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Arbuscular mycorrhizal symbiosis improves growth and antioxidative response of Stevia *rebaudiana* (Bert.) under salt stress.

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ABSTRACT

To investigate the possible role of arbuscular mycrrhizal fungi (AMF) in alleviating the negative effects of salinity on Stevia rebaudiana (Bert.), the regenerated plantlets in tissue culture was transferred to pots in greenhouse and inoculated with Glomus intraradices. Salinity caused a significant decrease in chlorophyll content, photosynthesis efficiency and enhanced the electrolyte leakage. The use of AMF in salt –affected plants resulted in improved all above mentioned characteristics. Hydrogen peroxide and malondialdehyde (MDA) contents increased in salt stressed plants while a reduction was observed due to AMF inoculation. CAT activity showed a significant increase up to 2 g/l and then followed by decline at 5 g/l NaCl in both AMF and non-AMF treated stevia, however, AMF inoculated plants maintained lower CAT activity at all salinity levels (2 and 5 g/l). Enhanced POX activities in salt- treated stevia plants were decreased by inoculation of plants with AMF. The addition of NaCl to stevia plants also resulted in an enhanced activity of SOD whilst, AMF plants maintained higher SOD activity at all salinity levels than those of non-AMF inoculated plants. AMF inoculation was capable of alleviating the damage caused by salinity on stevia plants by reducing oxidative stress and improving photosynthesis efficiency.

***Keywords*:** Arbuscular mycorrhizal fungi; salinity; Stevia rebaudiana; antioxidant enzymes; oxidative stress; photosynthesis

1. Introduction

Stevia (*Stevia rebaudiana* Bertoni) is a perennial shrub widely planted in many countries of the world with great potential as a natural sweetener source, up to 150 times sweeter than sugar, but with no calories. Therefore, it has been applied as a substitute for saccharose, and in the treatment of diabetes mellitus, obesity, and hypertension and in caries prevention[20,22]. According to reports by Hajar *et al*. (2014) stevia has successfully been able to adapt to different growing areas in terms of climate and soils [12]. However, for an economical production, crop irrigation is required[21]. Although stevia has shown different degrees of sensitivity or resistance against salinity, there is often a tendency for a relation between growth and salinity according to reports of the scientific literatures where in, the higher salinity level the less growth of stevia[6, 14, 25].

Because, around 40% of the arable lands in the world have insufficient rainfall to support economically viable agriculture, the influence of saline water irrigation on stevia growth has attracted more attention [28]. Plants grown in fields are surrounded by various microorganisms like bacteria and fungi that help and improve the plant growth under various stress conditions[7]. The application of Arbuscular mycorrhizal fungi (AMF) as a biologically based strategy could be an efficient alternative to mitigate the harmful effects of plant crops exposed to salt. AMFs alleviate salt stress by several possible mechanisms, including maintaining higher activities of antioxidant enzymes, improving nutrient uptake and providing a higher accumulation of osmosolutes[9,23]. Under salinity stress conditions, AMFs improve the uptake mechanism in plants by supplying the essential nutrients; thereby the plant recovers the water balance machinery, resulting in the enhancement of its tolerance against salt stress[1,3]. Some experiments have been conducted to assess the response of some plants such as *Panicum turgidum*[13], *Ephedra aphylla Forssk*[1],

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sweet basil[8], apple seedlings[32] and *Populus catayana*[31] to salinity and possible role of AMF in enhancing the salt tolerance. In Citrus plants, leaf area, photosynthesis and phosphorus uptake were enhanced by AMF colonization of plants[27]. According to reports of Reis *et al*. (2015), stevia is suitable to be grown in semiarid regions, if well irrigated, even with a relatively high salinity, but only one harvest is possible[22]. Therefore, it was hypothesized that, AM inoculation could be capable of alleviating the damage and restrictions caused by salinity stress on stevia plants by improving photosynthesis efficiency and reducing oxidative stress.

2. Materials and method

The plantlets were obtained according to Cantabella *et al*. (2017) [4] from micro-propagated stevia shoot cultures grown on Murashige and Skoog (MS) medium, supplemented with 3% (w/v) sucrose, 0.6% (w/v) agar and 0.4% (w/v) activated charcoal. For elongation and rooting, shoots with three internodes were transferred to half strength MS medium without growth regulator. Under these conditions, the shoots elongated and rooted in 7 weeks. All cultures were maintained at 25± 2ºC in a growth chamber under the controlled photoperiod of 16 h light and 8 h dark cycle with a light intensity of 3000- 4000 lux provided by white fluorescent lamps. The rooted shoots were taken out of the medium and washed with distilled water to remove medium attached to the roots. The plantlets were then acclimatized in pots containing a mixture of perlite and peat (1:2) in a controlled growth chamber under the same controlled photoperiod and temperature condition as mentioned above, for the nodal segment culture stage. After 8 weeks of acclimatization to in vivo conditions, the S. *rebaudiana* plants were transplanted to pots (2L) containing the same proportion of substrate used for acclimatization. Seven days later, the plant tips were cut in order to obtain the uniform size of all plants.

2.1 Plant inoculation

AMF treatments were inoculated with 250 g (per pot) soil inoculated with *Glomus intraradices*, by placing a thin layer of maycorrhizal inoculums around the roots of plantlets below the surface of the soil in pots directly prior to the growing of the stevia plants to promote fungal inoculation of plant roots. Counterparts of non-AM treatment were received volumetric sterilized soils free of spores.

2.2 Salinity treatment

The modified Hoagland's solution according to Zeng *et al*. (2013)[33], supplemented with different concentrations of NaCl to get concentrations of 0, 2 and 5 g/l were used for irrigation in normal pots as well as in pots with mycorrhizal inocolum. The rate of irrigation was 250 ml for each treatment twice a week with these solutions at the beginning of and throughout the experiment period. At the end of the experimental period (8 weeks), the plants were harvested for growth, physiological and biochemical estimation.

Chlorophyll determination, chlorophyll fluorescence and electrolyte leakage

Chlorophyll fluorescence was measured using a fluorometer (Walz, Effeltrich, Germany). The photochemical efficiency of PSII was calculated as the ratio Fv/Fm for each segment. The relative chlorophyll (Chl) content was measured with a portable leaf chlorophyll meter (SPAD 502, Minolta Co., Osaka, Japan). Electrolyte leakage (EL) was measured by using a conductivity meter according to Ozden et al. (2009)[18]. The samples were divided into equal sized pieces (0.3 g per treatment) and placed in culture vessels containing 15 ml of distilled water and were left for 24 h at room temperature. The initial conductance of the solution was measured using a conductivity meter. The tubes were then autoclaved at 115 º C for 10 min and final reading was taken following autoclaving and an additional 24 h incubation at room temperature. EL (%) was calculated using the following formula:

$$\text{EL (\%)} = \frac{\text{The initial conductance of the solution}}{\text{The final conductance of the solution}} \times 100$$

2.3 Oxidative stress parameters

Lipid peroxidation: (MDA) and H_2O_2 measurement

The concentration of malondialdehyde (MDA) which is a product of lipid peroxidation was assessed by the thiobarbituric acid (TBA) according to Wang *et al*. (2009) [30], as 1 g fresh leaves were detached from glasshouse grown seedlings, placed in a mortar containing 5 ml 0.6% TBA in 10% trichloroacetic acid (TCA) and ground with a pestle. The mixture was heated at 100 ºC for 15 min. These samples were cooled on ice for 5 min, then, the mixtures were centrifuged at 5,000 rpm for 10 min. The absorbances of the supernatant at 450, 532 and 600 nm wavelengths were recorded and MDA content was calculated on a fresh weight basis using the following formula:

$$(\text{nmol MDA g}^{-1}\text{ FW}) = 6.45\,(OD_{532} - OD_{600}) - 0.56\,(OD_{450}) \times 1000.$$

Hydrogen peroxide (H_2O_2) was assessed spectrophotometrically after the reaction with potassium iodide (KI), according to the method presented in Velikova and Loreto (2005) [29]. Leaf tissues (1 g) were ground and homogenized in a mortar containing 10 ml 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min. Afterwards, 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml reagent (1 M KI in fresh double distilled water) and then absorbance of the supernatant was read at 390 nm. The blank probe was prepared using 0.1 % TCA in the absence of leaf extract. The content of H_2O_2 was calculated applying a standard curve prepared by identified concentrations of hydrogen peroxide.

2.4 Antioxidative metabolism

2.4.1 Enzyme extraction and analysis

Frozen-leaf samples (0.2 g) were ground in liquid nitrogen and stored at -80 ºC until assay. The enzyme extract for superoxide dismutase (SOD), proxidase (POX) and catalase (CAT) was prepared by mixing frozen samples with 2 ml extraction buffer containing 0.1 M potassium phosphate buffer, pH 7.5 and 0.5 mM ethylenediamintetra acetic acid (EDTA). The extract was centrifuged for 20 min at 12,0009g and 4 ºC. Then the supernatant was used for enzymatic assay. Assay of SOD activity (expressed as unit per milligram of protein) was based on reduction of nitroblue tetrazolium (NBT) according to the method used by Padmaja *et al*. (2011) [19]. A complete reaction mixture contained 1 ml of the 125 mM sodium carbonate, 0.4 ml of 25 lM NBT and 0.2 ml of 0.1 mM EDTA added to 0.5 ml of plant extract. The reaction was initiated by adding 0.4 ml of 1 mM hydroxylamine hydrochloride and the absorbance was read at 560 nm using a spectrophotometer at 5 min intervals. Units of SOD were expressed as the amount of enzyme required for inhibiting the reduction of NBT by 50 %. Catalase activity was measured by the titrimetric method applied by Padmaja *et al*. (2011) [19]. The reaction mixture comprised of 5 ml of 300 lM phosphate buffer (pH 6.8) containing 100 lM hydrogen peroxide (H_2O_2) and 1 ml of plant extract was prepared and left at 25 ºC for 1 min. The reaction was stopped by adding 10 ml of 2 % sulfuric acid and residual H2O2 as titrated with potassium permanganate (0.01 N) till pink color was obtained. Enzyme activity was measured by calculating the decomposition of lM H_2O_2 per min per mg protein. Assay of peroxidase was also achieved according to the method used by Padmaja *et al*. (2011) [19]. 3.5 ml of phosphate buffer (pH 6.5) was taken into a clean, dry cuvette, 0.2 ml of plant extract and 0.1 ml of freshly prepared O-dianisidine solution was added to it at 28–30 ºC and absorbance was recorded at 430 nm. Then 0.2 ml of 0.2 mM H_2O_2 was added and mixed and then the absorbance was read at every 30 s intervals up to 3 min. A graph was plotted with an increase in absorbance against time. The enzyme activity was expressed per unit time per mg of protein.

2.4.2 Statistical analysis of data

Experiments were performed using a completely randomized design. All statistical analyses were carried out with SAS and MSTAT-C computer programs. The data were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by LSD test. Differences at $p \leq 0.05$ were considered as significant.

3. Results

The effect of arbuscular mycorrhizal fungi (AMF) G. *intraradices* on the chlorophyll content and photosynthesis efficiency of Stevia under different levels of NaCl induced salt stress is shown in **Figures 1**. Salinity treatments significantly lowered chlorophyll content and photosynthesis efficiency (Fv/Fm) of non-AMF inoculated plants (Fig. 1) and the rate of decrease was directly proportional to the salt concentrations. Maximum decrease of 19.40% and 27.40%, in chlorophyll content and photosynthesis efficiency, respectively was observed at 5 g/l NaCl. However, AMF

inoculated plants showed improved efficiency of photosynthesis and chlorophyll content at all salinity levels (2 and 5 g/l NaCl) as the increase of chlorophyll and photosynthesis efficiency of AMF inoculated stevia at 5 g/l NaCl induced salt stress was observed 56.52 and 47.61% respectively, more than non-AMF inoculated plants (Figs. 1 a and b). The data presented in **Figure 2** revealed that the electrolyte leakage (EL) of stevia plants was significantly increased by increasing salt levels, but the use of AMF alleviated the negative effect of salinity on electrolyte leakage significantly. Inoculation with AMF caused a 41% reduction of ion leakage in the stevia plants under 5 g / l of NaCl induced salt stress compared with non-AMF inoculated plants (**Figure 2**). Malondialdehyde (MDA) and H_2O_2 contents increased with an increased salt level irrespective of the AMF treatment used **(Figures 3**). However, these values in AMF-inoculated stevia plants were significantly lower in both the cases (MDA and H_2O_2) than the corresponding non-AMF inoculated plants, indicating higher lipid peroxidation in the non-AMF plants, thus supporting the role of AMF colonization in preventing oxidative stress. The content of chlorophyll and photosynthesis efficiency showed a significant negative correlation with MDA and H_2O_2 accumulation and electrolyte leakage (**Table 1**). Data related to the activities of CAT, POX and SOD are summarized in **Figures 4, 5** and **6**. The data in these figures indicated that salt stress caused significant changes in the activities of these antioxidant enzymes in stevia plants. Results showed an increase in CAT activity significantly up to 2 g/l and then followed by decline at 5 g/l NaCl in both AMF and non-AMF treated stevia, however, AMF inoculated stevia plants maintained lower CAT activity at all salinity levels (2 and 5 g/l) (**Figure 4**). POX activities were significantly higher in salt- treated stevia plants than in the non-treated plants (**Figure 5**), and the inoculated of plants with AMF resulted in decreased POX activities. The addition of NaCl induced salt stress to stevia plants also resulted in an enhanced activity of SOD whilst, AMF stevia plants maintained higher SOD activity at all salinity levels than those of non-AMF inoculated plants (**Figure 6**).

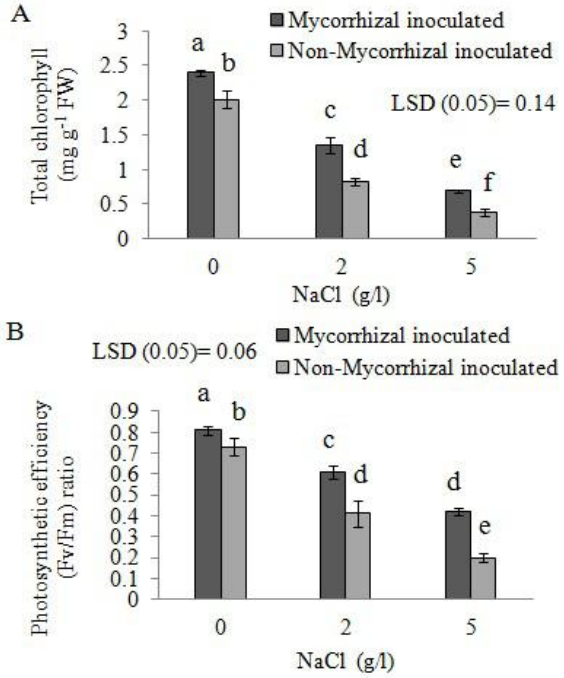

Figure 1; Total chlorophyll and photosynthetic efficiency in *Stevia rebaudiana* Bertoni plants inoculated with AMF or non-AMF subjected to three different levels of NaCl induced salt stress. Mean pairs for each treatment followed by different letters are significantly different ($P < 0.05$) by LSD test; n= 3.

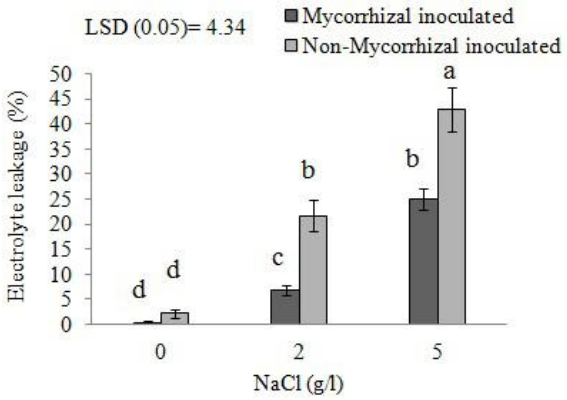

Figure 2; Electrolyte leakage in *Stevia rebaudiana* Bertoni plants inoculated with AMF or non-AMF subjected to three different levels of NaCl induced salt stress. Mean pairs for each treatments followed by different letters are significantly different ($P < 0.05$) by LSD test; n= 3.

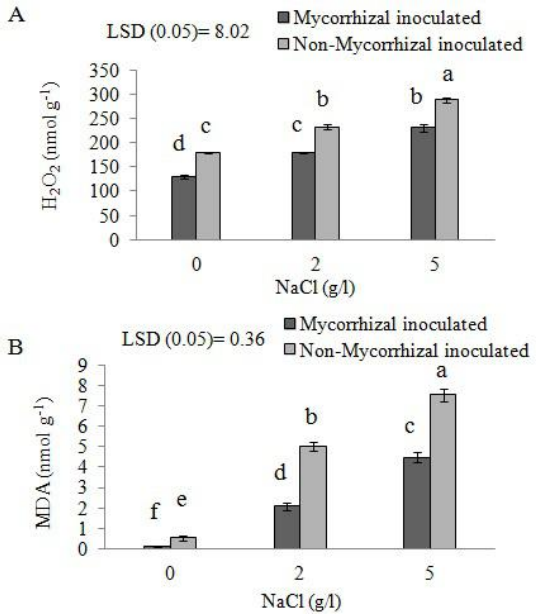

Figure 3; Oxidative stress indicated by H_2O_2 and MDA production in *Stevia rebaudiana* Bertoni plants inoculated with AMF or non-AMF subjected to three different levels of NaCl induced salt stress. Mean pairs for each treatments followed by different letters are significantly different ($P < 0.05$) by LSD test; n= 3**.**

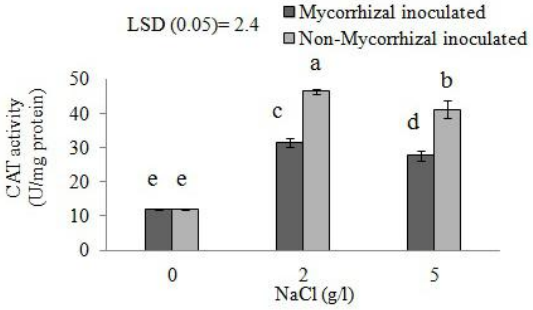

Figure 4; CAT activity in *Stevia rebaudiana* Bertoni plants inoculated with AMF or non-AMF subjected to three different levels of NaCl induced salt stress. Mean pairs for each treatments followed by different letters are significantly different ($P < 0.05$) by LSD test; n= 3**.**

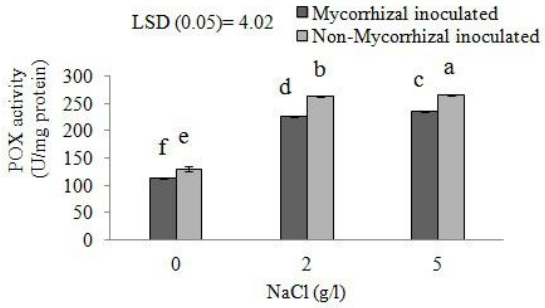

Figure 5; POX activity in *Stevia rebaudiana* Bertoni plants inoculated with AMF or non-AMF subjected to three different levels of NaCl induced salt stress. Mean pairs for each treatments followed by different letters are significantly different ($P < 0.05$) by LSD test; n= 3**.**

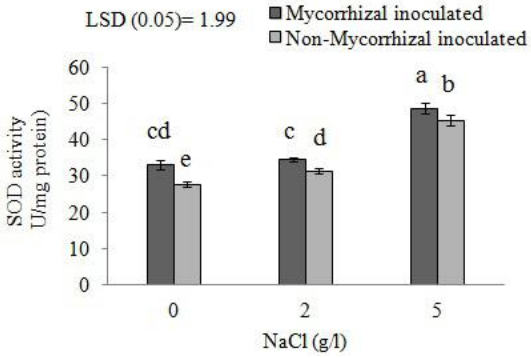

Figure 6; SOD activity in *Stevia rebaudiana* Bertoni plants inoculated with AMF or non-AMF subjected to three different levels of NaCl induced salt stress. Mean pairs for each treatments followed by different letters are significantly different ($P < 0.05$) by LSD test; n= 3**.**

	1	2	3	4	5	6	7	8
1- Total chlorophyll content	1	0.95**	-0.91**	-0.94**	-0.94**	-0.85**	-0.95**	-0.69**
2- Photosynthesis efficiency		1	-0.97**	-0.97**	-0.98**	-0.83**	-0.90**	-0.67**
3- Electrolyte leakage (EL)			1	0.95**	0.97**	0.74**	0.81**	0.74**
4- H_2O_2				1	0.96**	0.78**	0.84**	0.62**
5- MDA					1	0.85**	0.89**	0.67**
6-CAT						1	0.95**	0.34ns
7- POX							1	0.55*
8- SOD								1

**Correlation is significant at the 0.01 level, *Correlation is significant at the 0.05 level and ns Correlation is non- significant

Table1. Correlation coefficients among measured traits of AMF and non-AMF inoculated stevia plants under salt stress

The AMF inoculation of plants has been reported to have a considerable role in tolerance against adverse environmental conditions such as salt stress[1,8,10,13,17,32]. Salinity stress as shown in the result has affected some physiological attributes like chlorophyll content, photosynthesis efficiency of AMF and non-AMF inoculated stevia plants in a negative way, but those negative effects were more apparent in non-AMF inoculated plants. However, these physiological attributes clearly proves that the inoculation with mycorrhiza *Glomus intraradices* has a significant positive improving effect on stevia plants under control and salinity stressed conditions. Higher chlorophyll content in leaves of mycorrhizal plants under saline conditions has also been reported by various authors[1,2,5,11,24,26,34]. Similarly, Kumar *et al*. (2013) reported that AMF inoculation improved some physiological attributes such as chlorophyll content of Na_2SO_4 salt stressed *Jatropha* over non-inoculated plants[17]. Decline in the ratio of Fv/Fm (Chlorophyll fluorescence) in the leaves of stevia caused by salinity stress could be mitigated by AMF symbiosis, which was in line with the reports of Sheng *et al*. (2008) in maize plants[26], where the higher ratio of Fv/Fm was observed in the leaves of AMF treated plants than those of non-AMF inoculated plants. Improvement of chlorophyll activity by AMF inoculated stevia grown under salinity stress is in confirmation with the findings of Kadian *et al*. (2013) who reported that the improved chlorophyll content of Cyamopsis tetragonoloba (L.) inoculated by AMF may be due to increased activity of specific enzymes involved in its biosynthesis[15]. Our results of mitigation in electrolyte leakage (EL) of leaves due to inoculation of stevia plants with AMF under salinity stress are in confirmation with the findings of Kaya *et al*. (2009) where the enhanced EL in leaves of Capsicum annum as a result of NaCl induced salinity stress was ameliorated in AMF-inoculated plants [16]. According to our results, AMF inoculated plants exhibited salinity tolerance by showing reduced EL over non-AMF plants at all salinity levels. Salinity stress was able to affect key physiological and biochemical processes of stevia, as indicated by measurements of some indices, e.g., lipid peroxidation (MDA production), electrolyte leakage, photosynthesis and different antioxidant enzymes (SOD, POX and CAT).

Electrolyte leakage (EL) showed very significant negative correlation with photosynthesis efficiency (-0.98**) and very significant positive correlation with MDA production (0.97**) (**Table 1**) suggesting that, the effect of salinity was most deleterious in accordance with the results for Fv/Fm, EL and MDA production (lipid peroxidation). The increased EL showed a positive correlation with H_2O_2 and MDA accumulation, suggesting that the detrimental effects of salt stress were associated with oxidative stress. In this study, AMF-inoculated stevia exhibited lower H_2O_2 and MDA content than non-AMF –inoculated plants under salt stress, supporting the positive role of AMF fungi in ameliorating of NaCl induced salt stress. Higher efficiency of photosynthesis (Fv/Fm) in AMF – inoculated stevia plants contribute to NaCl induced salt stress tolerance through lowering the components of oxidative stress (H_2O_2 and MDA contents). Lower leakage values, H_2O_2 and MDA contents in AMF- inoculated stevia also supports the role of AMF fungi in enhancing the salt tolerance in stevia by activating the production of some antioxidant enzymes. Similar observations of the reduced of H_2O_2 and MDA contents caused by AMF inoculation which is possibly because of the increased activity

of antioxidants in AMF-inoculated plants grown under salinity stress, have earlier been reported by Hashem *et al*. (2015) in *Panicum turgidum* Forssk. Our results of salinity induced enhanced in the activities of SOD and POX in stevia corroborate with the findings of Hashem *et al*. (2015) for *Panicum turgidum* Forssk and Alqarawi *et al*. (2014) for *Ephedra aphylla* Forssk[1,13], however, further increase of these enzymes in AMF inoculated plants was observed only in terms of SOD activity, in the present study. Results of an increase in leaf CAT activity under low salinity followed by a decrease under high salinity in non-AMF and AMF inoculated stevia plants in our study support the findings of Yang *et al*. (2014)[32], however, in contrast to their results, mycorrhizal plants demonstrated lower activities of CAT and POX than non-mycorrhizal plants, meaning that AMF fungi could not induce more activities of these enzymes in stevia under salt stress. The results of alleviation of salinity-induced detrimental effects through arbuscular mycorrhizal fungus in this study come in compliance with those of other researchers in *Ephedra aphylla* Forssk (Alqarawi *et al*. 2014) and *Panicum turgidum* Forssk (Hashem *et al*. 2015)[1, 13].

5. Conclusion

NaCl stress resulted in the collapse of Stevia growth due to its evident impacts on the physiological and biochemical indices studied. Salt stress, also increased production of hydrogen peroxide and lipid peroxidation causing damage to the cell membrane. These results evidence that, AMF mitigated the salt stress induced alterations by its positive effect on photosynthesis and antioxidant defense system, thereby enhancing plant efficiency and protecting cells from oxidative damage. Our study demonstrated that to reduce the unfavorable effects of salinity on stevia plants, the use of AM technology could be considered as a biological method for improving stevia production under salinity stress.

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