Antioxidant Enzyme Activities in Arbuscular Mycorrhizal (*Glomus intraradices*) Fungus Inoculated and Non-inoculated Maize Plants Under Zinc Deficiency

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Abstract A greenhouse experiment was conducted to examine the changes in antioxidant enzyme activities of arbuscular mycorrhizal (AM) fungus *Glomus intraradices* Schenck and Smith inoculated (M+) and non-inoculated (M–) maize (*Zea mays* L.) plants (variety COHM5) under varying levels of zinc (0, 1.25, 2.5, 3.75 and 5.0 mg kg^{–1}). Roots and shoots sampled at 45 days after sowing (DAS) were estimated for its antioxidant enzymes (superoxide dismutase, peroxidase) IAA oxidase, polyphenol oxidase, acid phosphatase and nutritional status especially P and Zn concentrations. Mycorrhizal inoculation significantly ($P \leq 0.01$) increased all the four antioxidant enzymes in both roots and shoots at 45 DAS regardless of Zn levels. All enzyme activities except SOD increased progressively with increasing levels of Zn under M+ and M– conditions. The SOD activity got decreased in roots and shoots at 2.5 and 3.75 mg Zn kg^{–1}. Acid phosphatase activity in M+ roots and shoots were higher in all levels of Zn but the values decreased with increasing levels of Zn particularly in roots. Mycorrhizal fungus inoculated plants had higher P and Zn concentrations in both stages in comparison to non-inoculated plants. Our overall data suggest that mycorrhizal

symbiosis plays a vital role in enhancing activities of antioxidant enzymes and nutritional status that enables the host plant to sustain zinc deficient conditions.

Keywords Arbuscular mycorrhiza · Zinc · Maize · Antioxidant enzymes · Nutrition

Zinc deficiency in soils is widespread across the globe at varying intensities and particularly severe in arid and semi-arid regions where the soils are predominantly calcareous [1]. In India, the extent of Zn deficiency is reported as 60% and alarmingly increasing year by year [2] as a result of intensive agriculture, monocropping, continuous use of high analysis fertilizers and non-addition of organic manures. Zinc deficiency reduces crop yields besides lowering nutritional qualities of grains. One of the physiological disturbances caused by zinc deficiency in plants is the production of reactive oxygen species (ROS) [3] which damages cell membrane [4]. Though the production of ROS including superoxide radical and hydrogen peroxide is unavoidable in plant cells [5], the ROS can be detoxified by sufficient levels of Zn [6] as it involves in the activation and expression of genes responsible for detoxification [7]. Further the ROS can be effectively quenched by antioxidant enzymes such as peroxidase (POX), superoxide dismutase (SOD), catalase (CAT) and polyphenol oxidase (PPO) [8]. Superoxide dismutase, especially CuZn-SOD detoxify superoxide radical (O₂[–]) and protect plants from oxidative damage [9]. Deficient supply of Zn affects both the concentration and activity of CuZn-SOD [10] and thus the plants are exposed to oxidative damage.

Mycorrhizal symbiosis is known to offer protection against abiotic and biotic stresses as a secondary consequence of host plant nutritional improvement especially

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phosphorous [11]. The external mycelium of arbuscular mycorrhizal (AM) fungus explores larger soil volume beyond the rhizosphere and facilitates nutritional improvement of the host plant especially slowly diffusing nutrient ions such as P, Zn and Cu. Li et al. [12] demonstrated that mycorrhizal hyphal uptake and translocation of Cu to *Trifolium repens* L. contributed about 62% of the total Cu uptake and the mycorrhizal response was independent of the effects of P nutrition. Number of studies have clearly shown that Zn uptake via mycorrhizae is important for the alleviation of Zn deficiency in several plant species [13–15]. Therefore literatures suggest that there is a possibility of using mycorrhiza as a biological agent to alleviate Zn deficiencies in crops.

We hypothesized that mycorrhizal colonization in maize triggers antioxidant enzyme systems, which may be a factor related to the host plant tolerance to zinc deficient conditions. To test this hypothesis, we examined antioxidant enzymes such as SOD, peroxidase, polyphenol oxidase, and IAA oxidase, acid phosphatase and nutritional status in roots and shoots of inoculated and non-inoculated maize plants exposed to varying levels of Zn. To test the hypothesis, the physiological changes in host plant were assessed at 45 days after sowing.

Materials and Methods

Experimental Soil

The experimental soil was an Alfisol, sandy loam in texture, neutral in pH (7.4), free from salinity (0.04 dSm^{-1}) and it carried low organic carbon status (0.4%), available N (1.23 g kg^{-1}), available (NaHCO_3 -extractable) P (0.058 g kg^{-1}) and medium in available K (1.6 g kg^{-1}). The soil had extremely low status of available (DTPA extractable) Zn (0.63 mg kg^{-1}). The soil was drawn from a field, which was kept fallow for more than 3 years so as to reduce indigenous mycorrhizal population and to decompose root fragments of previous crop to eliminate propagules. Since the indigenous viable spore population was low ($<10 \text{ spores } 100 \text{ g}^{-1} \text{ soil}$) no attempt was made to fumigate the soil. Further, the greenhouse experiment was conducted to mimic the real time situation, so that the result from the experiment is directly applicable to field condition.

Greenhouse Experiment

A greenhouse experiment was conducted in a red sandy loam soil (Alfisol) at the Department of Soil Science and Agricultural Chemistry, Tamil Nadu Agricultural University, Coimbatore. Maize (var. COHM-5) plants were grown

in earthen pots of 10 kg capacity (30 cm diameter and 30 cm height) with five levels of Zn (0, 1.25, 2.5, 3.75 and 5.0 mg kg^{-1}) with AM (*Glomus intraradices* Schenck and Smith) or without AM inoculation. The above levels of Zn were applied as 0, 62.5, 125, 187.5, 250 mg of ZnSO_4 per pot. The exact quantity of ZnSO_4 was dissolved in 25 ml distilled water and poured in 5 different spots in each pot to have uniform distribution. There were 10 treatment combinations replicated 6 times in a randomized block design. One plant per pot was maintained throughout the experiment. The greenhouse had $24\text{--}28^\circ\text{C}$, light intensity ($800\text{--}1,000 \mu\text{mol}$ provided by natural light), relative humidity (60–65%) and 12-h photoperiod.

Vermiculite based mycorrhizal inoculum (*G. intraradices* TNAU-03-06) used in this study was provided by the Department of Microbiology of this university. This strain was cultured in maize plants and propagules comprised of infected root bits and spores were blended in sterile vermiculite. The inoculum with the spore density of 200 spores per gram was applied at 10 g pot^{-1} as a thin layer, 5 cm below seeds prior to sowing. Roots and shoots of each treatment sampled on 45 days after sowing (DAS) were analysed for enzyme activities, soluble proteins and nutrient status.

Mycorrhizal Colonization

The presence of arbuscules, vesicles, external hyphae and spores in roots sampled at 45 DAS were assessed [16] and the per cent colonization was worked out.

Enzyme Extraction and Assays

Superoxide dismutase (EC 1.15.1.1) in fresh roots and shoots were extracted with 0.2 M citrate phosphate buffer (pH 6.5) at 4°C and the SOD activity in the supernatant was determined by its ability to inhibit the photochemical reduction of nitro blue tetrazolium [17]. Peroxidase (EC 1.11.1.7) extracted with 0.1 M phosphate buffer (pH 7.0) was quantified [18]. Polyphenol Oxidase (EC 1.14.18.1) activity was determined [19]. IAA oxidase (EC 1.1.4) activity was measured as mg of unoxidised auxin in the fresh samples [18]. Acid phosphatase (EC 3.11.3.2) activity was measured as the amount of *p*-nitrophenol released during incubation [20].

Total Phenols

Fresh roots and shoots (500 mg) were macerated in a pestle and mortar with 10 ml of 80% ethanol and centrifuged at 10,000 rpm for 10 min [21]. The supernatant solution was evaporated to a dry powder and homogenized in 2.5 ml of distilled H_2O and mixed in 0.5 ml Folin–Ciocalteu

reagent. After 3 min of incubation, 2 ml of 20% (w/v) Na_2CO_3 was added and kept in boiling water for 1 min and cooled to room temperature. Then the absorbance was read at 650 nm and was compared with the standard curve prepared using catechol.

Soluble Proteins

Soluble proteins in roots and shoots were determined by the Folin phenol method [22] using bovine serum albumin as a standard. Two hundred and fifty milligrams of freeze dried root or shoot tissues were macerated with 10 ml phosphate buffer. One milliliter of supernatant solution was mixed with 5 ml alkaline copper tartrate reagent and kept for 30 min for the biuret reaction to take place. Soluble protein content was estimated by measuring the absorbance of blue colour at 660 nm that developed with Folin–Ciocalteu reagent.

Plant Nutrient Status

Maize roots and shoots sampled at 45 and 75 DAS for nutrient analysis were washed thoroughly, dried at 70°C, weighed and digested in triple acid mixture (9:2:1 nitric:sulphuric: perchloric acid) in a conical flask under a fumehood. The digested samples were diluted to 50 ml with distilled water. Phosphorus concentration of plant tissues was estimated using vanadomolybdo phosphoric acid yellow colour method [23]. Zinc concentrations were measured in the diluted plant extract directly in an atomic absorption spectrophotometer (Varian Spectra AA 220, Australia).

Statistical Analysis

A one-way analysis of variance (ANOVA) was done for all data and comparisons among means were made using DMRT (Duncan's Multiple Regression Test) test, calculated at $P < 0.05$. Statistical procedures were carried out with the software package IRRI stat (IRRI, Manila Philippines).

Results and Discussion

Mycorrhizal Colonization

Mycorrhizal colonization in AM fungus-inoculated plants was twice higher than non-inoculated plants regardless of levels of Zn application. M+ roots had the colonization percentage ranging from 59.0 to 66.6, while M– roots had only 18.5–26.5% colonization. Zinc nutrition had no consistent changes in colonization pattern of both M+ and M– roots.

Anti-oxidant Enzyme Activities (Tables 1, 2)

AM inoculated maize plants had significantly ($P \leq 0.05$) higher SOD activities in shoots (M– 129.2 U g^{-1} ; M+ 136.4 U g^{-1}) and roots showed no significant variation. Zinc application significantly ($P \leq 0.01$) increased the SOD activities in roots up to 2.5 mg kg^{-1} and declined thereafter, while in shoots the increase in SOD activities was recorded up to 1.25 mg kg^{-1} . Mycorrhizal inoculation and Zn application had significantly ($P \leq 0.01$) increased the peroxidase and polyphenol oxidase activities of maize roots and shoots at 45 DAS. Even under Zn 0 level, M+ shoots had higher peroxidase activities by 42% (0.881 Δ in $\text{OD g}^{-1} \text{min}^{-1}$) than M– shoots (0.514 Δ in $\text{OD g}^{-1} \text{min}^{-1}$). It is understandable that SOD activity is a defense response to abiotic stress conditions including Zn deficiency in soil and a protective mechanism to quench the reactive oxygen species [24, 25]. The increase in anti-oxidant enzyme activities of AM-inoculated plants may be attributed to contribution of hyphal transport of slowly diffusing micronutrient ions such as Zn and Cu which serve as co-factor for these enzymes. Li et al. [12] reported that the external mycelium of the mycorrhizal fungus proliferate beyond the rhizosphere and transports Cu to the tune of 62%. Under Zn deficient conditions, plants produce more reactive oxygen species [26] that can be quenched by antioxidant enzymes including peroxidase [27]. In this study, both zinc nutrition and mycorrhizal inoculation increased peroxidase activity indicating tolerance of plants to Zn deficiency. AM plants recorded increased peroxidase activity even at higher level of Zn application and this can be attributed to the plant's response to fungal colonization. In this study, polyphenol oxidase (PPO) activities of treatment carrying AM-inoculation with Zn 0 is comparable to Zn 5 mg kg^{-1} suggesting that mycorrhizal colonization enables the plants to produce PPO abundantly through increase in total phenols. Inoculation of AM-fungus significantly ($P \leq 0.01$) increased total phenol contents of roots by 49.2%. A strong correlation ($r^2 = 0.97^{**}$) has been observed between PPO and total phenols especially in maize roots. Mathur and Vyas [28] showed a considerable increase in PPO activity in AM fungus (*Glomus fasciculatum*) inoculated *Ziziphus xylopyrus*. Hao et al. [29] also suggested an increase in PPO activity with mycorrhizal inoculation as a defense mechanism of plants.

Arbuscular mycorrhiza (AM) fungal inoculated (M+) maize plants had significantly ($P \leq 0.01$) higher IAA oxidase activities (measured as unoxidised auxin) in roots and shoots at 45 DAS than non-inoculated (M–) plants. There is a close association between Zn nutrition and accumulation of auxin [30]. Our data indicate that the mycorrhizal symbiosis enhances auxin levels of host plant which may be associated with improved Zn nutrition.

Table 1 Means ($n = 3$) for enzyme activities in roots of maize at 45 days after sowing under different levels of Zn with (M+) or without (M-) arbuscular mycorrhizal fungal inoculation

Treatments (mg kg ⁻¹)	Superoxide dismutase (U g ⁻¹)		Peroxidase (Δ in OD/g/min)		Polyphenol oxidase (Δ in OD/g/min)		Total phenols (% of fresh weight)		IAA oxidase (mg of unoxidised auxin/g/h)		Acid phosphatase (μmol of p NP released/g/min)	
	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+
Zn 0	118 ^b (4.65)	121 ^b (5.05)	0.195 ^c (0.009)	0.264 ^{ab} (0.012)	1.82 ^d (0.069)	2.28 ^b (0.040)	0.197 ^c (0.017)	0.316 ^c (0.027)	0.796 ^d (0.016)	0.824 ^d (0.012)	0.135 ^f (0.003)	0.243 ^a (0.002)
Zn 1.25	120 ^b (3.81)	124 ^a (3.58)	0.221 ^c (0.012)	0.291 ^{ab} (0.016)	1.91 ^d (0.035)	2.34 ^b (0.058)	0.217 ^c (0.026)	0.358 ^b (0.030)	0.804 ^d (0.007)	0.875 ^c (0.016)	0.130 ^{fg} (0.002)	0.229 ^b (0.003)
Zn 2.5	121 ^b (3.64)	126 ^a (5.34)	0.243 ^c (0.010)	0.312 ^a (0.007)	2.20 ^c (0.052)	2.57 ^b (0.023)	0.256 ^d (0.0267)	0.384 ^b (0.026)	0.814 ^d (0.006)	0.902 ^{bc} (0.011)	0.123 ^g (0.001)	0.208 ^c (0.005)
Zn 3.75	111 ^c (5.23)	114 ^c (2.69)	0.258 ^{ab} (0.014)	0.334 ^a (0.014)	2.29 ^c (0.052)	2.81 ^a (0.040)	0.299 ^d (0.030)	0.421 ^a (0.036)	0.896 ^{bc} (0.006)	0.926 ^{ab} (0.010)	0.116 ^g (0.004)	0.189 ^d (0.003)
Zn 5.0	108 ^c (4.51)	112 ^c (4.13)	0.264 ^{ab} (0.016)	0.341 ^a (0.018)	2.31 ^c (0.075)	2.91 ^a (0.046)	0.313 ^c (0.024)	0.432 ^a (0.025)	0.922 ^b (0.008)	0.956 ^a (0.007)	0.115 ^g (0.003)	0.179 ^e (0.005)
ANOVA: M (mycorrhizal treatment), Z (Zn levels)												
M	NS		**		**		**		**		**	
Z	*		**		**		**		**		**	
M × Z	NS		NS		*		NS		*		**	

Values in parentheses indicate the standard error; and the levels of significance for ANOVA * $P \leq 0.05$; ** $P \leq 0.01$; NS not significant; means with different letters are significantly different based on DMRT

Table 2 Means ($n = 3$) for enzyme activities in shoots of maize at 45 days after sowing under different levels of Zn with (M+) or without (M-) arbuscular mycorrhizal fungal inoculation

Treatments (mg kg ⁻¹)	Superoxide dismutase (U g ⁻¹)		Peroxidase (Δ in OD/g/min)		Polyphenol oxidase (Δ in OD/g/min)		Total phenols (% of fresh weight)		IAA oxidase (mg of unoxidised auxin/g/h)		Acid phosphatase (μmol of p NP released/g/min)	
	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+
Zn 0	133 ^c (2.63)	146 ^a (6.32)	0.514 ^d (0.008)	0.881 ^b (0.035)	0.643 ^d (0.010)	0.898 ^b (0.012)	0.158 ^e (0.022)	0.181 ^d (0.014)	0.750 ^e (0.018)	0.830 ^b (0.023)	0.325 ^c (0.003)	0.376 ^{ab} (0.003)
Zn 1.25	136 ^c (3.52)	149 ^a (5.20)	0.549 ^d (0.020)	0.910 ^{ab} (0.025)	0.699 ^d (0.011)	0.913 ^b (0.018)	0.185 ^d (0.025)	0.211 ^c (0.015)	0.794 ^e (0.009)	0.896 ^b (0.019)	0.328 ^c (0.001)	0.377 ^{ab} (0.002)
Zn 2.5	135 ^c (7.16)	140 ^b (2.43)	0.601 ^c (0.024)	0.949 ^a (0.020)	0.743 ^c (0.012)	0.952 ^b (0.013)	0.227 ^c (0.021)	0.232 ^b (0.018)	0.882 ^b (0.012)	0.944 ^{ab} (0.017)	0.333 ^c (0.002)	0.379 ^a (0.003)
Zn 3.75	123 ^d (4.75)	128 ^d (4.88)	0.663 ^c (0.022)	0.991 ^a (0.017)	0.798 ^c (0.012)	1.075 ^a (0.093)	0.256 ^{ab} (0.023)	0.262 ^a (0.020)	0.902 ^{ab} (0.011)	0.970 ^a (0.012)	0.335 ^{bc} (0.002)	0.380 ^a (0.001)
Zn 5.0	119 ^e (6.12)	119 ^e (7.62)	0.789 ^b (0.024)	1.102 ^a (0.018)	0.899 ^b (0.010)	1.149 ^a (0.011)	0.266 ^a (0.031)	0.278 ^a (0.019)	0.926 ^{ab} (0.009)	0.980 ^a (0.009)	0.341 ^b (0.002)	0.381 ^a (0.002)
ANOVA: M (mycorrhizal treatment), Z (Zn levels)												
M	*		**		**		NS		**		**	
Z	**		**		**		**		**		**	
M × Z	NS		NS		NS		NS		NS		NS	

Values in parentheses indicate the standard error; and the levels of significance for ANOVA * $P \leq 0.05$; ** $P \leq 0.01$; NS not significant; means with different letters are significantly different based on DMRT

Table 3 Levels of significance for ANOVA for soluble proteins, zinc uptake in roots and shoots under different Zn levels (Z) with (M+) or without (M–) arbuscular mycorrhizal fungal inoculation

	M	Z	M × Z
Soluble proteins			
Roots	**	**	**
Shoots	**	**	NS
Zn uptake			
Roots	**	**	NS
Shoots	**	**	*

** $P \leq 0.01$; NS not significant

Mycorrhizal response to auxin level of plants was more pronounced under lower levels of zinc than at higher levels.

AM inoculated maize plants had significantly ($P \leq 0.01$) higher activities of acid phosphatase in roots and shoots at 45 DAS (Table 3). Inoculated plant (M+) roots had acid phosphatase activities (0.209μ moles of p NP released/g/min) two times higher than that of M– roots (0.13μ moles of p NP released/g/min) at 45 DAS. Higher acid phosphatase activities under AM inoculation had been reported in several crops such as onion [20], soybean [31] and alfalfa [32].

Soluble Proteins (Fig. 1)

AM-inoculation or Zn application had significantly ($P \leq 0.01$) increased soluble proteins in roots and shoots at 45 DAS (Fig. 1). Soluble protein contents increased progressively with Zn levels irrespective of inoculated or uninoculated treatments. The highest protein content in roots at 45 DAS was recorded in treatment receiving mycorrhizal inoculation in combination with Zn application @ 5 mg kg^{-1} .

The increase in soluble protein concentrations in AM-inoculated maize plants corresponded with earlier reports [28, 33]. Arines et al. [34] also detected two- to sixfold increase in soluble proteins in mycorrhizal clover roots. Zinc nutrition also enhanced the accumulation of soluble proteins in mycorrhizal plants which may be due to the fact that Zn is responsible for the synthesis of amino acids such as tryptophan and asparagine.

Nutrient Status in Plants (Fig. 2)

Inoculated maize plants (M+) had significantly ($P \leq 0.01$) higher Zn uptake than uninoculated (M–) plants regardless of Zn levels. M+ shoots and roots had higher Zn uptake (0.195 and $0.138 \text{ mg plant}^{-1}$) than M– shoots and roots (0.168 and $0.108 \text{ mg plant}^{-1}$). AM-fungus inoculated plants were nutritionally rich, which resulted in higher biomass production and Zn uptake even under Zn deficient conditions. The external mycelium of AM fungus proliferates extensively beyond the rhizosphere capable of translocating immobile ions such as PO_4^- and Zn^{2+} [11, 12]. The response to mycorrhizal inoculation was more pronounced under deficient levels of Zn than sufficient levels indicating that mycorrhizal association is more beneficial when the plants are exposed to Zn deficiency.

In summary, the present study shows that AM-fungus inoculation enhanced anti-oxidant enzyme activities which further increased with the addition of Zn. These favourable changes in conjunction with improved nutritional status of Zn assist the mycorrhizal plants to sustain Zn deficient conditions. The overall data suggest that AM association plays a major role in alleviating Zn deficiency in crops grown in semi-arid regions. Further studies are required to precisely predict the mechanism associated with the tolerance of mycorrhizal plants under Zn deficient conditions.

Fig. 1 Soluble protein concentrations ($n = 3$) in roots and shoots of maize plants harvested at 45 DAS under different levels of Zn with (M+) or without (M–) arbuscular mycorrhizal fungal inoculation. The data sets for roots and shoots were analyzed separately. Error bars represent standard errors of three replications

Fig. 2 Zn upake in roots and shoots ($n = 3$) of maize plants harvested at 45 DAS under different levels of Zn with (M+) or without (M–) arbuscular mycorrhizal fungal inoculation. The data sets for roots and shoots were analyzed separately. Error bars represent standard errors of three replications

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