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Mycorrhizal symbiosis to increase the grain micronutrient content in maize

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Abstract

Four sets of experiments (two each under open field and greenhouse conditions) were conducted in order to increase micronutrient concentrations in maize (*Zea mays* L.) grains using arbuscular mycorrhizal fungal (AMF) inoculation. 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 or without inoculum carrying *Glomus intraradices* Schenck & Smith) at the rate of 2g $plant^{-1}$ replicated four times in a factorial randomized block design (FRBD). Grains sampled from AMF+ and AMF- treatments were analyzed for their micronutrient concentrations (Fe, Mn, Cu and Zn) besides an anti-nutritional factor phytic acid. Further, the grain phytase activity was also measured to determine the bioavailability of micronutrients. In addition, soil samples were assessed for its available micronutrients at 45 and 75 days after seeding. The results revealed that AMF+ soils had significantly higher available micronutrients in comparison to AMF- soils. Increased availability of micronutrients in soil in combination with enhanced concentrations in plants assisted the mycorrhizal plants to maintain higher micronutrient concentrations in grains. AMF inoculated maize plants produced grains with 10-15% higher Fe and Zn concentrations while an anti-nutritional factor "phytic acid" decreased regardless of soil types. Overall, the data suggest that AMF inoculation is one of the potential factors assist in biofortification of grains with micronutrients besides circumventing the impact of anti-nutritional factors.

Keywords: Maize; grain; quality; micronutrients; iron; zinc; mycorrhiza.
Abbreviations : AMF - arbuscular mycorrhizal fungus, Cu – copper, DAS - days after seeding, DMRT- Duncun multiple range test, Fe- iron, FRBD – factorial randomized block design, Mn – manganese, Zn – zinc.

Introduction

Micronutrient deficiencies are widely prevalent in soils of arid and semi-arid regions where major part of the soils are calcareous which suppresses the availability **(**Khademi et al., 2006**)**. Food crops grown in such soils produce grains with deficient quantities of micronutrients that cause malnutrition in humans. It has been estimated that over 3 billion across the globe are affected by micronutrient deficiencies (Cakmak et al., 2010). Zinc deficiency in humans cause impairment in brain development and wound healing and increase the susceptibility to infectious diseases (Black et al., 2009). Iron deficiency causes anemic conditions in humans and reduces productivity in adults (Kennedy et al., 2003). Micronutrient malnutrition is a prime concern in developing countries where the deficiency causes widespread illness and diseases (WHO, 2002). In fact, micronutrient deficiency in soils affects the entire food chain. Recently, Prasad (2010) has reported that fodder crops cultivated in deficient soils fed to the cattle cause low birth weight and number of off-springs besides affecting the humans who consume the deficient milk. Even plants nourished with micronutrients adequately, the bioavailability of micronutrients in grains is being circumvented by an anti-nutritional factor phytic acid. It is well established that phytic acid involved in poly valent cation chelating activity and lack of phytase enzyme in mono-gastric animals and humans that reduces the absorption of dietary Zn and Fe (Hotz & Brown, 2004). Further, micronutrients get accumulated in aleurone layer of the grains that inhibit bioavailability of Zn and Fe (Brinch – Pedersen et al., 2007). Molar ratio of phytic acid to Zn or Fe is often referred as an indicator of bioavailability of micronutrients (Ryan et al., 2008). A strong correlation between phytic acid and total P concentration in seeds has been established (Erdal et al., 2002). These literatures suggest that mitigating micronutrient efficiencies in food grains is quite complex and alternate strategy to be evolved to improve the micronutrient status. A new approach of tackling the problem of micronutrient deficiencies in the diet is through biofortification. This approach has proved to be sustainable, can be implemented at a relatively low cost, is highly efficacious and has a large coverage, especially in the poorer regions of the world (Poletti et al., 2004). Arbuscular mycorrhizal fungi (AMF) are known to improve the host plant nutrition as a result of increased volume of soil being exploited by plants (Bolan, 1991). Consequently, root colonization by AMF often results in enhanced uptake of relatively immobile metal micronutrients, such as Cu, Zn and Fe (Li et al. 1991; Liu et al., 2000; Ryan & Angus, 2003). Recently, we have found that AMF colonization acidifies the

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

Parameters	Calcareous		Non-calcareous	
	Sterilized	Natural	Sterilized	Natural
Soil Texture	Clay Loam	Clay Loam	Sandy Loam	Sandy Loam
pH	8.38	8.39	7.16	7.20
EC (dS m^{-1})	0.45	0.45	0.03	0.04
Organic carbon (%)	0.41	0.42	0.26	0.26
Available N (kg ha^{-1})	186.2	186.2	226.2	226.2
Available P (kg ha^{-1})	16.8	16.6	19.1	19.6
Available K (kg ha^{-1})	415.0	412.4	262.6	258.4
DTPA Zn (mg g^{-1})	0.61	0.61	0.91	0.93
DTPA Fe (mg g^{-1})	1.71	1.67	34.3	36.2
Spore Count (Nos $100g^{-1}$)	0	8	0	21

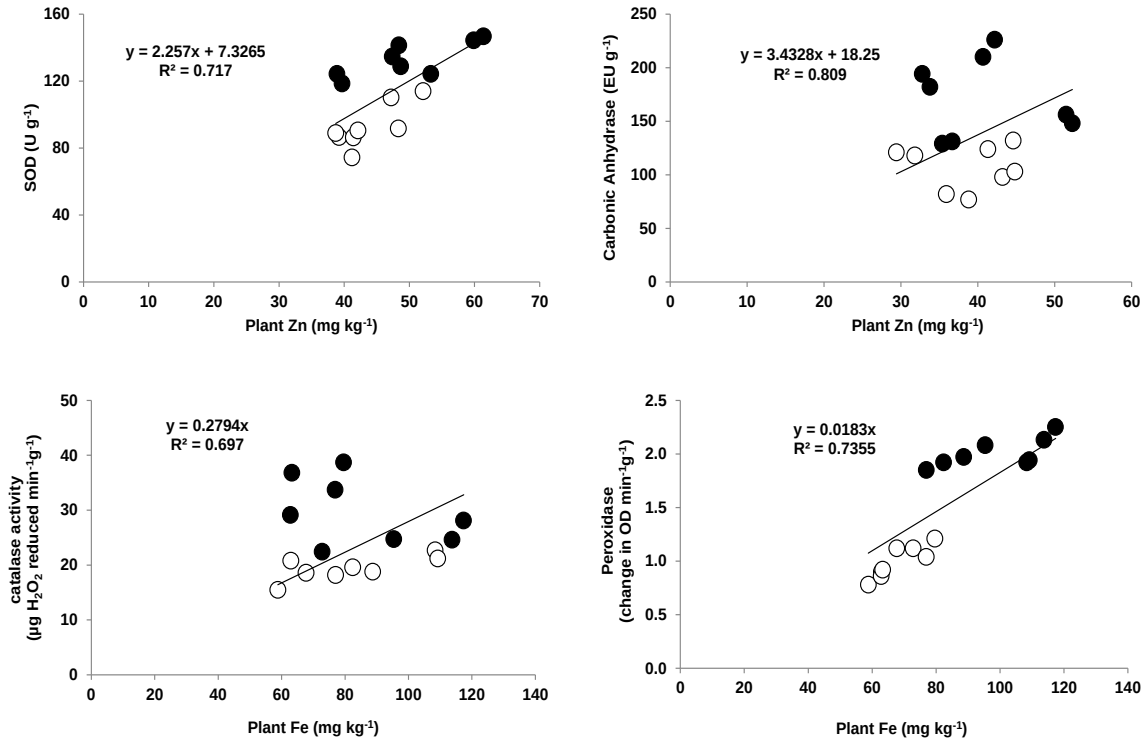

Fig 1 . Relationships between plant micronutrient status and plant enzymatic activities of arbuscular mycorrhiza inoculated (AMF+) and non- inoculated (AMF-) soils.

rhizosphere and provides ability for the host plant to utilize the tightly bound Zn by the external mycelium which unavailable for the uninoculated plants (Subramanian et al., 2009). Further, a synergistic interaction between the available P and available Zn has been reported earlier (Subramanian et al., 2008). The data suggest that mycorrhizal symbiosis may be a potential factor in alleviating Zn deficiency of host plants. This process may facilitate biofortification of grains with micronutrients. These papers have focused only on mycorrhiza-aided Zn nutrition in maize. However, there is a possibility that the associated micronutrients such Fe, Mn and Cu may also get altered in the presence or absence of mycorrhizal fungal inoculation. Further, the present study is intended to determine the anti-nutritional factor "phytic acid" that has an inhibitory effect on the bioavailability of micronutrients. The impacts of mycorrhizal fungal inoculation were assessed under two distinct soils (calcareous and non-calcareous) and conditions (sterilized and natural). Such arrangements would help to determine the significance of AMF inoculation on micronutrient contents in maize grains. This study hypothesized that arbuscular mycorrhizal fungal (AMF) inoculation assists in availability of micronutrients in the soil that facilitate uptake of these nutrients besides fortifying it in the grains. Further, the mycorrhizas may improve phytase activity in grains which inhibits the phytic acid concentrations while improving the bioavailability of micronutrients in grains. In this study, grains sampled from inoculated and uninoculated maize plants grown under two distinct soil types (calcareous and non-calcareous) and two distinct situations (natural and sterilized) were determined for its micronutrient content, phytic acid and phytase activity.

Results

Root colonization

Arbuscular mycorrhizal fungal (AMF) inoculation significantly ($P \leq 0.01$) increased the mycorrhizal colonization of maize plants grown under sterilized or unsterilized conditions of both Coimbatore (CBE) and Bhavanisagar (BSR) soils. However, natural soils had the mycorrhizal colonization in the range of 37-48% and 45-57%, in CBE and BSR soils, respectively (Table 2).

Micronutrient status of soil

The available (DTPA extractable) Fe and Zn concentrations in AMF+ soils were significantly ($P \leq 0.01$) higher than AMF- soils in both locations regardless of sterilized or natural conditions (Table 3). The available Fe concentrations of both AMF+ and AMF- 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.

Leaf chlorophyll and physiologically active Fe concentrations

Mycorrhizal plants had significantly ($P \leq 0.01$) higher chlorophyll and physiologically active Fe concentrations than uninoculated plants at both 45 and 75 DAS in calcareous and non-calcareous soils. Chlorophyll concentrations in AMF+ in calcareous soil were 5-7% and 18-19% higher in sterilized and natural soils, respectively. Similar trend of responses was observed in non-calcareous soil also (Table 4). 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 (Table 5) (calcareous soil $r^2 = 0.76$; non-calcareous soilr $r^2 = 0.87$).

Biochemical changes in plants

The catalase, peroxidase, SOD and carbonic anhydrase activities were significantly higher in AMF+ than AMF- plants and the increase was 14.5, 26.3, 31.2 and 12.6%, respectively, in comparison to non-mycorrhizal plants (Fig. 1). Correlation studies revealed a significant and strong correlation between micronutrient status and the respective key enzyme activities

Micronutrient concentrations in grains

AMF+ maize plants produced grains with significantly higher Fe, Mn, Cu and Zn concentrations under sterilized and natural soils conditions regardless of lime status. Grain Fe concentrations of AMF+ were nearly doubled and consistently higher than AMF- under calcareous (Fig. 2b) (M- 37.6; M+ 51.8 mg kg^{-1}) and non-calcareous (Fig. 2d) (M- 48.4; 55.5 mg kg^{-1}) soils under natural conditions in comparison to sterilized (Fig. 2a) (M- 21.7; M+ 29.0 mg kg^{-1}) and (Fig. 2c) (M- 23.6; M+ 35.2 mg kg^{-1}) in both soil conditions. Similar trend of response was observed for other micronutrients such as Mn, Cu (Table 6) and Zn (Figs 3 a-d).

Phytic acid concentrations

AMF+ plants produced grains with significantly ($P \leq 0.01$) lower phytic acid concentrations than AMF- plants in both calcareous (Fig. 4a-b) and non-calcareous (Fig. 4c-d) soils. The phytic acid concentrations in AMF+ 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 AMF- 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.

Phytase activity

AMF+ plants produced grains with significantly higher concentrations of phytase activities in calcareous (Fig. 5a) (M- 211.4; M+ 269.5 mg kg^{-1}) and non-calcareous (Fig. 5c) (M- 223.6; M+ 278.2 mg kg^{-1}) under sterilized soil conditions. Similar trend of response was also observed in natural soils (Fig. 5b and 5d) but less pronounced in comparison to sterilized soils.

Discussion

Two sets of pot experiments (sterilized soil) and two sets of field experiments (natural soils) were conducted in two distinct soil types namely calcareous and non-calcareus in order to assess the response of maize plants to mycorrhizal fungal inoculation. Our data clearly demonstrated that sterilization of both calcareous and non-calcareous soils had effectively eliminated the indigenous mycorrhizal population registering the lowest values in the range of 2-3%. 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. On the other hand, AMF inoculated plants had root colonization 10-15 times higher in comparison to AMF- plants. This suggests that the AMF inoculation had really helped the maize plants to maintain its higher mycorrhizal fungal colonization. Such response was less pronounced in natural soils where the increase was just three times indicating the need for AMF inoculation when the soil carries the least number of indigenous spores. Further, micronutrient fertilization (Fe & Zn) has improved the percentage of mycorrhizal fungal colonization which is associated with the formation of highly branched fibrous roots The results are in agreement with our earlier paper (Subramanian et al. 2008) that indicated higher percentage of colonization in Zn fertilized soils. In addition, 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. As consequence of higher percentage of AMF colonization in roots particularly in sterilized soils, AMF+ inoculation had increased the available Fe content by 12.6% while such increase was only 8.3% in natural soil conditions. The mycorrhiza-aided increase in available Fe content was obvious in calcareous soils where the lime induced Fe deficiency in very common. A negative correlation between lime status and available Fe has already been well established (Zuo et al., 2007). Even under non-calcareous soils, AMF inoculation increased the available Fe concentrations by 16% and 13% under sterilized and natural conditions, respectively.

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; Subramanian et al., 2009).

Mycorrhizal colonization is known to improve the chlorophyll concentrations in well watered and drought-stressed rose and maize plants (Augé et al., 1987; Subramanian et al. 1995). Mycorrhizal plants are nourished adequately with nutrients particularly Fe and Zn that may have helped in synthesis of chlorophyll molecules (Misra et

Table 2. Percentage of mycorrhizal colonization examined in the arbuscular mycorrhiza inoculated (AMF+) and non-inoculated (AMF-) root segments (n=100) of maize plants. 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	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^+
$Fe_{12.5}$ $Zn_{12.5}$	1.8^{c}	31.4^{b}	4.5^{c}	39.4^{b}	8.8^{d}	33.7^{b}	11.6^{c}	46.4^{b}	2.1^{d}	37.5^{b}	2.8^{d}	39.7^{c}	11.4^{de}	40.4^{c}	15.4^{d}	55.7^{cb}
Fe_{25} $Zn_{12.5}$	2.2^{c}	33.8^{b}	3.2^{c}	38.6^{b}	8.2^{d}	35.3^{b}	11.6^{c}	45.8^{b}	2.6^{d}	30.0^{c}	2.9^{d}	46.2^{ab}	10.7^{e}	42.4^{bc}	15.1^{d}	55.0^{c}
$Fe_{12.5}$ Zn_{25}	3.4^{c}	35.6^{b}	2.7^{c}	46.7^{a}	10.9^{c}	37.6^{a}	10.8^{c}	50.3^{a}	2.0^{d}	37.5^{b}	2.4^{d}	48.4^{a}	14.2^{d}	45.1^{b}	14.3^{d}	60.4^{a}
Fe_{25} Zn_{25}	2.0^{c}	40.5^{a}	2.4^{c}	47.5^{a}	11.5^{c}	42.2^{a}	9.2^{c}	48.1^{a}	2.8^{d}	40.0^{a}	3.0^{d}	44.1^{b}	14.9^{d}	50.6^{a}	12.6^{ed}	57.7^{ba}
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)																
CD(0.05)																
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		*	

Table 3. Available zinc (Zn) and iron (Fe) (mg kg^{-1}) concentrations examined in the soils 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	DTPA Zn (mg/kg)								DTPA Fe (mg/kg)							
	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^+
$Fe_{12.5}$ $Zn_{12.5}$	0.91^{d}	1.00^{b}	1.10^{d}	1.30^{b}	1.10^{f}	1.20d	1.33^{e}	1.64^{b}	1.02^{e}	1.20^{c}	1.12^{c}	1.27^{b}	35.0^{d}	42.6^{b}	42.2^{d}	50.6^{b}
Fe_{25} $Zn_{12.5}$	0.93^{d}	1.02^{b}	1.13^{d}	1.30^{b}	1.12^{f}	1.32c	1.37^{d}	1.65^{b}	1.13^{d}	1.32^{b}	1.27^{b}	1.42^{a}	36.7^{d}	48.3^{a}	44.4^{d}	55.8^{a}
$Fe_{12.5}$ Zn_{25}	1.04^{c}	1.17^{a}	1.25^{c}	1.49^{a}	1.17^{ed}	1.39b	1.38^{d}	1.82^{a}	1.02^{e}	1.17^{c}	1.14^{c}	1.27^{b}	35.2^{d}	42.6^{b}	42.5^{d}	50.3^{b}
Fe_{25} Zn_{25}	1.05^{c}	1.19^{a}	1.27^{c}	1.54^{a}	1.22^{d}	1.49a	1.49^{c}	1.85^{a}	1.15^{d}	1.37^{a}	1.29^{b}	1.47^{a}	39.4^{cb}	51.9^{a}	47.6^{cb}	59.2^{a}
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)																
M	**		**		**		**		**		**		**		**	
F	**		**		**		**		**		**		*		**	
Zn	*		*		**		**		**		**		**		*	
M×F	*		*		*		*		**		*		*		*	
F×Z	*		**		*		**		**		*		*		*	
M×Z	*		*		*		*		*		*		*		*	
M×F×Z	*		*		*		*		*		NS		*		**	

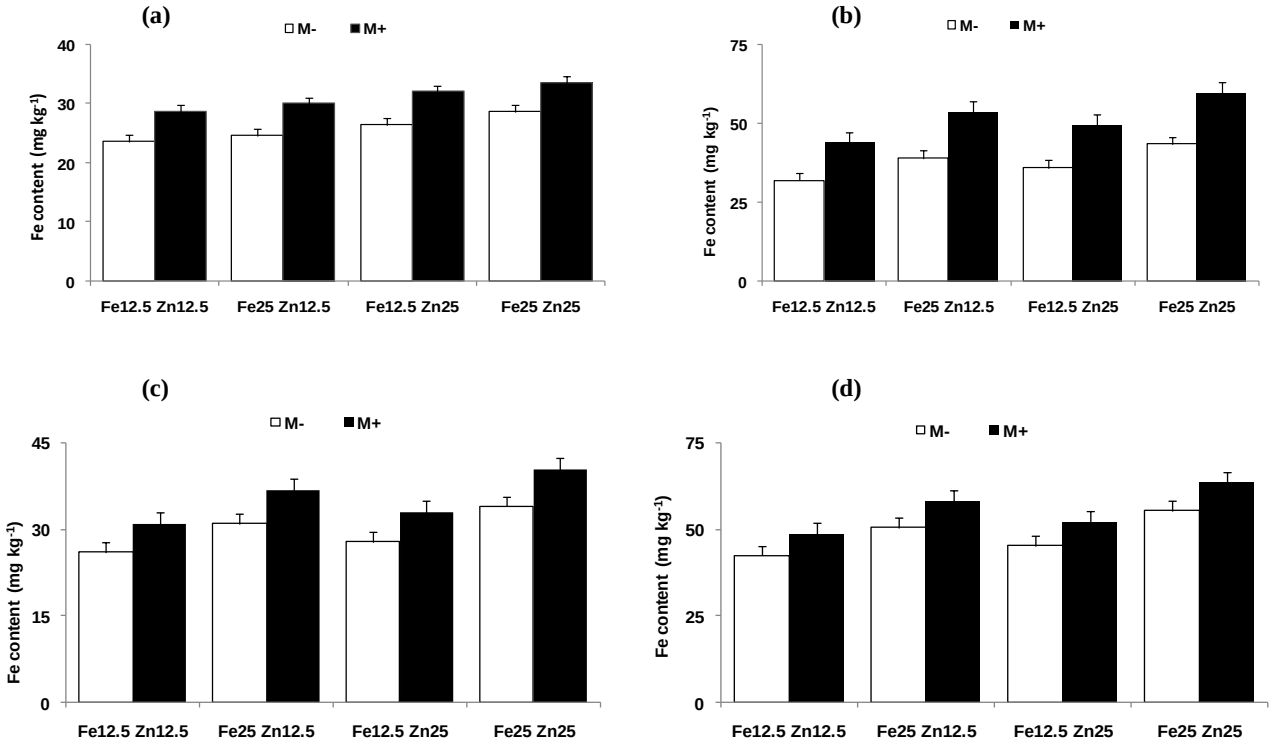

Fig 2. Iron concentration in grain (mg kg^{-1}) of arbuscular mycorrhizal fungus inoculated (AMF+) and uninoculated (AMF-) maize plants in calcareous sterilized (a) and natural soils (b) and non- calcareous sterilized (c) and natural soils (d). Error bars represent standard errors of four replications.

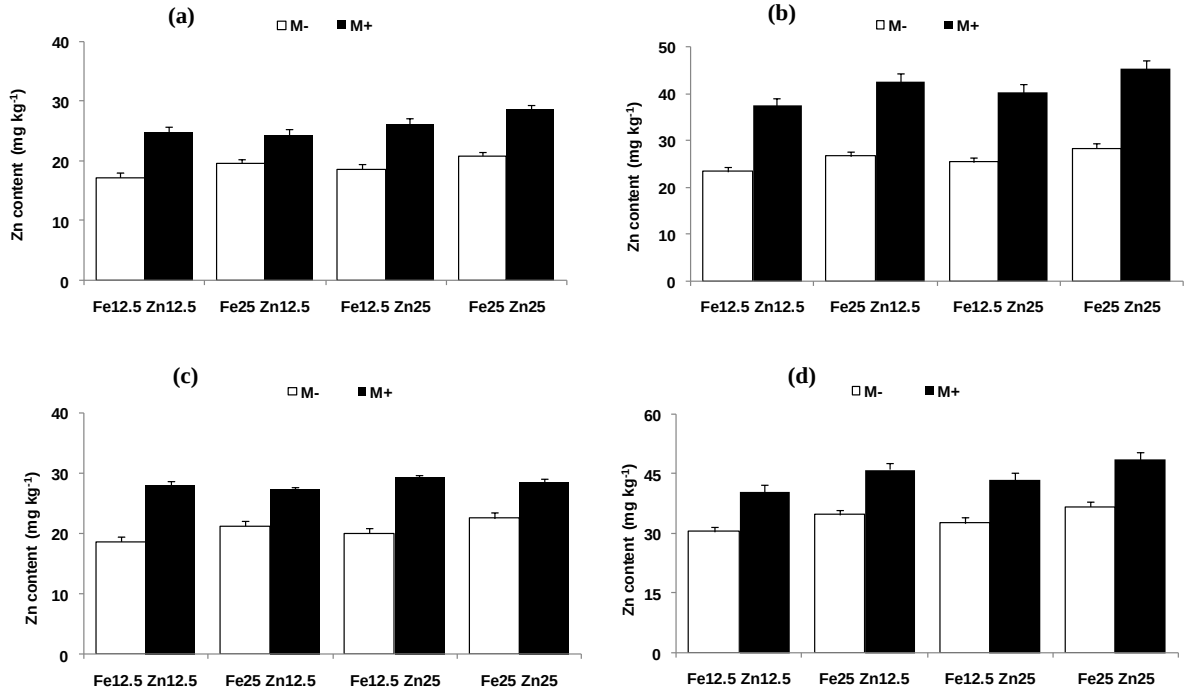

Fig 3. Zinc concentration in grain (mg kg^{-1}) of arbuscular mycorrhizal fungus inoculated (AMF+) and uninoculated (AMF-) maize plants in calcareous sterilized (a) and natural soils (b) and non-calcareous sterilized (c) and natural soils (d). Error bars represent standard errors of four replications.

al., 2005; Wang et al., 2008). The response to AMF inoculation was higher in natural than sterilized soils suggesting that natural soils have indigenous mycorrhizal population besides other helper bacteria associated in the rhizosphere. These microorganisms may have contributed for micronutrients which in turn assist in synthesis of chlorophylls. Such increase in chlorophyll concentration in AMF+ plants is closely associated with physiologically active Fe content (Zou at al. (2000; Suresh Kumar et al. 2011). The active Fe in leaves is recognized as a better nutritional iron indicator as suggested by Katyal and Sharma (1980) and Mengel et al. (1984).The improvement in host plant Fe and Zn nutrition is supported by the enzyme activities. The key enzymes involved in Fe nutrition such as catalase and peroxidase were consistently higher in AMF+ than AMF- plants regardless of sterilized or natural soils. Similar trend of response was also observed for Zn related enzymes namely super oxide dismutase (SOD) and carbonic anhydrase. A close correlation was established between Fe or Zn concentrations in plants versus respective enzymes. Our data corroborate with the observations made in earlier reports where we suggested enhanced enzyme activities of SOD activities in AMF+ plants (Subramanian et al. 2011). Higher Fe concentrations in grains of AMF+ 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. Our data clearly demonstrated that mycorrhizas improve Fe concentrations of maize irrespective of soil conditions. Similarly, Zn concentrations (Fig. 2a-2d) of maize grains were significantly higher for AMF treatments in both calcareous (36.3 mg kg^{-1}) and non-calcareous (39.7 mg kg^{-1}) soils than AMF- treatments (calcareous 22.6; non-calcareous 27.2 mg kg^{-1}). 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. The availability of Fe and Zn may have assisted the AMF+ plants to retain higher concentrations of physiologically active Fe besides enhanced enzyme activities. These physiological and biochemical attributes assist in maintaining higher concentrations of micronutrients while circumventing deficiencies in AMF+ plants that eventually resulted in accumulation of higher concentrations of Fe and Zn in grains of maize. There is no reported literature to support that mycorrhizal symbiosis has a potential to decrease phytic acid concentration. 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 results have been reported 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. Phytase activity in maize grains increased with the progression of course of germination AMF+ and AMF- maize plants. Abdel-Gawad et al. (2004) reported that barley and maize germ showed increases in phytase activity up to 96 h germination and thereafter the activity was decreased. Phytase activity at zero time compared with that of highest value during germination indicated increases by 5.4-, 4.6-, 7.3- and 6.9-folds for wheat, barley, sorghum and maize germ, respectively. Phytase activity of wheat, barley, oat and rye increased by 4.5-, 6.0-, 9.0- and 2.5-folds after three days germination compared to the initial activity at zero time (Bartnik and Szafrańska, 1987). Germination for 5 days increased the phytase activity in rye and barley up to 112% and 212%, respectively (Centeno et al., 2001). The present study had clearly demonstrated that Zn concentrations of AMF+ grains had increased by 12-15% which may have suppressed the phytic acid content while enhancing phytase activity. Major part of maize grains is being fed to poultry industry where the presence of phytic acid is considered as an anti-nutritional factor that circumvents the availability of micronutrients particularly Fe and Zn. In order to improve the bioavailability, commercially available enzyme is added to the feed to induce the bioavailability of micronutrients. This is one of the first studies comes up with a suggestion that mycorrhizas improve the quality of grains that possesses higher amounts phytase. More studies have to be undertaken in order to gain the insights into the biochemical mechanisms in the biosynthetic pathway of phytase production.

Materials and methods

Experimental soil

Field experiments were conducted in two locations one each at the Experimental Farms of Agricultural Research Station (ARS), Bhavanisagar (non-calcareous) and Tamil Nadu Agricultural University (TNAU), Coimbatore (calcareous), 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 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 attempts were made to fumigate the soil before field tests.

Field tests and greenhouse experiments

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 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 all the

Table 4. Total chlorophyll (mg/g of tissue) in the arbuscular mycorrhiza inoculated (AMF+) and non-inoculated (AMF-) maize plants. 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	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^+
$Fe_{12.5}$ $Zn_{12.5}$	1.81^{e}	1.96^{d}	1.80^{e}	1.95^{d}	2.24^{d}	2.50^{b}	2.69^{d}	3.04^{b}	2.06^{e}	2.38^{d}	1.89^{f}	2.20^{d}	2.52^{c}	2.81^{b}	3.02^{d}	3.42^{ba}
Fe_{25} $Zn_{12.5}$	1.93^{d}	2.07^{c}	1.93^{d}	2.13^{b}	2.08^{e}	2.41^{c}	2.49^{f}	2.96^{c}	2.35^{d}	2.44^{c}	2.08^{e}	2.16^{c}	2.34^{d}	2.71^{b}	2.80^{f}	3.33^{c}
$Fe_{12.5}$ Zn_{25}	2.09^{c}	2.08^{c}	2.07^{c}	2.15^{b}	2.09^{e}	2.63^{a}	2.60^{ed}	3.09^{b}	2.46^{c}	2.58^{b}	2.16^{c}	2.36^{b}	2.35^{d}	2.95^{a}	2.92^{ed}	3.47^{a}
Fe_{25} Zn_{25}	2.14^{b}	2.30^{a}	2.13^{b}	2.30^{a}	1.95^{f}	2.70^{a}	2.44^{f}	3.16^{a}	2.64^{b}	2.84^{a}	2.30^{b}	2.51^{a}	2.19^{e}	3.03^{a}	2.74^{f}	3.55^{a}
Mean	1.99	2.10	1.98	2.13	2.09	2.56	2.56	3.06	2.38	2.56	2.11	2.31	2.35	2.88	2.87	3.44
ANOVA: M (Mycorrhizal inoculation), F (Fe levels), Z (Zn levels)																
CD(0.05)																
M	**		**		**		**		**		**		**		**	
F	**		**		**		**		**		**		*		**	
Zn	**		*		*		**		*		*		*		**	
M×F	NS		NS		NS		*		*		*		NS		*	
F×Z	NS		*		*		NS		NS		*		*		NS	
M×Z	*		*		*		NS		*		*		*		NS	
M×F×Z	*		*		*		*		*		*		*		*	

Table 5. Physiologically active iron (mg/kg of tissue) in the arbuscular mycorrhiza inoculated (AMF+) and non-inoculated (AMF-) maize plants. 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	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^+
$Fe_{12.5}$ $Zn_{12.5}$	$2.96d^{c}$	7.63^{b}	4.90^{e}	10.3^{cb}	7.17^{bc}	10.8^{b}	9.49^{d}	12.5^{b}	11.1^{e}	21.0^{b}	16.5^{f}	20.9^{d}	16.2^{d}	22.4^{b}	24.7^{f}	32.6^{d}
Fe_{25} $Zn_{12.5}$	3.85^{c}	8.99^{a}	6.64^{d}	13.2^{a}	8.42^{c}	12.7^{a}	11.5^{c}	16.6^{a}	14.0^{c}	23.9^{a}	18.8^{ed}	24.3^{b}	21.7^{b}	31.2^{a}	33.3^{c}	38.1^{b}
$Fe_{12.5}$ Zn_{25}	3.44^{c}	7.26^{b}	4.75^{e}	$11.2b^{a}$	5.84^{d}	10.2^{b}	9.28^{d}	12.8^{b}	12.9^{d}	20.0^{b}	15.7^{f}	22.8^{cb}	15.4^{d}	21.7^{b}	32.1^{d}	35.6^{c}
Fe_{25} Zn_{25}	3.73^{c}	8.68^{a}	6.35^{d}	12.0^{a}	7.25^{bc}	12.2^{a}	11.1^{c}	15.8^{a}	14.5^{c}	24.7^{a}	19.5^{d}	26.8^{a}	20.2^{cb}	30.4^{a}	30.2^{ed}	41.9^{a}
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)																
CD(0.05)																
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	

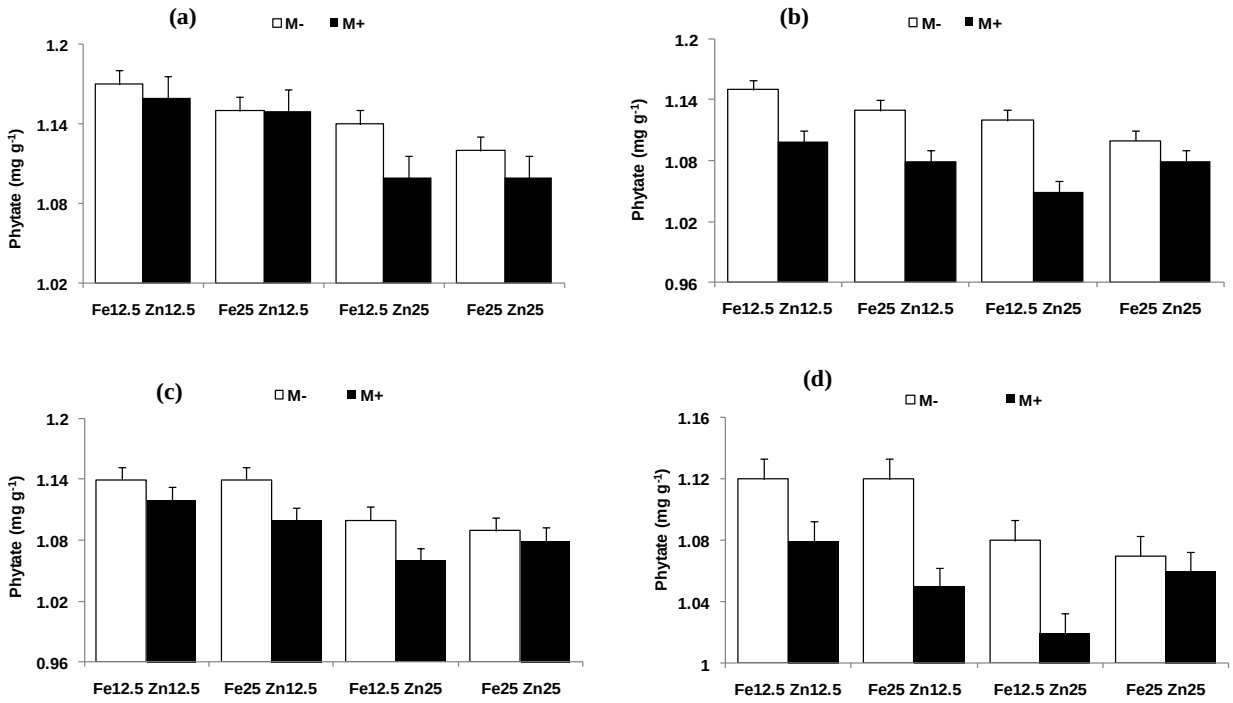

Fig 4. Phytate concentration in grain (mg g^{-1}) of arbuscular mycorrhizal fungus inoculated (AMF+) and uninoculated (AMF-) maize plants in calcareous sterilized (a) and natural soils (b) and non-calcareous sterilized (c) and natural soils (d). Error bars represent standard errors of four replications.

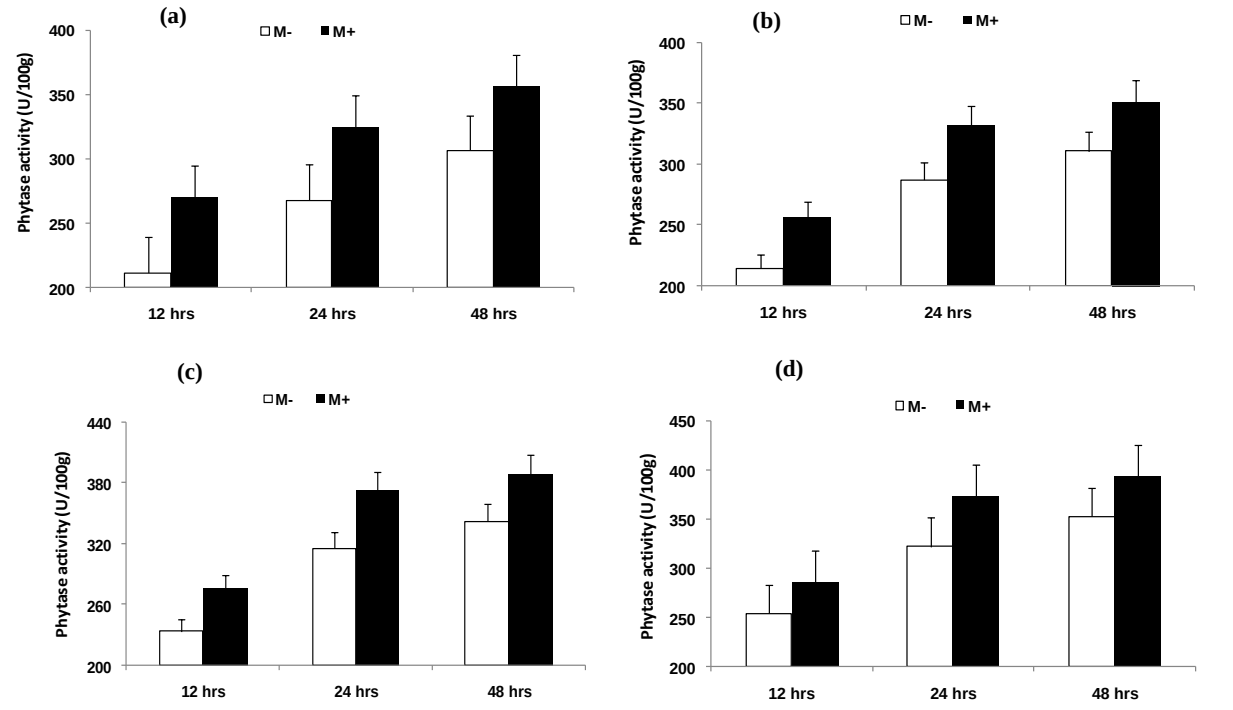

Fig 5. Phytase activity (mg $100g^{-1}$) of maize grain during the course of germination in arbuscular mycorrhizal fungus inoculated (AMF+) and uninoculated (AMF-) maize plants in calcareous sterilized (a) and natural soils (b) and non-calcareous sterilized (c) and natural soils (d). Error bars represent standard errors of four replications.

four experiments, root colonization, soil available micronutrients, plant micronutrient status, chlorophyll content, physiologically active Fe and grain Fe and Zn besides phytic acid and phytase activity were measured. The data collected were statistically analyzed using ANOVA and mean comparison test (DMRT-Duncan's Multiple Range Test).

Mycorrhizal colonization

Maize plant roots sampled from AMF+ and AMF- treatments were analyzed for their mycorrhizal colonization at 45 DAS (days after seeding). 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 0.5g, glycerol 500 ml, 1% HCl 50 ml and distilled water 450 ml) and destained (The stained roots are destained using destaining solution (glycerol 500 ml, 1% HCl 50 ml and distilled water 450 ml) (Except tryphan blue). Wash the stained roots repeatedly till the excess stain is removed). Root segments were observed under the 10 x lens microscope for the presence of any of the mycorrhizal structures such as arbuscules, vesicles, external hyphae and spores.

Chlorophyll content

One hundred mg fresh leaf samples were macerated with 10 ml of 80% acetone and centrifuged at 5000 rpm for 15 minutes. The supernatant was transferred to 25 ml volumetric flask and the volume was made up to the mark. The absorbance was measured at 645 and 663 nm to estimate total chlorophylls (Bruinsma, 1963).

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}.

Enzyme activities

Leaf samples collected from various treatments at 45 DAS were analyzed for catalase (Woodbury et al. 1971), peroxidase (Sadasivam and Manickam, 1996), super oxide dismutase (Beyer and Fridovich (1987)) and carbonic anhydrase (Gibson and Leece (1981)) using standard protocols

Micronutrient concentrations in grains

One g of powdered plant samples (roots, shoots) was or 0.5g grain samples mixed with 12 mL triple acid (HNO_3, H_2SO_4 and $HClO_4$ in 9:2:1) mixture and were 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, Mn, Cu and Zn concentrations. Blanks were maintained without adding sample.

Phytase activity assay

Phytase activity in grain samples was assayed as per the protocol suggested (Kim and Lei, 2005). Five gram ground grain samples (in triplicate) were placed in a 125-mL conical flask and 50 mL of 0.2 *M* citrate buffer (pH 5.5) was added, transferred to 50 ml falcon tubes, and centrifuged at 4°C at 15,000 rpm for 20 min. Two aliquots (0.2 mL each) of samples were taken in 10-mL test tubes and incubated in 37°C water bath for 5 min. The substrate 0.2 mL of 1% (wt/vol) sodium phytate in the selected buffer and pH to start the enzymatic hydrolysis of phytate, and incubate for 15 min at 37°C. The reaction was stopped by adding 0.4 mL of 15% trichloroacetic acid. The mixture was centrifuged at 2,000 rpm for 10 min and the supernatant fraction was transferred to a new tube. The supernatant fraction of 0.2 ml mixed with 1.8 mL of micro-pure water. Fresh ascorbic reagent (2.0 ml) was added to each tube and mixed well. The mixture was kept at 50°C for 15 min and taken to the room temperature. Absorbance of each sample solution was read at 820 nm, using water as the blank and the series diluted potassium phosphate solutions as standards. Calculate phytase activity per gram of grain. One unit of phytase is defined as the amount of enzyme required to release 1µmol of inorganic P/min from sodium phytate at 37°C. Because 5 g of diet is extracted in 50 mL of buffer, the dilution factor is 10.

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 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 and comparisons among means were made using LSD

Table 6. Cu and Mn concentrations in grain (mg kg^{-1}) of arbuscular mycorrhizal fungus inoculated (AMF+) and uninoculated (AMF-) maize plants. 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	Cu							Mn
	Calcareous				Non-calcareous			
	Sterilized		Natural		Sterilized		Natural	
	M^-	M^+	M^-	M^+	M^-	M^+	M^-	M^+
$Fe_{12.5}$ $Zn_{12.5}$	10.4d	13.2c	15.2d	18.4ab	45.2c	52.4a	50.7c	53.9b
Fe_{25} $Zn_{12.5}$	15.7ab	16.6a	18.3ab	18.1ab	51.4a	53.3a	52.5bc	56.4b
$Fe_{12.5}$ Zn_{25}	14.3bc	15.4ab	19.4a	22.3a	48.3ab	49.8a	54.2b	59.7a
Fe_{25} Zn_{25}	16.1a	16.9a	16.8bc	20.7a	49.6a	50.4a	58.3ab	61.4a
Mean	14.1	15.5	17.4	19.9	48.6	51.5	53.9	57.9
ANOVA: M (Mycorrhizal inoculation), F (Fe levels), Z (Zn levels)								
CD(0.05)								
M	**		**		**		**	
F	**		**		**		**	
Zn	**		**		**		*	
M×F	NS		*		*		NS	
F×Z	NS		NS		*		NS	
M×Z	NS		*		*		NS	
M×F×Z	NS		NS		NS		NS	

(least square difference) test, calculated at $p < 0.05$. Statistical procedures were carried out with the software package IRRI stat (IRRI, Manila, Philippines).

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 micronutrients 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. AMF 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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References

Abdel-Gawad AS, Ramadan BR, Omar MB, Oraby REA, (2004) Charactarization of phytases from cereal grains as affected by soaking and germination process and some inhibitors. The first international conference for food industries and biotechnology and associated fair. p 1-19

Akay A, Ertas N (2008) Farklı Çinko Seviyelerinin Nohutun Fitik Asit Miktarına Etkisi. Türkiye 10. Gıda Kongresi. 21-23 Mayıs, Erzurum (in Turkish)

Augé RM, Schekel KA, Wample RL (1987) Rose leaf elasticity in response to mycorrhizal colonization and drought acclimation. Physiol Plant 70: 175–182

Bartnik M, Szafranska I (1987) Changes in phytate content and phytase activity during the germination of some cereals. J Cereal Sci 5: 23-28

Beyer WF, Fridovich I (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem 161: 559–566

Black RE, Lindsay HA, Bhutta ZA, Caulfield LE, De Onnis M, Ezzati M,Mathers C, Rivera J (2008) Maternal and child undernutrition: global and regional exposures and health consequences. Lancet 371: 243-260.

Bolan NS (1991) A Critical Review on the Role of Mycorrhizal Fungi in the Uptake of Phosphorus by Plants. Plant Soil 134: 189–207.

Brinch-Pedersen H, Borg S, Tauris B, Holm PB (2007) Molecular genetics approaches to increasing mineral availability and vitamin content of cereals. J Cereal Sci 46: 308–326.

Bruinsma J (1963). The quantitative analysis of chlorophyll a and b in plant extracts. Photochem Photobiol 72: 241-249.

Cakmak I, Pfeiffer WH, McClafferty B (2010) Biofortification of durum wheat with zinc and iron. Cereal Chemistry 87, 10-20.

Caris C, Hawkins WHHJH, Römheld V, George E (1998) Studies of iron transport by arbuscular mycorrhizal hyphae from soil to peanut and sorghum plants. Mycorrhiza 8: 35–39

Centeno C, Viveros A, Brenes A, Canales R, Lozano A, Cuadra CD (2001) Effect of several germination conditions on total P, phytate P, phytase and acid phosphatase activities and inositol phosphate esters in rye and barley. J Agric Food Chem 49: 3208-3214

Dalpé Y (1993) Vesicular-arbuscular mycorrhiza. In: Carter MR (eds) Soil sampling and methods of analysis. Lewis Publishers, Boca Raton pp 287.

Davies NT, Reid H (1979) An evaluation of phytate, zinc, copper, iron and availability from soy based textured vegetable protein meat substitutes or meat extruders. Br J Nutr 41: 579.

DeKock P, Hall A, Inkson R (1979) Active iron in plant leaves. Ann Bot 43: 737-740.

Erdal I, Yilmaz A, Taban S, Eker S, Cakmak I, (2002) Phytic acid and phosphorus concentrations in seeds of wheat cultivars grown with and without zinc fertilization. J Plant Nutr 25, 113-127.

Gibbson TS, Leece DR (1981) Estimation of physiologically active Zn in maize by biochemical assay. Plant Soil 63: 395-406

Hotz C, Brown KH (2004) Assessment of the risk of zinc deficiency in populations and options for its control. Food Nutr Bull 25, 94-204.

Katyal JC, Sharma BD (1980) A new technique of plant analysis to resolve iron chlorosis. Plant Soil 55, 103-119

Kaya M, Kucukyumuk Z, Erdal I (2009) Phytase activity, phytic acid, zinc, phosphorous and protein contents in different chickpea genotypes in relation to nitrogen and zinc fertilization. Afr J Biotechnol. 8(18), p: 4508-4513

Kennedy G, Nantel G, Shetty P (2003) The scourge of "hidden hunger": global dimensions of micronutrient deficiencies. Food, Nutrition and Agriculture (FAO FNA) 32, 8-16

Khademi Z, Ahmad JA, Jones DL, Malakouti MJ (2006) The role of organic acids in manipulating nutrient levels in calcareous soils. 18th World Congress of Soil Science. July 9-15, 2006 - Philadelphia, Pennsylvania, USA

Kim TW, Lei XG (2005) An improved method for a rapid determination of phytase activity in animal feed. J Anim Sci 83:1062–1067.

Koide RT, Kabir Z (2000) Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolyse organic phosphate. New Phytol 148: 511-517.

Li XL, Marschner H, Romheld V (1991) Acquisition of phosphorus and copper by VA-mycorhizal hyphae and root to shoot transport in white clover. Plant Soil 136: 49-57.

Lindsay WL, Norvell WA (1978) Development of DTPA soil test for zinc, iron, manganese and copper. Soil Sci Soc Am J 42**:** 421-428.

Liu A, Hamel C, Hamilton RI, Ma BL, Smith DL (2000) Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels. Mycorrhiza 9: 331-336.

Mengel K, Breininger Th, Bulb W (1984) Bicarbonate, the most important factor inducing iron chlorosis in vine grapes on calcareous soil. Plant Soil 81: 333-344

Misra A, Srivastava AK, Srivastava NK, Khan A (2005) Zn acquisition and its role in growth, photosynthesis, photosynthetic pigments and biochemical changes in essential monoterpene oils of *Pelargonium graveolens.* Photosynthetica 43: 153-155.

Poletti S, Gruissen W, Sautter C (2004) The nutritional fortification of cereals. Curr Opin Biotechnol, 15, 162–165.

Prasad R (2010) Zinc biofortification of food grains in relation to food security and alleviation of zinc. Current science, 98(10): 1300-1304

Ryan MH, Angus JF (2003) Arbuscular mycorrhizal fungi increase zinc uptake but do not influence yield or P uptake of field crops in SE Australia. Plant Soil 250: 225-239.

Ryan MH, McInerney JK, Record IR, Angus JF (2008) Zinc bioavailability in wheat grain in relation to phosphorus fertiliser, crop sequence and mycorrhizal fungi. J Sci Food Agri, 88, 1208-1216.

Sadasivam S, Manickam A (996) Biochemical Methods. New Age International, New Delhi, India pp, 112-113.

Scholl W (1979) Erfahrungen mit der chlorose der weinreben in der Bundesrepublik Deutschland. Mitt Klosterneuburg 29, 186-193.

Subramanian KS, Charest C, Dwyer LM, Hamilton RI (1995) Arbuscular mycorrhizas and water relations in maize under drought stress at tasselling. New Phytol 129: 643–650

Subramanian KS, Bharathi C, Jegan RA (2008) Response of maize to mycorrhizal colonization at varying levels of zinc and phosphorus. Biol Fertil Soils 8, 317-328.

Subramanian KS, Tenshia V, Jayalakhshmi K, Ramachandran V (2009) Role of arbuscular mycorrhizal fungus (*Glomus intraradices*)- (fungus aided) in zinc nutrition of (in) maize. J Agric Biotech Sustain Dev 1: 029-038

Suresh Kumar R, Ganesh P, Tharmaraj K, Saranraj P (2011) Growth and development of blackgram (*Vigna mungo*) under foliar application of Panchagavya as organic source of nutrient. Curr Bot 2(3): 09-11

Wang M, Christie P, Xiao Z, Qin C, Wang P, Liu J, Xie Y, Xia R (2008) Arbuscular mycorrhizal enhancement of iron concentration by *Poncirus trifoliata* L. Raf and *Citrus reticulata* Blanco grown on sand medium under different pH. Biol Fertil Soils 45: 65–72

WHO (2002) The World Health Report 2002. Reducing Risks, Promoting Healthy Life. World Health Organization, Geneva, Switzerland, pp 1–230.

Woodbury W, Spencer AK, Stahman MA (1971) An improved procedure for using ferricyanide for detecting catalase isozymes. Anal Biochem 44: 301-305

Zuo Y, Ren L, Zhang F, Jiang RF (2007) Bicarbonate concentration as affected by soil water content controls iron nutrition of peanut plants in a calcareous soil. Plant Physiol Biochem 45: 357-364.

Zou YM, Zhang FS, Li XL, Cao YP (2000) Studies on the improvement in iron nutrition of peanut by intercropping maize on a calcareous soil, Plant Soil 220 pp 13–25.