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ALLEVIATION OF ADVERSE IMPACT OF SALINITY ON FABA BEAN (*VICIA FABA* L.) BY ARBUSCULAR MYCORRHIZAL FUNGI

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Abstract

The present study was conducted to assess the effect of different concentrations of sodium chloride (NaCl) in presence and absence of AMF on growth, physio-biochemical and enzymatic activity in faba bean (*Vicia faba*). Different concentrations of NaCl showed reduction in growth and yield parameters, which indicates the deleterious effects of salinity on the plant. The total spore count and colonization by arbuscular mycorrhizal fungi (AMF) is also decreasing at higher concentrations of NaCl. Application of AMF mitigates the effect of NaCl stress and improved the growth and yield in the present study. NaCl also decreased the nodulation as well as nodule activity and pigments content, however the supplementation of by AMF to plants treated with sodium chloride showed enhancement in nodule activity and pigment content. Polyamines (Putresciene, Spermidine, Spermine), acid and alkaline phosphates increased with increasing concentration of sodium chloride and application of by AMF showed further increase in the above phytoconstituents, proving the protective role of these phytoconstituents against salt stress. Salinity stress is responsible for the generation of reactive oxygen species, which lead to the membrane damage through lipid peroxidation in the present study. Maximum lipid peroxidation was observed at higher concentration of sodium chloride and AMF treatment minimized the effect of salinity on lipid peroxidation. To combat with the reactive oxygen species, plants upregulate the enzymatic antioxidants like superoxide dismutase, catalase, peroxidase and ascorbate peroxidase. As the concentration of sodium chloride increases the enzyme activity also increases and further increase was observed with supplementation of AMF to salt treated plants. Arbuscular mycorrhizal fungi also restores the potassium and calcium contents and maintain their ratio that was hampered with increasing concentration of sodium chloride in the present study. In conclusion, application of AMF could alleviate the negative effect of sodium chloride and can be used as a suitable approach for the salt affected soils.

Keywords: Salt stress; *Vicia faba*; growth, pigments; nodulation; polyamines; lipid peroxidation; enzymatic activity; elemental uptake.

Introduction

In developed and developing countries the intensive use of agricultural practices has led to the degradation of farming land and water supplies (Flowers, 2004) At one hand farming land is degrading and on the other hand the world population is increasing and is expected to be 8.5 billion over the next 25 years. Salinity greatly affected the crop production and has become a major threat to feed the population in near future (Anon, 2010). Saline soil with soluble salts affects plant growth, development and ultimately affects the yield. Salinity not only reduces yield of crops but also disrupts the ecological balance of the area (Aggarwal *et al*., 2012; Alqarawi *et al*., 2014a). Salinity stress lead to low osmotic potential of the soil solution, nutritional imbalance, specific ion effect or combination of all these factors hampers the metabolic activity of the plant (Alqarawi *et al*., 2014b). All the major biochemical reactions related to photosynthesis, protein synthesis etc. are affected by salinity stress (Ahmad *et al*., 2014). The morphological and yield parameters are affected by the increasing concentration of salt content in soil. Osmotic stress is a primary stress caused due to the excess of Na^+ and Cl^-. Salinity is also responsible for secondary stress known as oxidative stress and is caused by reactive oxygen species (ROS) (Ahmad *et al*., 2010). During oxidative stress, electrons that have high-energy state are transferred to molecular oxygen (O_2) to form reactive oxygen species (ROS) (Mittler, 2002). ROS, such as singlet oxygen (^1O), superoxide ions (O^{2-}) and peroxides, the most widely distributed being hydrogen peroxide (H_2O_2) very much toxic to biomolecules like membrane lipids, DNA/RNA, proteins etc. (Apel & Hirt, 2004; Triantaphylidès *et al*., 2008). Oxidative stress is resulted when generation of ROS exceeds the scavenging nature of antioxidants. The toxic effects of ROS are counteracted by enzymatic antioxidants like, superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX). These antioxidants quench the ROS and protects the macromolecules from damage. Normally, each cellular compartment contains more than one enzymatic activity that detoxifies a particular ROS.

Faba bean (*Vicia faba* L.) is an important leguminous crop and is cultivated in maximum parts of the world for human consumption. Cultivation of faba bean has been reported to increase the soil nitrogen compounds to a greater level (Hungria & Vargas, 2000). Being a good source of protein, it becomes the interest of many plant scientists to increase its production especially on marginal lands of the world. The growth and productivity of faba bean are affected by various abiotic stresses such as soil salinity.

World scientists are looking for alternatives that will help to maintain the crop yield under stressful conditions. One of the alternatives is use of arbuscular mycorrhizal (AM) fungi that acts as growth regulator and mitigates the harmful effects of salt stress on plants. AM fungi form symbiotic associations with most of the plants and enhance the tolerance capacity to withstand the abiotic stresses including salinity. AM fungi supply mineral nutrients to plants, especially phosphorus, which is precipitated by the ions such as Ca, Mg, Zn (Alqarawi *et al*., 2014c). AM fungi plays an important role in membrane stability, stimulated plants to produce their own defense compounds, enhance the photosynthetic pigments and maintains the osmotic and ionic balance of the cell.

The aim of this study was to evaluate the ability of AMF to improve growth and biomass yield in faba bean under different concentrations of NaCl. Different parameters related to NaCl stress like pigment system, nodulation, polyamines, antioxidants and elemental analysis have been studied in the present study.

Materials and Methods

The pot experiment: Seeds of faba bean (*Vicia faba* L.) variety Giza 716 were obtained from Agricultural Research Center, Giza, Egypt. This study was conducted under controlled conditions at the Botany and Microbiology Department, College of Science, Zagazig University, Zagazig, Egypt. The seeds were surface sterilized with sodium hypochlorite (0.5%, v/v) for 3 min, washed thoroughly with distilled water before germination on blotter. Healthy germinating seeds were transferred to pots (1 plant/pot) with normal soil in one set of experiments and soil of 2nd set of experiment with amended with arbuscular mycorrhiza fungi (AMF). The biofertilizer (*Rhizobium leguminosarum* bv. *viciae* Frank) was added to the germinated seeds as thin film of Peat Inoculant (2×10^8 CFU/g) at rate of 4 g Inoculant / Kg Seed. Hoagland's solution (Hoagland & Arnon 1950) was used for irrigation with different concentrations of NaCl to get concentration of 0, 50 and 100 mM. The NaCl solution was applied every alternate day and to maintain the moisture content of the soil, 200 ml of distilled water was applied to each pot every day. The seedlings were grown for eight weeks at 20 ± 1^oC with 12 h light (750 µmol m^{-2} S^{-1}) and 12 h dark photo-cycle, and RH of 70-75% after transplantation. The pot experiment was carried out by split-plot in randomized complete block design with five replications. At the end of pot experiment (8 week), the plants were harvested carefully, washed in distilled water, separated into shoots and roots. For the estimation of dry weight, the samples were dried at 70^oC for 48 h and dry weight was recorded. Leaf samples were used for estimation of photosynthetic pigments, free polyamine and enzymes activity. Fresh root samples were used for mycorrhizal studies.

The mycorrhizal inoculums: The selected AM fungi [*Funneliformis mosseae* (syn. *Glomus mosseae*); *Rhizophagus intraradices* (syn. *Glomus intraradices*), and *Claroideoglomus etunicatum* (syn. *Glomus etunicatum*)] were inoculated singly with wheat plants using autoclaved soil (clay:sand, 1:1, w/w) as a host plant for three generations in pot cultures in a greenhouse for propagation of fungal spores. The growth conditions were 25^oC day/20^oC night temperatures, 65% relative humidity, 16/8 h light/dark photo period cycle with a photosynthetic photon flux density of (750 µmol m^{-2} S^{-1}). Hoagland's solution without phosphorus source (free phosphorus) used for irrigation to enhance mycorrhizal colonization. AM fungal spores were extracted from the trap cultures as described above to develop pure AM fungal cultures. Fungal inoculums potential was determined by the most probable numbers method (Alexander, 1982), and each trap culture contained ~10.2 X 10^3 propagules per pot (1 Kg capacity). Fungal inoculums consisted of AM fungal spores, hyphae, and colonized root fragments. The mycorrhizal inoculum was added to the experimental soil as 10 g of trap soil culture (approx. 100 spores/g trap soil, M = 80%)/ pot (1Kg). Non mycorrhizal soil was used as reference.

Determination of growth parameters: Fresh weight (FW) of root and shoot was taken instantly after harvesting, whereas dry weight (DW) was determined by drying the plant samples at 70 °C for 72 h and then weighed.

Determination of arbuscular mycorrhizal colonization: The mycorrhizal spores were extracted from the experimental soil of each treatment by wet sieving and decanting method as described by Daniels & Skipper (1982) and modified by Utobo *et al*., (2011). Total population of mycorrhizal spore was calculated per hundred gram soil. The root system was washed carefully in ice-cold water (4^oC) to remove the adhering soil. The cleaning of roots was carried out in 10% (w/v) KOH followed by staining with trypan blue in lactophenol. The stained root segments (100 segment/treatment, approximately 25-30, 1 cm long root) were examined under light microscope at 400x magnification. The intensity of fungal infection (mycelium, vesicles and arbuscules) and development within the infected regions of the roots were calculated according to the following formula:

$$\% \text{ Colonization} = \frac{\text{Total number of AM positive segments}}{\text{Total number of segments studied}} \times 100$$

Extraction and estimation of leghemoglobin: Nodules (2.0 g) were ground to a fine powder in liquid N2 and five volumes (per g fresh weight) of 50 mM KPO4 (pH 7.4) buffer that contained 1 mM EDTA was added. The mixture was stirred until it thawed to a homogenate at temperature 2°C and then transferred to centrifuge tubes. Following centrifugation at 4°C and 10,000xg for 10 min. The Leghemoglobin-containing supernatant was collected and mixed with 50 mM KPO_4 (pH 7.4) buffer which contained 1 mM EDTA and the color intensity was recorded spectrophotometrically at 710 nm (T80 UV/VIS Spectrometer, PG Instruments Ltd, USA), 50 mM KPO_4 (pH 7.4) buffer which contained 1 mM EDT was used as blank (Keilin & Wang, 1945).

Determination of photosynthetic pigments: The photosynthetic pigments were extracted from leaves of faba bean plants in dimethyl sulfoxide (DMSO) as described by Hiscox & Israelstam (1979). Absorbance was determined spectrophotometrically at 480, 510, 645, 663 nm (T80 UV/VIS Spectrometer, PG Instruments Ltd, USA) and DMSO was used as blank.

Extraction and determination of free polyamine: Leaf sample (300 mg) were homogenized in liquid nitrogen. The homogenate was resuspended in 1.0 mL of perchloric acid 5% (v/v), incubated in ice for 30 min and centrifuged at 15,000Xg for 15 min. Pellet was discarded and the supernatant was kept at 20°C (solution C). The free polyamine was determined by HPLC according to the method described by Jiménez-Bremont *et al*., (2007).

Estimation of lipid peroxidation: Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid reaction as described by Heath & Packer (1968). Absorbance was recorded at 600 nm and the blank used was 1% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA). The concentration of MDA was calculated using an extinction coefficient of 155 mM cm^{-1}.

Determination of acid phosphatase and alkaline phosphatase activity: The activity of both acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) were determined quantitatively according to Gianinazzi-Pearson & Gianinazzi (1976). Acid phosphatase was assayed by adding 0.2 ml of enzyme extract to 1.0 ml of 5.5 mM p-nitrophenol phosphate in 5.5 mM citrate buffer (pH 4.8). In case of alkaline phosphatase, 0.05M tris-citrate (pH 8.5) was used instead of citrate buffer (pH 4.8) which was used in case of acid phosphatase. The reaction mixture was incubated at 37°C for 30 min., and then 10 ml of 200 mM NaOH was added to stop the reaction. Absorbance was recorded at 410 nm and the amount of p- nitrophenol released was estimated. The assay was performed in triplicate and acid phosphatase activity expressed as µmol p-nitrophenol released min^{-1} mg $protein^{-1}$.

Antioxidant enzymes assays: Fresh leaves (10 g) were homogenized in 50mM sodium phosphate buffer (pH 7.0) containing 1% PVP-40 (Polyvinylpyrrolidone) as described by Malik & Singh (1980). The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C and the supernatant was used for the assays of enzymes activity. Protein in the enzyme extract was estimated according to Lowry *et al*., (1951).

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium adopting the method of Bayer & Fridovich (1987). The reaction mixture consisted of 1 ml of enzyme extract, 40mM phosphate buffer (pH 7.8), 13mM methionine, 75mM NBT, 0.1mM EDTA, and 2µM riboflavin, which was added at the end. After mixing the contents, test tubes were shaken and placed 30 cm below light source consisting of two 20 W fluorescent lamps for 15 min. A tube with protein kept in the dark served as a blank, while the control tube was without the enzyme and kept in the light. The absorbance was measured at 540 nm. The activity of SOD is the measure of NBT reduction in light without protein minus NBT reduction in light with protein. One unit of activity is the amount of enzyme required to inhibit 50% initial reduction of NBT under light. The results were expressed as EU mg-1 protein.

A method of Samantary (2002) was employed for the assay of CAT (EC 1.11.1.6). The enzyme extract (1 ml) was added to 1 ml of H2O2 and 3 ml of 0.1 sodium phosphate buffer (pH 7.0). The reaction was discontinued by adding 10 ml of 2% H2SO4 after 1 min of incubation at 20oC. The reaction mixture was then titrated against 0.01 M KMnO4 to determine the quantity of H_2O_2 used by the enzyme. Enzyme activity was expressed as M H_2O_2 destroyed mg protein-1 min-1.

Peroxidase (POD) (EC 1.11.1.7) activity was assayed spectrophotometrically according to the method of Kar & Mishra (1976). The reaction mixture contained 0.5 ml of enzyme extract, 2.5 ml of 0.1M phosphate buffer (pH 7.0), 1.0 ml of 0.01M pyrogallol and 1.0 ml of 0.005M H_2O_2 were also added. After incubation, the reaction was stopped by adding 1.0 ml of 2.5N H_2SO_4. The amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm. The enzyme activity was expressed as EU mg^{-1} protein.

The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was assayed according to the method of Nakano & Asada (1981). The reaction mixture contained 1.0 ml of reaction buffer [potassium phosphate (pH 7.0) with 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2 and 0.1 ml of enzyme extract]. APX was assayed as decrease in absorbance at 290 nm of ascorbate. APX activity was expressed as unit mg-1 protein. For the calculation of APX enzyme activity, the extinction coefficient of 2.8 mM^{-1} cm^{-1} was used. One unit of enzyme was considered as the amount necessary to decompose 1 mol of substrate min^{-1} at 25°C.

Estimation of ion accumulation: A known weight of oven dry leaf sample were digested and Na^+, K^+ and Ca^{2+} were estimated according to the method of Wolf (1982) using a flame photometer Jenway Flame Photometer, Bibby Scientific Ltd-Stone-Staffs-St15 0SA–UK. Standard curve of each mineral (10-100 µg ml^{-1}) used as reference.

Statistical analysis: Two-way analysis (ANOVA) was used for statistical analysis followed by Duncan's Multiple Range Test (DMRT). The values obtained were the mean ± SE for five replicates in each group. P value at 0.05 was considered as significant.

Results

Growth and biomass yield: The AM fungi used in the present study were illustrated in Fig. 1. The results related to the effect of NaCl and AMF on morphological and yield parameters are depicted in Table 1. All the parameters showed decline with increasing concentrations of NaCl. The shoot length decreases to 40.23%, number of branches per plant to 43.65%, number of pods per plant to 81.60%, pod dry weight to 85.71% and seed weight per plant decreased to 83.16% at 100 mM NaCl stress. The application of AMF proved to have positive role in alleviating the NaCl stress. The decrease of only 25.76%, 5.97%, 40.53%. 35.51% and 45.54% in shoot length, number of branches per plant, number of pods per plant, pod dry weight and seed weight per plant respectively was observed at 100 mM NaCl in presence of AMF as compared to the control.

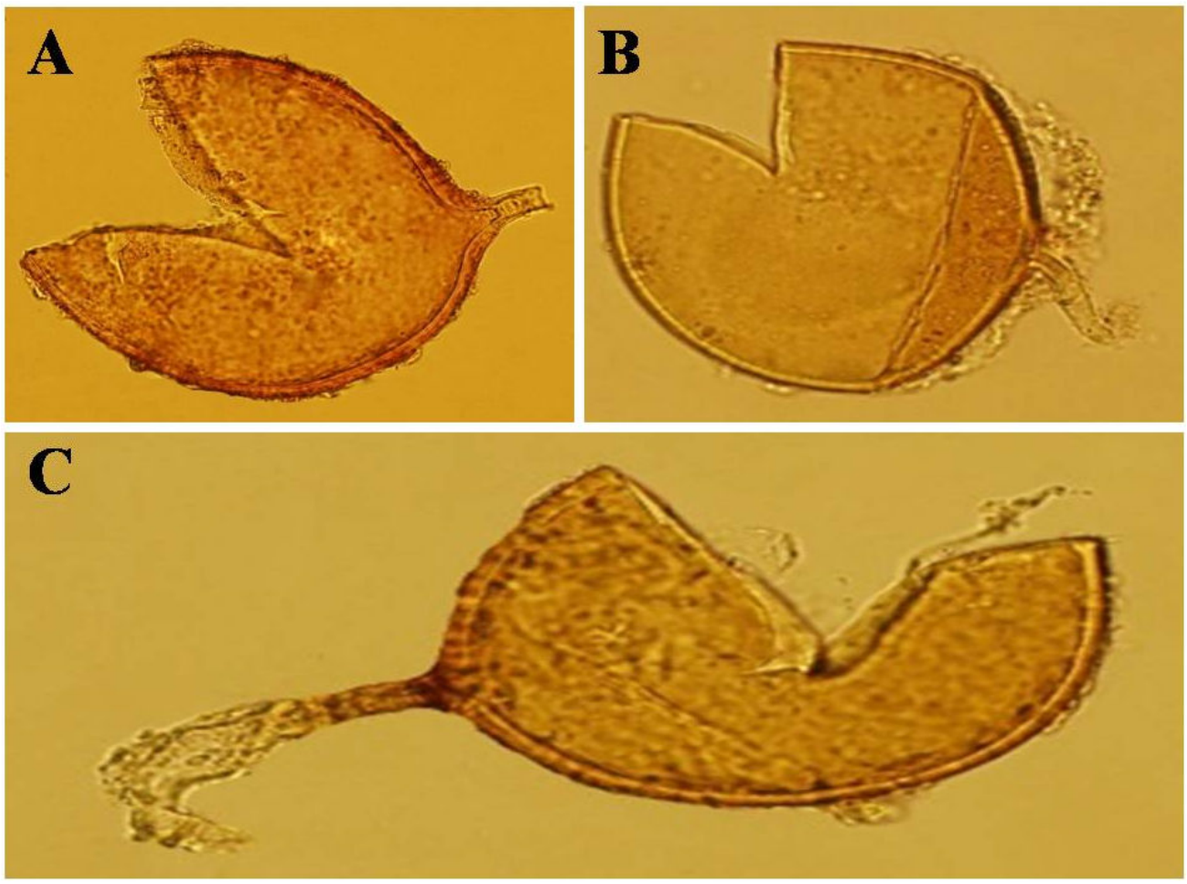

Fig. 1. Spores morphology of arbuscular mycorrhizal fungi used in this study. A, *Funneliformis mosseae* (syn. *Glomus mosseae*); B, *Rhizophagus intraradices* (syn. *Glomus intraradices*) and C, *Claroideoglomus etunicatum* (syn. *Glomus etunicatum*).

Table 1. Effect of NaCl in presence and absence of AMF on growth and biomass yield of *Vicia faba*. Data presented are the means ± SE (n = 5). Different letters next to the numbers indicate significant difference (p<0.05) among the treatments. Symbols $ and £ denote significant change between with and without AMF within the same treatment.

Treatments		Morphological and yield parameters				
Salinity (mM NaCl)	AM fungi	Shoot length (cm)	Number of branches plant^{-1}	Number of pods plant^{-1}	Pod dry weight (g)	Seed weight plant^{-1} (g)
Control	Without AMF	39.11 ± 1.55a$^{\$}$	2.68 ± 0.11a$^{\$}$	22.72 ± 1.13a$^{\$}$	2.45 ± 0.07a$^{\$}$	58.67 ± 2.27a$^{\$}$
	With AMF	43.38 ± 1.62a$^{£}$	4.88 ± 0.19a$^{£}$	29.71 ± 1.23a$^{£}$	4.98 ± 0.21a$^{£}$	102.35 ± 3.51a$^{£}$
50 mM	Without AMF	33.05 ± 1.33b$^{\$}$	2.14 ± 0.05b$^{\$}$	13.15 ± 0.73b$^{\$}$	0.91 ± 0.005b$^{\$}$	20.77 ± 1.06b$^{\$}$
	With AMF	36.88 ± 1.39b$^{£}$	3.47 ± 0.10b$^{£}$	22.04 ± 1.06b$^{£}$	2.68 ± 0.09b$^{£}$	62.52 ± 3.01b$^{£}$
100 mM	Without AMF	23.38 ± 1.12c$^{\$}$	1.51 ± 0.09c$^{\$}$	4.18 ± 0.21c$^{\$}$	0.35 ± 0.001c$^{\$}$	9.88 ± 0.52c$^{\$}$
	With AMF	29.04 ± 1.20c$^{£}$	2.52 ± 0.19c$^{£}$	13.51 ± 0.75c$^{£}$	1.58 ± 0.01c$^{£}$	31.95 ± 1.31c$^{£}$

Total spores and structural colonization: The total spores/100g soil increases at 50 mM NaCl. But higher concentration of NaCl (100 mM) decreased it to 81.92% as compared to control. The structural colonization also showed increase at 50 mM and decrease at 100 mM NaCl. The decrease of 70.02%, 84.55% and 86.79% in mycelium, vesicles and arbuscles respectively was observed at 100 mM NaCl stress (Table 2).

Nodulation and nodule activity: NaCl decreases nodule number to 53.08% and 90.82% at 50 and 100 mM NaCl stress alone as compared to control (Table 3). Plants treated with AMF showed increased nodule number to 48.12% and 68.00% at 50 and 100 mM with AMF respectively as compared to control. Nodule mass decreased to 26.08% at 50 mM and 86.95% at 100 mM NaCl alone. NaCl (50 and 100 mM) in combination with AMF showed increase of 29.16% and 66.66% in nodule mass respectively as compared to salt treatments alone. Leghemoglobin decreases with increasing concentration of NaCl. Maximum decrease of 88.47% was observed at 100 mM NaCl stress alone. When NaCl (100 mM) was applied with AMF an increase of 76.84% was observed in leghemoglobin as compared to salt stress alone.

Table 2. Effect of NaCl on total spores (spore /100 g soil) and structural colonization of AM fungi of faba bean (*Vicia faba* L.) plants. Different letters next to the numbers indicate significant difference ($p<0.05$) among the treatments.

Salinity (mM NaCl)	Total spore / 100 g soil	Structural colonization		
		Mycelium	Vesicles	Arbuscules
Control	201.25 ± 4.0a	74.62 ± 2.34a	66.37 ± 3.29a	51.12 ± 2.76a
50 mM	214.87 ± 4.11b	82.87 ± 2.52b	67.12 ± 3.32a	60.87 ± 3.01b
100 mM	36.37 ± 1.33c	22.37 ± 1.18c	10.25 ± 0.55b	6.75 ± 0.46c

Table 3. Effect of NaCl in presence and absence of AMF on nodulation and nodule activity of *Vicia faba*. Data presented are the means ± SE (n = 5). Different letters next to the numbers indicate significant difference ($p<0.05$) among the treatments. Symbols $ and £ denote significant change between with and without AMF within the same treatment.

Treatments		Nodulation and nodule activity		
Salinity (mM NaCl)	AM Fungi	Nodule number plant^{-1}	Nodule mass (g plant^{-1})	Leghemoglobin (mg g^{-1} nodule fresh wt)
Control	Without AMF	87.41 ± 2.42a$^{\$}$	0.23 ± 0.035a$^{\$}$	8.42 ± 0.85a$^{\$}$
	With AMF	126.62 ± 3.61a$^{£}$	0.31 ±0.042a$^{£}$	15.85 ± 1.31a$^{£}$
50 mM	Without AMF	41.01 ± 2.15b$^{\$}$	0.17 ±0.004b$^{\$}$	3.82 ± 0.39b$^{\$}$
	With AMF	79.05 ± 2.54b$^{£}$	0.24 ± 0.008b$^{£}$	6.91 ± 0.69b$^{£}$
100 mM	Without AMF	8.02 ± 0.72c$^{\$}$	0.03 ± 0.0002c$^{\$}$	0.97 ± 0.06c$^{\$}$
	With AMF	25.07 ± 1.34c$^{£}$	0.09 ± 0.0005c$^{£}$	4.19 ± 0.40c$^{£}$

Pigment content: Pigment content showed decrease with increasing concentration of NaCl. A decrease of 54.80%, 61.29%, 14.32%, 51.11% and 56.83% in chl 'a', chl 'b', chl a/b ratio, carotenoids and total pigments respectively at 100 mM NaCl (Table 4). NaCl supplemented with AMF enhanced the pigment content in the present study. A decrease of only 15.38% in chl 'a', 12.90% in chl 'b', 2.98% in total pigments was observed at 100 mM NaCl in combination with AMF.

Polyamines: The polyamine content was increased with increasing concentration of NaCl (Table 5). Maximum increase of 61.42%, 65.38% and 60.00% in putrescience, spermidine and spermine respectively is observed at 100 mM NaCl stress. Plants treated with NaCl (100 mM) in combination with AMF showed further increase of 46.76% in putrescience, 55.17% in spermidine and 50.00% in spermine as compared to plants treated with NaCl alone.

Lipid peroxidation: MDA content increases to 55.36% and 66.02% at 50 mM and 100 mM NaCl respectively as compared to control. However, treatment with AMF reduced the MDA content to 33.05% at 50 mM and 30.60% at 100 mM NaCl as compared to the plants treated with NaCl alone (Fig. 2).

Acid and alkaline phosphatases: The results related to the effect of NaCl and AMF on activity of phosphatase enzymes is depicted in Table 6. The acid phosphatase and alkaline phosphatase increases to 40.46% and 43.88% respectively at 100 mM NaCl stress as compared to control. Application of AMF increased the acid and alkaline phosphatase activity in control as well as in NaCl treated plants. The increase in acid phosphatase was less 27.05% and alkaline phosphatase was 37.45% at 100 mM NaCl supplemented with AMF as compared to control.

Antioxidants: The results pertaining to the effect of NaCl in presence and absence of AMF on antioxidant enzymes are presented in Fig. 3 A-D. SOD activity increased to 57.20% at 100 mM NaCl stress in present study. Plants treated with AMF in presence of NaCl (100 mM) showed maximum increase of 88.07% (Fig. 3A). Catalase activity increased to 42.21% at 100 mM NaCl stress. The CAT activity further increased to 74.29% on treatment with 100 mM NaCl in presence of AMF as compared to control (Fig. 3B). An increase of 46.25% in POD activity was observed at 100 mM NaCl alone. Plants treated with AMF in presence of NaCl (100 mM) showed further increase in POD activity to 62.28% as compared to control (Fig. 3C). Plants exposed to maximum concentration of NaCl (100 mM) showed an increase of 21.77% only in APX. However in combination with AMF the increase in APX was 47.30% as compared to the control (Fig. 3 D).

Elemental content: Na^+ content increased to 48.48% and 60.99% at 50 and 100 mM NaCl respectively as compared to control. Potassium (K^+) showed decrease with increase in concentration of NaCl. At 50 mM NaCl, a decrease of 23.89% and 53.74% decrease at 100 mM NaCl alone is observed in present study. However NaCl (100 mM) treated plants in presence of AMF showed less decrease of 26.56% in K^+ content as compared to control (Table 7). An increase of 60.37% and 81.73% in Na/K ratio at 50 and 100 mM NaCl respectively was observed in present study. Same concentration of NaCl when applied with AMF showed less increase in Na/K ratio. Plants treated with 50 mM and 100 mM NaCl showed 20.28% and 50.72% decrease in Ca^{2+} accumulation, however, when 100 mM NaCl in combination with AMF was applied to plants, only 20.86% of decrease was observed in Ca^{2+} accumulation in present study (Table 7).

Table 4. Effect of NaCl in presence and absence of AMF on pigment system of *Vicia faba*. Data presented are the means ± SE (n = 5). Different letters next to the numbers indicate significant difference (p<0.05) among the treatments. Symbols $ and £ denote significant change between with and without AMF within the same treatment.

Treatments		Pigment content (mg g^{-1} fresh wt)				
Salinity (mM NaCl)	AM Fungi	Chl 'a'	Chl 'b'	Chl a/b ratio	Carotenoids	Total pigments
Control	Without AMF	1.04 ± 0.11a$^{\$}$	0.31 ± 0.04a$^{\$}$	3.35 ± 0.32a$^{\$}$	0.45 ± 0.05a$^{\$}$	1.39 ± 0.88a$^{\$}$
	With AMF	1.35 ± 0.23a$^{£}$	0.57 ± 0.07a$^{£}$	2.36 ± 0.21a$^{£}$	0.52 ± 0.07a$^{£}$	1.99 ± 0.71a$^{£}$
50 mM	Without AMF	0.71 ± 0.07b$^{\$}$	0.21 ± 0.01b$^{\$}$	3.38 ± 0.32b$^{\$}$	0.32 ± 0.03b$^{\$}$	0.94 ± 0.16b$^{\$}$
	With AMF	1.10 ± 0.15b$^{£}$	0.38 ± 0.05b$^{£}$	2.89 ± 0.28b$^{£}$	0.40 ± 0.04b$^{£}$	1.53 ± 0.62b$^{£}$
100 mM	Without AMF	0.47 ± 0.03c$^{\$}$	0.12 ± 0.002c$^{\$}$	3.91 ± 0.40c$^{\$}$	0.22 ± 0.02c$^{\$}$	0.60 ± 0.07c$^{\$}$
	With AMF	0.88 ± 0.08c$^{£}$	0.27 ± 0.03c$^{£}$	3.25 ± 0.30c$^{£}$	0.31 ± 0.03c$^{£}$	1.18 ± 0.09c$^{£}$

Table 5. Effect of NaCl in presence and absence of AMF on polyamines content (mg g^{-1} fresh wt) of *Vicia faba*. Data presented are the means ± SE (n = 5). Different letters next to the numbers indicate significant difference (p<0.05) among the treatments. Symbols $ and £ denote significant change between with and without AMF within the same treatment.

Salinity (mM NaCl)	Polyamines content (mg/g fresh wt)					
	Without AMF			With AMF		
	Putresciene	Spermidine	Spermine	Putresciene	Spermidine	Spermine
Control	0.54 ± 0.05a$^{\$}$	0.09 ± 0.002a$^{\$}$	0.02 ± 0.001a$^{\$}$	1.44 ± 0.11a$^{£}$	0.33 ± 0.03a$^{£}$	0.05 ± 0.0005a$^{£}$
50 mM	0.97 ± 0.09b$^{\$}$	0.21 ± 0.01b$^{\$}$	0.03 ± 0.002b$^{\$}$	2.19 ± 0.21b$^{£}$	0.50 ± 0.04b$^{£}$	0.07 ± 0.001b$^{£}$
100 mM	1.40 ± 0.10c$^{\$}$	0.26 ± 0.03c$^{\$}$	0.05 ± 0.004c$^{\$}$	2.63 ± 0.27c$^{£}$	0.58 ± 0.06c$^{£}$	0.10 ± 0.002c$^{£}$

Table 6. Effect of NaCl in presence and absence of AMF on activity of phosphates enzymes (Unit) of *Vicia faba*. Data presented are the means ± SE (n = 5). Different letters next to the numbers indicate significant difference (p<0.05) among the treatments. Symbols $ and £ denote significant change between with and without AMF within the same treatment.

Treatments	Activity of phosphates enzymes (Unit)			
	Without AMF		With AMF	
Salinity (mM NaCl)	Acid phosphates	Alkaline phosphates	Acid phosphates	Alkaline phosphates
Control	691.75 ± 5.63a$^{\$}$	213.30 ± 3.69a$^{\$}$	1010.25 ± 6.07a$^{£}$	242.65 ± 3.43a$^{£}$
50 mM	921.46 ± 6.91b$^{\$}$	289.63 ± 3.92b$^{\$}$	1227.07 ± 8.27b$^{£}$	332.39 ± 4.11b$^{£}$
100 mM	1161.98 ± 7.89c$^{\$}$	380.14 ± 4.37c$^{\$}$	1384.87 ± 9.81c$^{£}$	387.97 ± 4.41c$^{£}$

Table 7. Effect of NaCl in presence and absence of AMF on elemental content (mg g^{-1} dry wt) of *Vicia faba*. Different letters next to the numbers indicate significant difference (p<0.05) among the treatments. Symbols $ and £ denote significant change between with and without AMF within the same treatment.

Treatments		Elements content (mg/g dry wt)			
Salinity (mM NaCl)	AM Fungi	Na^+	K^+	Na/K	Ca^{2+}
Control	Without AMF	10.20 ± 1.07a$^{\$}$	24.73 ± 1.35a$^{\$}$	0.42 ± 0.05a$^{\$}$	3.45 ± 0.36a$^{\$}$
	With AMF	12.77 ± 1.15a$^{£}$	35.33 ± 1.48a$^{£}$	0.36 ± 0.03a$^{£}$	4.57 ± 0.45a$^{£}$
50 mM	Without AMF	19.80± 1 .24b$^{\$}$	18.82 ± 1.21b$^{\$}$	1.06 ± 0.11b$^{\$}$	2.75 ± 0.29b$^{\$}$
	With AMF	24.66 ± 1.31b$^{£}$	26.84 ± 1.38b$^{£}$	0.92 ± 0.08b$^{£}$	3.80 ± 0.40b$^{£}$
100 mM	Without AMF	26.15 ± 1.36c$^{\$}$	11.44 ± 1.11c$^{\$}$	2.30 ± 0.27c$^{\$}$	1.70 ± 0.14c$^{\$}$
	With AMF	32.03 ± 1.42c$^{£}$	18.16 ± 1.19c$^{£}$	1.77 ± 0.15c$^{£}$	2.73 ± 0.28c$^{£}$

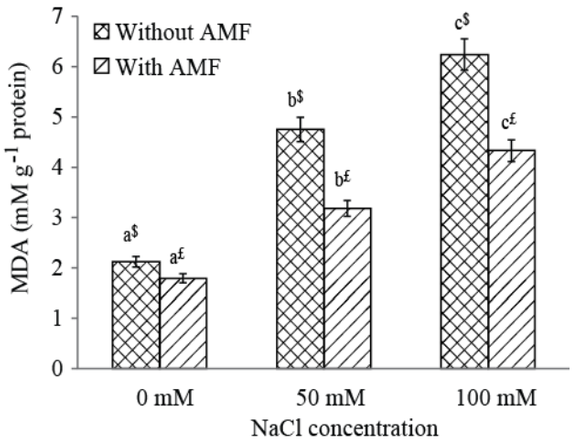

Fig. 2. Effect of NaCl in presence and absence of AMF on MDA content (mM g^{-1} protein) in *Vicia faba*. Data presented are the means ± SE (n = 5). Different letters indicate significant difference (p<0.05) among the treatments. Symbols $ and £ denote significant change between with and without AMF within the same treatment.

Discussion

Growth and biomass yield: Salt stress decreased the plant growth and productivity of major crop species. NaCl decreased the biomass yield in present study and may be due to the increase in osmotic stress and low uptake of potential elements. Decrease in biomass yield due to NaCl is also reported by Rasool *et al*., (2013) in chickpea; Ahmad *et al*., (2012a) in mustard and Alqarawi *et al*., (2014b) in *Ephedra alata*. Application of AMF have been shown to alleviate the negative effect of NaCl and the results are in accordance with Al-Kariki *et al*., (2006); Ying-Ning *et al*., (2013) and Alqarawi *et al*., (2014c). AM fungi improve plant growth and yield as it provides adequate supply of mineral nutrients particularly phosphorous (Marschner, 1986; Al-Kariki *et al*., 2000).

Arbuscular mycorrhizal colonization: NaCl reduced the root colonization by AMF (Giri *et al*., 2007). Decrease in hyphal length due to salt stress is also reported by Juniper & Abbott (2006). The suppression of root colonization may be due to the sensitivity of hypha to NaCl. That is why high concentration of NaCl hampers the colonization.

Nodulation and nodule activity: Our results of reduced nodulation and nodule activity corroborates with the findings of Djilianov *et al*., (2003) who showed reduced number of nitrogen-fixation nodules under salinity stress in alfalfa. Salinity is responsible for less nodule formation, less weight, less leghemoglobin and reduced nitrogenase activity (Bouhmouch *et al*., 2005; Soussi *et al*., 1999; Egamberdieva *et al*., 2013a,b; 2014). The number of nodules decreased with the increase in salt stress have also been reported by Rao *et al*., (2002) in chickpea. Plants under salinity stress showed reduced root growth, lower number of root hairs and deformed root hairs which are the potential sites of infection (Miransari & Smith, 2009). Nodule formation needs normal root hairs and not the deformed one. For the successful nodulation high number of rhizobia is necessary which are reduced under salinity stress (Tu, 1981). Density of root hairs under salt stress decreases in *Hordeum vulgare* (Shabala *et al*., 2003); and *Arabidopsis thaliana* (Halperin *et al*., 2003); *Ephedra alata* (Alqarawi *et al*., 2014b) and *Ephedra aphylla* (Alqarawi *et al*., (2014c). Decrease in Ca^{2+} have major role in decrease of number of root hairs and their extension. Negative effect of salt on the root hairs could be alleviated or even completely reversed by increasing the extracellular concentration of calcium. Direction of root hair growth is determined by an intracellular calcium gradient in *A. thaliana* (Bibikova *et al*., 1997). AM fungi helps in uptake of calcium and maintain the nodule activity as compared to salt treated plants alone. A decrease in leghemoglobin content in the nodule is also reported by Bergersen (1981) and Delgado *et al*., (1994) in legumes. Ashraf & Bashir (2003) showed production and accumulation of osmolytes such as proline, glycinebetaine that counteracts osmotic stress induced by NaCl, thus, increases the nodule functioning under salinity stress.

Pigment system: Increase in NaCl concentration decreases the pigment content in present study and the results are in accordance with the findings of Rasool *et al*., (2013) in chickpea and Ahmad *et al*., (2012a) in mustard. Salt stress disturbs the enzyme activities responsible for chlorophyll synthesis hence the decrease in chlorophyll content (Parida & Das 2005). Salt treated plants inoculated with AMF showed less decrease in pigment system and are reported by different researchers (Colla *et al*., 2008; Zuccarini, 2007). AM fungi provides Mg to the chlorophyll molecules which was hampered by NaCl stress (Giri *et al*., 2004; Zuccarini *et al*., 2007). Zhu *et al*., (2010) reported that maize plants inoculated with *Glomus etunicatum* showed decreased Na^{+} level. The increased photosynthetic pigments by mycorrhizal colonization in plants is due to the inhibition of Na transport which leads to better functioning of photosynthetic machinery (García-Garrido & Ocampo 2002; Borde *et al*., 2010; Alqarawi *et al*., (2014b).

Polyamine content: Polyamines (putrescine, spermidine, spermine), are the widely distributed of N containing organic molecules, and have a positive role in alleviating the abiotic stress (Alcázar *et al*., 2010; Groppa & Benavides 2008; Ahmad *et al*., 2012b). In the present study NaCl increases the putrescine, spermidine, spermine. Santa- Cruz *et al*., (1997) reported the (Spd+Spm): Put ratios increased with salinity in the salt-tolerant tomato species (*Lycopersicon pennellii*, Carrel D'Arcy) but not in the salt-sensitive tomato species (*L. esculentum*). Yamaguchi *et al*., (2006) also suggested the protective role of Spm as its addition suppressed the salt sensitivity in Spm deficient mutants. Application of AMF further increases these polyamines and suggests the defensive roles against NaCl stress. Some of the observations suggest that PAs play a role in stabilizing membranes, scavenging free radicals, nucleic acids and protein synthesis, RNAse, protease and other enzyme activities, and interacting with hormones, phytochromes, and ethylene biosynthesis (Slocum *et al*., 1984; Galston & Tiburcio 1991). Because of these numerous biological interactions of PAs in plant systems, it has been difficult to determine their precise role in plant growth and development (Kaur-Sawhney *et al*., 2003).

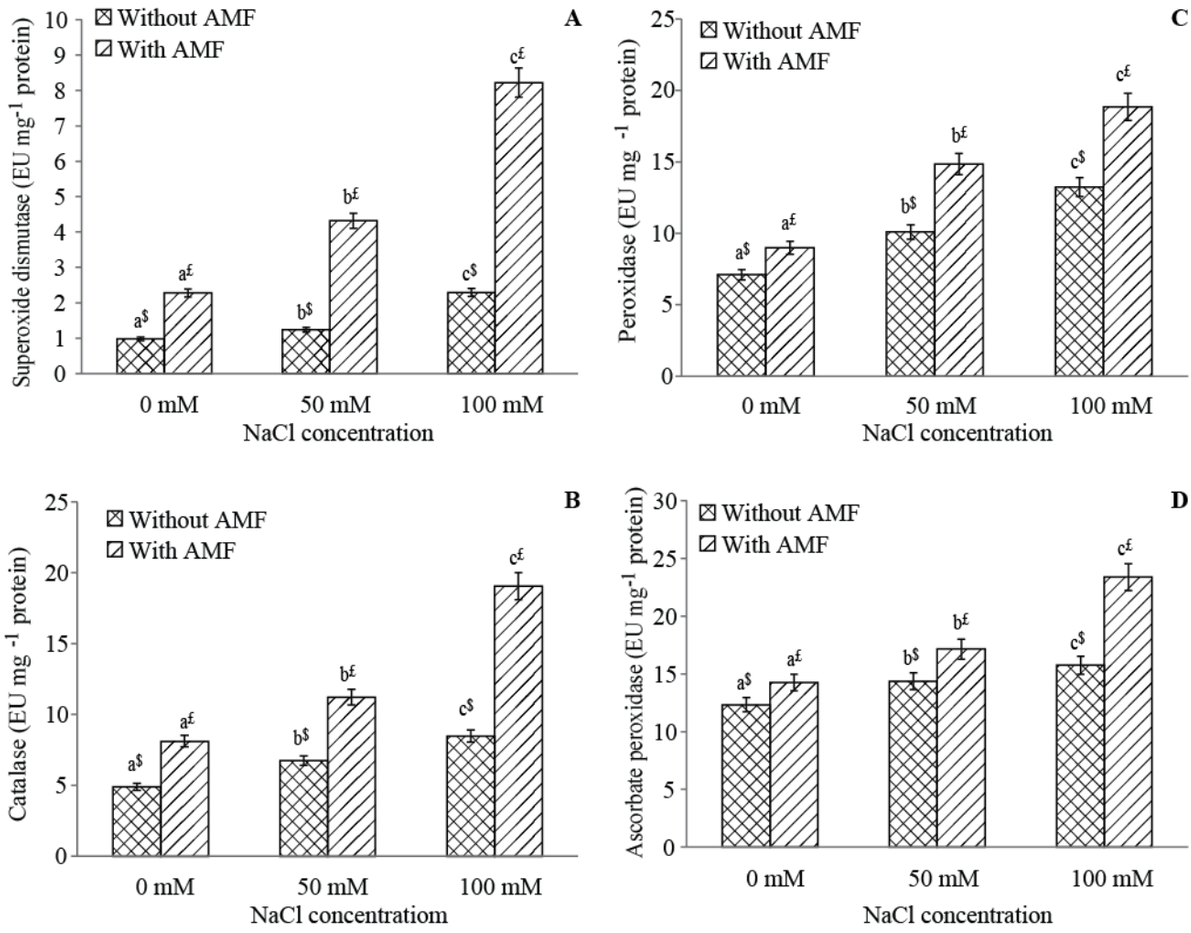

Fig. 3. Effect of NaCl in presence and absence of AMF on SOD (EU mg^{-1} protein) (A), CAT (EU mg^{-1} protein) (B), POD (EU mg^{-1} protein) (C), APX (EU mg^{-1} protein) (D) in *Vicia faba*. Data presented are the means ± SE (n = 5). Different letters indicate significant difference (p<0.05) among the treatments. Symbols $ and £ denote significant change between with and without AMF within the same treatment.

Lipid peroxidation (MDA content): Increase in lipid peroxidation by NaCl in the present study corroborates with the findings of Rasool *et al*., (2013) in chickpea and Ahmad *et al*., (2012a) in mustard. NaCl induced ROS which reacts with lipid membrane and gives rise to lipid hydroperoxides. Mehrizi *et al*., (2012) also reported increase in lipid peroxidation with increasing concentration of NaCl in *Rosmarinus officinalis*. Plants treated with NaCl in combination with AMF showed less increase in lipid peroxidation. One of the reason might be antioxidant enzymes are able to scavenge the ROS before reacting with membrane lipids and minimizes peroxidation of lipids. Another reason might be upregulation of stress related proteins like glutathione S-transferase (GST), glutathione dependent formaldehyde dehydrogenase (FALDH) and peroxidase. These proteins also help in quenching of ROS and maintains the cell membrane integrity.

Acid and Alkaline phosphatase: Acid phosphatase activity increases with increase in NaCl concentration in the present study corroborates with the findings of Stephen *et al*., (1994) and Olmos & Hellin (1997) in pea and Ehsanpour & Amini (2003) in alfalfa. Deficiency of phosphorous is responsible for the increase in acid phosphatase activity (Barrett-Lennard 1982; Dracup *et al*., 1984). The higher acid phosphatase activity might be due to the high resistance of the pre-existent acid phosphatase to stress induced degradation or due to stress-stimulated new acid phosphatase synthesis (Pan & Chen, 1988). Alkaline phosphatase also increased with increased concentration of salt in the present study. Rai & Sharma (2006) also reported increase in alkaline phosphatase in Anabaena doliolum. Jain *et al*., (2004) showed increase in NaCl increases the acid and alkaline phosphatase in pearlmillet seeds. NaCl hampers the uptake of nutrients including phosphorous from the soil. The insoluble form of phosphatase becomes soluble by the activity of the cellular phosphatases and helps in osmotic adjustment (Fincher 1989; Jain *et al*., 2004). It has been shown root phosphatase activity increases when soil P availability decreases (Fujita *et al*., 2010). AMF provides the P content to the plant and the activity of the cellular phosphatases decreases and similar results have been reported by Beltrano *et al*., (2013) in pepper.

Antioxidants: Antioxidant enzyme activity increase under salt stress in present study corroborates with the

findings of Rasool *et al*., (2013) in chickpea and Ahmad *et al*., (2012a) in mustard. A great number of literature showed that salinity stress increases the antioxidant enzyme activity. NaCl treated plants in combination with AMF further increases the activity as compared to the NaCl treated plants alone (Wu *et al*., 2010; He *et al*., 2007; Huang *et al*., 2008). Superoxide dismutase acts as the first line of defense and dismutates the oxygen radicals to hydrogen peroxide and oxygen. The hydrogen peroxide produced is removed by the action of catalases, peroxidases and ascorbate peroxidases. Arbuscular mycorrhizas themselves possessed various SOD genes to up-regulate stress tolerance and decrease the accumulation of H_2O_2 as compared to non-mycorrhizal plants, indicating lower oxidative damage in the colonized plants (Wu *et al*., 2010; Hajiboland *et al*., 2012).

Elemental accumulation: NaCl stress resulted in more accumulation of Na^+ and decreased uptake of potassium and calcium in the present study. The main effect of salinity stress is imbalance of many important ions (Bhosale & Shinde 2011; Iqbal & Ashraf 2013). According to Zheng *et al*., (2008) the ionic balance has a key role in photosynthesis and other metabolic activities of the cell. Plants treated with NaCl in combination with AMF showed less effect on ionic balance and mitigates the deficiency of K and Ca and also increases K/Na ratio and the results are in accordance with Mohammad *et al*., (2003) and Audet & Charest (2006). Alqarawi *et al*., 2014c also reported that AM fungal symbiosis plays a key role in higher K^+ accumulation and hence higher K/Na ratio in mycorrhizal plants. AMF spread the mycelium deep in to the soil and absorb water and minerals for the host plant and increases the efficiency of the plant to withstand the NaCl stress.

Conclusion

In conclusion, NaCl stress negatively effects growth, biochemical attributes, nodulation and nodule activity, and enzymatic activities. However the application of AMF mitigates the negative effect of NaCl on faba beans. The polyamines are the nitrogenious compounds and have been found to be effective in allevating the NaCl stress. Antioxidant enzyme activity proved to be the main defense for the faba beans against NaCl stress in present study. Mycorrhiza treated faba bean have showed more increase in antioxidants and improvement in nutrient uptake. AMF spreads its hypha deep in to the soil and increases the mineral uptake, water content, which leads to decrease in toxic effect of NaCl in the cell. Using AMF as an alternative way in decreasing the NaCl stress in plants will be more beneficial as it maintains the soil fertility and the yield.

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