Original Research Article

Arbuscular Mycorrhizal Fungus Inoculation on Antioxidant Enzyme Activities in Maize Plants at Different Levels of Fe and Zn Fertilization

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Greenhouse and field experiments were conducted to study the changes of 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 (12.5 and 25 kg ha^{-1}) iron (12.5 and 25 kg ha^{-1}). Roots and shoots sampled at 45 and 75 days after sowing (DAS) were estimated for its antioxidant enzymes superoxide dismutase, peroxidase, IAA oxidase, polyphenol oxidase, acid phosphatase and nutritional status especially Fe and Zn concentrations. Mycorrhizal inoculation significantly ($P \leq 0.01$) increased all the antioxidant enzymes in both roots and shoots at 45 and 75 DAS regardless of Fe and Zn levels. All enzyme activities except SOD increased progressively with increasing levels of Fe and Zn under M+ and M− conditions. Acid phosphatase activity in M+ roots and shoots were higher in all levels of Zn and Fe but the values decreased with increasing levels of Zn particularly in roots. Mycorrhizal fungus inoculated plants had higher Fe and Zn concentrations in both stages in comparison to non-inoculated plants. 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 and iron deficient conditions.

Introduction

Zinc is an essential mineral nutrient and a cofactor of over 300 enzymes and proteins involved in cell division, nucleic acid metabolism and protein synthesis (Marschner, 1986). Zinc deficient soils can be easily treated with zinc fertilizers to provide an adequate supply of zinc to crops. When the supply of plant – available zinc is inadequate, crop yield is reduced and the quality of crop products is frequently impaired. In plants, Zinc plays a key role as a structural constituent or regulatory co-factor of a wide range of different enzymes and plant species are affected by zinc deficiency on a wide range of soil types in most agricultural regions of the world. Activated oxygen species (AOS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), are formed as by-products of normal metabolism in different cellular organelles (Scandalios 1993). A number of studies have clearly shown that Zn uptake via mycorrhizae is important for the alleviation of Zn deficiency in several plant species (Evans

and Miller, 1988; Sylvia *et al.,* 1993). These literatures suggest that there is a possibility of using mycorrhiza as a biological agent to alleviate Zn deficiencies in crops.

Arbuscular mycorrhizal (AM) fungi can colonize the roots of most vascular plants and can develop a complex system of extraradical hyphae under natural conditions. AM fungi can initiate a defense – like response when colonizing some host roots (Morandi *et al.,* 1984). Mycorrhizae may help plants to thrive in Mediterranean semi-arid ecosystems, where the water deficit seriously limits plant growth, by altering antioxidant enzyme activities (Requena *et al.,* 2001). The effect of mycorrhizal inoculation on SOD isozymes in mycorrhizal roots of red clover (Palma *et al.,* 1993) and *Pisum sativum* L. (Arines *et al.,* 1994) plants and on the SOD activity in shoots of mycorrhizal *Lactuca sativa* L. plants (Ruiz-Lozano *et al.,* 1996). The activity levels of some antioxidant enzymes have been investigated in roots and nodules of mycorrhizal soybean plants (Porcel *et al.,* 2003).

The oxidative stress involves many physiological and biochemical changes (Magnoli *et al.,* 1999) during root infection changes in enzyme activities and damage to membrane permeability. AM are known to enhance plant uptake of phosphate (P) and other mineral nutrients under certain conditions (Abbott and Robson, 1984). The effects of P and micro-nutrient levels on development of an arbuscular mycorrhizal fungus (AMF) and uptake of Zn, Cu, Mn and Fe by maize (*Zea mays* L.) (Faber *et al.,* 1990). Phosphatases are important for P nutrition of plants especially when there is storage of inorganic P in the soil. Phosphatase activity in the rhizosphere or soil solution may originate from plant roots (Tarafdar and Jungk, 1987; Dinkelaker and Marschner, 1992). The plant – mycorrhizal fungus

symbiosis results from a number of changes in gene expression, metabolism and morphogenesis (Bonfante and perotto 1995).

We hypothesized that mycorrhizal colonization to promote physiological changes in host plants by increasing plant metabolic changes and antioxidant enzyme activities. To test this hypothesis, we examined antioxidant enzymes such as SOD, CAT, CAase, 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. The progressive physiological changes in host plant were assessed at 45 and 75 days after sowing.

Materials and Methods

Experimental Soil

Field experiments were conducted in two locations one each at the Experimental Farms of Agricultural Research Station (ARS), Bhavanisagar and Tamil Nadu Agricultural University (TNAU), Coimbatore, under natural conditions. In the same two locations, soil samples were collected, processed and autoclaved in order to eliminate the indigenous mycorrhizal fungal population. Simultaneously, greenhouse experiments were undertaken in the sterilized soils. The calcareous soil was an Inceptisol, sandy loam in texture, alkaline in pH (8.4), free from salinity (0.34 d Sm^{-1}) and carried low organic carbon status (3.2 g kg^{-1}), available N (220.4 kg ha^{-1}) and available ($NaHCO_3$-extractable) P (9.6 kg ha^{-1}) and is medium in available K (224 kg ha^{-1}). The soil had extremely low status of available (Diethylene Triamine Penta Acetic Acid-extractable) Zn (0.63 mg kg^{-1}) and Fe (0.86 mg kg^{-1}). The experimental soil of non-calcareous 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}). And high in available Fe (36.2 mg kg^{-1})

Both field tests and greenhouse experiments had the same set of treatments. Treatments consisted of two levels of FeSO$_4$ (12.5 and 25 kg ha^{-1}) and two levels of ZnSO$_4$ (12.5 and 25 kg ha^{-1}) in the presence or absence of arbuscular mycorrhizal fungal (AMF) inoculation. There were 8 treatment combinations replicated four times in a factorial randomized block design (FRBD). The AMF inoculum carrying *Glomus intraradices* (2 g) was applied at the base of the seed hole just prior to sowing. Vermiculite based mycorrhizal inoculums (*Glomus intraradices* TNAU-11-08) 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. Maize hybrid seeds (COMH-5) was sown on the inoculum layer of soil. Germination percentage was nearly 95% on the seventh day of sowing. Half the dose of N (100 kg ha^{-1}) and full dose of P (100 kg ha^{-1}) and K (100 kg ha^{-1}) were applied in the form of urea, single superphosphate and muriate of potash, respectively, as basal at the time of sowing. In addition, two levels of Fe as FeSO$_4$ and Zn as ZnSO$_4$ were applied as per treatment. In all the four experiments, root colonization, plant chlorophyll content, biochemical and physiological changes were recorded at 45 and 75 DAS.

Chlorophyll

Fresh leaf samples (250 mg) were macerated in a pestle and mortar with 10 mL of 80% acetone and centrifuged at 5000 rpm for 10 min. The supernatant was collected and the volume was made up to 25 mL using 80% acetone and the chlorophyll content was obtained by measuring the OD at 663 nm in a spectrophotometer (Varian Cary 50 UV-visible spectrophotometer) (Bruinsma, 1963). The chlorophyll content of samples was expressed as mg g^{-1} of fresh leaves.

Soluble proteins

Soluble proteins in shoots were determined by the Folin phenol method (Lowry *et al.,* 1951) using bovine serum albumin (BSA) as a standard. 250 mg of root or leaf tissue were macerated with 10 mL 0.2 M phosphate buffer and centrifuged at 3000 rpm for 10 min. One mL of supernatant solution was mixed with 5 mL alkaline copper tartarate reagent (2% Na$_2$CO$_3$ in 0.1 N NaOH and 0.5% CuSO$_4$ in 1% sodium potassium tartarate mixed in 50:1) and kept for 30 min for the biuret reaction to take place. Soluble proteins content was estimated by measuring the absorbance of blue colour that developed with Folin Ciocalteau reagent (1 part of Folin Ciocalteau reagent mixed with 2 parts of distilled water) at 660 nm in spectrophotometer (Varian Cary 50 UV-visible spectrophotometer). The soluble proteins content was expressed as mg g^{-1}.

Total Phenols

Fresh 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. The supernatant solution was evaporated to a dry powder and homogenized in 2.5 ml of distilled H$_2$O and mixed in 0.5 ml Folin–Ciocalteau reagent. After 3 min of incubation, 2 ml of 20% (w/v) Na$_2$CO$_3$ 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.

Peroxidase activity

Fresh shoots of 500 mg were macerated with 0.1M phosphate buffer, pH 7.0, in a pre-chilled pestle and mortar at 4°C. The homogenate was centrifuged at 5,000 rpm for 15 minutes. One ml of supernatant solution was taken for assay and mixed with 3 ml of 0.05 M pyrogallol and 0.5 ml 30% hydrogen peroxide. The change in absorbance was measured at 425 nm in 30 seconds interval up to 120 seconds and the enzyme activity was reported as change in OD $min^{-1}g^{-1}$ of sample. Reaction mixture containing pyrogallol and hydrogen peroxide without enzyme extract constituted the blank (Sadasivam and Manickam, 1996).

IAA oxidase

IAA oxidase activity was measured as mg of unoxidised auxin in the fresh samples as suggested (Sadasivam and Manickam, 1996). Fresh shoots of 500 mg were macerated with 0.1M phosphate buffer, pH 7.0, in a pre-chilled pestle and mortar at 4°C. The homogenate was centrifuged at 5,000 rpm for 15 min. One ml of supernatant was taken for assay and mixed with 1 ml of extraction buffer (0.1 M phosphate buffer) and 1ml of 10 ppm auxin solution. The mixture was kept in dark for 1 hr and added with 8 ml of Garden-Weber reagent and the absorbance was measured at 540 nm and compared with standard curve prepared using auxin solution of 20 to 100 ppm.

Acid phosphatase

The acid phosphatase activity was measured as described by Dodd *et al.,* (1987). Freeze dried shoots (100 mg) were ground in pre-chilled pestle and mortar with 10 ml of 0.2 M sodium acetate buffer (pH 5.0). The enzyme extract was centrifuged at 5000 rpm for 15 minutes. Supernatant enzyme extract of 0.1 ml was incubated for 5 minutes with assay mixture containing 0.4 ml of 10 mM ρ-nitrophenol phosphate and 0.5 ml of extraction buffer. The reaction was terminated by the addition of 2 ml 200 mM Na_2CO_3. The resultant yellow chromophore was measured at 405 nm in a spectrophotometer. Acid phosphatase activity was expressed as the amount of ρ-nitrophenol produced per gram of tissue in one hour.

Superoxide dismutase

Fresh shoots of 500 mg was macerated with 10 ml 0.2 M citrate phosphate buffer (pH 6.5) at 4° C. The homogenate was centrifuged at 10,000 rpm for 30 minutes. The SOD activity in the supernatant was determined by its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) as suggested by Beyer and Fridovich (1987). One ml of supernatant was mixed with 3 ml assay mixture (50 mM sodium phosphate buffer, 13 mM methionine, 75 µ M NBT and 0.1 mM EDTA) in a test tube. At the end, 2µM riboflavin (0.01 ml) was added and mixed thoroughly. The tubes were illuminated for 7 minutes in an aluminum foil lined box containing fluorescent lamps. Blank was run without enzyme extract. The change in absorbance was measured at 560 nm. The decrease in NBT reduction was calculated from the blank and sample absorbance values and 50% decrease in NBT reduction was reported as 1 unit of SOD.

Catalase activity

Catalase activity of leaf was estimated according to Woodbury *et al.,* (1971) and expressed as µg H_2O_2 reduced $min^{-1}g^{-1}$ fresh weight. Fresh shoots of 500 mg of leaf sample were macerated with 10 ml of phosphate buffer. The content was Centrifuge at 3000 rpm for 10 minutes. 1ml of each supernatant was taken in 5 beakers. To this 5 ml of 1.5% sodium perborate and 1.5ml of phosphate

buffer was added. Later 10ml of 2 N sulphuric acids at the time interval of 1 minute, 2 minute, 3 minute, and 4 minutes was added after enzyme extract in first four beakers respectively being added. In the final beaker, 10ml of sulphuric acid was added before addition of enzyme extract. This beaker was kept as blank for comparison. The content in the beaker was titrated against 0.05 N $KMnO_4$. The end point was development of pink colour which persisted for 30 seconds. The volume of $KMnO_4$ consumed was noted.

Carbonic anhydrase

Carbonic anhydrase (CA) was estimated by the method of Gibson and Leece (1981). CA was extracted from triplicate 500 mg samples of fresh leaf tissue with 5 ml 100 mM Tris-SO_4 pH 8.3, containing I mM EDTA and 100 mM 2-morcaptoethanol, using a chilled mortar and pestle. Acid washed sand (1 g) was added to aid grinding.

The extract was filtered through moist Miracloth, and the carbonic anhydrase activity of 0.1 ml was measured at 0°C by the veronal -indicator method. Extracts with a reaction time of less than 10 seconds were diluted with extraction buffer and reassayed. Controls, which consisted of 0.1 ml buffer in place of enzyme extract, generally completed reaction in 100-110 seconds. Each extract was assayed three times.

Extracts with a reaction time of less than 10 seconds were diluted with extraction buffer and reassayed. Controls, which consisted of 0.1 ml buffer in place of enzyme extract, generally completed reaction in 100-110 seconds. Each extract was assayed three times. Carbonic anhydrase activity was expressed on a fresh weight basis using the formula: EU/g = [10(Tb-Te)-1]/g, where Tb= time for the uncatalyzed reaction and Te = time for the catalyzed reaction

Plant nutrient status

Maize 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. Zinc concentrations were measured in the diluted plant extract directly in an atomic absorption spectrophotometer (Varian Spectra AA 220, Australia).

Statistical analysis

A two-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

Chlorophyll

Mycorrhizal plants (M+) had significantly higher concentration of chlorophyll at both soils under sterilized and natural conditions over uninoculated plants (Table 1). Fe and Zn levels produced a significant difference in chlorophyll concentration

Soluble proteins

The soluble proteins M+ shoots was significantly ($P \leq 0.01$) higher than M- shoots and the increase was exhibited in all the levels of Fe and Zn levels at both stages in calcareous and non-calcareous soil under sterilized (calcareous M- 45.5; M+ 51.4, non-calcareous M- 50.3; M+ 54.6 mg g^{-1}) and natural

(calcareous M- 46.1; M+ 56.4, non-calcareous M- 43.1; M+ 52.7 mg g^{-1}) conditions (Table 1). With the progression of growth, both M- and M+ plants had higher soluble proteins in shoots in both calcareous and non-calcareous soil under sterilized condition.

Total phenols

Mycorrhizal plants registered significantly ($P \leq 0.01$) higher phenol concentration than the un-inoculated plants irrespective of the Fe and Zn levels (Table 2). Mycorrhizal inoculation resulted in the improving the total phenol concentration in soil by 27.8 and 37.0% in calcareous soil of both sterilized and natural conditions and 34.8 and 46.7% in non-calcareous soil of either sterilized or natural conditions at 45 DAS in comparison to M-plants.

Acid phosphatase activity

Acid phosphatase activity of M+ shoots was significantly ($P \leq 0.01$) higher than the uninoculated maize plants irrespective of the Fe and Zn levels (Table 2). Mycorrhizal inoculation resulted in the increasing the acid phosphatase activity of maize plants by 13.4 and 9.1% in calcareous soil of both sterilized and natural conditions and 11.7 and 8.3% in non-calcareous soil of sterilized and natural conditions.

Peroxidase (POX) activities

Mycorrhiza inoculation increased the POX activities in maize plant shoots significantly ($P \leq 0.01$) higher than the uninoculated maize plants irrespective of the Fe and Zn levels (Fig.1a. Application of Fe and Zn also significantly increased the POX activities at both stages in calcareous and non-calcareous soil. A significantly higher POX activity in maize plants was recorded by Fe$_{25}$ Zn$_{25}$ followed by Fe$_{12.5}$ Zn$_{25}$ whereas lowest recorded in Fe$_{12.5}$ Zn$_{12.5}$

at both soils of sterilized and natural condition and both stages.

Poly phenol oxidase (PPO) activities

The PPO activities in M+ shoots was significantly ($P \leq 0.01$) higher than M- plants and the increase was exhibited in all the levels of Fe and Zn in calcareous and non-calcareous soil under sterilized and natural conditions (Table 3). With the progression of growth, both M- and M+ plants had higher PPO activity of both calcareous and non-calcareous soil under sterilized or natural conditions.

Indole acetic acid (IAA) oxidase activities

Mycorrhizal shoots significantly ($P \leq 0.01$) had higher IAA oxidase than M- plants irrespective of the Fe and Zn levels at both stages in calcareous and non-calcareous soil under sterilized (calcareous M- 173.0; M+ 187.7 Change in OD/min/g, non-calcareous M- 167.1; M+ 188.7 Change in OD/min/g) and natural (calcareous M- 162.2; M+ 181.0 Change in OD/min/g, non-calcareous M- 167.1; M+ 198.9 Change in OD/min/g) condition (Table 3). Application of Fe and Zn fertilizers also significantly increased the IAA oxidase activities at both stages in crop under sterilized and natural condition.

Super oxide dismutase (SOD)

The SOD activity in M+ shoots was significantly ($P \leq 0.01$) higher than M- shoots and the increase was exhibited in all the levels of Fe and Zn at both stages in calcareous and non-calcareous soil under sterilized (calcareous M- 76.8; M+ 97.3, non-calcareous M- 98.4; M+ 109.6 U g^{-1}) and natural (calcareous M- 129.6; M+ 141.7, non-calcareous M- 124.5; M+ 130.9 U g^{-1}) conditions and interactions was significant in sterilized and natural condition (Table 3). With the progression of growth, both M- and

M+ plants had higher SOD activity in both calcareous and non-calcareous soil under sterilized or natural conditions.

Catalase activity (CAT)

Mycorrhizal inoculated plants recorded significantly ($P \leq 0.01$) higher catalase activity than the uninoculated irrespective of the Fe and Zn levels at both stages in calcareous and non-calcareous soil under sterilized and natural conditions and the interactions were not significant (Table 4). Mycorrhizal inoculation resulted in the improving the catalase activity in plants by 14.5 and 7.9% in calcareous soil of both sterilized and natural conditions and 16.9 and 8.2% in non-calcareous soil of either sterilized or natural conditions at 45 DAS in comparison to M- soil.

Fig.1 Peroxidase activity (a), shoot Zn (b) and Fe (b) content (mg kg^{-1}) of arbuscular mycorrhizal fungus inoculated (AMF+) (filled bars) and uninoculated (AMF-) (Empty bars) maize plants (n = 3) under two levels of Fe (12.5 and 25 kg FeSO$_4$ ha^{-1}) and two levels of Zn (12.5 And 25 kg ZnSO$_4$ ha^{-1}) in soils. Error bars represent standard errors of three replications

(a)

(b)
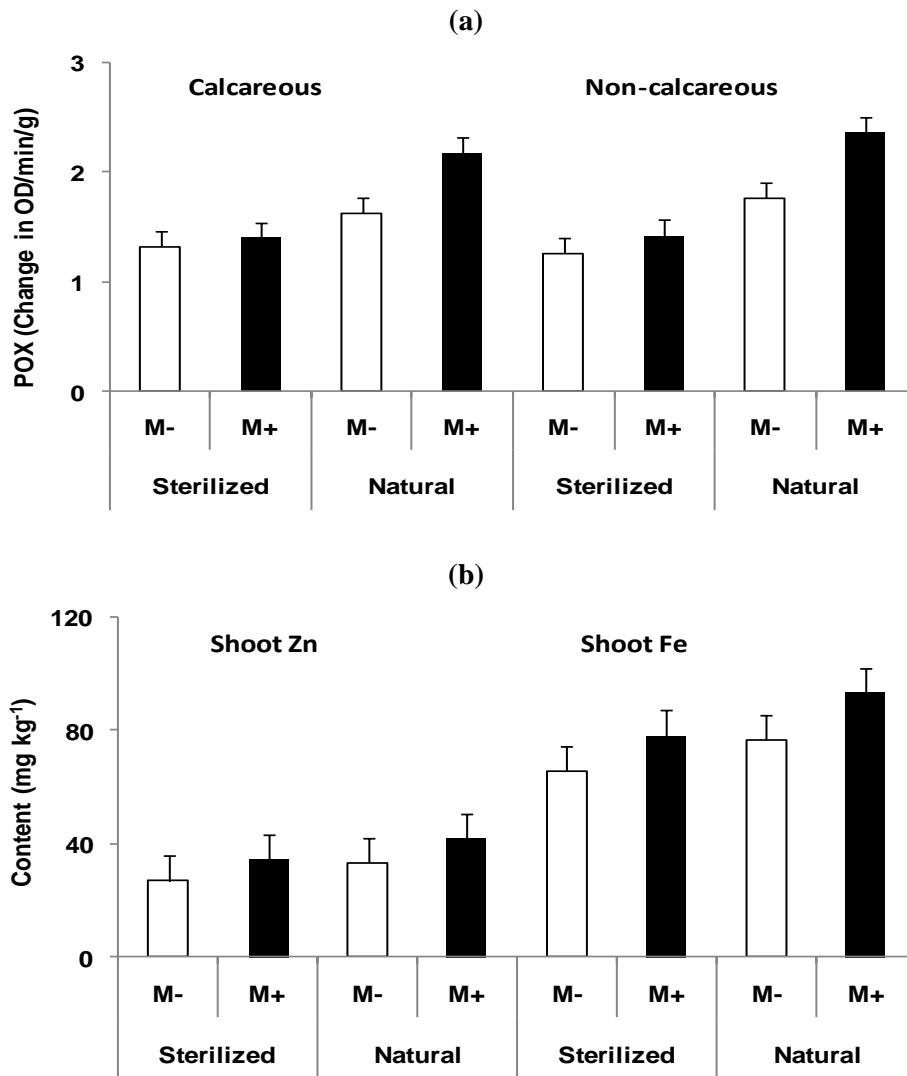
Table.1 Total chlorophyll and soluble protein concentrations examined in the shoots of arbuscular mycorrhiza inoculated (AMF+) and non-inoculated (AMF-). The levels of significance for ANOVA, * = $P \leq 0.05$; ** = $P \leq 0.01$; NS = Not significant. Means followed by a common letter are not significantly different at the 5% level by DMRT

Treatments	Total chlorophyll (mg g⁻¹ of tissue)								Soluble protein (mg g⁻¹ of tissue)							
	Calcareous				Non-calcareous				Calcareous				Non-calcareous			
	Sterilized		Natural		Sterilized		Natural		Sterilized		Natural		Sterilized		Natural	
	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺
Fe₁₂.₅ Zn₁₂.₅	1.81ᶜ	1.96ᶜ	2.24ᵇ	2.50ᵃ	1.89ᶜ	2.20ᵇ	2.06ᶜ	2.38ᵇ	24.8ᶜ	39.7ᵃ	44.9ᶜᵇ	47.2ᵇ	45.6ᶜ	47.5ᶜ	51.4ᶜᵈ	65.1ᵇ
Fe₂₅ Zn₁₂.₅	1.93ᶜ	2.07ᵇ	2.08ᵇ	2.41ᵃᵇ	2.08ᵇᶜ	2.16ᵇ	2.35ᵇ	2.44ᵇ	24.7ᶜ	39.4ᵃ	43.5ᶜᵇ	49.1ᵇ	50.3ᵇᶜ	52.4ᵇ	53.3ᶜᵈ	71.2ᵃ
Fe₁₂.₅ Zn₂₅	2.09ᵇ	2.08ᵇ	2.09ᵇ	2.63ᵃ	2.16ᵇ	2.36ᵃ	2.46ᵇ	2.58ᵃ	39.7ᵃ	40.0ᵃ	47.4ᵇ	57.3ᵃ	51.7ᵇ	58.2ᵃ	53.8ᶜᵈ	73.4ᵃ
Fe₂₅ Zn₂₅	2.14ᵇ	2.30ᵃ	1.95ᵇ	2.70ᵃ	2.30ᵃᵇ	2.51ᵃ	2.64ᵃ	2.84ᵃ	35.8ᵃᵇ	42.0ᵃ	46.3ᵇ	52.1ᵃᵇ	53.5ᵇ	60.3ᵃ	58.7ᶜ	68.3ᵃᵇ
Mean	1.99	2.10	2.09	2.56	2.11	2.31	2.38	2.56	31.3	40.3	45.5	51.4	50.3	54.6	54.3	69.5
ANOVA: **M** (Mycorrhizal inoculation), **F** (Fe levels), **Z** (Zn levels)																
M	**		**		**		**		**		**		**		**	
F	**		**		*		*		*		*		**		**	
Zn	**		**		*		**		*		*		*		*	
M×F	*		*		*		*		*		NS		*		NS	
F×Z	*		**		NS		NS		NS		*		*		*	
M×Z	*		*		NS		*		NS		*		NS		*	
M×F×Z	NS		*		NS		NS		NS		*		*		*	

Table.2 Total phenols and acid phosphatase activity examined in the shoots of arbuscular mycorrhiza inoculated (AMF+) and non-inoculated (AMF-). The levels of significance for ANOVA, * = $P \leq 0.05$; ** = $P \leq 0.01$; NS = Not significant. Means followed by a common letter are not significantly different at the 5% level by DMRT

Treatments	Total phenols (% fresh weight)								Acid phosphatase activity (µg of ρNP g⁻¹ min⁻¹)							
	Calcareous				Non-calcareous				Calcareous				Non-calcareous			
	Sterilized		Natural		Sterilized		Natural		Sterilized		Natural		Sterilized		Natural	
	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺	M⁻	M⁺
Fe₁₂.₅ Zn₁₂.₅	0.10ᶜᵈ	0.14ᶜ	0.38ᵇ	0.52ᵃ	0.12ᵇ	0.27ᵃ	0.46ᵇ	0.62ᵃ	0.86ᵃ	0.93ᵃ	1.10ᵃ	1.14ᵃ	1.15ᵇ	1.27ᵃ	1.20ᵃ	1.17ᵃᵇ
Fe₂₅ Zn₁₂.₅	0.12ᶜ	0.19ᵃ	0.42ᵇ	0.60ᵃ	0.16ᵇ	0.32ᵃ	0.49ᵇ	0.69ᵃ	0.76ᵃᵇ	0.99ᵃ	1.09ᵃᵇ	1.24ᵃ	1.15ᵇ	1.25ᵃ	1.26ᵃ	1.31ᵃ
Fe₁₂.₅ Zn₂₅	0.14ᶜ	0.18ᵃ	0.44ᵇ	0.54ᵃ	0.17ᵇ	0.30ᵃ	0.54ᵇ	0.65ᵃ	0.86ᵃ	0.99ᵃ	1.13ᵃ	1.20ᵃ	1.11ᵇ	1.31ᵃ	1.11ᵇ	1.25ᵃ
Fe₂₅ Zn₂₅	0.17ᵃᵇ	0.20ᵃ	0.46ᵇ	0.65ᵃ	0.20ᵇ	0.30ᵃ	0.45ᵇ	0.75ᵃ	0.87ᵃ	0.97ᵃ	1.09ᵃᵇ	1.26ᵃ	1.12ᵇ	1.28ᵃ	1.18ᵃᵇ	1.38ᵃ
Mean	0.13	0.18	0.43	0.58	0.16	0.30	0.48	0.68	0.84	0.97	1.10	1.21	1.13	1.28	1.19	1.28
ANOVA: **M** (Mycorrhizal inoculation), **F** (Fe levels), **Z** (Zn levels)																
M	**		**		**		**		**		*		**		**	
F	**		**		**		**		*		*		*		*	
Zn	**		*		*		**		*		NS		*		*	
M×F	NS		NS		NS		*		NS		*		NS		NS	
F×Z	NS		*		*		NS		NS		NS		NS		NS	
M×Z	*		*		*		NS		NS		NS		NS		NS	
M×F×Z	*		*		*		*		NS		*		NS		NS	

Table.3 IAA oxidase activities and super oxide dismutase examined in the shoots of arbuscular mycorrhiza inoculated (AMF+) and non-inoculated (AMF-). The levels of significance for ANOVA, * = $P \le 0.05$; ** = $P \le 0.01$; NS = Not significant. Means followed by a common letter are not significantly different at the 5% level by DMRT

Treatments	IAA oxidase activities (Change in OD/min/g)								super oxide dismutase (U g^{-1})							
	Calcareous				Non-calcareous				Calcareous				Non-calcareous			
	Sterilized		Natural		Sterilized		Natural		Sterilized		Natural		Sterilized		Natural	
	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$
Fe$_{12.5}$ Zn$_{12.5}$	138.1^c	144.4^b	157.4^{bc}	180.8^a	141.1^b	149.4^{ab}	151.7^{bc}	189.7^a	72.5^{ab}	86.6^a	108.4^{ab}	124.2^a	88.6^b	96.1^a	112.4^c	123.5^b
Fe$_{25}$ Zn$_{12.5}$	134.3^c	160.2^a	154.4^{bc}	173.1^a	143.3^b	165.2^a	161.4^b	187.8^a	70.9^{ab}	86.4^a	109.2^{ab}	118.5^a	83.5^b	93.2^{ab}	116.2^c	123.2^b
Fe$_{12.5}$ Zn$_{25}$	146.7^b	168.2^a	167.2^b	187.8^a	139.8^{bc}	171.2^a	163.4^b	195.5^a	81.2^a	89.0^a	113.8^{ab}	128.7^a	99.0^a	111.3^a	131.8^a	136.4^a
Fe$_{25}$ Zn$_{25}$	145.8^b	165.7^a	169.8^b	182.3^a	140.6^b	166.7^a	167.7^b	181.9^a	82.6^a	91.7^a	117.4^a	124.2^a	101.6^a	109.2^a	137.5^a	140.3^a
Mean	141.2	159.6	162.2	181.0	141.2	163.1	161.1	188.7	76.8	88.4	112.2	123.9	93.2	102.5	124.5	130.9
ANOVA: M (Mycorrhizal inoculation), F (Fe levels), Z (Zn levels)																
M	**		**		**		**		**		**		**		**	
F	**		*		**		**		**		**		*		*	
Zn	**		*		**		*		**		**		**		*	
M×F	NS		NS		NS		*		NS		NS		NS		*	
F×Z	*		NS		NS		*		*		NS		NS		*	
M×Z	NS		*		NS		NS		NS		*		NS		NS	
M×F×Z	NS		*		NS		NS		NS		NS		NS		NS	

Table.4 Catalase activity and Carbonic Anhydrase activity examined in the shoots of arbuscular mycorrhiza inoculated (AMF+) and non-inoculated (AMF-). The levels of significance for ANOVA, * = $P \le 0.05$; ** = $P \le 0.01$; NS = Not significant. Means followed by a common letter are not significantly different at the 5% level by DMRT

Treatments	Catalase activity (μg H$_2$O$_2$ reduced min^{-1}g^{-1})								Carbonic Anhydrase (EU g^{-1} of fresh tissue)							
	Calcareous				Non-calcareous				Calcareous				Non-calcareous			
	Sterilized		Natural		Sterilized		Natural		Sterilized		Natural		Sterilized		Natural	
	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$	M$^-$	M$^+$
Fe$_{12.5}$ Zn$_{12.5}$	13.2^b	15.5^{ab}	26.2^b	29.1^{ab}	16.5^{ab}	16.8^{ab}	30.4^{ab}	32.7^{ab}	121^c	131^b	82^c	86^c	215^{bc}	241^b	184^c	203^b
Fe$_{25}$ Zn$_{12.5}$	17.1^a	20.8^a	30.4^a	36.8^a	17.2^{ab}	20.2^a	33.2^{ab}	35.4^a	118^c	140^a	77^c	97^b	221^{bc}	249^b	196^c	216^b
Fe$_{12.5}$ Zn$_{25}$	16.6^a	18.6^a	28.2^{ab}	33.7^a	17.7^{ab}	21.6^a	36.8^a	39.6^a	129^b	148^a	98^b	114^a	232^b	291^a	215^b	237^a
Fe$_{25}$ Zn$_{25}$	18.4^a	22.4^a	33.5^a	38.7^a	19.4^a	25.8^a	39.5^a	42.4^a	131^b	152^a	103^{ab}	106^a	236^b	290^a	243^a	253^a
Mean	16.3	19.3	29.6	34.6	17.7	21.1	35.0	37.5	125	143	90	101	226	268	210	227
ANOVA: M (Mycorrhizal inoculation), F (Fe levels), Z (Zn levels)																
M	**		**		**		**		**		**		**		**	
F	**		**		**		**		NS		**		NS		*	
Zn	*		*		*		*		*		**		*		**	
M×F	NS		**		NS		*		NS		*		*		*	
F×Z	NS		NS		*		NS		NS		*		NS		*	
M×Z	NS		NS		NS		NS		NS		NS		NS		NS	
M×F×Z	*		NS		*		*		NS		NS		NS		NS	

Carbonic anhydrase activity

The carbonic anhydrase activity of M+ shoots was significantly ($P \leq 0.01$) higher than M- shoots and the increase was exhibited in all the levels of Fe and Zn at both stages in calcareous and non-calcareous soil under sterilized (calcareous M- 125; M+ 143, non-calcareous M- 226; M+ 268 EU g^{-1}) and natural (calcareous M- 203; M+ 223, non-calcareous M- 386; M+ 396 EU g^{-1}) conditions (Table 4).

Shoot Zn content

The Zn concentration in M+ shoots was significantly ($P \leq 0.01$) higher than M- shoots and the increase was exhibited in all the levels of Fe and Zn levels in both calcareous and non-calcareous soils regardless of sterilized (calcareous M- 27.0; M+ 34.5, non-calcareous M- 33.3; M+ 41.9 mg kg^{-1}) and natural (calcareous M- 37.4; M+ 45., non-calcareous M- 47.3; M+ 56.7 mg kg^{-1}) conditions (Fig. 1b).

Shoot Fe content

Mycorrhizal plants had significantly ($P \leq 0.01$) higher Fe concentration than M- plants irrespective of the Fe and Zn levels in both calcareous and non-calcareous soils regardless of sterilized and natural conditions (Fig. 1b). Mycorrhizal inoculation resulted in improving the shoot Fe concentration by 20.0 and 14.3% in calcareous soil of both sterilized and natural conditions and 10.2 and 8.6% in non-calcareous soil of sterilized and natural conditions at 75 DAS in comparison to M- shoots.

Total Chlorophyll

Mycorrhizal symbiosis appears to alter the physiology of plants as a result of enhanced chlorophyll content. As mycorrhizal plants are known to be nutritionally rich and nourished with both macro and micronutrients that may have helped plants to retain higher amounts of chlorophyll content. Subramanian *et al.,* (1995, 1997) showed that mycorrhizal inoculated plants had retained higher amount of chlorophyll content under drought conditions. Similar trend of results was also reported under well watered conditions.

Plant metabolic changes

Increasing attention is being given to the study of the biochemical process involved in the mycorrhization. Important changes were proposed in the plant metabolism after the establishment of symbiosis (Arines *et al.,* 1993; Subramanian and Charest, 1995; Subramanian and Charest, 2004). Some studies also suggested that mycorrhizal infection causes changes in the biochemical constitution of the host plant. The data from this experiment revealed that the physiology of the maize plant was highly affected by the presence of the fungal symbiosis.

The mycorrhizal colonization increased the shoot soluble sugars, proteins and chlorophyll contents irrespective of the fertility gradients. Among the fertility gradients only the treatment with combined application of fertilizers with farmyard manure was significantly notable. Similarly the findings of Tejada and Gonzalez (2006) who observed highest values of these parameters with combined application of inorganic fertilizers and crushed cotton gin compost and the lowest values in control plots.

Soluble Proteins

In this study, soluble proteins concentrations in AM- inoculated maize roots increased was higher than uninoculated plants. These proteins may play a role in acquisition and assimilation of Zn which is yet to be explored. Further, Zn nutrition 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 asparagin (Dumas *et al.,* 1990; Simoneau *et al.,* 1994).

Krishna and Bagyaraj (1983) reported that total sugar and protein content of mycorrhizal groundnut plants were higher throughout the growth period than the non mycorrhizal plants. Subramainan *et al.,* (1995) also observed that the mycorrhizal inoculation enabled the plants to retain considerable amount of sugars and proteins especially in drought sensitive cultivar. Similarly, Wright *et al.,* (1996) also reported the amount of sugars in the leaves of mycorrhizal plants was significantly different to that in the leaves of non-mycorrhizal plants at any stage during experiment; the amount of soluble protein was higher in mycorrhizal compared to non-mycorrhizal plants. This may be because mycorrhizal fungi increased the uptake of P and they may also increase the synthesis of carbon compounds (Demir, 2004). The data on soluble protein of this experiment is in line with the above findings.

The increase in soluble protein concentrations in mycorrhizal inoculated maize plants corresponded with earlier reports (Mathur and Vyas, 1996; Subramanian and Charest, 1998). Arines *et al.,* (1993) also detected two to six fold increases 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.

Changes in anti-oxidant enzyme activities

Mycorrhizal symbiosis causes important changes in plant metabolism and growth (Arines *et al.,* 1994), although very little is known of the biochemical mechanisms involved in this mycorrhizal association. The shoot / root ratio is of importance when studying the degree of AM fungi effectiveness on the host plant (Tobar *et al.,* 1994). In the present work, the results obtained showed increased anti-oxidant enzyme activities (peroxidase, polyphenol oxidase and IAA oxidase) besides increased acid phosphatase, soluble proteins and nutritional status due to inoculation of arbuscular mycorrhizal (AM) fungus (*Glomus intraradices*) in maize while compared to non-mycorrhizal plants. The activities of enzymes and nutrient concentration in inoculated and uninoculated maize plants increased with incremental levels of Zn application but the values were consistently higher for inoculated treatments. This work support the view that increased antioxidant enzyme activities could be involved, in the beneficial effects of mycorrhizal colonization.

Plants grown in calcareous soil are exposed to wide range of nutrient depressed especially Fe and Zn. Management of Ca induced micronutrient deficiencies very difficult to correct due to fact that the addition of fertilizers will get fixed or converted into insoluble form. The micronutrient deficient plants are often produce ROS that causes lipid peroxidation in cell wall and associated impacts on photosynthesis. This study clearly demonstrated that mycorrhizal inoculated plants are less stressed by micronutrient deficiencies as indicated by the enhanced activities of anti-oxidant enzyme systems. Subramanian *et al.,* (2011) showed that mycorrhizal symbiosis triggers anti-oxidant system that effectively quench the ROS and protect the plants from oxidative damage. These data in conjunction with literature published is quite evident that mycorrhizal symbiosis can help the plants withstand micronutrient deficiencies as a result of anti-oxidant enzyme activities.

Mycorrhizal fungi are known to enhance water absorption by plants grown under water-deficit conditions, owing to altered root morphology or contributions by soil hyphae (Auge 2001).The enzymes superoxide dismutases (SOD), and catalases (CAT) and peroxidases are involved in the detoxification of (O_2^-), hydrogen peroxide (H_2O_2), respectively thereby preventing the formation of (OH^-) hydroxyl radicals (Jimenez *et al.,* 1997).In this study, the peroxidase activity of AM fungus (*Glomus intraradices*) inoculated maize plants increased with incremental levels of zinc application and the results are in agreement with the findings of Ghorbani *et al.,* (2004) who have reported enhanced peroxidase activity of AM-fungus inoculated soybean.

Poly phenol oxidase (PPO) activities have frequently been suggested to participate in plant defence against pests and pathogens (Thipyapong *et al.,* 1995, 1997). In this study, PPO was found to be abundant (*Glomus intraradices*) inoculated plants with the interaction effect of zinc at various levels compared to other AM fungus species inoculated plants. There are reports pointing out that PPO is prominent amongst the defence proteins induced in crop plants (Constabel *et al.,* 1995; Constabel and Ryan, 1998). There are also reports that inoculation with AM fungi may induce an increase in PPO activity and accumulation of defense proteins (Peter, 1989). Mathur and Vyas (1996) showed a considerable increase in PPO activity in AM fungus (*Glomus fasciculatum*) inoculated *Ziziphus xylopyrus*

IAA oxidase activity measured as mg of unoxidised auxin increased with mycorrhizal colonization or Zn nutrition. Auxins are widespread in nature and are produced by plants and mycorrhizal fungi (Slankis, 1974). There is a close association between Zn nutrition and accumulation of auxin (Tsui,

1948). It is observed in our study that mycorrhizal colonization improves the level of auxin in host plants in association with incremental level of zinc nutrition. As mycorrhizal symbiosis assists in improving Zn status and auxin levels of the host plant, the dry matter production of inoculated plants were consistently higher despite the height of the plants were similar (data not shown).

Nutrient Status in Plants

In the present study mycorrhizal inoculation had significantly increased the micronutrient content and uptake by maize plants over the non mycorrhizal maize plants in all levels of Fe and Zn in calcareous and non-calcareous soil regardless of sterilized or natural conditions. The results obtained by earlier workers on plant micronutrient content and its uptake on mycorrhizal inoculation are discussed below. Though uptake of micronutrient usually dominates consideration of the mycorrhizal association, it has become increasingly apparent that mycorrhiza can be important in the uptake of other nutrients by the host plant. Uptake of Zn, Cu, Fe, N, K, Ca and Mg has been reported as being enhanced (Smith and Read, 1997; Clark and Zeto, 2000).

Mycorrhizal fungus inoculated plants were nutritionally rich, which resulted in higher biomass production and Zn uptake even under Zn deficient conditions. The external mycelium of mycorrhizal fungus proliferates extensively beyond the rhizosphere capable of translocating immobile ions such as PO^{4-} and Zn^{2+} (Li *et al.,* 1991; Jakobsen *et al.,* 1992). 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. The effectiveness of mycorrhizal root absorption is increased by external hyphae

(Bürkert and Robson, 1994). Under conditions of low micronutrient level, arbuscular mycorrhizal fungus hyphae enhanced uptake of Fe, most likely by improved scavenging of this element. The results are in agreement with those of Clark and Zeto (1996), who found that mycorrhizae improved Fe uptake in maize grown in Fe-deficient soils. Hamel *et al.,* (2000) also recorded increase in micronutrient content due to inoculation of *G. intraradices* in tomato plants. Uptake of nutrients by plants not only depends on the availability of nutrients in soil solution, but also on the effectiveness of root systems for absorption.

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