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(RESEARCH ARTICLE)

Arbuscular mycorrhizal influence on oxidative stress in French bean (*Phaseolus vulgaris*) under drought conditions

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Abstract

We investigated the antioxidant response in French bean (*Phaseolus vulgaris*) seedlings in a symbiotic interaction with Arbuscular mycorrhiza fungi (AMF) under drought and studied both shooting and rooting tissues to detect the targeted tissue for AMF effects induced under drought. AMF and Non-AMF French bean seedlings were grown under control (well-watered: WW) and drought-stress (DS) conditions to study their tolerance response by assessing various physiological and biochemical parameters. AMF plants appeared to be sheltered from drought as seen by their higher leaf water potential and production of shoot-biomass. The AMF shoots accumulated less proline than those of non-AMF, while the opposite was observed in roots. Also, in DS AMF plants, lipid peroxides were 60 percent lower than in DS non - AMF plants. However, no significant correlation could be established between antioxidant enzyme activity and the low lipid oxidative damage in AMF plants due to which it appears that the symbiont fungus enhances osmotic adjustment first in the root system, which promotes a water potential gradient that favors entry of water into roots from the soil. This enables increased leaf water potential in DS-AMF plants keeping plants sheltered from oxidative damage, and all these collective effects increase the drought hardiness of seedlings.

Keywords: Arbuscular mycorrhiza fungi; Drought-stress tolerance; French bean

1. Introduction

Drought is regarded a major abiotic factor that limits the growth and yield in plants1 and Arbuscular mycorrhizal symbiosis has been seen to protect host plants from its detrimental effects [2]. Although mycorrhizal effects on plant water relations are not as dramatic and consistent as those on acquisition and host growth, it is accepted that modest changes, if sustained, can have meaningful effects on plant fitness3. Several studies on the topic have demonstrated that the contribution of the AMF symbiosis to plant drought tolerance results from a combination of physical, nutritional, physiological, and cellular effects [4]. This seems to be true in many instances of tissue hydration among AMF and non-AMF vegetation where one group either soaks up more or loses much less water as the soil dries5. However, this is not the only mechanism by which AMF symbiosis enhances drought tolerance in plants. Other mechanisms have been proposed, such as greater osmotic adjustment and leaf hydration or condensed oxidative damage by the reactive oxygen species (ROS) generated during drought [4]. In fact, it has been shown that mycorrhizal colonization and drought interact in modifying free amino acid and sugar pools in roots. In leaves of mycorrhizal basil plants, a greater osmotic adjustment was reported during a lethal drought period than in non - mycorrhizal plants [5]. Similarly, AMF plants showed delayed reduction in leaf water potential (Ψ) during DS and leaf Ψ eventually resumed to a non - AMF level in AMF faster than non - AMF maize plants after drought relief [6]. In contrast, when water was not limiting, leaf Ψwas similar in AMF and non-AMF plants [7]. Lettuce plants having AMF displayed enhanced activity of (SOD) under DS and

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this is associated with plant defense against drought [8]. In the same way, AMF soybean plants subjected to drought had lower oxidative damage to lipids and proteins in nodules than non-AMF plants, and this was linked to protection against nodule senescence [9,10]. Whilst numerous studies targeted the biochemical and physiological molecular responses of shoot tissue against DS, very few have investigated the root tissue simultaneously, even though the roots are the prime site of drought perception, playing a vital role in recovery and resistance from drought. Root length and structure are key features for evaluating the functioning and yield especially during limited availability of water as the root system is dynamically involved in receiving and sending DS signal that appears to be shifted to the entire plant through ethylene growth regulators or ABA which induce closure of stomata.

2. Material and methods

2.1. Experimental design

The experimentation consisted of a complete randomized block design with two inoculation treatments: (i) non - mycorrhizal control plants; and (ii) mycorrhizal plants inoculated with the fungus Glomus intraradices. Five replicates of each treatment were done with 20 pots (one plant per pot) so half of them were cultivated under well-watered conditions throughout the entire experiment while the other half were drought-stressed for 05 days before harvest.

2.2. Biological materials

The soil sample was taken from Experimental Station, Bangalore University Garden. (Bangalore, India) and was sterilized by steaming at 100 ºC for 1h. The soil pH was of 8.1 (water, 10 g soil in25 ml water); and contained 1.81% organic matter, the nutrient concentrations being (mg/kg): N, 2.5; P, 6.2 (NaHCO3-extractable P); K, 132.0 and its composition was 35.8% sand, 43.6% silt, and 20.5% clay. French bean (*Phaseolus vulgaris S*-9) seeds were sterilized in a 1.5% H2O2 solution for 5s, followed by several sterile water wash to eliminate any traces that would interfere with seed germination, and sowed in plastic pots containing 600 g of the sterilized sand-cocopeat mixture. Plants were grown in a controlled environment of 25/18 °C day/night temperatures with 16h photoperiod under 70-80% relative humidity. Mycorrhizal inoculum consisting of soil, spores, mycelia, and colonized root fragments in large scale in an open-pot culture of Zea mays L. The AMF species was Glomus intraradices isolate EEZ 6, BEG 121. Five-gram culture extract was added below the French bean seedlings while sowing to the applicable pots.

2.3. Parameters measured

2.3.1. Biomass production & Symbiotic development

20 DAS, shoot dry weight (DW) was measured after drying at 70°C for 48h. The roots were treated in 10% KOH (W/V) and stained with 0.05% trypan blue in lactophenol (v/v), according to Phillips and Hayman (1970), the % of mycorrhizal root infection was assessed by fungal colonization [11].

2.3.2. Relative water content

Leaf discs of 10 nm diameter of fresh weight (FW), soaked in distilled water at 25ºC for 4 h to determine the turgid weight (TW), then oven dried at 80 ºC for 24 h for dry weight (DW). RWC measured according to Turner and Kramer; RWC= (FW-DW) X100/TW-DW.

2.3.3. Proline, Ascorbic acid, and total soluble sugars

The quantification of total soluble sugars and free proline was done using 1g fresh root and leaf tissue. Spectrophotometric analysis was done for proline estimation by ninhydrin reaction at 515nm [12]. Ascorbic acid estimation was carried using an assay mixture that consisted of 1.0 ml of brominated sample extract, 2.0 ml of distilled water, 1.0 ml of DNPH reagent and 1-2 drops of thiourea. After incubation at 37 °C for 3 h, the orange-red osozone crystals formed were dissolved by the addition of 7 ml 80% sulphuric acid and absorbance was read at 540 nm after 30 min. The Soluble sugars levels were analyzed by anthrone method.

2.3.4. H_2O_2 concentration and lipid oxidative damage

0.5g aliquots of roots and leaves were homogenized in a chilled mortar using 25mM HCl and filtered through four layers of nylon cloth for estimation of H_2O_2 concentration. The pH of the supernatant was adjusted to neutral for H_2O_2 quantification by 4-aminoantipyrine method [13]. Extraction of Lipid peroxides was carried out by grinding 0.5g plant tissue in ice-cold condition followed by addition of 6ml 100mM potassium phosphate buffer (pH 7.0). Homogenates were sieved through cheesecloth and subjected to 20 min centrifugation at 15000g. 1 ml reaction mixture [15% w/v

trichloroacetic acid (TCA), 0.25N HCl, 0.1% (w/v) butyl hydroxyl toluene and 0.375% (w/v) 2-thiobarbituric acid (TBA)] was added to 200 ml of supernatant and incubated for 30 min at 100 °C for the chromogen formation [14]. The tubes were then centrifuged for 5 min at 800 g and spectrophotometric reading was noted at 532 nm using the supernatant. The relative extent of lipid peroxidation was evaluated according to Halliwell and Gutteridge by estimating the amount of TBARS (2-thiobarbituric acid-reactive substances) and expressing in terms of MDA equivalents [15]. The standardization curve was constructed with 0.1-10 nmol MDA. For each sample blank and control was made where the sample was substituted with extraction medium and TBA substituted with 0.25N HCl respectively.

2.3.5. Preparation of enzyme extracts

1g (FW) plant tissue was used for enzyme extraction by homogenization at 0-4^{0}C with 50 mg polyvinyl polypyrrolidone and 10 ml of 50 mM Na-phosphate buffer (pH 7.8) having 0.1 mM EDTA for catalase (CAT), superoxide dismutase (SOD) and peroxidase (APX) [16]. For glutathione reductase (GR) the above medium provided with 10 mM β-mercaptoethanol was used [17]. Extracts were filtered through four nylon cloth layers and centrifuged for 20 min at 20,000 g, 0 - 4 °C. For subsequent enzymatic assays, the supernatants were kept at -20 ° C. Dye binding method was used for soluble protein estimation [18].

2.3.6. Enzyme assays

Total SOD (EC 1.15.1.1) activity was measured based on the ability of SOD to inhibit nitroblue tetrazolium (NBT) reduction by photochemically generated superoxide radicals according to Beyer and Fridovich [19]. One unit of SOD has been defined as the amount of enzyme needed to inhibit NBT's reduction rate by 50 %.at room temperature. *Catalase* (CAT; E.C. No.1.11.1.6): CAT activity was calculated by following A_{240} decline as H_2O_2 (ε =36M^{-1} cm^{-1}) was catabolized, according to the method [20]. For the in-gel assay, CAT isozymes were separated on non-denaturing acrylamide gels (9%) at 100 V for 2 h at 4 °C. Electrophoresed gels were soaked in 3.27 mM H_2O_2, for 15 min, rinsed with water, and stained in 2% solution of ferric chloride and potassium ferricyanide to visualize the bands.

Glutathione reductase *(GR; E.C.No.1.6.4.2):* GR was assayed spectrophotometrically by monitoring the GSSG dependent NADPH oxidation. GR isozymes were separated on non-denaturing acrylamide gels (9%) at 100 V for 2 h at 4 °C. Gels were soaked in 50 mM Tris-HCl buffer, (pH 7.5) containing 10 mg MTT, 10 mg 2, 6- dichlorophenolindophenol, 3.4 nM GSSG, and 0.4 mM NADPH.

Peroxidase activity (POX; EC No. 1.11.1.1): Peroxidase activity was measured spectrophotometrically at 470 nm (ε =26.6 $mM^{-1}cm^{-1}$) using H_2O_2 as substrate. One unit of peroxidase was defined as the amount of enzyme that caused the formation of 1 mM of tetra-guaiacol per minute. The native PAGE of POX was performed on 9% native acrylamide gels with (pH 8.8). Gels were stained with o-dianisidine-HCl in acetate buffer (pH 5.5) for 30 min RT, followed by 100 mM H_2O_2 until visible bands developed.

β-Amylase (E.C. No. 3.2.1.1): The reaction mixture consists 0.5ml of 2.0% starch and 0.5 ml of enzyme extract and the activity was monitored spectrophotometrically. β- amylase isozymes were visualized starch followed by incubation in 0.025% acidified iodine solution for 5 min.

Polyphenol oxidase: The poly phenol oxidase activity was monitored in absorbance at 495 nm min-1mg^{-1} using catechol as substrate.

3. Results

In plants not supplied with AMF inoculum, no colonization was observed. Under WW conditions plant biomass, mycorrhizal colonization and shoot dry weight of AMF and non-AMF seedlings were comparable (Table-1), but leaf RWC was found higher in AMF plants than in non-AMF seedlings. Drought stress decreased RWC of leaves (Table 2) and plant growth in both cases (42% reduction in AMF and 57% reduction in non-AMF plants). DS AMF plants showed 26% higher shoot DW in comparison to non-AMF plants. However, under both WW and DS conditions root DW in AMF and non-AMF plants remained the same.

Table 1 Shoot and root tissues of DW and mid-day leaf Ψin AMF or non- AMF French bean seedlings under WW and DS conditions

Description	Shoot tissue DWp (g plant-1)	Root tissue DWp (g plant-1)	Leaf ψp (MPa)
AMF seedlings			
WW	2.32 [a]	1.97 [a]	-2.42 [c]
DS	1.69 [b]	1.62 [b]	-2.89 [b]
Non-AMF seedlings			
WW	1.31 [a]	1.3 [a]	-2.56 [c]
DS	0.61 [c]	1.49 [b]	-3.42[a]
Significance of sources of variation			
M	**	Not significant	*
W	***	**	***
M x W	*	Not significant	*

(P<0.05) as determined by Duncan's multiple-range test (n=5).

3.1. Accumulation of proline and total soluble sugars

Proline accumulation increased significantly in roots and shoots as a result of DS and AMF plants accumulated 14.5% more proline in roots and 39.3% less proline in shoots than non-AMF plants (Figure 1, 2 and Table 2). Total soluble sugars were higher in roots of AMF plants grown under WW conditions than in non-AMF plants (Figure 3, 4 and Table 2). DS increased the accumulation of sugar in both cases, but under such conditions ANOVA found no significant differences between them. Under WW conditions, total soluble sugar in shoots was similar in both AMF and non - AMF plants, whereas drought stress augmented the sugar levels of non - AMF French bean by 115 %, while AMF plants exhibited a similar sugar levels to WW plants.

Table 2 Significance of the sources of variation for soluble sugar (SS) & proline levels, and for oxidative stress to lipids (ODL)

	Soluble Sugar		Proline		ODL	
M	a	*	*a	*	**	*
W	**	***	***	*	*	*
M x W	ns	ns	*	*	ns	ns

a *P<0.05; **P<0.01; ***P<0.001; ns, not significant

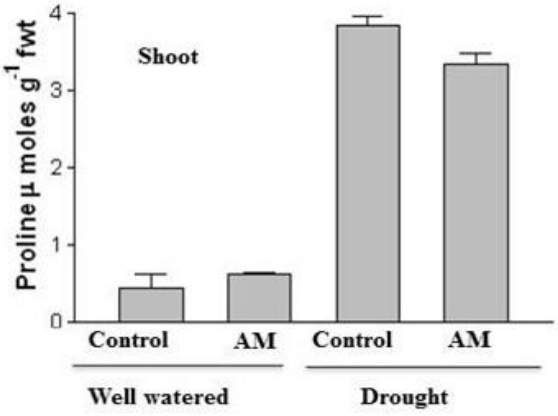

Figure 1 Proline (µmol /g Fresh weight) in shoot tissue of AMF or non-AMF French bean grown under WW and DS conditions

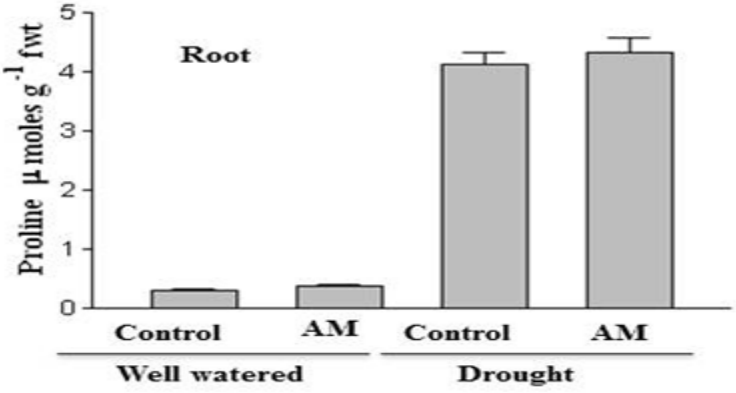

Figure 2 Proline (µmol g-1 FW) in roots of AMF and non-AMF French bean during WW and DS conditions

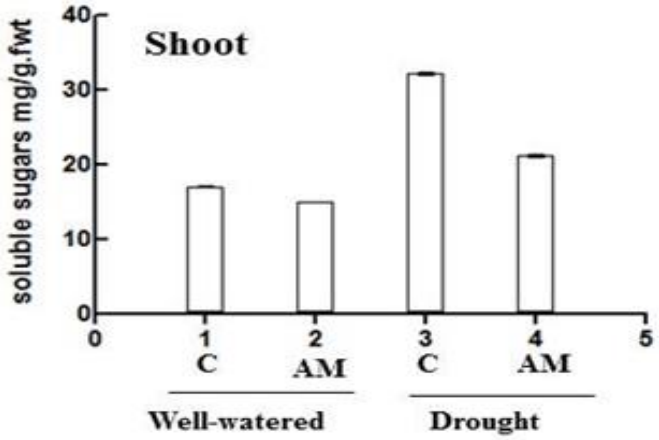

Figure 3 Soluble levels (mg g-1 Fresh Weight) in the shoot of AMF and non-AMF French bean during WW and DS conditions.

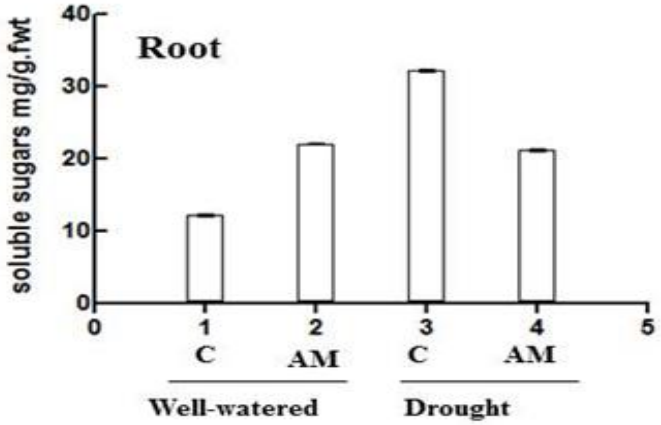

Figure 4 Soluble levels (mg g-1 FW) in roots of AMF and non-AMF French bean during WW and DS conditions**.**

3.2. Hydrogen peroxide accumulation and oxidative damage to lipids

Among all the treatments there was no significant difference in the concentration of H_2O_2 in both roots and lipid peroxidation shoots. Roots in all cases accumulated about 150nmol H_2O_2 per 1g FW, while shoots accumulated 10 times more. le increased only in the roots of non-AMF plants as a result of DS (Table 2). Under both water conditions, AMF plants showed similar levels of lipid peroxidation. DS AMF plant roots exhibited 13 percent fewer lipid peroxides than

DS non - AMF plant roots. The different responses of AMF and non-AMF plants were more evident in shoots. Drought enhanced significant raise in lipid peroxidation in non-AMF plants by 77%, while, in shoot tissue of AMF plants remained modest. In any case, under drought conditions, shoots of AMF plants had 50% fewer lipid peroxidation than shoots of non-AMF plants (Fig 5,6).

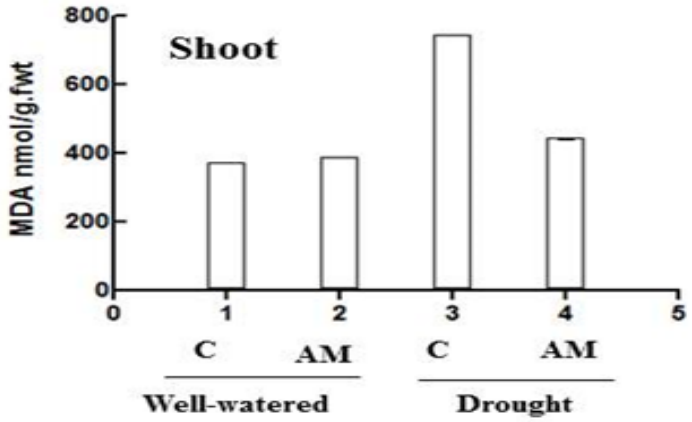

Figure 5 Lipid Peroxidation (nmol MDA g-1 FW) in shoots of AMF and non-AMF French bean during WW and DS conditions

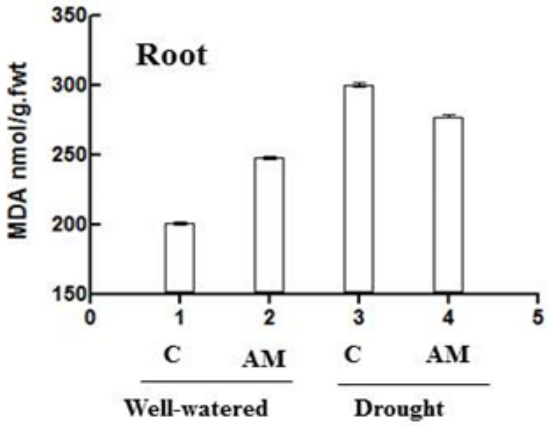

Figure 6 Lipid peroxidation (nmol MDA g-1 FW) in roots of AMF and non-AMF French bean during WW and DS conditions.

3.3. Antioxidant activities

SOD levels were similar in roots in the various treatments, with the exception of DS AMF roots, significantly lower SOD (Table 3). In shoots, AMF plants had lower SOD level than non - AMF plants when grown under WW conditions and increased activity when grown under conditions of DS. CAT levels showed an opposite response in roots and in shoots (Table 3). In roots, CAT levels only increased in AMF plants as a consequence of drought while in shoots, the CAT activity of AMF plants was significantly higher than in non-AMF plants under WW conditions, but the CAT levels of AMF plants decreased under DS conditions, attainment a similar value to that of non - AMF plants. POX was at all times higher in non-AMF plants than in AMF plants (Table 3). DS enhanced the POX activity in shooting tissue of both AMF and non-AMF plants in comparison to WW conditions. AMF roots had considerably less APX irrespective of whether they were grown under WW or DS conditions. GR activity was significantly increased by the stress of drought in non - AMF roots and decreased in AMF roots (Table 3). DS AMF and DS non - AMF shoots had analogous GR activities, while GR activity increased 40 times in non - AMF shoots compared to AMF plants under WW environment. Poly phenol-oxidase activity was increased by 1.8, fold in roots and 1 1.8-fold in shoots with respect to their water treated samples (Table 3).

Table 3 Antioxidant enzymes activities in roots and shoots of AMF or non-AMF French bean seedlings grown under WW and DS conditions

	Superoxide dismutase		**Catalase** [a]		**Peroxidase** [a]		**Glutathione reducatse**[a]	
Treatments	R	S	R	S	R	S	R	S
AMF French bean								
Well-watered	10.23 [a]	1.62 [c]	2.33 [b]	1.42 [a]	1.52 [c]	5.92 [c]	2.64 [b]	1.23 [c]
Drought stress	6.09 [b]	1.41 [b]	4.34 [a]	0.27 [c]	1.68 [c]	17.17 [b]	1.64 [c]	1.93 [b]
Non- AMF French bean								
Well-watered	8.73 [p]	2.93 [a]	4.74 [a, b]	1.84 [b]	1.62 [a]	13.5 [b]	1.53 [b, c]	4.09 [p]
DS	9.09 [p]	1.59 [c]	6.07 [p]	2.4 [b, c]	2.24 [b]	23.74 [p]	2.36 [p]	2.44 [b]
Significance of sources of variation								
Moderate	*	**	ns	*	**	**	*	*
Watered	*	***	*	*	*	**	*	*
Moderatex Watered	ns	*	ns	ns	ns	*	ns	ns

R- Root and S-Shoot; Means followed by the same letter are not significantly different (P<0.05) as determined by Duncan's multiple-range test (n=5). Significance of the sources of variation is also displayed. **P<0.05; **P<0.01; ***P<0.001; ns, not significant*

4. Discussion

Drought has extreme possessions on crop yield. Even plants with an optimal supply of water undergo transitory water deficit, where the water loss by transpiration cannot be compensated by water absorption. It has been shown that arbuscular mycorrhizal symbiosis increases plant tolerance to water shortage, though the precise mechanisms involved are silent debated [21]. Our study examined biochemical and physiological aspects of drought resistance and water relations in DS AMF and non - AMF plants. AMF plants exhibited higher tolerance against the imposed DS than non - AMF French bean, as evidenced by their increased production of biomass (27 %), higher leaf Ψ and lower lipid peroxidation under such conditions. It has been seen that plants under DS accumulate the amino acid proline and organic osmolytes such as sugars, which are known to combat conditions of drought. In WW condition, the elevated sugar levels in AMF roots may be due to the sink effect of the mycorrhizal fungus demanding shooting tissue sugar. Under drought, in both treatments, the sugar levels in roots was similar, suggesting an osmotic adjustment. While in shoot tissue of DS AMF French bean sugar levels were relatively lower than in non- AMF plants. Schellembaum et al., [22] recommended that *Glomus intraradices* could be a strong competitor for root - allocated carbon under conditions that limit photosynthesis. This explains the lower amounts of sugars in leaves of DS AMF French bean. One more rationalization is that AMF shoots were less stressed by DS than non-AMF shoots. The lower buildup of hexose sugars might indicate that the AMF plants have avoided DS more successfully. The other osmo-regulator measured in this investigation- proline was also accumulated less in AMF plant shoots than in non - AMF plants. The hypothesis is also supported by higher leaf Ψ in DS AMF French bean (-2.9 MPa) than in non-AMF French bean (-3.5 MPa). On the other hand, proline collected more in roots of AMF French bean than in non - AMF plants. The buildup of total soluble sugars and proline in the roots may perhaps have facilitated an osmotic mechanism for the root to maintain a favorable gradient for the entry of water into the roots, resulting in a lower stress injury in the plant. Besides being an osmo-protectant, proline serves as an energy sink to regulate redox potential as a hydroxyl radical scavenger consequently reducing acidity in the cell. And since DS also induces oxidative stress causing many drought-induced degenerative reactions, proline proves to be important for AMF French bean under drought. Membrane lipid oxidation is a consistent hint of abandoned free - radical creation and therefore oxidative stress. The amount of lipid peroxides in roots and shoots was therefore quantified. In roots, oxidative damage in DS AMF plants was 15% lesser than in DS non-AMF plants. In case of shoots, membrane damage/lipid peroxidation in DS AMF French bean was 55% lower than in DS non-AMF plants. Peculiarly, in all treatments of our study the concentration of H_2O_2 measured was similar. It should be recalled, however, that H_2O_2 would contribute in almost all aerobic biochemistry and is produced in abundant quantities, even under optimal conditions, by several enzyme systems. The negative power and signaling potential of H_2O_2 is used as an actual means of defense under certain circumstances [23]. Enzyme activity of four antioxidant enzymes was determined and correlated to lipid oxidative damage. It was seen that there was no correlation between the antioxidant activity and the reduction of membrane damage in DS AMF plants. Furthermore, only shoot APX and shoot SOD levels showed a

substantial communication with mycorrhizae and water conditions, whereas no significant correlation could be deduced for the other activity levels. The results of the four antioxidant enzymes are consistent with the results seen in roots of soybean inoculated with *G. mosseae*. The only exclusion in the present study involving *Glomus intraradices* was seen with respect to the GR activity which was found to be lower in roots of DS AMF plants than in non-AMF plants, though in the earlier study, linking *Glomus mosseae*, the Glutatione reductase activity enlarged in AMF plants [24]. However, it is important to bear in mind that the AMF fungi used were different in both studies and their differing behavior has often been reported in connection with several plant enzyme activity. Contrary to the Glutatione reductase activity, the lesser lipid oxidative damage in the AMF plants appears to be a persistent effect of AMF symbiosis, independent of fungi involved in the association [10, 24]. Apart from the above-mentioned mechanisms for drought tolerance, the AMF contribution could have also happened through drought evasion mechanisms such as hyphal water absorption or increased water uptake due to mycorrhizal changes in root morphology or soil structure. These mycorrhizal effects might allow plants to remain hydrated as the soil dries. This is suggested by the current study data, for example, the elevated mid-day leaf Ψ in AMF as compared to non - AMF plants ; less proline and soluble sugar accumulation in AMF shoots than in non - AMF plants, and lesser lipid peroxidation in AMF as compared to non - AMF plants, which have no correlation with enhanced antioxidant activity. The overall study indicates that AMF symbiosis affects both the root and shoots tissues through drought avoidance and drought-tolerance mechanisms.

5. Conclusion

The present investigations insights the stable antioxidant system operated/induced under drought stressed French bean AMF seedlings.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

Praba and Sharadamma N conducted experiments, Nagesh babu is written the manuscript

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