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Effect of Arbuscular Mycorrhizal Fungi on Growth and Antioxidant Activity in *Gmelina arborea* Roxb. under Salt Stress Condition

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Abstract

Gmelina arborea Roxb. is medicinally and economically important tree species were selected for study. An experiment was conducted to determine the influence of arbuscular mycorrhizal (AM) fungus *Glomus fasciculatum* on salt stress tolerance of tree species *Gmelina arborea*. Mycorrhizal and nonmycorrhizal seedlings were treated with 100 mM and 200 mM concentration of NaCl. *G. fasciculatum* treated plant showed increase in fresh and dry weight, greater percentage of mycorrhizal colonization, higher accumulation of proline and chlorophyll content with increasing levels of salinity. *G. fasciculatum* colonization significantly increased tolerance of salinity, acid phosphatases, and Proline content and also antioxidant enzymes like peroxidase, catalase and superoxide dismutase at all levels of salinity treatments of *Gmelina* plants in comparison with non-mycorrhizal salinity treated plants. These results demonstrate that AM fungus (*G. fasciculatum*) is very effective in strengthening the tolerance of *Gmelina arborea* grown in arid and semi arid areas.

Keywords: Catalase, *Gmelina arborea*, mycorrhizal fungi, peroxidase, salinity, superoxide dismutase

Introduction

Salinity is a major problem in arid and semiarid tropics. In India about 8.6 Mha of land area is affected by soil salinity (Pathak, 2000). Salt alters a wide array of metabolic processes, culminating in stunted growth, and reduced enzyme activities and biochemical constituents (Muthukumarasamy and Panneerselvam, 1997). Proline is most common osmolyte in plants under stress conditions (Hasegawa *et al*., 2000) and act as a mediator of osmotic adjustment (Ashraf and Foolad, 2007) and serve as a hydroxyl radical scavenger (Alia *et al.,* 1995).

There is accumulating evidence that production of reactive oxygen species (ROS) is a major damaging factor in plants exposed to different environmental stresses, including salinity (Hernandez *et al.,* 1995). Plants have evolved specific protective mechanisms, involving antioxidant molecules and enzymes in order to defend themselves against oxidants (Jiang and Zhang 2002; Nunez *et al.,* 2003, 2004).

Antioxidant mechanisms may provide a strategy to enhance salt tolerance in plants. Peroxidase (POX) and catalase (CAT) are involved in the defense mechanisms of plants in response to pathogens either by their participation in cell wall reinforcement, or by their antioxidant role in the oxidative stress generated during plant pathogen interaction (Mehdy, 1994). Cells under salt stress initially accumulate salts as free osmotica, however, a toxic specific ion effect appears once a certain threshold level of Na and/or Cl accumulation has been reached integrity, enzymatic activity, protein and nucleic metabolism (Hasegawa *et al.,* 2000; Mansour and Salama, 2004; Zhu 2001, 2002). Plants under stress produce some defense mechanisms to protect themselves from the harmful effect of oxidative stress. ROS scavenging is one among the common defense response against abiotic stresses (Vranova *et al.,* 2002). ROS scavenging depends on the detoxification mechanism provided by an integrated system of non- enzymatically reduced molecules like ascorbate, glutathione and enzymatic antioxidants (Prochazkova *et al.,* 2001; Shrivali *et al.,* 2003). The primary antioxidant enzyme which converts superoxide to H_2O_2 and oxygen is superoxide dismutase (SOD) (Alscher *et al.,* 2002). The key enzyme involved in H_2O_2 scavenging is also catalase which decomposes H_2O_2 to water and oxygen. SOD and CAT are considered key components in the antioxidant response system as they regulate the cellular concentration of O_2^- and H_2O_2 (Van Breusegem *et al.,* 2001).

Arbuscular mycorrhizal (AM) fungi are mutualistic symbiosis provides direct physical link between soils to plant root (Barea and Jeffries, 1995; Gaur and Adholeya, 2004). It is known that AM fungi can enhance plant growth and production under different conditions, including various soil stresses (Daei *et al.,* 2009; Dudhane *et al.,* 2011; Evelin *et al.,* 2009; Gaur and Adholeya 2004; Hildebrandt *et al.,* 2007; Miransari *et al.,* 2008)

The past decade has witnessed the rapid increase in interest in agroforestry and plantation as a land use practice across India. Of the tree species currently being tested for agro forestry or plantation in India, *Gmelina arborea* Roxb., is one moderately fast growing indigenous tree used for the purpose of timber, fuel and pulp production. The

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biodiversity of AM colonization and AM fungi in different forest tree species were studied in India (Kumar *et al.,* 2000; Verma and Jamaluddin, 1995).

Hence the present studies were undertaken in an attempt to improve the survival and growth of *Gmelina arborea* plants in saline soil condition using AM fungi.

Materials and methods

Plant material, AM inoculum and experimental design

Spores of Mycorrhizal fungus *Glomus fasciculatum* (Thaxter) Gerd. and Trappe Emend. Walker and Koske were isolated from rhizospheric soil of 15 year old *Gmelina arborea* plant from Botanical garden, Department of Botany, University of Pune, Maharashtra, India by wet sieving and decanting method (Gerdemann and Nicholson, 1963), identified with the help of Manual (Schenck and Perez, 1990). The spores were multiplied on Bajara for two months and used as an inoculum (soil containing spores AM colonized roots and extraradical mycelium) for the treatment of *Gmelina arborea* seedlings. Non-mycorrhizal plants consist of same inoculum but autoclaved for 1 hr at 121°C. The seedlings were grown in soil (autoclaved for1hr at 121°C) from seeds of *Gmelina arborea* (brought from Synergy farm, Pasure, Tal-Bhor. Dist- Pune, Maharashtra, India).

Experimental design

The experimental design consisted of six treatments having non- AM inoculated and AM inoculated with three salinity levels (NaCl: 0, 100 mM in 100 ml D.W. and 200 mM in 100 ml Distilled Water) per 3 kg of sterilized soil. Pots were arranged in completely randomized block design. Six replicates of each treatment were grown; total 36 pots (three plants/pot) were arranged. Two-month-old seedlings of *Gmelina* were used for salinity experiment. NaCl was used for salinity stress. After 30 days of AM inoculation, NaCl treatment was given at eight days interval and it was continued till the last observation was taken. Observations were recorded after 45, 75 and 100 days after AM inoculation.

Morphological parameters

The analyzed morphological parameters were: shoot length, root length, fresh weight, dry weight, leaf area. The roots were cleared and stained by using the methods by Phillips and Hayman (1970) and the percentage of mycorrhizal colonization was estimated by the methods by gridline intersect method (Giovannetti and Mosse, 1980). At each salinity level, the mycorrhizal dependency (M.D.) of the plants was calculated according to Gerdemann (1975) as:

$$\text{M.D.} = \frac{\text{Dry weight mycorrhizal plant at a particular level of salinity}}{\text{Dry weight nonmycorrhizal plant at the same level of salinity}} \times 100$$

Physiological and biochemical parameters

Total chlorophyll (Arnon, 1949), acid phosphatase (Lowry *et al.,* 1954) and proline (Bates *et al.,* 1973) were estimated in the AM inoculated seedlings.

Protein extraction

Fresh tissue was ground in 3 ml of Extraction buffer containing 10 ml of extraction buffer containing 9.460 ml distilled water, 500 µl 1M Tris acetate buffer, 20 µl Triton X 100, 200 mg PVP, 1 µl 100 mM PMSF, and 2 µl of 0.5 M Sodium EDTA. the homogenate was centrifuged at 18000 g at 5°C for 10 min and the supernatant was used for enzyme assay. Protein concentration was estimated according to Lowry *et al.* (1954) using Bovine Serum Albumin as standard.

Determination of antioxidant enzymes

Guiacol peroxidase (GPX) specific activity was determined by Putter (1974), superoxide dismutase (SOD) activity was determined by Beauchamp and Fridovich (1971). Catalase (CAT) activities were determined by the degradation of H_2O_2 described by Aebi (1984).

Statistical analysis

Statistical analysis was performed using one- way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). The values are mean ± standard deviation for six treatments in each group. P values ≤ 0.05 were considered significant.

Result and discussion

Salinity is one of the major limitations on crop productivity and quality in the world. Hoorn *et al.* (2002) have shown that the negative effects of salinity are reduced growth rate and biomass, smaller leaves, osmotic effects, nutritional deficiency as well as mineral disorders. In *Gmelina* we observed that shoots and root length was consequently increased after 45 and 75 days of AM inoculation in mycorrhizal *Gmelina* plants as compared to nonmycorrhizal *Gmelina* under low salinity level (100 mM). At high salinity level (200 mM) shoot and root length decreased as compared to low salinity level in mycorrhizal as well as nonmycorrhizal plants (Tab. 1). Ghollarata and Raiesi (2007) showed similar results in clover plants.As compared to nonmycorrhizal plants fresh and dry weight of mycorrhizal *Gmelina* plants increased at all salinity (100- 200 mM) levels after 45 and 75 days of AM inoculation. After 100 days of AM inoculation and at high salinity level (200 mM), fresh and dry weight decreased in nonmycorrhizal plants as well as mycorrhizal plants (Tab. 1). It was observed that symbiotic association between AM fungus *Glomus fasciculatum* and *Gmelina* strengthened in saline environment by increasing shoot and root length and plant total biomass. Chulan and Martin (1992) reported

Tab. 1. Morphological parameters of *Gmelina arborea* Roxb after 45, 75 and 100 days after AM inoculation

Treatments		Shoot lengt (cm)	Root lengt (cm)	Fresh weight (gm)	Dry weight (gm)	Percent root colonization
C	45days	12.16±0.471b	9.66±1.699b	0.837±0.040d	0.352±0.005e	0.00
	75days	12.50±0.408b	15.66±2.494b	2.683±0.644c	0.548±0.082d	0.00
	100days	14.66±2.494c	17.73±0.736c	4.122±0.876b	0.672±0.014f	0.00
C+1S	45days	14.00±0.471b	10.66±1.24b	2.404±0.041c	1.039±0.041d	0.00
	75days	14.80±0.616b	17.66±2.494b	3.146±1.256bc	1.037±0.022c	0.00
	100days	16.00±1.080c	21.50±0.408b	4.024±0.033b	1.384±0.013d	0.00
C+2S	45days	14.16±0.623b	9.66±1.699b	2.533±0.049c	1.177±0.040c	0.00
	75days	14.00±0.816b	15.33±1.247b	3.097±0.698bc	1.254±0.115c	0.00
	100days	15.00±2.160c	18.20±0.355c	3.853±0.138b	1.081±0.004e	0.00
Gf	45days	26.50±1.779a	20.33±1.69a	4.709±0.163b	2.020±0.005b	76.66±4.714b
	75days	26.66±3.858a	27.00±1.632a	4.574±0.832abc	2.080±0.029b	86.66±4.714a
	100days	30.33±1.433a	34.33±3.091a	7.005±0.408a	2.132±0.009c	93.33±9.428a
Gf+1S	45days	26.33±2.054a	22.66±1.24a	4.468±0.266b	2.310±0.033a	86.66±4.714a
	75days	28.00±3.741a	29.00±1.632a	5.236±1.288ab	2.261±0.213b	93.33±4.714a
	100days	33.50±2.160a	34.66±2.054a	6.336±1.406a	3.357±0.009a	86.66±4.714a
Gf+2S	45days	25.00±1.632a	23.33±1.24a	5.509±0.454a	2.375±0.021a	83.33±4.714ab
	75days	27.00±1.632a	27.33±1.699a	6.389±1.479a	2.566±0.090a	83.33±12.47a
	100days	25.00±0.816b	34.00±0.408a	6.015±0.271a	3.267±0.015b	86.66±12.47a

Note- Data was analyzed by Duncan's multiple new range test and different small alphabetical letters indicate significant differences at $p \leq 0.05$ level. C-Control, C+1S- Control with first salinity level, C+2S- Control with second salinity level, Gf- *G. fasciculatum*, Gf +1S- *G fasciculatum* with first salinity level, Gf +2S- *G fasciculatum* with second salinity level, first salinity level-100 mM NaCl, second salinity level-200 mM NaCl

a significant shoot dry weight increment when *Theobroma cacao* was inoculated with VA-mycorrhiza.

After 45, 75 and 100 days of AM inoculation percent AM root colonization in under non saline condition was 76%, 86% and 93% respectively. Under low salinity levels percent root colonization was 86%, 93% and 86% respectively, similarly in under high salinity levels 83%, 83% and 86% respectively (Tab. 1). In our study percent root colonization increased at low salinity level. Percent root colonization was reduced with increasing salinity level and days of salinity treatments. Previous research has shown that salinity may reduce mycorrhizal colonization by inhibiting the germination of spores (Hirrel and Gerdemann, 1980).

In mycorrhizal *Gmelina* plants (Gf), Mycorrhizal Dependency was found to higher as compared to first (100 mM) and second (200 mM) salinity level , but decreased with increasing time duration (45, 75 and 100 days of AM inoculation) (Fig. 1). Mycorrhizal dependency was increased in mycorrhizal *Gmelina* plants under high salinity condition. Thus, it could be concluded that the benefits of symbiotic association between AM fungi and *Gmelina* plants increased under salinity conditions.

In *Gmelina*, after 45 and 75 days of inoculation the total chlorophyll content was significantly increased in mycorrhizal plants at all levels of salinity. But after 100 days of AM inoculation total chlorophyll content was decreased in high salinity level in mycorrhizal plants (Fig. 2). It was

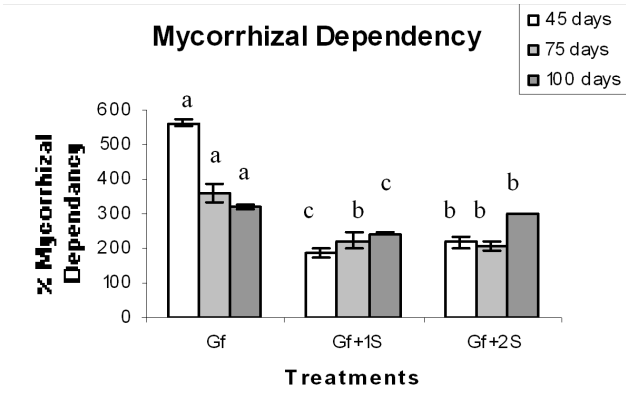

Fig. 1. Mycorrhizal dependency in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

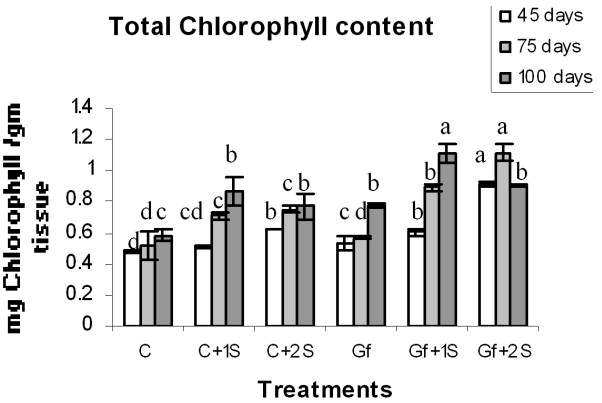

Fig. 2. Total chlorophyll content in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

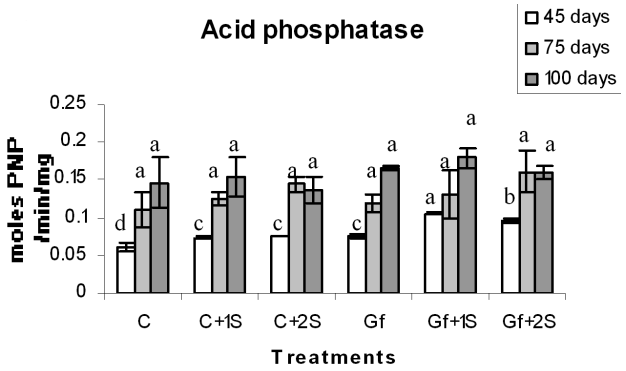

Fig. 3. Acid Phosphatase activity in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

shown that the chlorophyll content depends on the salinity level as well; on average, it was higher in inoculated plants, as already observed by some authors (Abdel and Mohamedin, 2000; Diaz and Garza, 2006). Moreover, inoculated plants under salt stress reach levels of photosynthetic capacity (estimated by the chlorophyll content) even superior to those of non-stressed plants, showing that in this respect mycorrhization is capable to fully counterbalance salt stress.

In *Gmelina* acid phosphatase activity significantly increased in mycorrhizal *Gmelina* plants as compared to nonmycorrhizal control plants at low and high salinity level (100 mM and 200 mM) after 45, 75 and 100 days of AM inoculation. (Fig. 3). Increased in acid phosphatase activity was similar to the earlier findings of AM-inoculated *Moringa concanensis* plants (Panwar and Vyas, 2002). Selvaraj (1998) found that due to inoculation of AM fungi, *G. fasciculatum*, acid and alkaline phosphatase activity increased in leaves and roots of *Prosopis juliflora*. The increased the phosphatase activity in mycorrhizal bajra plants leading to increase in phosphate uptake under salinity stress condition. These findings indicate that the effect of AM fungi on phosphorus uptake constitute one of the main mechanisms for increasing plant tolerance to salinity (Rabie and Almadini, 2005).

A number of nitrogen containing compounds accumulate in plants exposed to saline stress. The specific nitrogen containing compounds that accumulate in saline environments vary with plant species. Many plants accumulate Proline as a nontoxic and protective osmolyte under saline conditions. (Jain *et al.,* 2001; Pujol *et al.,* 2001.) In the present study accumulation of Proline in non-mycorrhizal and mycorrhizal *Gmelina* plants increased significantly by raising salinity. In *Gmelina*, shoot and root Proline activity increased in mycorrhizal plants as compared to nonmycorrhizal plants at low salinity (100 mM) after 45 and 75 days of inoculation. But at high salinity level (200 mM) and after 100 days of AM inoculation shoot as well as root Proline activity decreased in non AM inoculated control plants (C +2S) as well as AM inoculated plants (Gf +2S) (Fig. 4 and 5). Wang *et al.* (2003) reported that Proline accumulation in plants might be a symptom of stress in less salinity- tolerance species and its contribution to osmotic adjustment was apparently negligible as compared with K^+. Based on this data, we conclude that non-mycorrhizal *Gmelina* plants are less salinity tolerant to saline conditions as compared to AM *Gmelina* plants. *Lotus glaber* is known to accumulate high levels of Proline in response to salinity (Maiale *et al.*, 2004).

In our experiment we observed that, as compared to nonmycorrhizal *Gmelina* plants shoot POX activity of mycorrhizal *Gmelina* plants increased at all salinity (100-200 mM) levels after 45, 75 and 100 days of AM inoculation. But after 100 days of AM inoculation at high salinity level (200 mM) shoot POX activity was found to decrease in nonmycorrhizal *Gmelina* plants as well as AM inoculated plants (Fig. 6). Root POX activity consequently increased after 45 days of AM inoculation in all levels of salinity in mycorrhizal *Gmelina* plants as compared to nonmycorrhizal plants. After 75 and 100 days of AM inoculation root POX activity was found to be decreased in mycorrhizal *Gmelina* plants at high salinity levels. (Fig. 7)

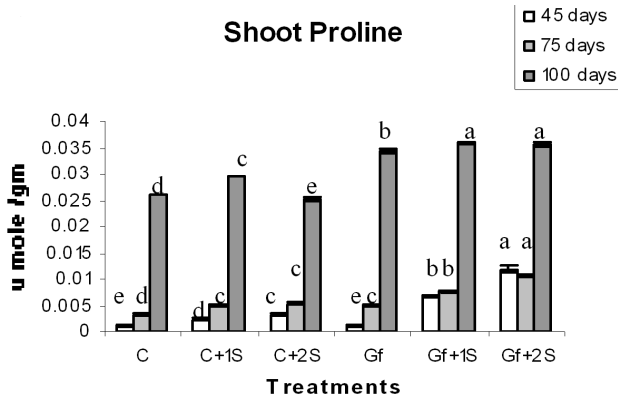

Fig. 4. Shoot proline content in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

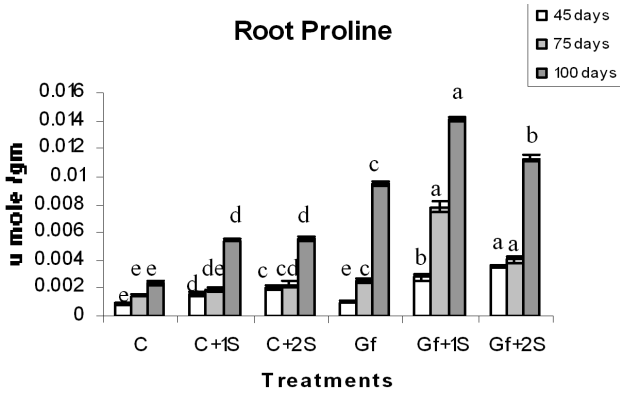

Fig. 5. Root proline content in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

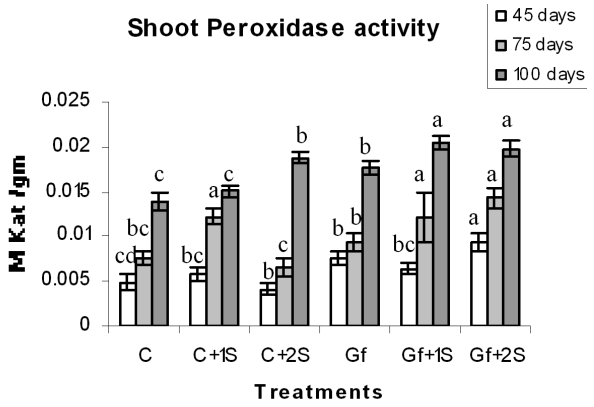

Fig. 6. Shoot peroxidase activity in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

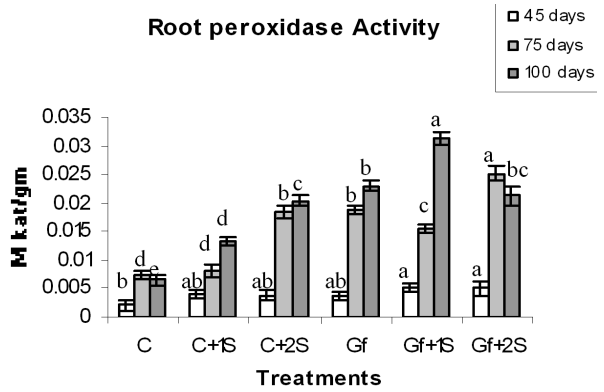

Fig. 7. Root peroxidase activity in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

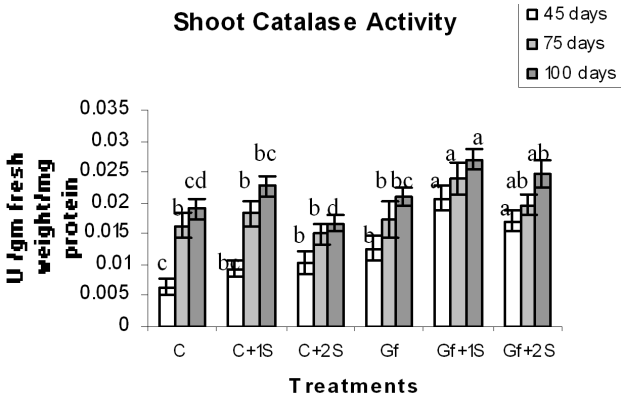

Fig. 8. Shoot catalase activity in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

The steady-state levels of ROS in plant cells may be determined by the balance between activities of SODs, POXs and CATs (Mittler, 2002). It has been reported that SOD specific activities were higher in roots and shoots of lettuce (*Lactuca sativa*) colonized by *Glomus mosseae* than in non-mycorrhizal roots, under drought stress (Ruiz-Lozano *et al*., 1996).

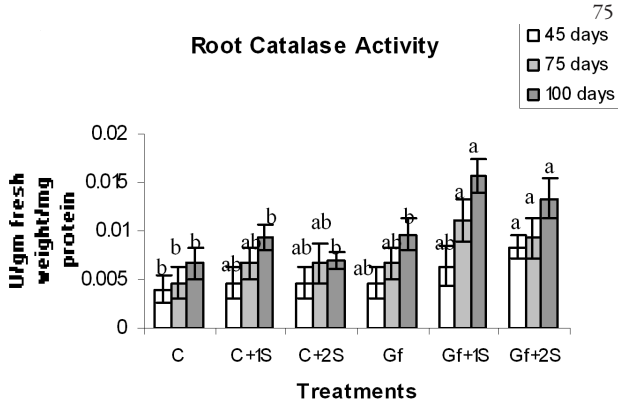

Fig. 9. Root catalase activity in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

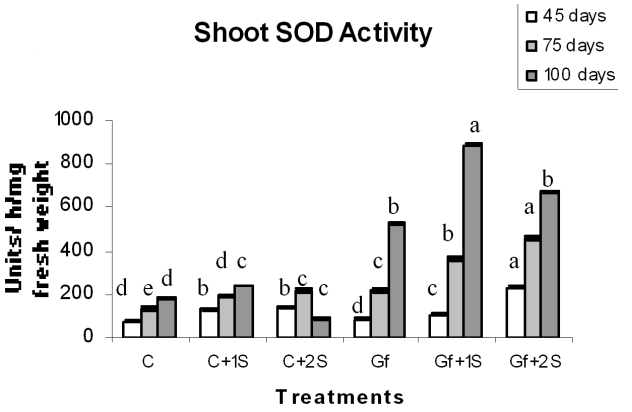

Fig. 10. Shoot superoxide dismutase activity in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

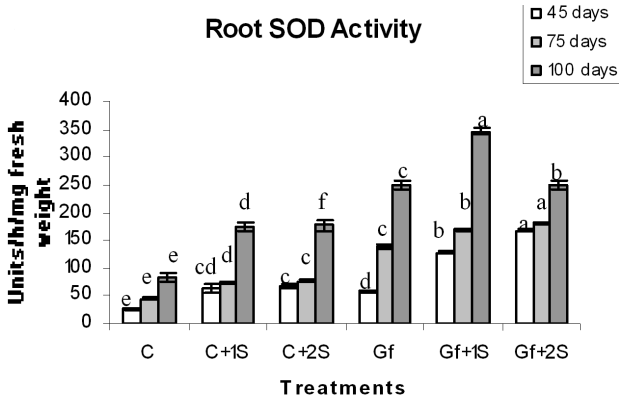

Fig. 11. Root superoxide dismutase activity in *Gmelina arborea* Roxb inoculated with *Glomus fasciculatum* after 45, 75 and 100 days after AM inoculation

In tomato and citrus, salt-tolerance is attributed to the increased activities of SOD, Ascorbate peroxidase (APX), and CAT (Gueta-Dahan *et al.,* 1997; Mittova *et al.,* 2004). In *Gmelina* we have obtained similar results. An increase in the activity of SOD, POX, and CAT during salinity was observed in 45 days and 75 days of AM inoculation but at high salinity level ROS enzyme activity was found to

decrease after 100 day of AM inoculated *Gmelina* plant. Zhong Qun *et al.* (2007) detected the growth parameters, cell membrane osmosis and the activities of SOD, POD, APX and CAT in roots of AM and non-AM tomato under NaCl and normal condition. They also evaluated the effects of these enzymes in ROS scavenging on the enhanced salt tolerance by AMF.

Gmelina showed higher shoot and root catalase activity in mycorrhizal *Gmelina* plants as compared to nonmycorrhizal *Gmelina* plants under zero salinity and low salinity levels after 45 ,75 and 100 days of AM inoculation. But in high salinity level after 45, 75 and 100 days of inoculation *Gmelina* plants showed decreased in catalase activity but it was higher in mycorrhizal as compared to nonmycorrhizal inoculated *Gmelina* plants. (Fig. 8 and 9)

The antioxidant enzymes such as SOD, CAT, and POX showed deviation in their activities under salinity condition. In different parts of the plants, the enzyme activity varied greatly. High salinity reduced SOD activity in both root and leaves, but low salinity led to an increase in root SOD activity (Jaleel *et al.,* 2007). Our results showed that, as compared to nonmycorrhizal *Gmelina* plants shoot and root SOD activity of AM inoculated plants increased at all salinity (100-200 mM) levels after 45 and 75 days of AM inoculation. After 100 days of AM inoculation at high salinity level (200 mM) shoot SOD activity was found to decrease in nonmycorrhizal as well as mycorrhizal *Gmelina* plants, but mycorrhizal plants showed higher activity than the nonmycorrhizal plants. (Fig. 10 and 11)

Plants with high concentration of antioxidants have been reported to have greater resistance to this oxidative damage (Borde *et al.,* 2011; Dionisio-Sese and Tobita, 1998; Jiang and Zhang, 2002). Salinity results higher activity of CAT (Dionisio-Sese and Tobita, 1998), POD (Gossett *et al.,* 1994) and SOD (Sudhakar *et al.,* 2001). In *Gmelina arborea* we found increased growth and also antioxidant activities in *Glomus fasciculatum* inoculated plants.

Conclusions

In conclusion, the results confirm that arbuscular mycorrhizal fungi alleviate the detrimental effect of salinity through improved water and nutrient uptake especially P through AM hyphae and colonized roots of *Gmelina*. This suggests that phosphatases might be involved in P transfer and uptake mechanism which leads to higher P from saline soil. Another mechanism through osmotic adjustment under saline condition by increasing water uptake by AM fungal hyphae. From our study revealed that exposure of mycorrhizal *Gmelina* plants to salinity resulted in significant induction of antioxidative enzyme activities such as SOD, POX and CAT that could help the plants protect themselves from the oxidative effects of the ROS. This cumulative effect increases the physiological performance and tolerance of the *Gmelina* plants under saline condition. Hence mycorrhizal inoculation to *Gmelina* plant for better survival under saline condition and use as in agroforestry system under reclamation of saline land.

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