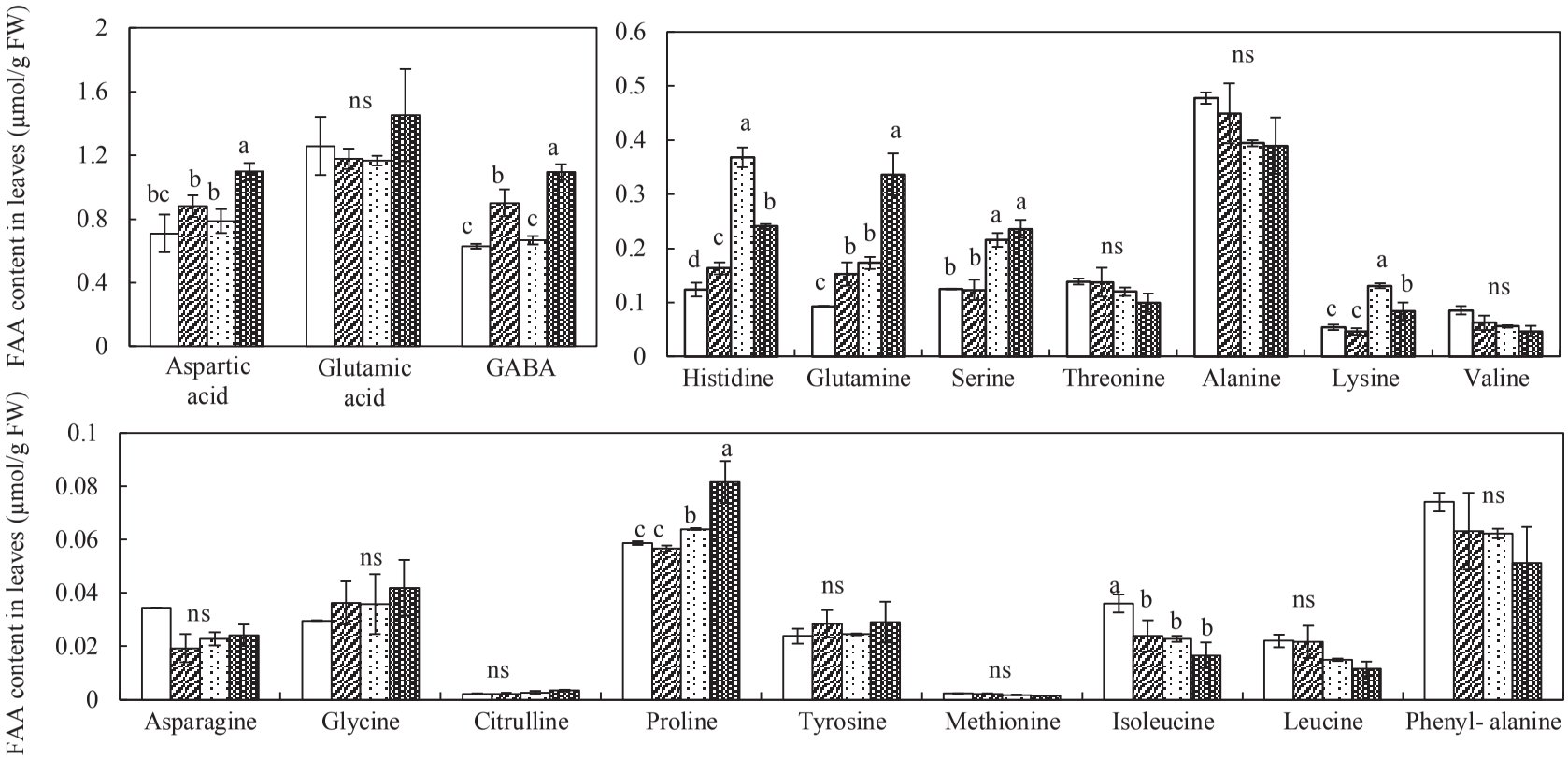


Fig. 40. Free amino acid (FAA) contents in leaves of strawberry plants. ☐ , N; ▨ , AMF; ⁝ , N + NaCl; ▩ , AMF + NaCl. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

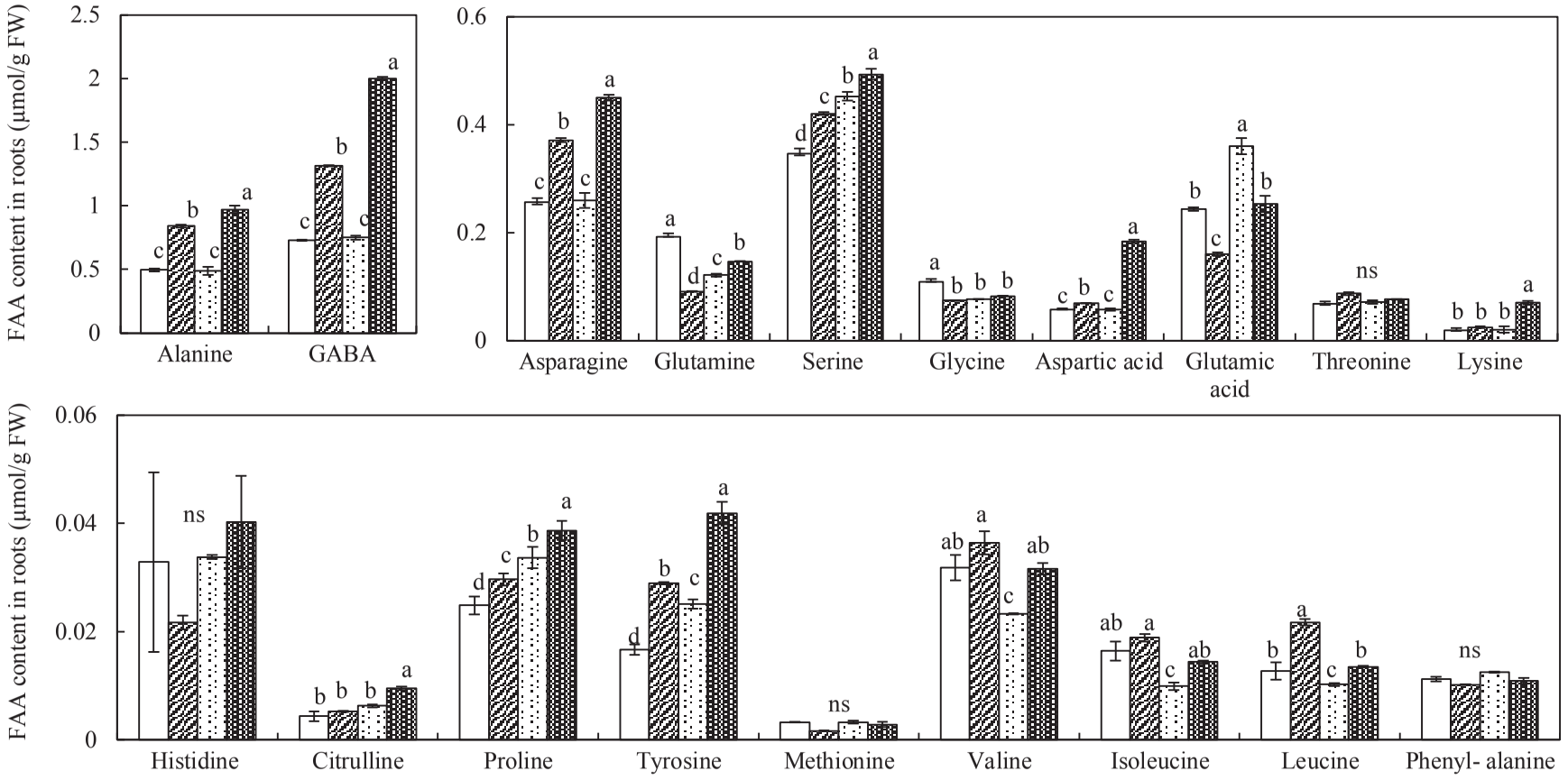


Fig. 41. Free amino acid (FAA) contents in roots of strawberry plants. ☐ , N; ▨ , AMF; ⁝⁝ , N + NaCl; ▩ , AMF + NaCl. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

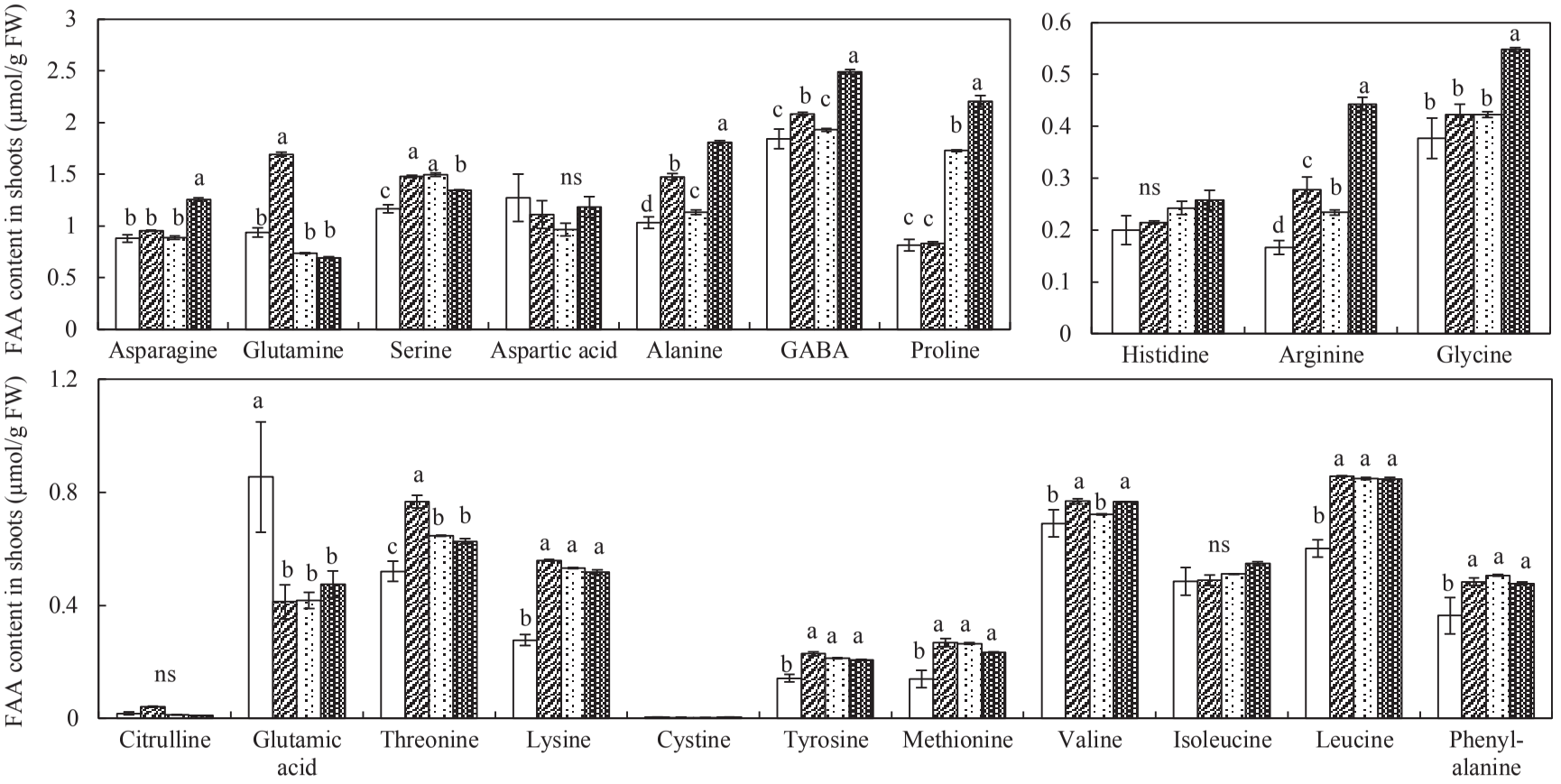


Fig. 42. Free amino acid (FAA) contents in shoots of asparagus plants. ☐, N; ▨, AMF; ⁝⁝, N + NaCl; ▩, AMF + NaCl. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

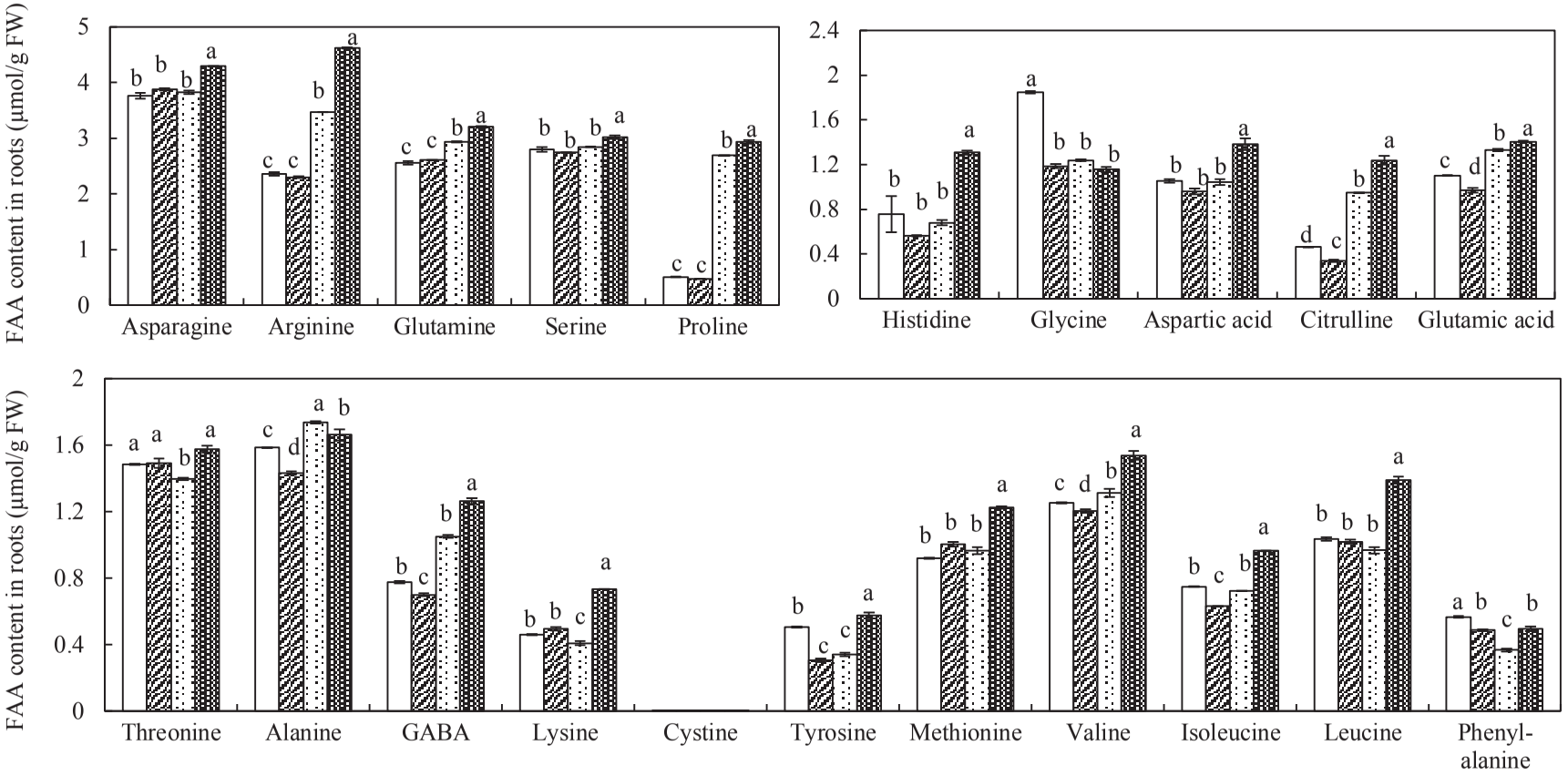


Fig. 43. Free amino acid (FAA) contents in roots of asparagus plants. □, N; ▨, AMF; ⁝⁝, N + NaCl; ▩, AMF + NaCl. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

Discussion

In the present study, microscope assessment confirmed that colonization occurred in mycorrhizal plants, which showed a significant decrease under salt treatment for strawberry plants. Plants with the non-inoculation treatment were not colonized by AMF. The presence of excess salt has a suppressive effect on mycorrhizal fungal hypha growth, or hyphal spreading after initial infection and spore germination (Talaat and Shawky, 2014). The decrease of colonization level in this study may be because of such suppressive effect of salt on fungi.

As for the plant growth, dry weight of shoots and roots decreased under salt stress, however, mycorrhizal plants had a higher dry weight when compared with non-mycorrhizal plants, suggesting the growth improvement through AMF application under stress.

In this study, total free amino acid content significantly increased in mycorrhizal plants under salt stress. The higher content of total free amino acids in mycorrhizal pepper plants compared to non-mycorrhizal plants under salt stress showed salt tolerance (Latef and Chaoxing, 2014). The free amino acids serve as important osmoprotectant for alleviating osmotic stress in plants under salt conditions (Evelin et al., 2009). Therefore, the increase in total amino acid content in mycorrhizal strawberry and asparagus plants may function as compatible solute for reducing salt stress.

Moreover, in this experiment, aspartic acid, GABA, glutamine, and proline contents increased in leaves of mycorrhizal plants under salt stress. In plants, GABA plays significant roles in various physiological processes like nitrogen storage, oxidative stress protection and osmoregulation and acts as a signalling molecule in stress response (Saito et al., 2008). In addition, in plants, aspartic acid is an amino acid that serves as precursor to several other amino acids such as arginine, methionine, threonine and isoleucine (Rawia et al., 2011). Thus, higher content of GABA and other amino acids potentially act as compatible solute in reducing osmotic stress under high salt conditions.

Root concentrations of alanine, GABA, asparagine, serine, aspartic acid, proline and tyrosine increased in mycorrhizal plants, whereas, their contents did not change or slightly increased in non-mycorrhizal roots under salt stress. A significant increase in alanine and GABA contents was observed in mycorrhizal roots under both no-salt and salt stress conditions. According to Sato et al. (2006), the increasing concentration of amino acids such as aspartic acid, asparagine and glutamine under salinity represents an active physiological response to a decrease in water potential. The higher aspartic acid, asparagine, and glutamine contents under salt stress were also responsible for the elevated nitrogen level in plants (Ashraf and Harris, 2004). Reports have stated that improved nitrogen nutrition may help in reducing the toxic effects of Na^+ by reducing its uptake (Evelin et al., 2009). Subsequently, the increase in aspartic acid and asparagine are evidence of higher production of alanine and GABA (Queiroz et al., 2012). In this study, a significant increase in alanine and GABA content in mycorrhizal plants under salt stress would be associated with elevated aspartic acid and asparagine contents. Moreover, in this study we focused on the increase of amino acids under salt stress; however, further investigation into amino acids that did not increase or only increased slightly is required for better understanding of the mechanism.

Proline acts as an osmoprotectant in plants subjected to salt stress (Kishor et al., 1995), and its concentration increases in mycorrhizal soybean plants under different levels of salt stress as observed by Sharifi et al. (2007). Conversely, Porcel et al. (2012) and Latef and Chaoxing (2014) stated that non-mycorrhizal plants accumulate higher proline than mycorrhizal plants under salt stress. In this study, mycorrhizal plants had higher proline content than non-mycorrhizal plants after salt stress. However, prior to salt stress treatment in leaves, proline content was same and in roots it was slightly increased than non-mycorrhizal plants. Proline contents increased after salt stress in both mycorrhizal and non-mycorrhizal plants. Moreover, non-mycorrhizal plants also had higher proline content than mycorrhizal

plants were also reported previously. Latef and Chaoxing (2014) also suggested that the accumulation of proline can serve as a symptom of salt stress or as a sensor of salt tolerance rather than AMF inoculation. So, on the basis of the results of this study, it can be suggested that proline accumulation may associated with salt stress and AMF can increase its quantity under salt stress. On the contrary, proline was less concentrated than other amino acids such as aspartic acid, GABA, and alanine, which all had larger contents. Hence, these amino acids play significant roles in the adaptation of mycorrhizal plants under salt stress. In addition, most of the amino acids in both plants increased in mycorrhizal roots, which is the primary site of salt stress and salt tolerance of plants.

Increased accumulation of organic osmolytes contributes to enhance the stress tolerance of plants (Ahanger et al., 2014). Furthermore, the accumulation of carbohydrates during stressed conditions enhances stress tolerance by maintaining the membrane structure and reducing the chances of ROS formation (Masood et al., 2013; Ahanger et al., 2014). In the present study, the soluble sugar content was also increased in AMF inoculated plants under salt stress. In addition, to this increased accumulation of soluble sugars including monosaccharides and disaccharides was also evident in salt stressed plants and the accumulation was also enhanced by AMF inoculation. Therefore, higher contents of soluble sugars and several amino acids in roots may contribute to the adaptation of the plants to salinity stress by functioning as compatible solute.

In conclusion, AMF inoculation alleviates salt stress by increasing dry weight and increased free amino acid and sugar contents in strawberry and asparagus plants. In addition, the increase in the concentration of several free amino acids in roots is associated with the accumulation of compatible solutes for increasing salt tolerance.

Chapter 1 - Conclusion

In this study, we investigated the establishment of salt tolerance improvement method by arbuscular mycorrhizal fungi (AMF) in several vegetables and elucidation of the mechanisms of salt tolerance improvement. Findings of the study suggested that AMF application alleviated salinity stress in asparagus, tomato and strawberry plants through, better growth and maintained chlorophyll content under salinity stress. Except phosphate contents where it increased in mycorrhizal plants, no significant changes were observed in other mineral contents through AMF application. On the other hand, the lower Na^+ content and Na^+/K^+ ratio in all the investigated site such as, young shoots, old shoots, young leaves, old leave and young petioles and old petioles of tested plants were also suggested salinity tolerance through AMF application. AMF association also can effectively manage oxidative and osmotic stress in plants under salinity stress. The higher antioxidant activity and accumulation of several free amino acids and sugar as compatible solute in plants under stress condition is responsible for oxidative and osmotic stress reduction. Under salinity condition, the excess Na^+ accumulated mostly in older organ of three vegetables but the plant parts were different according with vegetables. Moreover, this accumulation process was depends on plants species and AMF association have no effect with this process. The excess Na localization analysis through SEM-EDX inside strawberry petiole and root tissues revealed, Na decreased in AMF inoculated plants however, the accumulation site (around the vascular bundle) was not particularly different between AMF inoculated and non-inoculated plants in both petiole and root tissues. So, suppression of Na absorption through roots might be the mechanism of salt stress alleviation in AMF inoculated plants rather than to control regulation of Na localization. Roots cell wall cellulose and lignin contents might be act as an apoplast barrier for this Na suppression. Possible salinity stress mechanism through AMF application present in a layout in Fig. 41. The physiochemical responses were observed in this study which

proved AMF as an effective biocontrol tool for the salinity stress management in vegetable plants. The AMF association not only improved the growth but also alleviated salinity stress. In horticultural aspects this findings has significant importance as sustainable agricultural practice. In green house and protective cultivation salinity occurs due to anthropogenic activities with environmental factors that disrupt the hydrologic balance of the soil between water applied (irrigation or rainfall) and water used by crops (transpiration). And the initial effects of salt stress on plant are the reduction of growth rate. In early development stage is sensitive for mostly horticultural crops. So, AMF application in early growth stage can make the horticultural crops stronger in the nursery or green house and improved performance under saline condition. Previously, we confirmed that in field experiment of asparagus plants, AMF association improved growth under 100 mM NaCl salinity condition. In our experiment we also confirmed this phenomenon. The benefit of AMF introduction in the early plant stage prevailed for a long time and expected to better yield over time. Information on the tolerance mechanism is useful for developing new cultivation procedures that are adaptable in salinity environments. Although, defining salinity tolerance is quite difficult because of the complex nature of salt stress and the wide range of plant responses. The use of exogenous protectants such as AMF under salt stress condition has been found to be very much effective to alleviate salt induced damages.

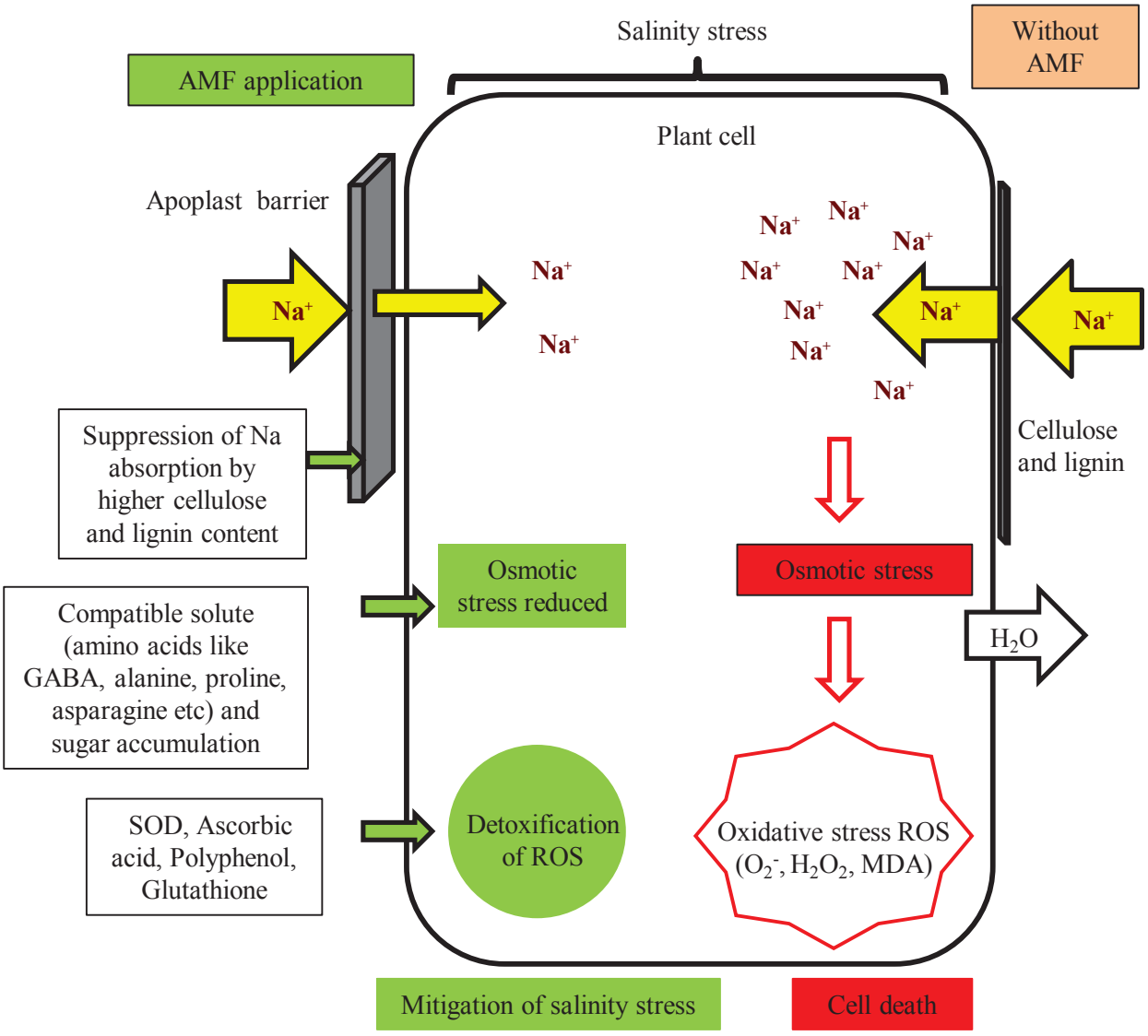


Fig. 44. Possible salinity stress mitigation mechanism in mycorrhizal plants.

CHAPTER 2

Cross-protection to salinity and disease with antioxidant changes in mycorrhizal vegetable plants

Introduction

Among abiotic stresses, soil salinity is considered as one of the most limiting factors for plant growth, and it has been estimated that 7% of the global land is higher salt concentrations which lead to perturbation of plant growth and development (Porcel et al., 2012; Ruiz-Lozano et al., 2012). During salinity stress within a plant, major processes, such as protein synthesis, energy, lipid metabolism and photosynthesis are disrupted (Evelin et al., 2009). On the other hand, salt stress also increases susceptibility of plants towards various phytopathogens and causes disease such as; stem and fruit blight of peppers (Sanogo, 2004), fusarium wilt on strawberry (Myeong et al., 2005), foot and root rot of cucumber (Egamberdieva et al., 2011) etc. Therefore, the response of plants to a single stress can be very different from that of plants to the conditions encountered in the field in which a number of different stressors often occur simultaneously (Rizhsky et al., 2004). So, methods which provide protection of plants from several stress conditions independent from plants are very important in agricultural production.

Arbuscular mycorrhizal fungi (AMF) are the most prevalent type of mycorrhizal fungi and form a mycorrhizal symbiosis with a wide range of vascular plants including many important crop species (Ruiz-Lozano et al., 2012). Also, they can alleviate plant stress caused by abiotic as well as biotic factors. Mycorrhizal symbiosis has been demonstrated to increase salinity tolerance in plants such as tomato and maize (Al-Karaki, 2000; Sheng et al., 2011) nevertheless, many unclear points remain in mycorrhizal salt tolerance mechanism (Evelin et al., 2009; Ruiz-Lozano et al., 2012). On the other hand, biocontrol effect of AMF has been observed in plant species against causal pathogens and most of were consisting of soil-borne fungal pathogens like species of *Macrophomina, Phytophthora, Pythium, Rhizoctonia* etc. (Pozo and Azcon-Aguilar, 2007; Jung et al., 2012). However, the exact mechanisms by which AMF colonization confers the protective effect are not completely understood. Furthermore,

very little attention has been paid to how AMF symbiosis response to combination stresses in plants.

In asparagus cultivation, Fusarium root rot disease caused by *Fusarium oxyporum* f. sp. *asparagi* (Foa) is a major factor for asparagus decline (Elmer, 2015). This factor is difficult to control because no resistant cultivars or disinfecting method has been developed. Chemical control of Fusarium diseases was also attempted by treatment of sodium chloride (Reid et al., 2001). However, it remains unclear what the mechanisms of disease tolerance are against pathogens in excess NaCl and AMF treated asparagus plants.

Strawberry cultivation is suitable for both greenhouse and opens field condition and it has high capability to adapt to diverse ecologic conditions. In particular, salinization is a serious problem in greenhouse conditions due to the fact that a certain area of space is used continuously and intensively with the intense use of salt included fertilizers and high evaporation induced gradual build up salt in plants root gone (Yildirim et al., 2009). On the other hand, Fusarium wilt caused by *Fusarium oxysporum* f. sp. *fragariae* (Fof) under salinization aggravates the strawberry production (Myeong et al., 2005). Therefore, it is important to develop strategies against salt stress and Fusarium wilt under salinity to maintain potential yields in strawberry cultivation.

Accumulations of reactive oxygen species (ROS) in plant body as a result of different environmental stresses causes significant damage to essential macromolecules such as photosynthesis apparatus, pigments, protein, nucleic acid and lipid. To overcome this negative consequence of ROS, plants have evolved various protective mechanisms either to reduce or completely eliminate antioxidative abilities to produce antioxidative enzymes and substances under environmental stresses (Sahoo et al., 2007). However, the accumulation of ROS and the antioxidant activities under dual stresses through mycorrhization have not been reported. So, the aim of this study was to check cross-protection against the combination of

salt stress and disease caused by *Fusarium* spp. in mycorrhizal strawberry and asparagus plants association with antioxidative changes.

Materials and methods

Plant materials and AMF inoculation: In this experiment asparagus (*Asparagus officinalis* L., cv. Welcome) seeds and strawberry (*Fragaria* × *ananassa* Duch., cv. Tochiotome) plants were planted in plastic pots (13.5×27.0×15.5 cm for asparagus, 10.5 in diameter for strawberry) containing autoclaved (121ºC, 1.2 kg/cm^2, 30 min) commercial potting media SM-2 (Ibigawa Industry Co. Ltd., Japan) (Fig. 45). The potting media contain Canadian sphagnum peat moss 85%, perlite, vermiculite, dolomitic and calcitic limestone and wetting agent. In the meantime, plants were inoculated with AMF (*Gigaspora margarita*) inocula 5 g/plant for mycorrhizal plants and autoclaved inocula for non-mycorrhizal plants. Commercial AMF inocula (supplied by Centralgrass Co. Ltd. Tokyo, Japan) were used in this study. One weeks after plantation, plants were fertilized with a slow release granular fertilizer (Long total 70 type, JCAM AGRI Co. Ltd., Japan) at the rate of N:P:K = 13:11:13, 1 g/pot and grown in a greenhouse at 25 ± 3ºC with a 12-13 h photoperiod (750-1000 µmol/m^2/s) and 60-70% relative humidity.

Treatment with NaCl: The plants were subjected to salinity stress with 200 mM NaCl solution after nine (strawberry) and fourteen (asparagus) weeks of AMF inoculation. Plants were irrigated with NaCl solution (50 ml/plant) for twelve days. The no salt-treated/control plants received an equal amount of distilled water. After salinity stress, half of both non-mycorrhizal and mycorrhizal plants were uprooted and rest of the plants were used for inoculation of *Fusarium* spp. pathogen.

Inoculation of *Fusarium oxysporum* f. sp. *fragariae* (Fof) and *Fusarium oxyporum* f. sp. *asparagi* (Foa): The isolate of Fof strain (2S, derived from the diseased strawberry plants) and Foa (MAFF305556) were grown on potato-dextrose agar medium and incubated at 25ºC

for 2 weeks in dark condition to prompt sporulation. The conidia were harvested in potato-sucrose liquid media and incubated at 25ºC in the dark for 7 days. The conidial suspension was sieved (45 µm) and the concentration adjusted to 10^6 conidia/ml. Then both non-mycorrhizal and mycorrhizal plants were inoculated with 50 ml/plant of conidial suspension on to the soil. Symptoms of disease were evaluated 3 weeks after Fof and Foa inoculation.

Experimental setup: This experiment contains eight treatments for both plants; non-mycorrhizal (N), AMF (mycorrhizal), N + NaCl, AMF + NaCl, N + Fof/Foa, AMF + Fof/Foa, N + NaCl + Fof/Foa, AMF + NaCl + Fof/Foa; each treatment contains 10 plants with three replications arranged in completely randomized design.

Plant growth and mycorrhizal colonization: The dry weight of shoots and roots of strawberry and asparagus (10) plants were measured after drying plant materials at 60-70ºC for 24 h. AMF colonization levels were checked after salt stress and both Fof and Foa inoculation. In this case, lateral roots were preserved with 70% ethanol and stained with trypan blue according to Philips and Hayman (1970). The rate of AMF colonization in 1cm segments of lateral roots (RFCSL) was calculated. Hence, RFCSL expresses the percentage of 1cm AMF-colonized segments to the total 1cm segments of all lateral roots; the number of total segments was approx. 50/plant. Average colonization level was calculated from the values of 20 plants.

Estimation of symptoms of Fusarium wilt and determination of population on strawberry plants: Three weeks after Fof inoculation with salt stress, the disease symptoms were categorized into five degrees: percentages of roots with the lesion in a plant: 0, no symptom; 1, <25%; 2, 25-50%; 3, 50-75%; 4, 75-100%. The disease index was calculated by following formula:

$$\text{Disease index} = \frac{\sum(\text{number of plants} \times \text{the number of the degree in symptom})}{\text{total number of plants} \times 5\ (\text{maximum degree in symptom})} \times 100$$

Three weeks after Fof inoculation, rhizosphere soil and roots were sampled. One gram root sample was diluted to 10^{-4} and 1g rhizosphere soil sample was diluted to 10^{-6} with distilled water. Komada's medium (Table 7) selective for *Fusarium oxysporum* (Komada, 1975) was used to determine the population expressed as colony forming units (CFU) at 25ºC for 5 days in the dark condition.

Estimation of symptoms of Fusarium root rot and determination of population on asparagus plants: Three weeks after Foa inoculation with salt stress, the disease symptoms were categorized into five degrees: of percentages of roots with the lesion in a plant: 0, no symptom; 1, <25%; 2, 25-50%; 3, 50-75%; 4, 75-100%. The disease index was calculated by following formula:

$$\text{Disease index} = \frac{\sum(\text{number of plants} \times \text{the number of the degree in symptom})}{\text{total number of plants} \times 5\ (\text{maximum degree in symptom})} \times 100$$

Two weeks after Foa inoculation, rhizosphere soil and roots were sampled. One gram root sample was diluted to 10^{-4} and 1g rhizosphere soil sample was diluted to 10^{-6} with distilled water. Komada's medium was used to determine the population expressed as colony forming units (CFU) at 25ºC for 5 days in the dark condition.

Na^+ and K^+ content and analysis of antioxidants activities: Plants of all treatments were partitioned into shoots/leaves and roots and all samples were frozen in liquid nitrogen until use. Na^+ and K^+ contents in shoots/leaves and roots after NaCl treatment were measured using Compact Na^+ (B-722) and K^+ (B-731) meter, (Horiba Ltd., Tokyo, Japan). Antioxidant activities were analyzed by the following methods. Superoxide dismutase (SOD), ascorbate peroxidase (APX), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and ascorbic acid contents was determined according to the method of Beauchamp and Fridovich (1971) (Fig. 4), Wu et al. (2006) (Fig. 46), Bruits and Bucar (2000) (Fig. 5) and Mukherjee

and Choudhuri (1983) (Fig. 6), respectively.

Statistical analysis: AMF colonization, Dry weights, antioxidants analysis and others were analyzed by Tukey's test at $P \leq 0.05$. All analyses were performed using XLSTAT 2012 pro statistical analysis software (Addinsoft, New York).

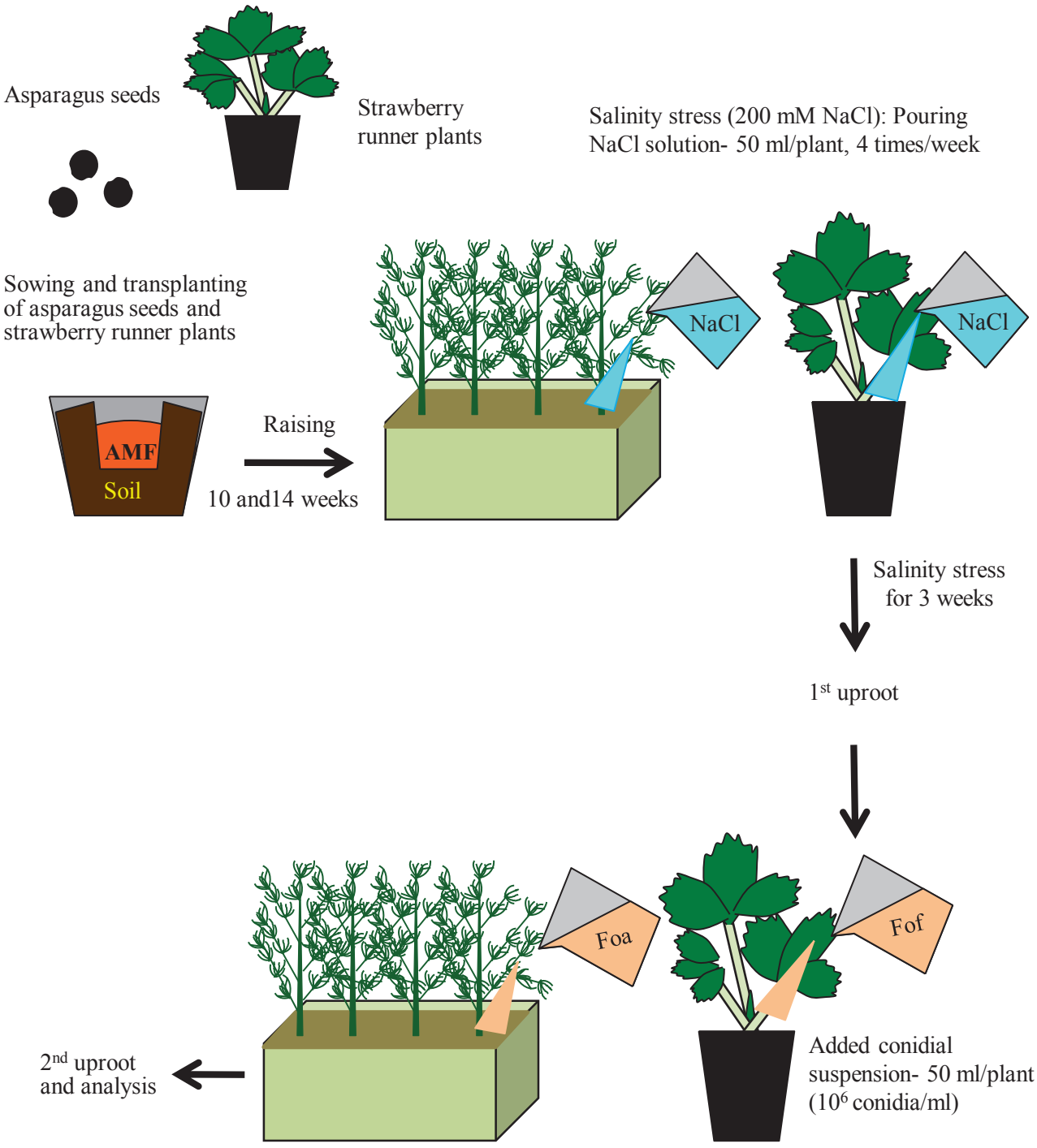


Fig. 45. AMF application, NaCl treatment and Fusarium application in asparagus and strawberry plants. AMF, arbuscular mycorrhizal fungus; NaCl, NaCl 200 mM; Fof, *Fusarium oxysporum* f. sp. *fragariae*; Foa, *Fusarium oxysporum* f. sp. *asparagi*.

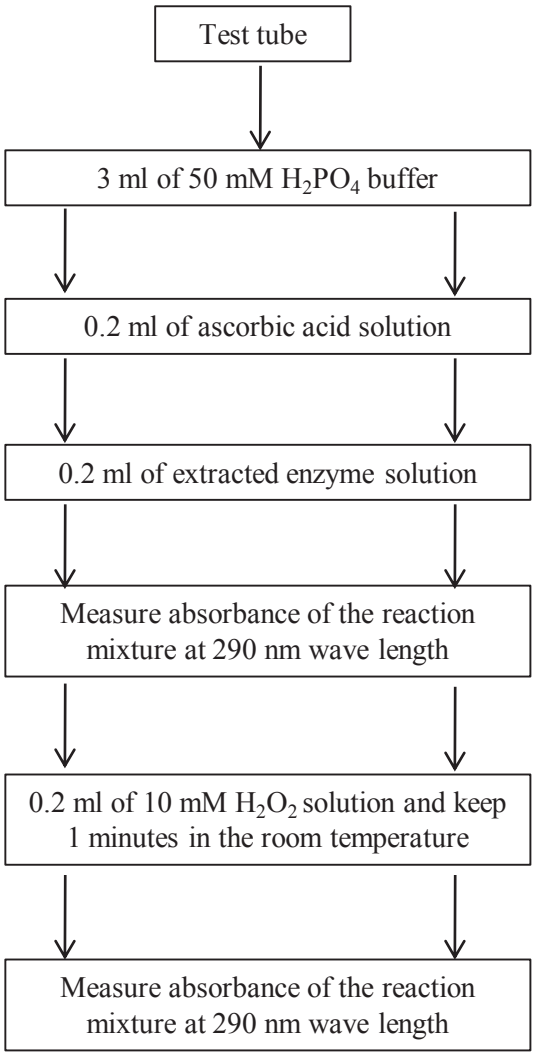


Fig. 46. Flow diagram of the procedures in APX analysis.

Chemicals/compounds	Quantities (g/l)	Remarks
K_2HPO_4	1.0	
KCl	0.5	
$MgSO_4 \cdot 7H_2O$	0.5	
Fe-Na-EDTA	0.01	
L-asparagine	2.0	Pentachloronitrobenzen, $Na_2B_4O_7.10H_2O$, cholic acid sodium salt and streptomycin sulphate was added finally when the medium was cooled after autoclaved. Finally, pH was adjusted to 3.8±0.2 with 10% H_3PO_4.
D-galactose	20.0	
Agar	15.0	
Pentachloronitrobenzene	1.0	
$Na_2B_4O_7 \cdot 10H_2O$	10.0	
Cholic acid sodium salt	0.5	
Streptomycin sulphate	0.3	

Results

Plant growth response: The dry weight of shoots and roots in all the mycorrhizal strawberry and asparagus plants became significantly higher than non-mycorrhizal plants in without stress, salinity, disease and dual stress conditions except roots of strawberry plants under salinity stress (Fig. 47, 48, 49).

AMF colonization: The microscope observation confirmed AMF colonization successfully occurred in all inoculated plants and non-inoculated plants had no colonization (Fig. 50). Highest colonization occurred in without stress condition. After salinity stress, colonization reduced significantly in strawberry plants, whereas in asparagus it was not significantly decreased. After pathogen inoculation in both plants, colonization reduced in single and with dual stress condition.

Disease incidence, index and population of Fof and Foa in roots and rhizosphere soil: Two weeks after Fof and Foa inoculation under with and without salinity conditions, disease symptoms were observed in all the plants. Hence, disease incidence reached 100% (severity level: 1, 2 and 3) in non-mycorrhizal strawberry plants and about 70% (severity level: 1) in mycorrhizal plants under without salinity condition, while in dual stress conditions, disease severity level increased 4 in 85% and 3 in 40% in non-mycorrhizal and mycorrhizal plants, respectively (Fig. 51A). The disease index was significantly reduced in mycorrhizal plants than the non-mycorrhizal plants under both with and without salt stress (Fig. 51B). In asparagus, disease incidence reached 100% in both non-mycorrhizal and mycorrhizal plants {severity level: 1(60%) and 2(40%) for non-mycorrhizal and severity level: 1(90%) and 2 (10%) in mycorrhizal plants} (Fig. 52A). On the other hand, under salinity condition, disease severity level increased 4 in 65%, 3 in 15% and 2 in 10% of non-mycorrhizal plants. In mycorrhizal plants, about 72% had severity 2 and 28% had severity level 3. In addition, the disease index was significantly reduced in mycorrhizal plants than the non-mycorrhizal plants

under both with and without salt stress (Fig. 52B). As for population of Fof and Foa in strawberry and asparagus plants roots and rhizosphere soil, CFU were lower in mycorrhizal plants compared to non-mycorrhizal plants under both no salt and salt conditions (Table 8 and 9).

Na^+ content and Na^+/K^+ ratio: Salt application increased Na^+ content in shoots/leaves and roots of asparagus and strawberry plants in both AMF inoculated and non-inoculated conditions (Fig. 53). However, mycorrhizal plants accumulated lesser Na^+ than non-mycorrhizal plants. In the case of Na^+/K^+ ratio mycorrhizal plants had lower ratio compared to non-mycorrhizal plants under salinity stress (Fig. 54).

Antioxidant activities: As for antioxidants activities, mycorrhizal plants significantly enhanced the enzymatic antioxidants SOD and APX activity in the leaves/shoots and roots under salinity, disease and dual stress conditions in comparison to non-mycorrhizal plants for both strawberry and asparagus (Fig. 55 and 56). Regarding the DPPH radical scavenging activity in leaves/shoots and roots mycorrhizal plants had higher activity compared to non-mycorrhizal plants under without stress, salinity, disease and dual stress conditions (Fig. 57). Similarly, non-enzymatic antioxidant, ascorbic acid contents significantly increased in leaves/shoots and roots of mycorrhizal plants under salinity, disease and dual stress conditions for both plants (Fig. 58).

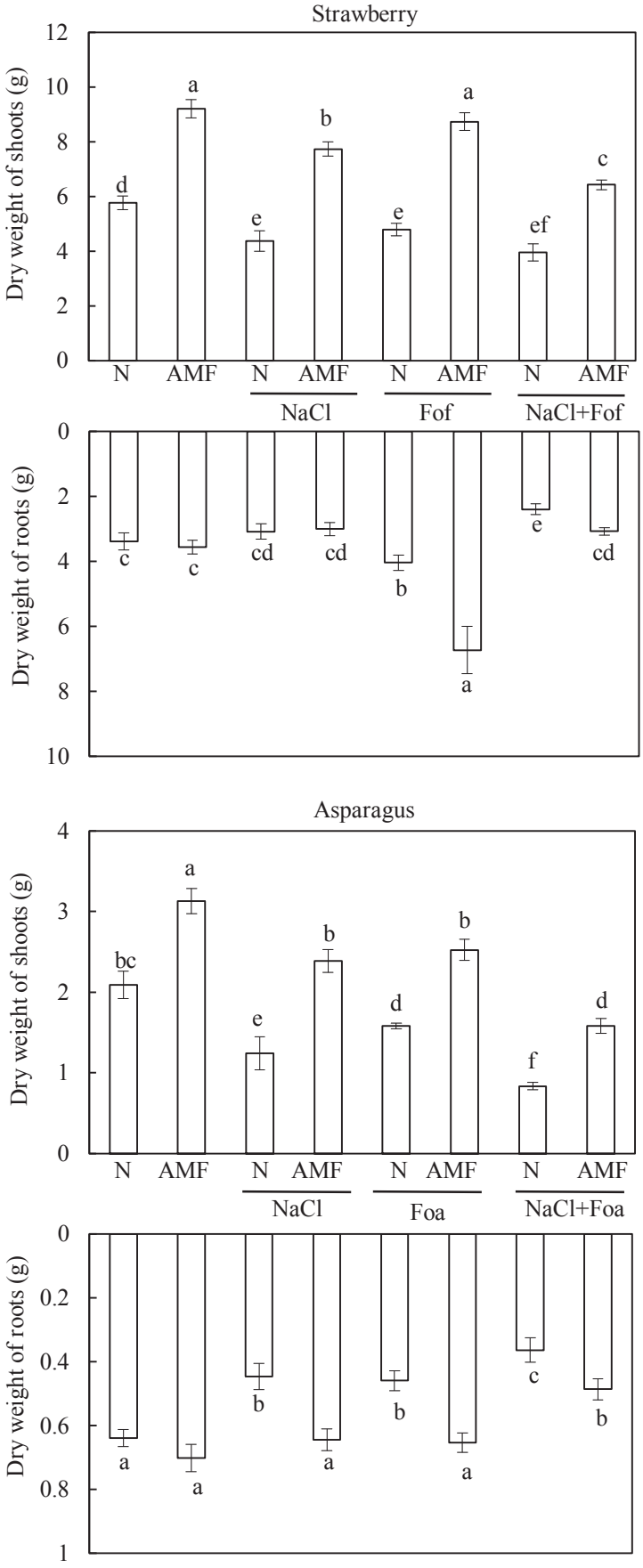


Fig. 47. Dry weight of shoots and roots in strawberry and asparagus plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl; Fof, *Fusarium oxysporum* f. sp. *fragariae*; Foa, *Fusarium oxysporum* f. sp. *asparagi*. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

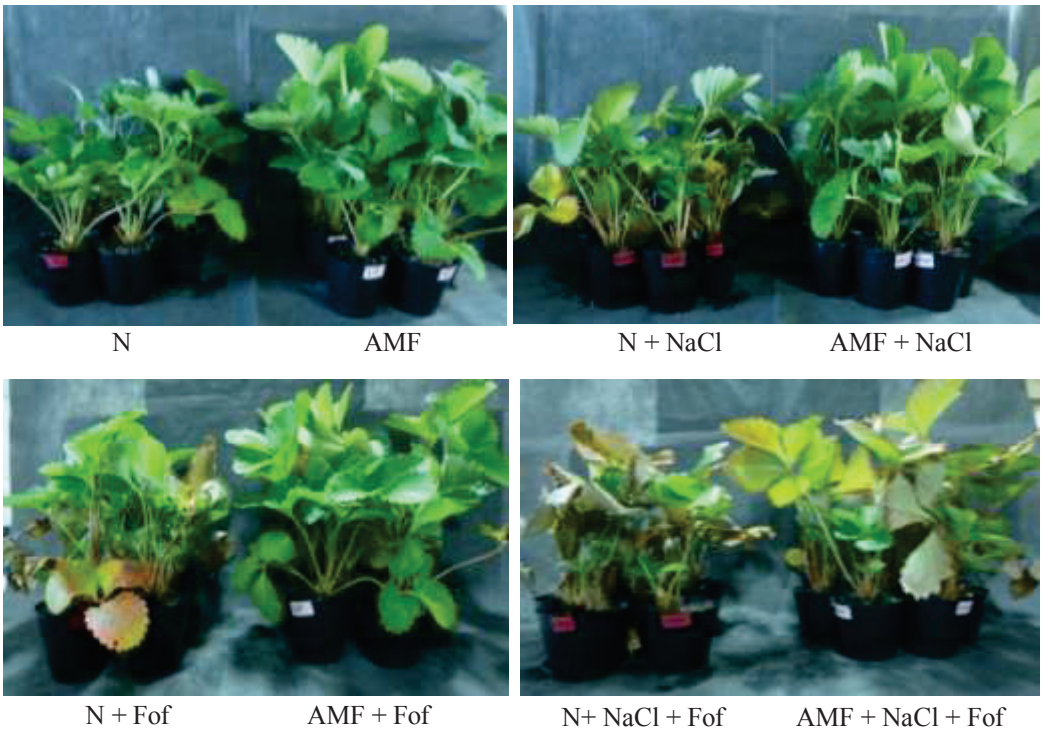


Fig. 48. Effect of AMF symbiosis on Fusarium wilt under salinity stress in strawberry plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; Fof, *Fusarium oxysporum* f. sp. *fragariae*; NaCl, NaCl 200 mM.

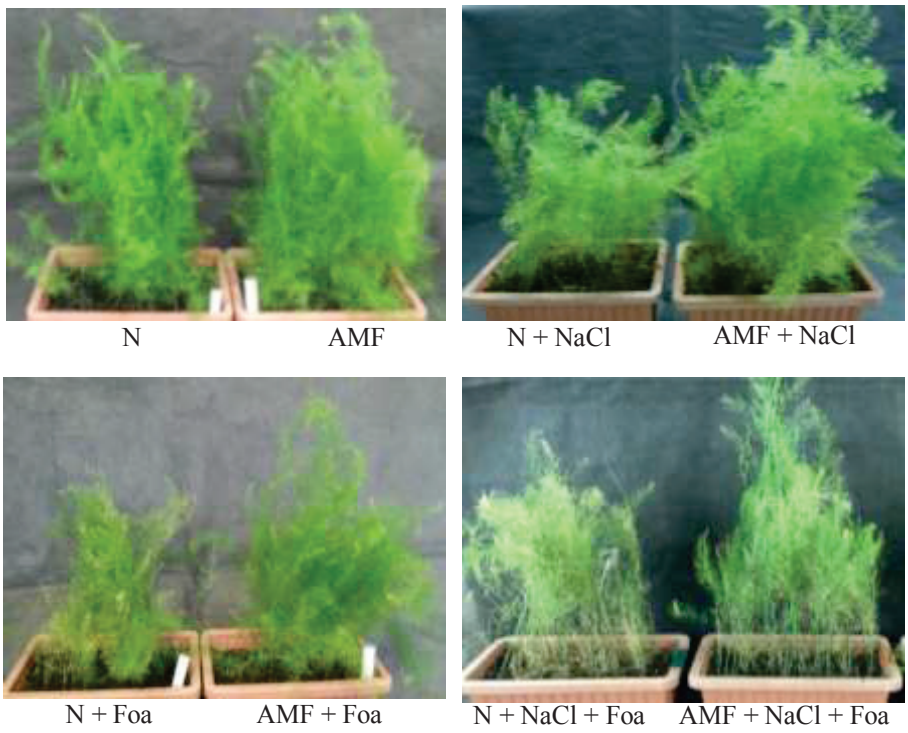


Fig. 49. Effect of AMF symbiosis on Fusarium root rot under salinity stress in asparagus plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; Foa, *Fusarium oxysporum* f. sp. *asparagi*; NaCl, NaCl 200 mM.

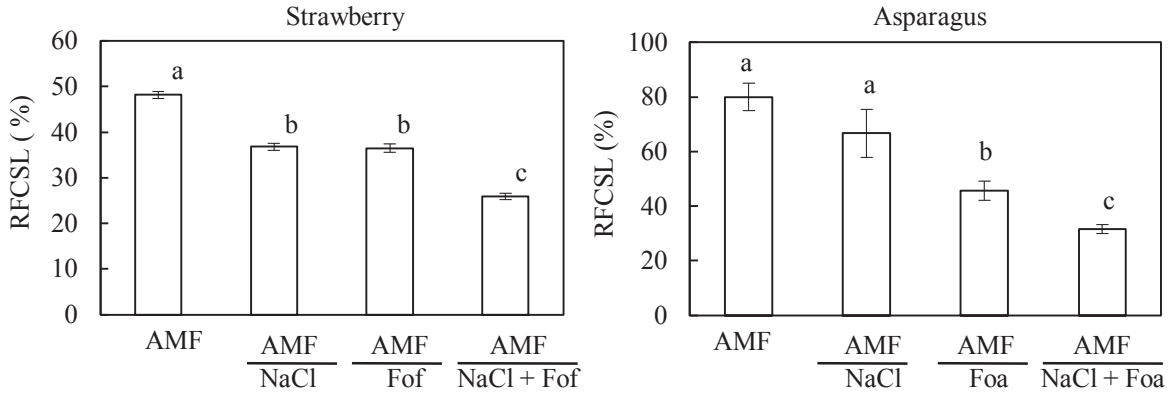


Fig. 50. AMF colonization level (RFCSL) in strawberry and asparagus plants. AMF, mycorrhizal plants; NaCl, 200 mM NaCl; Fof, *Fusarium oxysporum* f. sp. *fragariae*; Foa, *Fusarium oxysporum* f. sp. *asparagi*. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

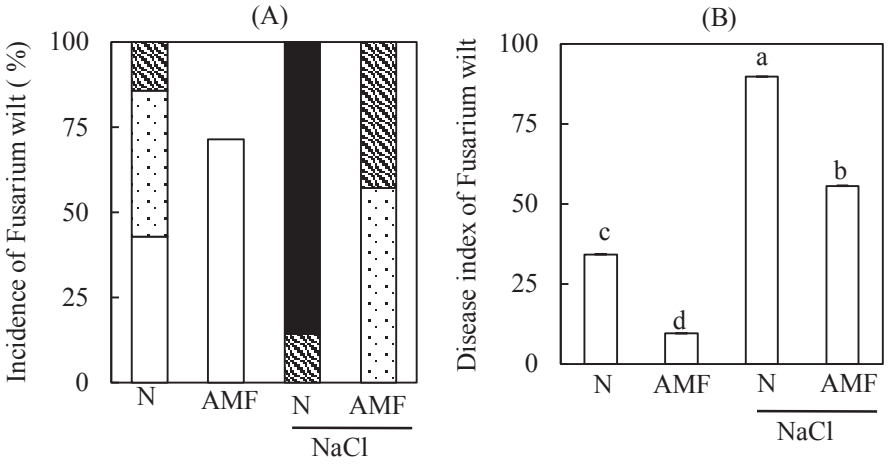


Fig. 51. Incidence (A) and disease index (B) of Fusarium wilt in strawberry roots. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl; Fof, *Fusarium oxysporum* f. sp. *fragariae*. Ratio of diseased roots in a root system: □ , <25% ; ⊡ , 25-50%; ▨ , 50-75%; ■ , 75-100%. Bars represent standard errors (n=40). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

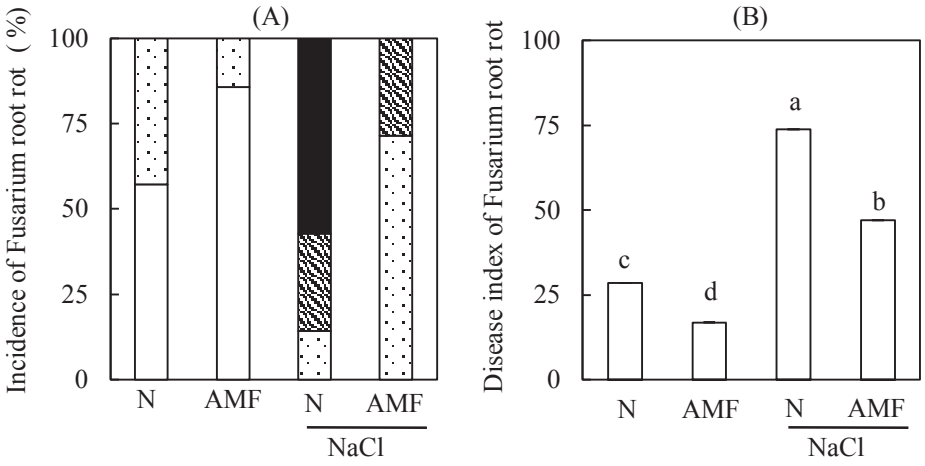


Fig. 52. Incidence (A) and disease index (B) of Fusarium root rot in asparagus. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl; Foa, *Fusarium oxysporum* f. sp. *asparagi*. Ratio of diseased roots in a root system: □, <25%; ⬚, 25-50%; ▨, 50-75%; ■, 75-100%. Bars represent standard errors (n=40). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

Table 8. Population of *Fusarium oxysporum* f. sp. *fragariae* in strawberry roots and rhizosphere soil.

Treatments	CFU ($\times 10^4$/g fresh roots)	CFU ($\times 10^6$/g dry soil)
N	51.5 ± 4.6a	66.25 ± 1.8a
AMF	35.0 ± 1.7b	51.0 ± 0.8c
N+NaCl	29.5 ± 1.8c	55.6 ± 2.0b
AMF+NaCl	17.5 ± 1.2d	40.5 ± 2.6d

Values are means ± SE (n=10). N, non-mycorrhizal; AMF, mycorrhizal plants; NaCl, NaCl 200 mM; CFU, colony forming unit. Data within the same column followed by different letters indicate significant difference according to Tukey's test ($P \leq 0.05$).

Table 9. Population of *Fusarium oxysporum* f. sp. *asparagi* in asparagus roots and rhizosphere soil.

Treatments	CFU ($\times 10^4$/g fresh roots)	CFU ($\times 10^6$/g dry soil)
N	61.5 ± 1.6a	96.2 ± 1.8a
AMF	42.0 ± 1.9c	61.0 ± 2.8c
N + NaCl	49.5 ± 2.8b	72.5 ± 2.0b
AMF + NaCl	35.5 ± 1.2d	53.5 ± 2.6d

Values are means ± SE (n=10). N, non-mycorrhizal plant; AMF, mycorrhizal plants; NaCl, 200 mM NaCl; CFU, colony forming unit. Data within the same column followed by different letters indicate significant difference according to Tukey's test ($P \leq 0.05$).

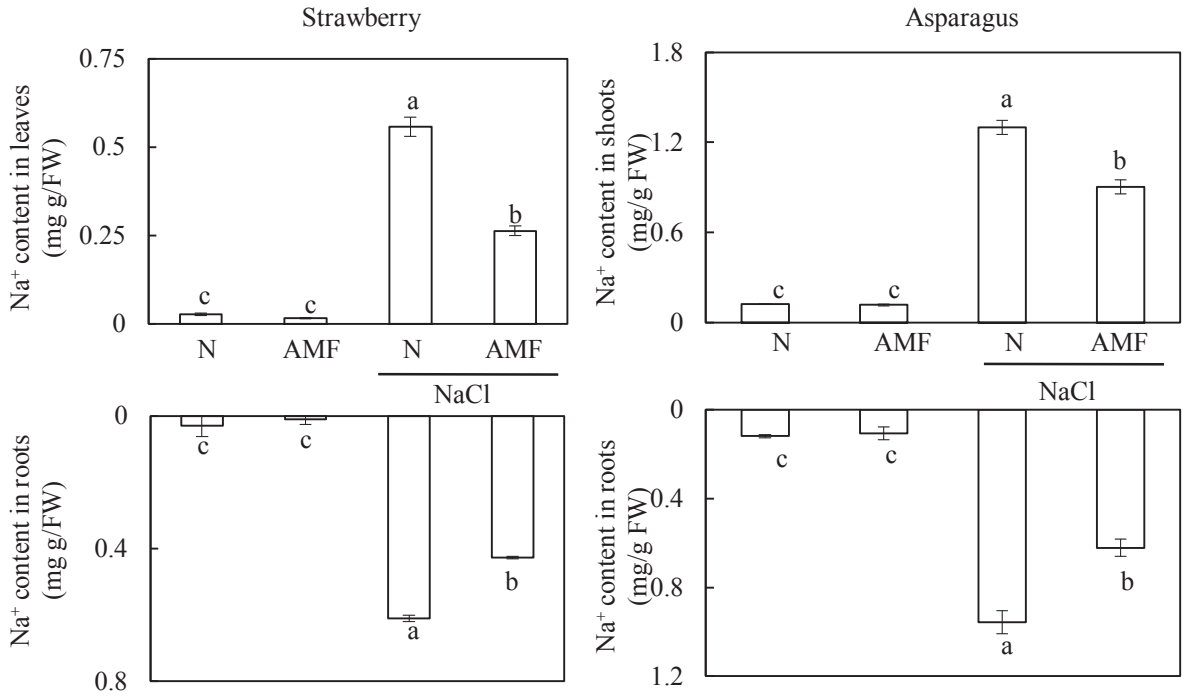


Fig. 53. Na^+ content in leaves/shoots and roots of strawberry and asparagus plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

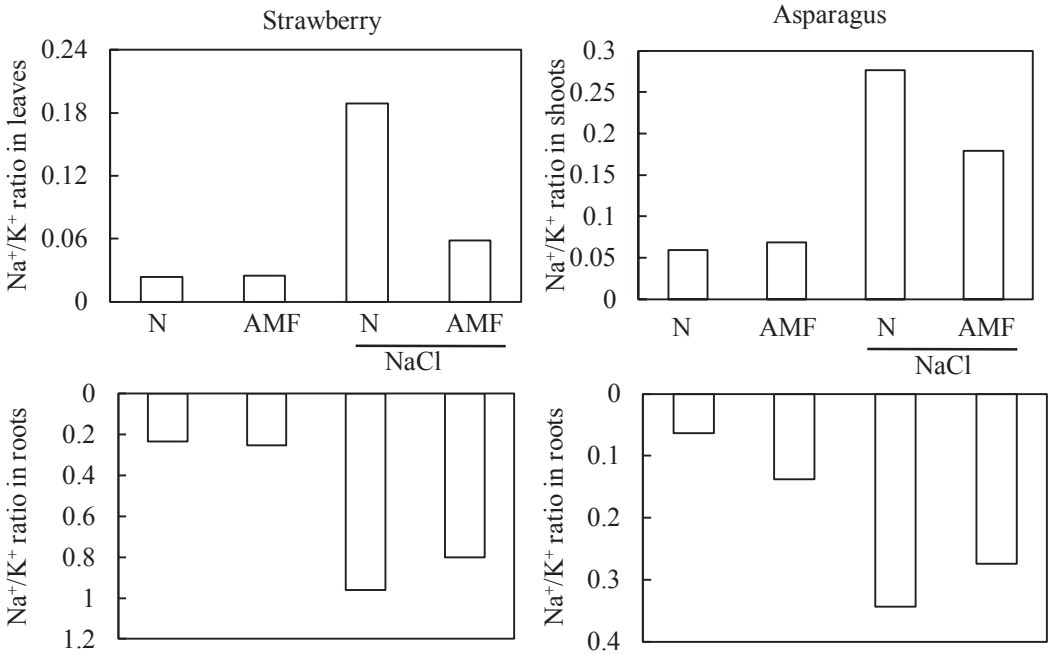


Fig. 54. Na^+/K^+ ratio in leaves/shoots and roots in strawberry and asparagus plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl.

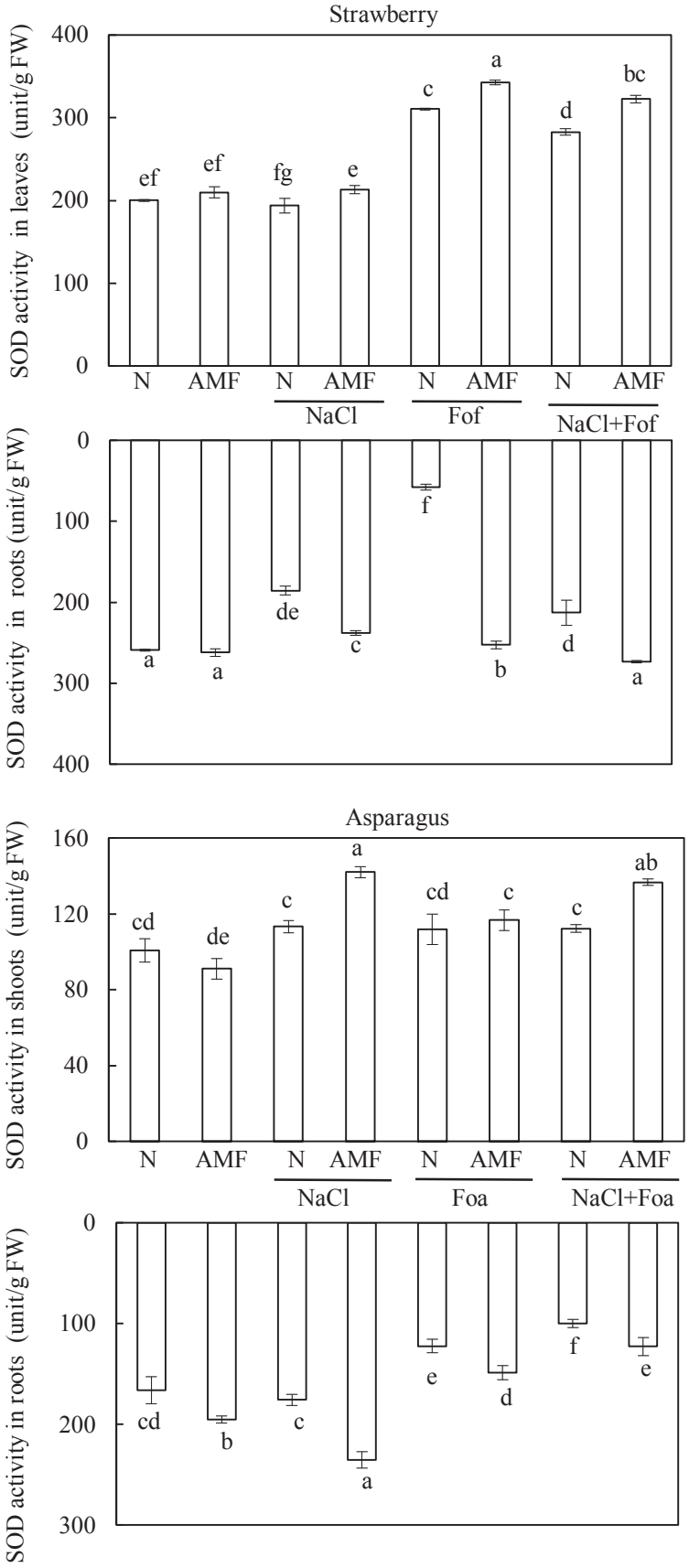


Fig. 55. Superoxide dismutase (SOD) activity in leaves/shoots and roots of strawberry and asparagus plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl; Fof, *Fusarium oxysporum* f. sp. *fragariae*; Foa, *Fusarium oxysporum* f. sp. *asparagi*. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

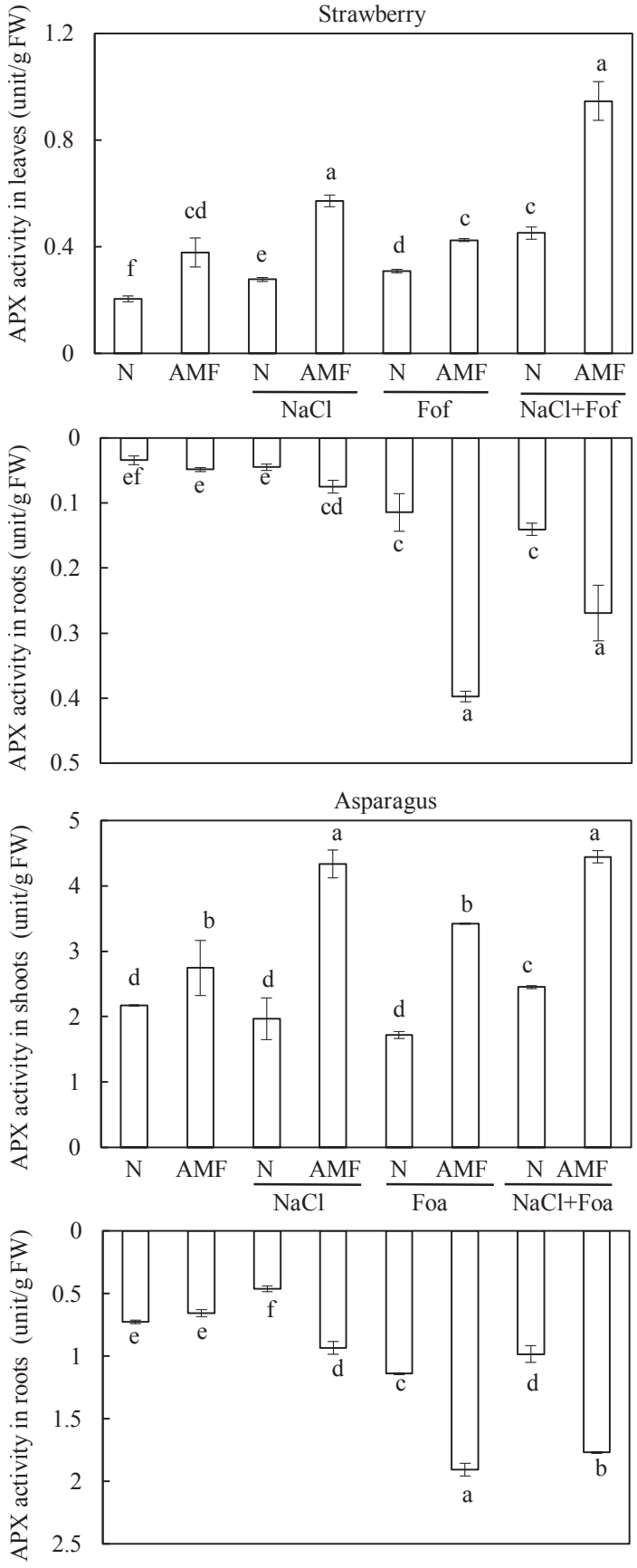


Fig. 56. Ascorbate peroxidase (APX) activity in leaves/shoots and roots of strawberry and asparagus plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl; Fof, *Fusarium oxysporum* f. sp. *fragariae*; Foa, *Fusarium oxysporum* f. sp. *asparagi*. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

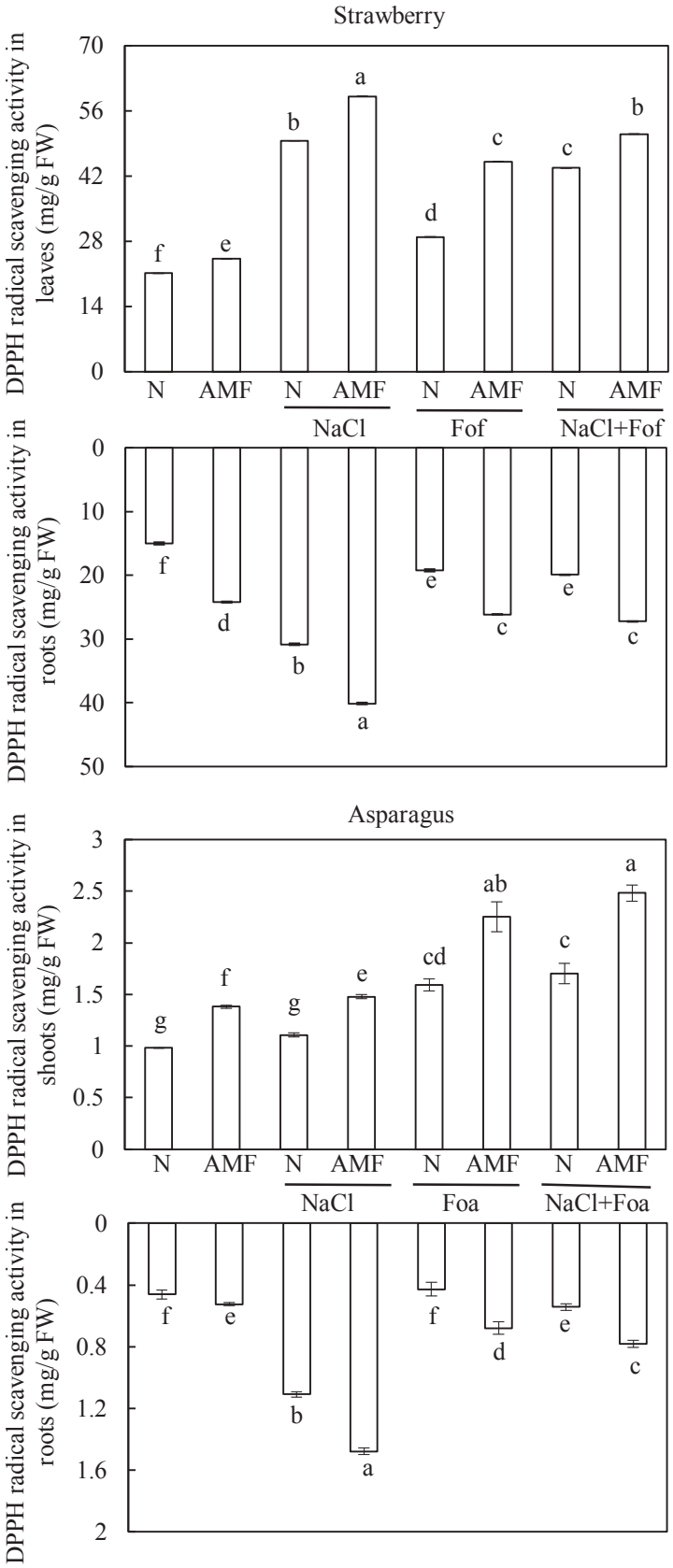


Fig. 57. DPPH radical scavenging activity in leaves/shoots and roots of strawberry and asparagus plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl; Fof, *Fusarium oxysporum* f. sp. *fragariae*; Foa, *Fusarium oxysporum* f. sp. *asparagi*. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

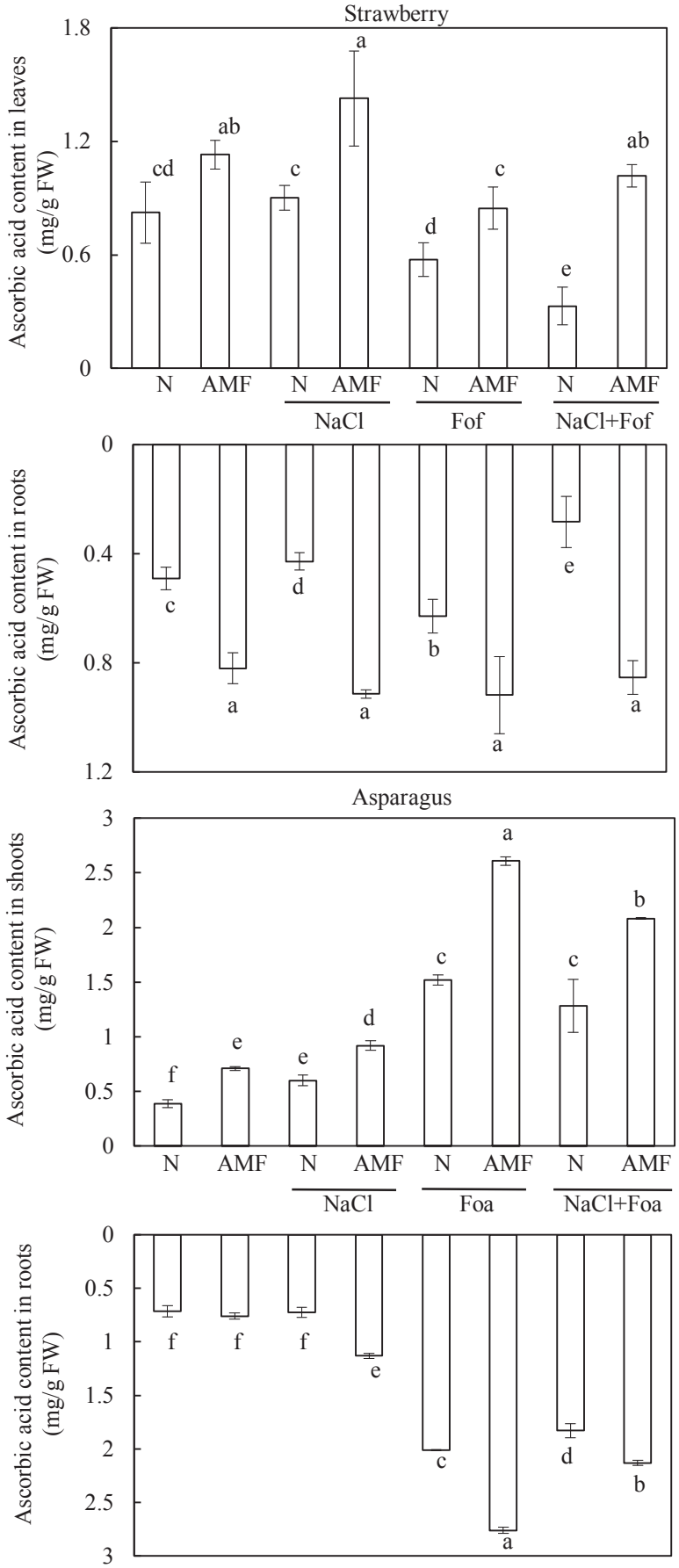


Fig. 58. Ascorbic acid content in leaves/shoots and roots of strawberry and asparagus plants. N, non-mycorrhizal plants; AMF, mycorrhizal plants; NaCl, 200 mM NaCl; Fof, *Fusarium oxysporum* f. sp. *fragariae*; Foa, *Fusarium oxysporum* f. sp. *asparagi*. Bars represent standard errors (n=10). Columns denoted by different letters indicate significant according to Tukey's test ($P \leq 0.05$).

Discussion

The colonization level was significantly lower after salinity, disease and dual stress conditions. This decreased pattern of colonization most likely due to the direct effect of stress factors like salinity and *Fusarium oxysporum* on fungal hyphae growth and spore germination. The dry weight of shoots and roots in all the mycorrhizal plants became significantly higher than non-mycorrhizal plants in salinity, disease and dual stress conditions. The higher dry matter in mycorrhizal plants, suggesting the growth promoting effect through AMF symbiosis appeared in strawberry and asparagus plants. So, pre-inoculation of AMF attenuated single and dual stresses. In Na^+ content and Na^+/K^+ ratio, AMF inoculated plants accumulated lesser amount Na^+ and had low Na^+/K^+ ratio compared to non-inoculated plants under salinity stress. This indicates, excess Na^+ did not entered in AMF inoculated plants and may protect plants from toxic excess Na^+ stress. Under high salinity, low Na^+ and higher K^+ content in mycorrhizal plants were also reported by Latef and Chaoxing (2011) in tomato plants. Higher accumulation of K^+ under salt condition indicates an ionic balance of cytoplasm or Na^+ efflux from plants (Giri et al., 2007). In resume, mycorrhizal strawberry and asparagus plants under salinity stress modifies the absorption of Na^+ and K^+ significantly and alleviates Na^+ toxicity.

The disease incidence and index and population of Fof and Foa were lower in AMF inoculated plants. AMF colonization can decrease the development of fungal root pathogens and severity of disease was mentioned in chili (Alejo-Iturvide et al., 2008) and cyclamen (Maya and Matsubara, 2013b). This study also noticed that under salinity condition, the fungal population decreased in roots and rhizosphere soil in non-mycorrhizal and mycorrhizal plants for both species. Beyond this decrease, the disease severity increased in both non-mycorrhizal and mycorrhizal plants, whereas, mycorrhizal plants showed lower severity than control plants. The reason behind this might be the salinity stress previously weakens plants.

However, mycorrhizal plants reduce salinity and maintain plant growth after pathogen inoculation. The lower CFU is supposed to be explained partially by fungistasis with NaCl and the infection competition between AMF and pathogen. So, AMF reduced disease incidence and the causal pathogen population in rhizosphere after salt stress and subsequently disease stress. Hence, it is suggested that AMF induced cross-protection to salt stress and Fusarium diseases in strawberry and asparagus plants.

Both biotic and abiotic stresses differentially affect plant processes that lead to loss of cellular homeostasis accompanied by the formation of ROS which causes oxidative damage to membrane lipids, proteins and nucleic acids (Srivalli et al., 2003). SOD and APX are important enzymatic antioxidants and ascorbic acid is the effective non-enzymatic antioxidants. Subsequently, DPPH radical scavenging activity is a rapid, simple and inexpensive method to measure the antioxidant capacity in biological compounds and involves the use of the free-radical DPPH (Marxen et al. 2007). They can efficiently prevent the accumulation of O_2^-, OH^-, H_2O_2 and minimize the toxic effects of ROS. SOD activity is considered the most important key enzyme in antioxidative abilities in plants to detoxify superoxide (O_2^-) under any stress condition (Fridovich, 1986). Stimulation of plant SOD activity or enzyme-encoding genes by the AMF symbiosis and more availability of ascorbic acid for reduction of H_2O_2 to H_2O might be the possible causes for higher SOD and APX activity in mycorrhizal plants (Foyer and Halliwell, 1976; Ruiz-Lozano et al., 2012). In addition, a greater response of DPPH radical scavenging activity and higher ascorbic acid content were found in mycorrhizal cyclamen plants under heat stress (Maya and Matsubara, 2013a). The growth of mycorrhizal strawberry and asparagus plants were more vigorous than non-mycorrhizal plants, while the antioxidants productions were higher in mycorrhizal plants. Indeed, plant size affects the overall plant physiology, so that higher amounts of antioxidants in mycorrhizal plants could be associated with improved plant growth, nutrition, and

oxidative damage reduction.

In conclusion, AMF colonization enhanced plants growth, reduced Na^+ accumulation and lower disease incidence compared with non-inoculated plants under salinity, disease and dual stresses. Moreover, the activity of enzymatic antioxidants and higher production of non-enzymatic antioxidants in mycorrhizal plants under single and dual stress conditions imply that the AMF symbiosis can alleviate ROS damages. So, application of AMF could be a successful candidate for protecting plants from dual stress conditions.

Chapter 2 - Conclusion

It is generally true that plant responses to a given environmental stress can be complicated by multiple stresses. In the field, a number of different stresses can occur simultaneously. Such as, the salt stress increases susceptibility of plants towards various phytopathogens and causes diseases. Here we observed the mycorrhiza mediated cross protection from salinity and disease caused by *Fusarium* spp. in strawberry and asparagus plants. Mycorrhizal association showed better plant growth, lower disease incidence and index both salinity and disease conditions. The AMF application also maintained good nutrient status of plants by reducing the excess Na^+ absorption and lower Na^+/K^+ ratio under salt stress. As well as, mycorrhization effectively manage the oxidative stress that increased in plant cell under salinity, disease and dual stress conditions. Mycorrhizal association increased both enzymatic and non-enzymatic antioxidants under single and dual stress conditions. In addition, AMF inoculation reduced the fungal population in the root zone of strawberry and asparagus plants. So, the mechanisms underlying this dual stress tolerance development is increment of antioxidant activities with AMF application. The fungistasis with NaCl and infection competition with AMF might be also responsible for low fungal invasion on plants. In this aspect, in a previous experiment of asparagus declined field affected by *Fusarim oxysporum* f. sp *asparagi* were try to control through NaCl and AMF application. The research found NaCl is a stress factor (100 mM) rather than fungal infection suppression (50 mM) when it was in excess manner in root zone. However, AMF application improved plant growth and reduced withered plants with fungal population at root zone compared with NaCl treatments. Therefore, AMF application induced cross protection from salinity and disease in strawberry and asparagus plants. This knowledge is important to develop sustainable strategies for successful utilization of AMF to improve plant health under a variety of stress conditions.

Summary

Salinity stress is one of the most devastating agricultural problem especially for vegetable cultivation in the Asian region. Because most of the vegetables are sensitive to salinity and soil desalination is time-consuming and costly in practical means. Salinity affects the establishment, growth and development of crops by creating osmotic stress, ion stress and oxidative stress that disrupts the major intracellular processes like photosynthesis, protein and energy metabolism etc. In this study, we investigated the establishment of salinity tolerance improvement method by arbuscular mycorrhizal fungi (AMF) in several vegetables and elucidation of the mechanisms of salinity tolerance improvement. AMF make a symbiotic relationship with the host plant and they can survive under salinity condition. So, the use of AMF for salinity stress amelioration especially in vegetable cultivation could be a cost effective and eco-friendly approach.

The asparagus (*Asparagus officinalis* L., cv. Welcome), tomato (*Solanum* l*ycopersicum* L., cv. Momotaro 8) and strawberry (*Fragaria* × *ananassa* Dutch., cv. Tochiotome) plants were treated with 200 mM NaCl solution. After three weeks under salinity stress, all the plants reduced dry weight, showed leaves and stem browning including low chlorophyll (a+b) content compared to non-treated plants. On the other hand, AMF (*Gigaspora margarita* and *Glomus fasciculatum*) application alleviated salinity stress in all three plants through, increases dry weight, inhibition of browning of leaves and maintained chlorophyll content. Subsequently, the Na^+ content and the Na^+/K^+ ratio were also lower in all the investigated site such as, young and old shoots, leaves, petioles of asparagus, tomato and strawberry plants under salt stress. No significant changes were observed in mineral contents through AMF application except phosphate where, it increased in mycorrhizal plants. Excess Na^+ accumulated mainly in the older organs-like, old petioles and shoots of strawberry and asparagus plants compared to younger parts and it was common for AMF inoculated plants.

To check the excess Na localization inside strawberry petiole and root tissues through scanning electron microscope and energy dispersive x-ray spectroscopy (SEM-EDX) line scan, a nano-suit technique was used for sample preparation. The analysis showed number of Na decreased in mycorrhizal plants however, the accumulation site (around the vascular bundle) was not particularly different between AMF inoculated and non-inoculated plants. So, suppression of Na absorption through roots might be the mechanism of salt stress alleviation in mycorrhizal plants rather than to control regulation of Na localization. In this aspect, fiber components of strawberry root tissues revealed that cellulose and lignin contents were higher in mycorrhizal plants whereas, the pectin content had no significant changed. The fiber components (higher cellulose and lignin contents) of mycorrhizal roots may be act as the apoplast barrier for suppression of Na influx.

As for the ion stress reduction through antioxidants, AMF inoculation reduced MDA and H_2O_2 content compared to non-inoculated plants under salinity stress for all three plants. The SOD and DPPH radical scavenging activity increased in AMF inoculated plants, particularly in shoots for all three types under salt stress. The antioxidant substances such as ascorbic acid, glutathione and polyphenol contents were also increased in AMF inoculated plants, particularly in roots of all three vegetables under salinity stress. So, AMF inoculation reduced oxidative stress in all three vegetables and it has influenced on both antioxidant enzyme and substances. Subsequently, in the salt affected field the diseases caused by Fusarium spp. were severely damaged crop production. Therefore, the cross protection to salinity and disease of strawberry and asparagus plants showed, AMF inoculation reduced disease incidence and index and also increased dry weight under both salinity and disease conditions. As well as, the antioxidants activities were also increased in AMF inoculated plants under salinity, disease and both stress conditions. These results suggested mycorrhization enhanced plant growth by mitigating oxidative stress, resulted in the protection of plants from dual stress conditions.

As for the osmotic stress response through compatible solute such as amino acid analysis using LC-MS in strawberry and asparagus plants showed, total amino acid increased in AMF inoculated plants under salt stress. The several free amino acids such as GABA, asparagines, aspartic acid and alanine etc, increased in particularly roots of AMF inoculated plants than non-inoculated plants. Also in free sugar analysis, accumulation of sucrose, glucose and fructose in strawberries treated with NaCl was confirmed in the mycorrhizal plants. From the above facts, it was suggested that increase of several free amino acids and free sugar content in the mycorrhizal plants under salt stress acted as acceleration of compatible solute accumulation.

In this study, we established a method to improve salinity tolerance independent from vegetable species through AMF application and basic knowledge's on ion stress and osmotic stress response in salinity tolerance induction mechanism was obtained.

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