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Mycorrhizal symbiosis and bioavailability of micronutrients in maize grain

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Abstract

Field experiments were conducted in calcareous and non-calcareous soils in order to study the biofortification of Fe and Zn in maize grain using arbuscular mycorrhizal fungal (AMF) symbiosis. Treatments consisted of two levels of $FeSO_4$ (12.5 and 25 kg ha^{-1}), two levels of $ZnSO_4$ (12.5 and 25 kg ha^{-1}) and two mycorrhizal treatments [with (M+) or without (M-)] inoculum carrying *Glomus intraradices*) replicated four times in a factorial RBD. The results revealed that AMF colonization significantly increased soil available Fe (M- 1.9; M+ 2.1 mg kg^{-1}) and Zn (M- 4.16; M+ 4.50 mg kg^{-1}). Siderophore production in M+ plants (51.4 μmol cm^{-3} hr) were higher than M- plants (39.5 μmol cm^{-3} hr) and the increase observed irrespective of levels of Fe and Zn. Increased availability of Fe and Zn in soil in combination with enhanced concentrations in plants assisted M+ plants to maintain higher micronutrient contents in grains (Fe M- 31.2, M+ 35.3; Zn M- 45.1, M+ 52.4 mg kg^{-1}). Mycorrhizal plants produced grains with had 10-15% higher Fe and Zn contents while anti-nutritional factor “phytic acid” had decreased (M- 1.13; M+ 1.07 mg g^{-1}). Overall, the data suggest that mycorrhizal fungal inoculation assists in biofortification kernels with Fe and Zn besides circumventing the impact of anti-nutritional factors.

Keywords: arbuscular mycorrhizal fungal (AMF), maize (*Zea mays* L), soil iron and zinc, nutritional quality, biofortification

Introduction

Micronutrient malnutrition is most prevalent in developing countries and deficiencies of Fe, Zn, and vitamin A are among the ten leading causes of illness and diseases in low-income countries (WHO, 2002). Widespread micronutrient malnutrition has enormous socio–economic consequences, resulting in increased mortality and morbidity, impaired growth, development and learning ability in infants and children, and loss in work capacity of adults; these in turn undermine economic growth and perpetuate poverty. Tackling micronutrient malnutrition is considered to be among the best investments that will generate a high return in socio-economic benefits (The World Bank, 2006).

Zinc and iron deficiencies are the most common micronutrient deficiencies in human populations affecting health of over three billion people worldwide (Welch and Graham, 2004; Cakmak et al, 2010). According to a report published by the World Health Organization in 2002, deficiencies of Zn and Fe ranked fifth and sixth in terms of leading disease causing of high mortality in developing countries (WHO, 2002). Zinc deficiency causes impairments in brain development and wound healing and increases susceptibility to infectious diseases including diarrhoea, pneumonia and malaria by weakening the immune system (Black et al, 2008). Iron deficiency impairs physical growth, mental development and learning capacity in children, reduces reproductivity in adults and represents the most common cause of anemia (Kennedy et al, 2003). In most cases, Zn and Fe deficiencies are caused by inadequate dietary intake of Zn and Fe (Welch and Graham, 2004). In many countries, wheat is the main component of the diet and responsible for more than 50% of the daily caloric intake (Cakmak, 2008). Wheat is, however, inherently too poor in Zn and Fe to meet the recommended dietary allowances for human-beings and also rich in anti-nutritional factor “phytic acid” which inhibits the bioavailability of micronutrients (Welch and Graham, 2004; Cakmak et al, 2010).The current Recommended Dietary Allowance (RDA) for Zn and Fe average daily level of intake sufficient to meet the nutrient requirements is 11 and 8 mg day^{-1} respectively.

Biofortification is a process in which plants are allowed to take up the minerals (Fe and Zn) from the soil and immobilize them in the grains so as to produce nutritionally rich grains that support dietary requirement of humans. This approach has proved to be sustainable, relatively low cost, highly efficacious and large coverage (Poletti et al, 2004). One of the biological means to mitigate micronutrient deficiency is by exploiting naturally occurring mycorrhizal symbiosis. Arbuscular mycorrhizal fungal (AMF) association is known to facilitate uptake of slowly diffusing nutrient ions such as phosphorus, zinc and copper

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by the external mycelium (Li et al, 1991; Sylvia et al, 1993; Subramanian and Charest, 1995; Subramanian et al, 2008; 2009). Besides hyphal transport of Zn, mycorrhizal symbiosis orchestrates soil biochemical changes such as increased phosphatase (Tarafdar and Marschner, 1994; Kim et al, 1998; Kandeler et al, 2002) and dehydrogenase (Wamberg et al, 2003) activities, enhanced biomass carbon contents (Hamel et al, 1991; Kim et al, 1998) and secretion of a unique glycoprotein ''glomalin'' by the hyphae (Wright and Upadhyaya, 1998) in the rhizosphere that may assist in promoting availability of Zn. The micronutrient improvement in mycorrhizal plants is always associated with rhizosphere acidification (Dodd et al, 1987), more external mycelium in the soil (Jakobsen et al, 1992) and soil biochemical changes (Subramanian and Charest, 2007). Besides, host plants retain the large green leaf area (Subramanian et al, 1997) and chlorophyll concentration (Subramanian and Charest, 1995; Augé, 2001) under the water deficit conditions.

Habashy and Abo-Zide (2005) showed that the availability of micronutrients (Fe, Mn, and Zn) was positively affected by inoculation with AM fungi when compared to the uninoculated treatments. DTPA extractable Fe and Mn were slightly affected by AM fungi inoculation than that uninoculated one. In addition, the DTPA extractable Zn was also increased in the soil treated with AM. In the presence of mycorrhizal fungi, a decrease in Fe concentration was observed in soybean (Pacovsky and Fuller, 1988), whereas for maize an increase of shoot Fe concentration was described (Clark and Zeto, 1996) and total Fe uptake by soybean and maize was increased in mycorrhizal plants (Lambert et al, 1979). Caris et al (1998) reported that the Fe concentration in shoots and were significantly higher in mycorrhizal than non-mycorrhizal sorghum plants. This study hypothesizes that AMF colonization acidifies the rhizosphere that assists in improving the availability of Fe and Zn. Further, root architecture modifications may facilitate uptake of micronutrients which eventually resulted in biofortification of maize kernels.

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. The details of soil characteristics are given in Table 1. Briefly, the ARS soil had red sandy loam texture, neutral pH, free from salinity and low in organic status and low, medium and high in available N, P and K, respectively. The TNAU soil had clay loam texture, alkaline pH, and low in available N and medium in available P and K, respectively. The indigenous mycorrhizal fungal spore populations in ARS and TNAU soils were 21 and 8 100 g^{-1}, respectively. Since the native inoculums load was low, no attempt was made to fumigate the soil before field tests.

Field experiments

Both field 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 (M+ and M-) inoculation. There were eight 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 inoculum (*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) were sown on the inoculum layer of soil. Germination percentage was nearly 95% on the seventh day of sowing. Half the dose of N (75 kg ha^{-1}) and full dose of P (75 kg ha^{-1}) and K (75 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 the two sets of experiments, root colonization, soil available micronutrients, siderophore concentration, plant micronutrient status, physiologically active Fe and grain Fe and Zn besides phytic acid was measured.

Mycorrhizal colonization

Maize plant roots sampled from M+ and M- treatments were analyzed for their mycorrhizal colonization at 45 DAS. The roots were uprooted along with a ball of earth without disturbing the neighboring plants by a spade. The roots were repeatedly washed with tap water until they are free from dirt and soil particles. The root segments of 1 cm length in 100 numbers were cut per treatment, and estimated for mycorrhizal colonization following Dalpé (1993). Before mounting the root segments on slides, they were bleached with 2.5% KOH, acidified in 1% HCl and stained in 0.05% tryphan blue solution (tryphan blue 0.5 g, glycerol 500 ml, 1% HCl 50 ml and distilled water 450 ml) and destained. Root segments were observed under the

Table 1 - Initial soil characteristics of the calcareous and non-calcareous soils

Parameter	Calcareous	Non-calcareous
Soil Texture	Clay Loam	Sandy Loam
pH	8.39	7.20
EC (dS m^{-1})	0.45	0.04
Organic carbon (%)	0.42	0.26
Available N (kg ha^{-1})	186.2	226.2
Available P (kg ha^{-1})	16.6	19.6
Available K (kg ha^{-1})	412.4	258.4
DTPA Zn (mg g^{-1})	0.61	0.93
DTPA Fe (mg g^{-1})	1.67	36.2
Spore Count (Nos 100g^{-1})	8	21

Table 2 - Percentage of mycorrhizal colonization examined in the arbuscular mycorrhiza inoculated (M+) and non-inoculated (M-) root segments (n=100) of maize plants at 45 and 75 days after sowing (DAS) under varying Fe and Zn levels.

Treatments	Calcareous								Non-calcareous							
	Sterilized				Natural				Sterilized				Natural			
	45 DAS		75 DAS		45 DAS		75 DAS		45 DAS		75 DAS		45 DAS		75 DAS	
	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+
$Fe_{12.5}$ $Zn_{12.5}$	1.8e	31.4c	4.5c	39.4b	8.8e	33.7c	11.6c	46.4b	2.1d	37.5b	2.8c	39.7a	11.4e	40.4cb	15.4c	55.7b
Fe_{25} $Zn_{12.5}$	2.2e	33.8b	3.2d	38.6b	8.2e	35.3b	11.6c	45.8b	2.6d	30.0c	2.9c	46.2a	10.7e	42.4b	15.1c	55.0ba
$Fe_{12.5}$ Zn_{25}	3.4d	35.6b	2.7d	46.7a	10.9d	37.6b	10.8c	50.3a	2.0d	37.5b	2.4c	48.4a	14.2d	45.1b	14.3c	60.4a
Fe_{25} Zn_{25}	2.0e	40.5a	2.4d	47.5a	11.5d	42.2a	9.2d	48.1a	2.8d	40.0a	3.0c	44.1ba	14.9d	50.6a	12.6c	57.7a
Mean	2.4	35.3	3.2	43.1	9.9	37.2	10.8	47.7	2.4	36.3	2.8	44.6	12.8	44.6	14.4	57.2

ANOVA: M (Mycorrhizal inoculation), F (Fe levels), Z (Zn levels)

	Calcareous Sterilized 45 DAS	Calcareous Sterilized 75 DAS	Calcareous Natural 45 DAS	Calcareous Natural 75 DAS	Non-calcareous Sterilized 45 DAS	Non-calcareous Sterilized 75 DAS	Non-calcareous Natural 45 DAS	Non-calcareous Natural 75 DAS
M	**	**	**	**	**	**	**	**
F	**	**	**	*	**	**	**	**
Zn	**	**	**	**	*	*	**	**
M×F	**	*	*	NS	*	NS	*	NS
F×Z	*	*	*	NS	NS	NS	*	NS
M×Z	*	*	*	*	NS	NS	*	*
M×F×Z	NS	*	NS	*	NS	NS	NS	*

*P ≤ 0.05; **P ≤ 0.01; NS = Not significant

10 x lens microscope for the presence of any of the mycorrhizal structures such as arbuscules, vesicles, external hyphae and spores.

Physiologically active iron (Fe^{2+})

Fresh leaves (100 mg) sampled at 45 and 75 days after sowing were washed in dH_2O, air dried and incubated in 1.5% 1–10 orthophenanthroline solution for 16 h with continuous stirring at 25 ± 1°C. The contents were filtered through Whatman No 1 filter paper and the absorbance of the resulting solution was read at 510 nm (Katyal and Sharma, 1980). A standard curve for iron was prepared using varying concentrations of ferrous ammonium sulfate ranging from 5 to 150 µg ml^{-1}.

Micronutrient concentrations in grains

One g of powdered plant samples (roots, shoots) or 0.5 g grain samples were mixed with 12 ml triple acid (HNO_3, H_2SO_4 and $HClO_4$ in 9:2:1) mixture and kept overnight for cold digestion. The digested samples were kept on a sand bath till the samples become colourless. The digested samples were diluted up to 50 ml using dH_2O and were stored for further nutrients analysis. The Fe and Zn concentrations were determined by a standard protocol described by Lindsay and Norwell (1956). The diluted samples were fed to an Atomic Absorption Spectrometer (Varian Spectra AA 220, Australia) to determine Fe and Zn concentrations. Blanks were maintained without adding sample.

Estimation of phytic acid

Phytic acid was estimated by the method of Davies and Reid (1979). One g of material was ground and extracted with HNO_3 by continuous shaking, filtered and made up to suitable volume with water. To 1.4 ml of the filtrate, 1 ml of ferric ammonium sulphate (21.6 mg in 100 ml water) was added, mixed and placed in a boiling water bath for 20 min. The contents were cooled and 5 ml of isoamyl alcohol was added and mixed. To this, 0.1 ml ammonia solution was added, shaken thoroughly and centrifuged at 3000 rpm for 10 min. The alcoholic layer was separated and the colour intensity was read at 465 nm

Table 3 - Available zinc (Zn) and iron (Fe) (mg kg^{-1}) concentrations examined in the soils of arbuscular mycorrhiza inoculated (M+) and non-inoculated (M-) soils at 45 days after sowing (DAS) under varying Fe and Zn levels.

Treatments	DTPA Zn (mg kg^{-1})								DTPA Fe (mg kg^{-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+
$Fe_{12.5}$ $Zn_{12.5}$	0.91d	1.00c	1.10c	1.30b	1.10d	1.20c	1.33d	1.64b	1.02d	1.20b	1.12c	1.27b	35.0dc	42.6b	42.2d	50.6b
Fe_{25} $Zn_{12.5}$	0.93d	1.02c	1.13c	1.30b	1.12d	1.32b	1.37d	1.65b	1.13cb	1.32a	1.27b	1.42a	36.7c	48.3a	44.4c	55.8a
$Fe_{12.5}$ Zn_{25}	1.04b	1.17a	1.25b	1.49a	1.17c	1.39b	1.38d	1.82a	1.02d	1.17b	1.14c	1.27b	35.2dc	42.6b	42.5d	50.3b
Fe_{25} Zn_{25}	1.05b	1.19a	1.27b	1.54a	1.22c	1.49a	1.49c	1.85a	1.15b	1.37a	1.29b	1.47a	39.4c	51.9a	47.6cb	59.2a
Mean	0.98	1.10	1.18	1.40	1.15	1.35	1.39	1.74	1.07	1.26	1.20	1.35	36.6	46.3	44.1	53.9

ANOVA: M (Mycorrhizal inoculation), F (Fe levels), Z (Zn levels)

CD(0.05)

	Zn Calcareous Sterilized	Zn Calcareous Natural	Zn Non calcareous Sterilized	Zn Non calcareous Natural	Fe Calcareous Sterilized	Fe Calcareous Natural	Fe Non calcareous Sterilized	Fe Non calcareous Natural
M	**	**	**	**	**	**	**	**
F	**	**	**	**	**	**	*	**
Zn	*	*	**	**	**	**	**	*
M×F	*	*	*	*	**	*	*	*
F×Z	*	**	*	**	**	*	*	*
M×Z	*	*	*	*	*	*	*	*
M×F×Z	*	*	*	*	*	NS	*	**

*P ≤ 0.05; **P ≤ 0.01; NS = Not significant

Table 4 - Siderophores (µmol cm^{-3} h^{-1}) concentration in the arbuscular mycorrhiza inoculated (M+) and non-inoculated (M-) maize plants at 45 and 75 days after sowing (DAS) under varying Fe and Zn levels.

Treatments	Calcareous								Non calcareous							
	Sterilized				Natural				Sterilized				Natural			
	45 DAS		75 DAS		45 DAS		75 DAS		45 DAS		75 DAS		45 DAS		75 DAS	
	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+
$Fe_{12.5}$ $Zn_{12.5}$	40.6b	47.8a	41.2b	51.7a	46.1c	58.3a	46.8b	63.1a	13.4c	22.4a	13.6ba	20.3a	15.2c	27.3a	15.4b	24.8a
Fe_{25} $Zn_{12.5}$	38.4cb	46.2a	38.3b	50.7a	43.6c	56.4a	43.5b	61.9a	11.8c	20.2a	12.5b	19.5a	13.4c	24.6a	14.2b	23.8a
$Fe_{12.5}$ Zn_{25}	36.4c	43.1b	40.0b	51.4a	41.4dc	52.6a	45.4b	62.7a	11.5c	17.1b	10.8b	17.1a	13.1c	20.9b	12.3b	20.9a
Fe_{25} Zn_{25}	34.5c	41.8b	37.0b	49.8a	39.2d	51.0b	42.1b	60.8a	10.8c	16.4b	9.8b	15.3b	12.3c	20.0b	11.1c	18.7ba
Mean	37.5	44.7	39.1	50.9	42.6	54.6	44.5	62.1	11.9	19.0	11.7	18.1	13.5	23.2	13.3	22.1

ANOVA: M (Mycorrhizal inoculation), F (Fe levels), Z (Zn levels)

	Calcareous Sterilized 45 DAS	Calcareous Sterilized 75 DAS	Calcareous Natural 45 DAS	Calcareous Natural 75 DAS	Non calcareous Sterilized 45 DAS	Non calcareous Sterilized 75 DAS	Non calcareous Natural 45 DAS	Non calcareous Natural 75 DAS
M	**	**	**	**	**	**	**	**
F	**	**	**	**	**	*	**	*
Zn	*	*	*	*	*	*	*	*
M×F	*	*	*	*	*	NS	*	NS
F×Z	NS	*	*	*	*	NS	NS	NS
M×Z	NS	NS	NS	NS	NS	NS	NS	NS
M×F×Z	NS	NS	*	*	NS	NS	NS	NS

*P ≤ 0.05; **P ≤ 0.01; NS = Not significant

against amyl alcohol blank after 15 min. Sodium phytate standards were run along with the sample. The results were expressed as mg phytic acid g dry wt^{-1}.

Soil available micronutrient status

Soil available Fe and Zn was extracted by mixing 10 g of soil sample with 20 ml DTPA extractant (13.1 ml triethanolamine, 1.967 g DTPA, and 1.47 g $CaCl_2$ mixed together, made up to 1 l and adjusted to pH 7.3) for 2 h and filtered through Whatman# 42 filter paper, and the absorbance was read in an atomic absorption spectrophotometer (Spectra AA220, Varian). The Fe and Zn concentrations were determined by a standard protocol described by Lindsay and Norwell (1978).

Statistical analysis

A two-way analysis of variance (ANOVA) was done for all data set and the entire set of data had fulfilled the assumptions of ANOVA. None of the tables had required transformations of the data before carrying out ANOVA. The data collected from the field sites (Coimbatore and Bhavanisagar) were analyzed separately. Despite the fact that the experimental design had only three replications, care was taken to record the observations from 5 plants in each replication. Mean Comparison test (Duncan's Multiple Range Test, DMRT) was done for the significantl values at p < 0.05. Statistical procedures were carried out with the software package IRRI stat (IRRI, Manila, Philippines).

Results and Discussion

Mycorrhizal colonization

The experiments were undertaken in order to study the effect of mycorrhizal inoculation on improving the availability of micronutrients (Fe and Zn), enhancing the host plant nutritional status which in thus fortification of micronutrients in grain which circumventing phytic acid "anti-nutritional" factors. The data on soil, plant and mycorrhizal parameters have taken statistically analyzed and the results obtained are critically discussed. Arbuscular mycorrhizal fungal (M+) inoculation significantly (P ≤ 0.01) increased the mycorrhizal colonization of maize plants grown

Table 5 - Physiologically active iron (mg kg^{-1} of tissue) in the arbuscular mycorrhiza inoculated (M+) and non-inoculated (M-) maize plants at 45 and 75 days after sowing (DAS) under varying Fe and Zn levels.

Treatments	Calcareous								Non calcareous							
	Sterilized				Natural				Sterilized				Natural			
	45 DAS		75 DAS		45 DAS		75 DAS		45 DAS		75 DAS		45 DAS		75 DAS	
	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+	M-	M+
$Fe_{12.5}$ $Zn_{12.5}$	2.96b	7.63a	4.90b	10.3a	7.17b	10.8a	9.49b	12.5b	11.1d	21.0b	16.5cb	20.9b	16.2c	22.4b	24.7c	32.6b
Fe_{25} $Zn_{12.5}$	3.85b	8.99a	6.64b	13.2a	8.42ba	12.7a	11.5b	16.6a	14.0c	23.9a	18.8b	24.3a	21.7b	31.2a	33.3b	38.1a
$Fe_{12.5}$ Zn_{25}	3.44b	7.26a	4.75b	11.2a	5.84b	10.2a	9.28b	12.8b	12.9c	20.0b	15.7cb	22.8ba	15.4c	21.7b	32.1b	35.6ba
Fe_{25} Zn_{25}	3.73b	8.68a	6.35b	12.0a	7.25b	12.2a	11.1b	15.8a	14.5c	24.7a	19.5b	26.8a	20.2b	30.4a	30.2b	41.9a
Mean	3.50	8.14	5.66	11.7	7.17	11.5	10.4	14.4	13.1	22.4	17.6	23.7	18.4	26.4	30.1	37.1

ANOVA: M (Mycorrhizal inoculation), F (Fe levels), Z (Zn levels)

	Calcareous Sterilized 45 DAS	Calcareous Sterilized 75 DAS	Calcareous Natural 45 DAS	Calcareous Natural 75 DAS	Non calcareous Sterilized 45 DAS	Non calcareous Sterilized 75 DAS	Non calcareous Natural 45 DAS	Non calcareous Natural 75 DAS
M	**	**	**	**	**	**	**	**
F	**	**	**	**	**	**	**	**
Zn	**	**	**	*	**	**	**	*
M×F	NS	*	*	NS	*	*	NS	NS
F×Z	NS	NS	*	NS	*	NS	*	NS
M×Z	NS	*	*	NS	NS	NS	NS	*
M×F×Z	NS	NS	NS	NS	NS	NS	NS	NS

*P ≤ 0.05; **P ≤ 0.01; NS = Not significant

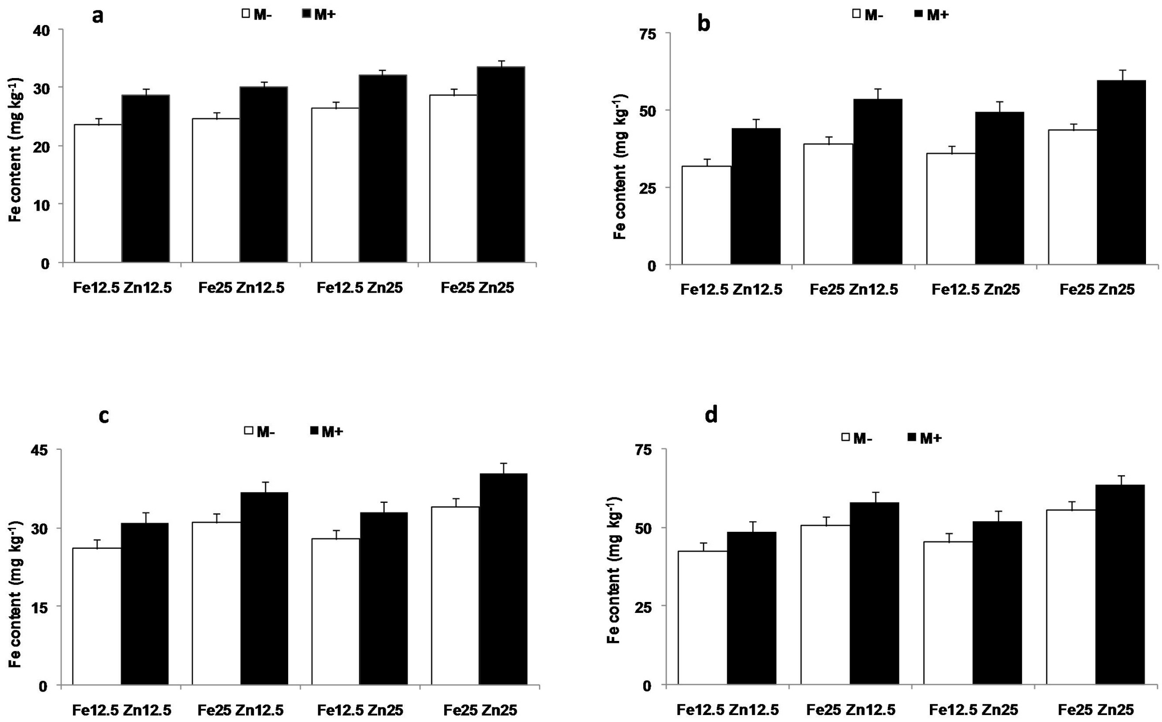

Figure 1 - Iron concentration of grain (mg kg^{-1}) of arbuscular mycorrhizal fungus inoculated (AM+) and uninoculated (AM-) maize plants under two levels of FeSO4 (12.5 and 25 kg ha^{-1}) and two levels of $ZnSO_4$ (12.5 and 25 kg ha^{-1}) in calcareous (A) and natural soils (B) and non-calcareous sterilized (C) and natural soils (D). Error bars represent standard errors of four replications.

under sterilized or unsterilized conditions of both calcareous and non-calcareous soils (Table 2). However, natural soils had the mycorrhizal colonization in the range of 37-48% and 45-57%, in calcareous and non-calcareous soils, respectively. Iron and zinc application had a little effect on root colonization under sterilized or unsterilized conditions in both calcareous and non-calcareous soils. The sterilization of the experimental soils eliminated indigenous mycorrhizal population which resulted in less than 5% of the root segments exhibiting mycorrhizal colonization. The data are in conformity with the observations of Wang et al (2008) who have reported no colonization in citrus plants grown in sterilized soils. Further, addition of both Fe and Zn singly or in combination improved the percentage of mycorrhizal fungal colonization regardless of calcareous or non-calcareous soils. Zinc fertilization is known to promote the production of highly branched fibrous roots of maize that facilitate mycorrhizal colonization. Subramanian et al (2008) have shown that Zn fertilization improved the root biomass of both mycorrhizal and non-mycorrhizal maize plants but the response was more pronounced for M+ plants. Since the experimental soils of both locations were deficient in Zn (less than 1 mg kg^{-1}), the fertilization would have helped in alleviating Zn deficiency besides promoting root growth. Further, Fe fertilization has shown to improve colonization of *Glomus versiforme* in citrus plants. These data suggest that micronutrient fertilization assists root growth and mycorrhizal colonization.

Soil available micronutrient status

The available (DTPA extractable) Zn and Fe concentrations in M+ soils were significantly ($P \leq 0.01$) higher than M- soils in both calcareous and non-calcareous regardless of sterilized or natural conditions (Table 3). The available Fe concentrations of both M+ and M- soils had 30-40 times lower values in calcareous soils in comparison to non-calcareous soils suggesting that there is a strong inhibitory effect of free lime status on the availability of Fe. A negative correlation between lime status and available Fe has already been well established (Zuo et al, 2007). The data clearly indicated that the introduced AMF species Glomus intraradices inoculation had consistent effects on availability of micronutrients in soil regardless of free lime status of soils. Subramanian et al (2009) have shown that the mycorrhizal colonization facilitates acidification of rhizosphere, solubilization of tightly bound residual form of zinc besides hyphal transport of metallic micronutrients collectively contribute for the availability. Rhizosphere of mycorrhiza colonized citrus plants assists in acidification and increased the root ferric chelate reductase activity in combination with hyphal transport helped the acquisition by the host plant. Our study in conjunction with reported literature are in conformity with the observations of earlier reports (Koide and Kabir, 2000;

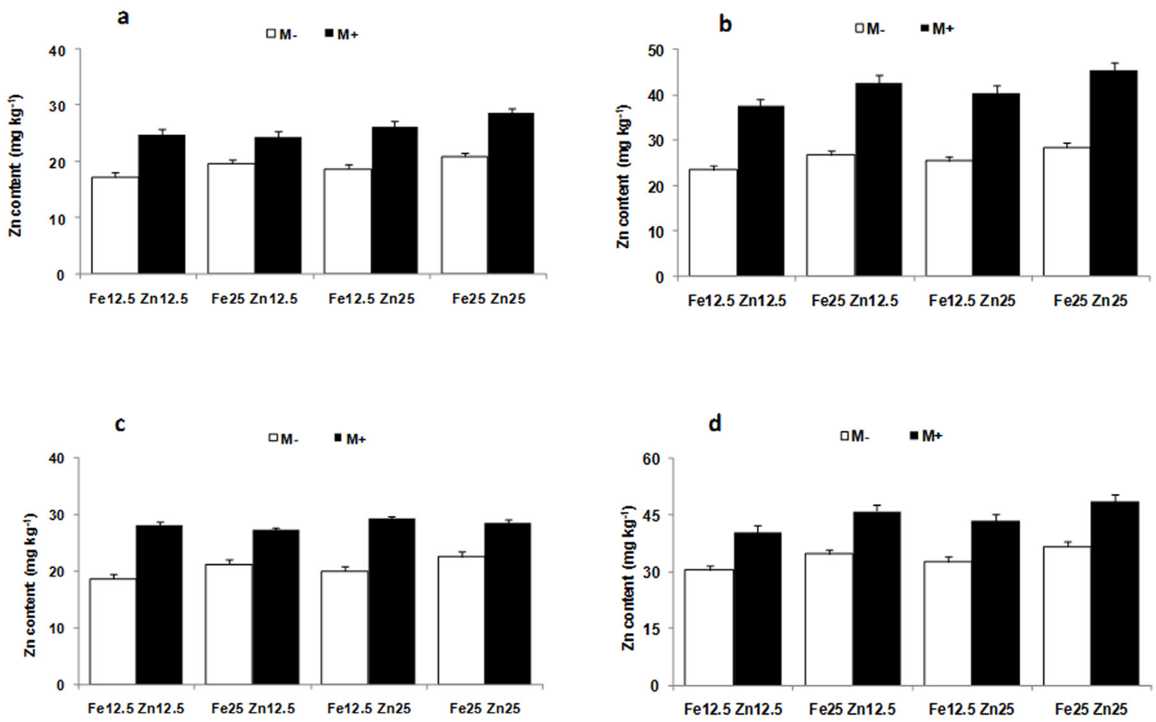

Figure 2 - Zinc concentration of grain (mg kg^{-1}) of arbuscular mycorrhizal fungus inoculated (AMF+) and uninoculated (AMF-) maize plants under two levels of $FeSO_4$ (12.5 and 25 kg ha^{-1}) and two levels of $ZnSO_4$ (12.5 and 25 kg ha^{-1}) in calcareous sterilized (A) and natural soils (B) and non-calcareous sterilized (C) and natural soils (D). Error bars represent standard errors of four replications.

Subramanian et al, 2008).

Siderophore concentration

Mycorrhizal fungal inoculated roots significantly produced higher ($P \leq 0.01$) siderophore concentrations than non-mycorrhizal roots in both stages of calcareous and non-calcareous soil (Table 4). With the progression of plant growth stages on both soils, M+ soil had higher siderophore production status while M- soil had consistently lower siderophore production under both soil conditions. Mycorrhizal fungus inoculated soil had significantly ($P \leq 0.01$) higher siderophore production in calcareous soil compared to the non-calcareous soil (calcareous M- 44.5; M+ 62.1 µmol cm^{-3} h^{-1}, non-calcareous M- 13.3; M+ 22.1 µmol cm^{-3} h^{-1}) conditions.

Mycorrhizal symbiosis enhances the production of mugenic acids which serve as a chelating agent that favors availability of micronutrients particularly in calcareous soils where the availability is very much restricted. Similar results were reported by Lindermann (1992) and he stated that an arbuscular mycorrhizal grass species, which showed greater Fe uptake than non-mycorrhizal controls, tested positively when bioassayed for hydroxymate siderophores (Haselwandter, 1995). Even higher siderophore concentrations may be reached in microenvironments such as biofilms, unless pH depression and/or anaerobic conditions in the microenvironment increase the solubility of iron, depressing siderophore production. Siderophores facilitate Fe uptake to both microbial flora and higher plants. Ericoid mycorrhizal fungi produce siderophore (Landeweert et al, 2001; Howard, 2004). Ericoid mycorrhizal fungi release ferricrocin or fusigen as the main siderophores. Ferricrocin was also shown to be produced by the ectomycorrhizal fungi *Cenococcum geophilum* and *Hebeloma crustuliniforme*.

Arbuscular mycorrhizal fungi are reported to enhance Fe-uptake rates of associated host plants, which can be taken as an indication that mycorrhizal siderophores of a yet unknown structure may be involved (Haselwandter, 2008). Enhancement of siderophores and/or phytosiderophores per unit volume of root in mycorrhizal plants suggests that mycorrhizal fungi may secrete siderophore by themselves and/or induce plant root to produce more phytosiderophore (Aliasgharzad et al, 2009).

Active Fe content

Mycorrhizal plants had significantly ($P \leq 0.01$) higher physiologically active Fe concentrations than non-mycorrhizal plants at both 45 and 75 DAS in calcareous and non-calcareous soils (Table 5). The physiologically active Fe content in plants appears to play a vital role in chlorophyll synthesis. In this study, a strong correlation between physiologically active Fe and chlorophyll concentration has been established

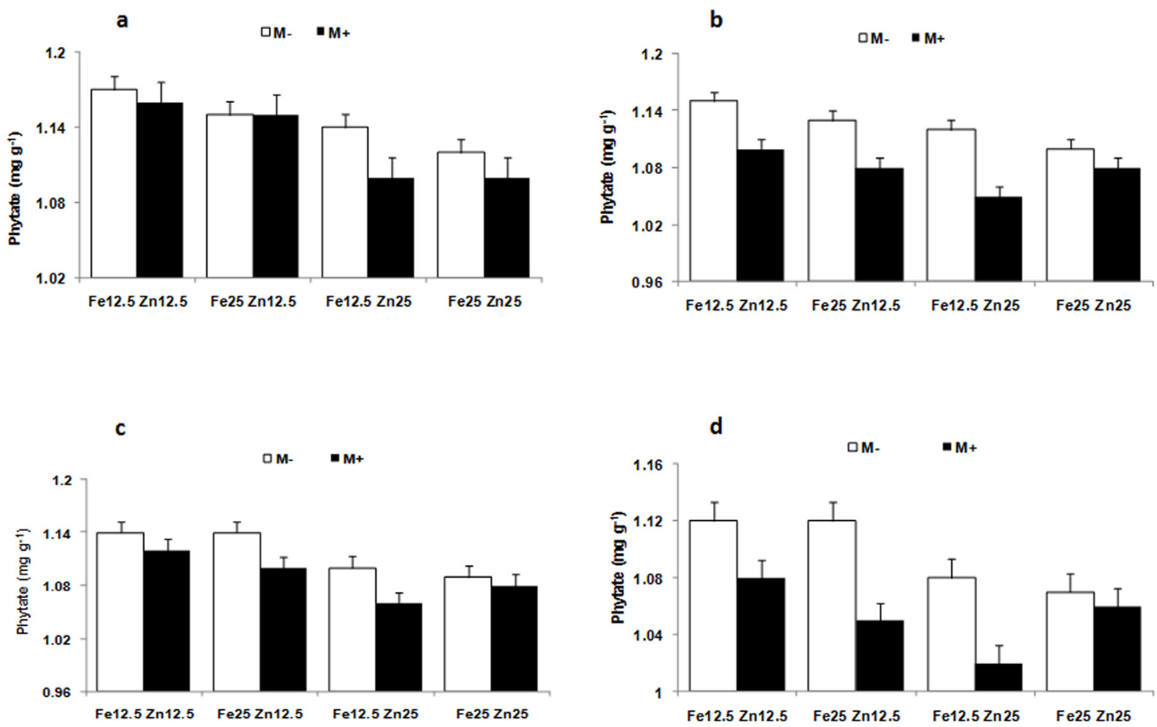

Figure 3 - Phytate concentration of grain (mg g^{-1}) of arbuscular mycorrhizal fungus inoculated (AMF+) and uninoculated (AMF-) maize plants under two levels of $FeSO_4$ (12.5 and 25 kg ha^{-1}) and two levels of $ZnSO_4$ (12.5 and 25 kg ha^{-1}) in calcareous sterilized (A) and natural soils (B) and non-calcareous sterilized (C) and natural soils (D). Error bars represent standard errors of four replications.

(Table 5) (Calcareous soil r^2 = 0.76; Non-calcareous soil r^2 = 0.873). Our data are in agreement with the observations Zou at al (2000) who have reported a strong correlation between active Fe and chlorophyll concentrations. Chlorophyll synthesis in the plants is directly related to the availability of the physiologically active Fe and micronutrients in plants available form (Suresh Kumar et al, 2011). Fe nutrition in plants, the concentration of active iron in leaves is recognized as a better nutritional iron indicator than total iron and has been also suggested by Scholl (1979), Dekock (1979), Katyal and Sharma (1980), and Mengel et al (1984). Higher Fe concentrations in grains of M+ plants may be attributed to the hyphal transport of Fe and besides improved plant available Fe that may have supported Fe nutrition of maize plants and fortification of grains (Caris et al, 1998). In addition to the hyphal transport, mycorrhizal fungi produce Fe siderophores that may favour chelation and availability of Fe.

Iron and zinc concentrations in grains

M+ maize plants produced grains with significantly higher Fe concentrations under sterilized and natural soils conditions regardless of lime status. Grain Fe concentrations of M+ were nearly doubled and consistently higher than M- under calcareous (Figure 1A-1D) (M- 37.6; M+ 51.8 mg kg^{-1}) and non-calcareous (M- 48.4; 55.5 mg kg^{-1}) soils under natural conditions in comparison to sterilized calcareous (M- 21.7; M+ 29.0 mg kg^{-1}) and non-calcareous (M- 23.6; M+ 35.2 mg kg^{-1}). Similarly, Zn concentrations (Figure 2A-2D) of maize grains were significantly higher for mycorrhizal treatments in both calcareous (36.3 mg kg^{-1}) and non-calcareous (39.7 mg kg^{-1}) soils than M- treatments (Calcareous 22.6; non-calcareous 27.2 mg kg^{-1}). Our data clearly demonstrated that mycorrhizas improve Fe concentrations of maize irrespective of soil conditions. The data have shown that mycorrhizal symbiosis has a potential to enhance grain Zn concentrations to the tune of 13-15 mg per kg grains. Such response has already been reported earlier. Our earlier experimental data have shown improved Zn concentrations in maize grains as a result of hyphal transport, acidification of rhizosphere and synergistic interaction with P (Subramanian et al, 2008; 2009).

Phytic acid concentrations

Mycorrhiza inoculated plants produced grains with significantly ($P \leq 0.01$) lower phytic acid concentrations than M- plants in both calcareous (Figure 3A-3D) and non-calcareous soils. The phytic acid concentrations in M+ grains in calcareous soil were 1.12 and 1.07 mg g^{-1} which were 5-6% and 5-7.5% lower in sterilized and natural soils, respectively, in comparison to M- grains (sterilized 1.10; natural 1.05 mg g^{-1}). Similar trends were observed in non-calcareous soils but the values were lower than calcareous soils.

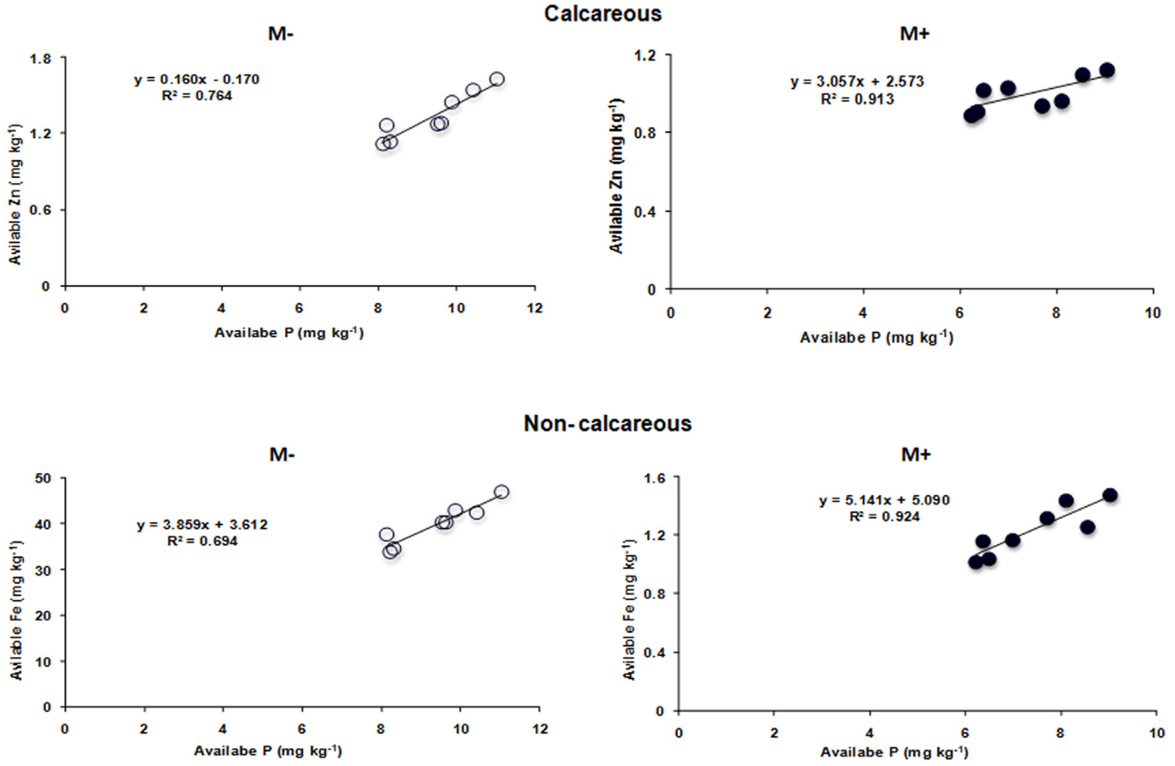

Figure 4 - Correlations between available micronutrients (Fe and Zn) and P of the arbuscular mycorrhiza inoculated (M+) and non- inoculated soils.

There is no reported literature to support that mycorrhizal symbiosis has a potential to decrease phytic acid concentrations. But, indirectly, mycorrhizas are well known to promote the availability of Zn is soils as well as in grains which is widely considered as an inhibitory factor. Akay and Ertas (2008) have indicated that the chickpea genotypes rich in Zn have a negative correlation with phytic acid concentrations. Similar observation has made by Ryan et al (2008). Our present study has clearly shown an increase in grain Zn which may have suppressed the phytic acid concentrations. A strong negative correlation between grain Zn concentrations and phytic acid content has been established (Kaya et al, 2009). Since mycorrhizal symbiosis facilitates accumulation of Zn concentrations in grains which may suppress the phytic acid content.

Conclusion

Overall, the four sets of greenhouse and field experimental data unequivocally demonstrated that mycorrhizal symbiosis facilitates the availability of both Fe and Zn. The synergistic interaction between these two nutrients may assist in enhanced uptake of iron and zinc which eventually gets remobilized into developing grains. Since mycorrhizal fungal inoculation is one of the potential factors assist in biofortification kernels with minerals besides circumventing the impact of anti-nutritional factors. Mycorrhizal symbiosis is a potential factor to be considered to achieve nutritional security in the context of severity of micronutrient deficiencies in arid and semi-arid regions.

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