Australian Journal of Crop Science AJCS
AJCS 8(5):655-665 (2014) ISSN:1835-2707

Role of arbuscular mycorrhizal (*Glomus intraradices*) fungus inoculation on Zn nutrition in grains of field grown maize

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Abstract

Bioavailability of zinc (Zn) concentrations in maize grains is low causing malnutrition in humans. This study is aimed to use mycorrhizal fungal inoculation as one of the biological strategies to improve Zn concentrations in field grown maize. Treatments consisted of three levels of Zn (0, 2.5 and 5 kg Zn ha^{-1}), two levels P (30 and 60 P kg ha^{-1}) and two mycorrhizal fungal inoculation with (AMF+) and without arbuscular mycorrhizal (AMF-) fungus (*Glomus intraradices* Schenck & Smith) replicated three times in a FRBD. AMF+ plants had significantly ($P \leq 0.01$) higher root length (AMF- 16.8; AMF+ 23.5 cm) and volume, leaf area and chlorophyll concentrations regardless of P or Zn fertilization but the response to AMF inoculation was higher at lower levels of Zn fertilization. Maize grains of AMF+ plants had higher Zn and tryptophan concentrations by 15 and 8.6%, respectively, in comparison to AMF- plants. The plant available Zn concentration in soil had a highly significant correlation with Zn content in roots (r = 0.93), shoots (r = 0.91) and grains (r = 0.91). AMF symbiosis enhances Zn supply to the host plant by improving the available Zn and P enabling the plant to maintain higher nutritional status and produce grains fortified with Zn and tryptophan concentrations in field grown maize.

Keywords: Arbuscular Mycorrhizal Fungus, Chlorophyll, Maize, Nutritional Quality, Zinc, Biofortification.
Abbreviations: AMF-Arbuscular Mycorrhizal Fungus, FRBD-Factorial Randomized Block Design, DTPA-Diethylene Triamine Penta Acetic Acid, COHM-Coimbatore Hybrid Maize, P-Phosphorus, Zn- Zinc.

Introduction

Zinc deficiency in soil is a global concern that affects yield and quality of grain crops grown over 50% of the arable lands (Alloway, 2001). The magnitude of Zn deficiency is high in calcareous soils of arid and semi-arid tropical regions (Marschner, 1995) where major portion of added Zn got fixed. In India, agricultural practices such as imbalanced use of fertilizers and exclusion of organic manures are believed to be responsible for the widespread Zn deficiency in soils which closely associated with grain yield reduction (Singh et al., 2005). Zinc deficiency in soil reduces yield and qualities of grains (Cakmak et al., 1998). It has been estimated that about two billion people are affected by Zn deficiency where people are exposed to cereal based diets with deficient level of Zn (Graham and Welch, 1996; Welch and Graham, 2002). Improving zinc content in food grains is given prime importance to ensure nutritional security. Despite Zn fertilization is a commonly recommended practice in cereal production systems, Zn use efficiency by crops rarely exceeds 1% and the major portion got fixed in soils (Mandal and Mandal, 1986). This caused an accumulation of Zn in total pool but the bioavailability is too low, suggesting that there is a need to adopt strategies to transform tightly bound Zn into plant available forms. *Arbuscular mycorrhizal* fungi (AMF) form symbiotic association which is ubiquitous and known to improve the nutritional status of the host plants by facilitating absorption of relatively immobile micronutrients such as Zn and Cu besides P (Li et al., 1991; Jakobsen et al., 1992; Liu et al., 2000; Ryan and Angus, 2003; Subramanian et al., 2009). Mycorrhizal plants have the ability to explore larger soil volume beyond the rhizosphere through external mycelium that assists in nutrient transport (Jakobsen et al., 1992; Subramanian and Charest, 1999; Liu et al., 2000). In addition to the hyphal transport, the rhizosphere of mycorrhizal plants maintains acidic pH (Dodd et al., 1987) facilitates solubilization of tightly bound Zn (Subramanian et al., 2009) besides synergistic interaction with phosphorus (Subramanian et al., 2008). These changes improve the availability of Zn in soil that may help in biofortification of micronutrients in grains. Cakmak (2008) suggested that agronomic biofortification of Zn is of great importance in enriching grains of cereal crops thereby malnutrition of Zn and its associated ill-effects on human health can be circumvented. Though several greenhouse experiments have proved the significance of mycorrhizal symbiosis in crop nutrition, limited works have been done under open field conditions which may be of practical significance to evolve suitable strategy in alleviating Zn deficiency besides enriching grains with Zn. We hypothesized that AMF symbiosis improves

Zn nutrition of cereals (maize) as a secondary consequence of synergistic interaction with P nutrition. The response to AMF inoculation may vary with Zn or P fertilization levels. In order to address this, AMF inoculated and uninoculated maize plants were subjected to varying levels of Zn and P fertilization under open field conditions.

Results

Mycorrhizal colonization

AMF inoculated treatments had significantly ($P \leq 0.01$) higher percentage of colonization in all the three locations (Coimbatore, Vagarai and Bhavanisagar) regardless of Zn or P fertilization. Among the three locations, Bhavanisagar registered the highest mean mycorrhizal colonization of 45.5% in inoculated treatments (Table 1). The increase in levels of Zn or P application had significantly increased the mycorrhizal colonization of both AMF inoculated and uninoculated maize plants in all the three locations.

Root volume and mass

The root volume of AMF+ plants were significantly ($P \leq 0.01$) higher than AMF- plants at 45 DAS at all levels of P and Zn application in all the three locations (Coimbatore, Bhavanisagar, Vagarai). The average root volume of AMF+ plants (96.3 cm^3) was 35-36% higher than the AMF- plants (61.4 cm^3). The response to mycorrhizal inoculation was more pronounced in P60 than P30 and the values were higher by 1.8, 1.8 and 1.9 times in Coimbatore, Vagarai and Bhavanisagar, respectively. Application of Zn at graded levels significantly increased the root volume of both AMF+ and AMF- plants (data not shown). Root masses of AMF+ plants were significantly ($P \leq 0.01$) higher in all the three locations and the increase were to the tune of 4.37 to 4.80 % in comparison to AMF- treatment. Both AMF- and AMF+ plants of P60 had higher root masses than P30 (Fig. 1).

Shoot mass

Shoot dry mass of AMF plants were significantly ($P \leq 0.01$) higher than uninoculated plants at 45 days after sowing at all levels of P and Zn in all the three locations (Fig. 1). Among them, Bhavanisagar registered the highest shoot dry mass followed by Coimbatore and Vagarai. Incremental levels of P and Zn fertilization progressively increased the shoot mass of AMF inoculated and uninoculated plants but the response was more pronounced under lower levels of Zn.

Leaf area and chlorophyll content

The leaf area of AMF plants were significantly ($P \leq 0.01$) higher than uninoculated plants at 45 DAS at all levels of P and Zn application in all the three locations (Table 2). The leaf area of AMF plants was 6-35% higher than the uninoculated plants. The response to mycorrhizal inoculation was more pronounced in P60 than P30 and the values were higher by 1.4, 1.2 and 1.2 times in Coimbatore, Vagarai and Bhavanisagar, respectively. Application of Zn at graded levels significantly increased the leaf area of both AMF and uninoculated plants. The chlorophyll content of AMF plants were significantly ($P \leq 0.01$) higher in all the three locations and the increase were to the tune of 2.5 to 6.1% in comparison to uninoculated treatment. Mycorrhizal plants of P60 had higher chlorophyll content than P30.

Nutrient status in plants

Phosphorus concentrations

Mycorrhizal plants had significantly ($P \leq 0.05$) higher P concentrations of shoots and roots at 45 DAS of observation at all the three locations (Table 3; Fig. 2a). Inoculated roots (AMF) had higher P concentrations than uninoculated (AMF−) roots at both P30 and P60 levels. A similar trend was observed for AMF shoots as well. Phosphorus concentrations increased significantly ($P \leq 0.05$) with increasing levels of Zn or P of AMF plants. Among the three locations, Coimbatore registered the highest P concentrations (both root and shoot) followed by Bhavanisagar and Vagarai.

Zinc concentrations

AMF inoculated maize plants (AMF+) had significantly ($P \leq 0.05$) higher Zn concentrations than uninoculated (AMF-) plants irrespective of incremental levels P or Zn (Table 4; 2b). A higher level of P (P60) increased Zn concentrations of AMF inoculated and uninoculated roots and the response was less pronounced in P30. Among the three locations, the response to AMF inoculation was more obvious in Bhavanisagar than Vagarai and Coimbatore.

Acid phosphatase activity and nutrient status of the soil
Acid phosphatase activity

Arbuscular mycorrhizal fungus (AMF) inoculated treatments had significantly ($P \leq 0.01$) higher activities of acid phosphatase in soil at all the three locations (Coimbatore, Vagarai and Bhavanisagar) regardless of Zn or P fertilization. The increase in acid phosphatase activities was higher in AMF inoculated soils than uninoculated treatments. Among the three locations, Vagarai registered the highest mean acid phosphatase activities of 19.6% in inoculated treatments (Table 6). The increase in levels of Zn or P application had significantly increased the acid phosphatase activities of both AMF inoculated and uninoculated soils in all the three locations.

Soil P and Zn

The available P status of AMF inoculated soils was significantly ($P \leq 0.05$) higher than uninoculated soils regardless of varying levels of P or Zn application in all the three locations (Table 6). Among them, Coimbatore registered the highest available P concentration followed by Bhavanisagar and Vagarai. The response to AMF inoculation was more pronounced in P60 than P30 and the values were higher by 1.6, 1.1 and 1.8 times in Coimbatore, Vagarai and Bhavanisagar, respectively. Incremental levels of Zn progressively increased the available P status of both AMF and uninoculated soils, but the values were consistently higher for inoculated soils. The available (DTPA-extractable)

Table 1. Initial soil characteristics of the three experimental locations.

Parameter	Coimbatore	Vagarai	Bhavanisagar
Soil Texture	Clay Loam	Sandy Clay Loam	Sandy Loam
pH	8.25	7.54	7.25
EC (dS m^{-1})	0.67	0.07	0.14
Organic carbon (%)	0.32	0.40	0.21
Available N (kg ha^{-1})	233.2	196.3	272.4
Available P (kg ha^{-1})	12.6	14.5	18.9
Available K (kg ha^{-1})	222.7	263.2	284.6
DTPA Zn (mg kg^{-1})	0.56	1.21	0.93
Spore Count (Nos $100g^{-1}$)	6±0.6	5±0.5	10±1.1

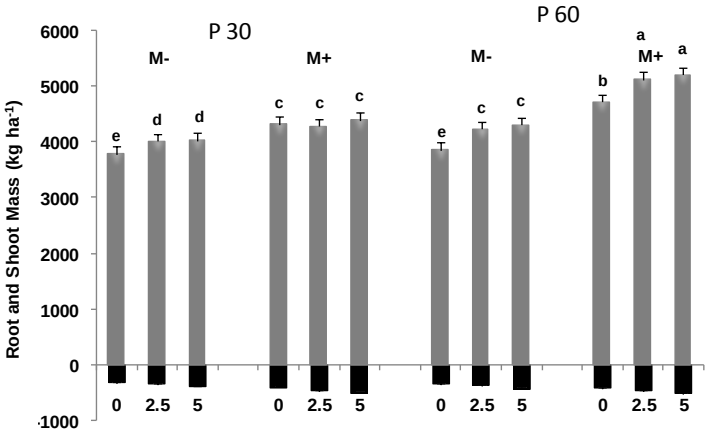

Fig 1. Shoot (solid grey bars above the X axis) and root (solid black bars below the X axis) masses (kg ha^{-1}) of arbuscular mycorrhizal fungus (AMF+) inoculated and uninoculated (AMF-) maize plants (n = 45) under two levels of P (30 and 60 kg P ha^{-1}) and three levels of Zn (0, 2.5 and 5.0 kg Zn ha^{-1}).

Zn increased significantly ($P \leq 0.05$) with AMF inoculation under varying levels of P or Zn at all the three locations viz., Coimbatore, Vagarai and Bhavanisagar. Among them, Coimbatore registered the highest DTPA Zn- followed by Bhavanisagar and Vagarai. Inoculated (AMF+) soils had higher available Zn by 22% and 30% under P30 and P60, respectively, in comparison to uninoculated soils.

Grain yield and nutrient contents

Arbuscular mycorrhizal fungus (AMF+) inoculated plants produced significantly ($P \leq 0.05$) higher grain yield than uninoculated (AMF-) plants regardless of P or Zn levels in all the three locations viz., Coimbatore, Vagarai and Bhavanisagar (Fig. 3a–c). Grain yields of AMF+ plants were higher by 12%, 8 % and 14% in Coimbatore, Vagarai and Bhavanisagar, respectively. Further, incremental levels of Zn application correspondingly and significantly ($P \leq 0.05$) increased the grain yields of AMF and uninoculated plants, but the response was more pronounced at higher levels of added P. Application of P at P60 had higher P and Zn contents in grains by 32.8% and 30.9%, and such increases in P30 were only 9.9% and 9.4%, respectively, in comparison to their respective uninoculated treatments (Fig. 4a-c).

Discussion

The increase in levels of Zn or P application had significantly increased the mycorrhizal colonization of both uninoculated and AMF maize plants. Other field studies have shown no significant effects of Zn addition on AMF colonization in tomato plants (Cavagnaro et al., 2010). The data are in agreement with the observations of Ortas et al. (2002) who have reported that responses to the inoculation of AM fungus *Glomus clarum* increases with Zn and P fertilization in *Citrus aurantium*. In our earlier studies, we found that response of AMF inoculation increased with incremental levels of either P or Zn fertilization (Subramanian et al., 2008). This may be due to the advancement of growth stages in maize that depleted the available nutrients particularly P which favoured the AMF colonization. In contrast, high levels of P had an inhibitory effect on AMF colonization as determined by the external mycelium (Kothari et al., 1991; Liu et al., 2000). Further, a negative correlation between AMF colonization and excess Zn fertilization has been established (Gildon and Tinker, 1983: Boyle and Paul, 1988). On the other hand, the AMF colonization was the highest even at the toxic level of 250 mg Zn kg^{-1} soil suggesting the potential role of arbuscular mycorrhiza in reduction of metal contamination (Audet and Charest, 2006). Since the experimental soils had extremely low in available P and Zn status, the addition of incremental levels had a positive effect on AMF colonization regardless of variations in locations. The AMF response to added P was more pronounced at P60 than P30. This may be attributed to the extensive branching and proliferation of roots associated with P fertilization which may have helped the AMF plants to produce larger volume and mass of roots. Our data are in conformity with the observations of Subramanian et al. (2008). Root volume of P60 fertilized AMF inoculated and uninoculated plants were nearly twice as much as

Table 2. Percentage of mycorrhizal colonization examined in the arbuscular mycorrhiza inoculated (AM+) and non-inoculated (AM-) root segments (n=100) of maize plants at 45 days after sowing (DAS) under varying P and Zn levels. The levels of significance for ANOVA, * = $P \leq 0.05$; ** = $P \leq 0.01$; NS = Not significant. Statistics was done for each location separately. Means followed by a common letter are not significantly different at the 5% level by DMRT.

Treatments	Coimbatore		Vagarai		Bhavanisagar	
(kg ha $^{-1}$)	AM-	AM+	AM-	AM+	AM-	AM+
P30						
Zn0	7.9^{h}	31.3^{d}	10.0^{f}	34.7^{c}	8.0^{i}	41.0^{e}
Zn2.5	9.0^{g}	34.0^{c}	9.7^{h}	38.7^{c}	7.3^{h}	44.0^{d}
Zn5.0	10.1^{f}	35.7^{b}	11.0^{e}	40.0^{b}	7.3^{h}	45.7^{c}
P60						
Zn0	9.7^{f}	34.3^{c}	9.3^{h}	40.0^{b}	9.0^{g}	44.3^{d}
Zn2.5	9.7^{f}	34.0^{c}	8.3^{h}	40.7^{b}	9.0^{g}	48.0^{b}
Zn5.0	11.0^{e}	38.0^{a}	9.7^{g}	44.7^{a}	10.3^{f}	49.7^{a}
CD (P=0.05)						
s	**		**		**	
P	*		**		*	
Zn	**		*		*	
MXP	*		*		**	
PXZn	*		*		**	
MXZn	*		**		**	
MXPXZn	NS		NS		*	

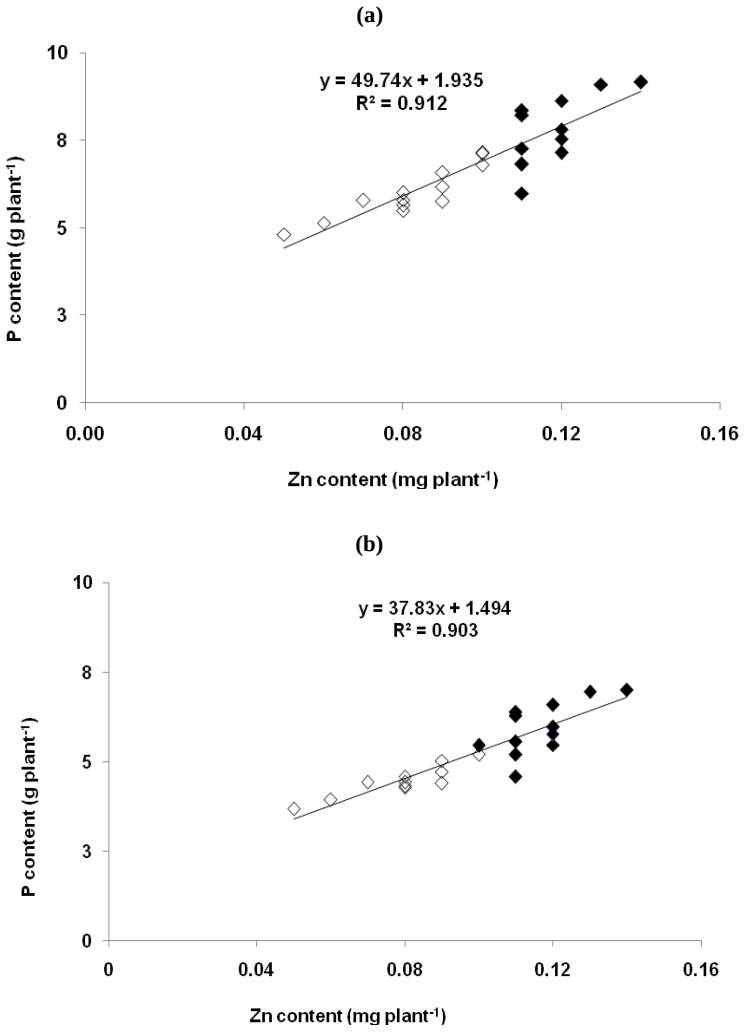

Fig 2. Correlations between Zn and P contents in shoots (a) and roots (b) of the arbuscular mycorrhizal fungus inoculated (AMF+) (filled diamond) and uninoculated (AMF-) (empty diamond) maize plants.

that of P30 plants irrespective of Zn levels. Incremental levels of Zn addition progressively increased the root architecture of AMF and uninoculated plants but the response was more exhibited under lower Zn levels. The extensive root growth of AMF plants can be attributed to the improved P nutrition. The enhanced supply of P by AMF symbiosis has been unequivocally demonstrated (Jakobsen et al., 1992; Asmah, 1995; Hetrick et al., 1996; Smith and Read, 1997; Subramanian et al., 2006). The synergistic interaction between P and Zn has already been reported (Subramanian et al., 2008) which may also assist in higher root biomass production. In contrast, at early stage of crop growth, AMF plants showed an increase in root mass while the shoot masses were similar due to the utilization of carbon for establishment of functional symbiosis (Fitter, 1988; Jakobsen and Rosendahl, 1990). In contrast, Kothari et al. (1991) who obtained no response in terms of shoot growth of maize to AMF inoculation in the early stages. Arbuscular mycorrhizal fungal colonization would have stimulated leaf expansion as a result of improved Zn nutritional status (Cakmak et al., 1998) or better water use efficiency (Subramanian and Charest, 1995). As expected, zinc application progressively increased the leaf area of both AMF inoculated and uninoculated plants. Similar observations were made in AMF maize plants grown under well-watered and drought-stressed conditions (Subramanian and Charest, 1995). The response to added Zn was more pronounced in uninoculated than AMF+ plants. The increase in chlorophyll content of AMF plants can be attributed to enhanced Zn nutrition as Zn promotes the development of photosynthetic pigments (Misra et al., 2005). A close relationship between P concentrations and chlorophyll has already been established. Since, Zn deficient plants are known to exhibit chlorotic streaks on the leaves, chlorophyll measurement is quite appropriate to justify the beneficial role AMF symbiosis in promoting Zn nutritional status of the host plant.The increase in P concentration in AMF inoculated plants can be attributed to the extensive root development and hyphae that reduce the distance for diffusion of nutrients thus enhancing the nutrient absorption. The enhanced acid phosphatase activities in mycorrhizal plants (Dodd et al., 1987) may assist in releasing P which in turn transported by the external mycelium (Jakobsen et al., 1992) and resulted in the enhancement of nutritional status of host plants. The hyphal transport of Zn to the host plant has been reported as 60 % (Liu et al., 2000). Increasing levels of Zn significantly increased the Zn concentrations of roots and shoots at 45 DAS. There was a strong positive relationship between Zn and P contents in the roots (r = 0.95) and the shoots (r = 0.91), which confirms the synergistic interaction among P and Zn aided by AMF colonization (Subramanian et al., 2008). In this experiment, AMF inoculation resulted in increased acid phosphatase activity in the soil under varying levels of Zn and P. Similarly, Wang et al. (2006) had shown the enhanced phosphatase activity by AMF colonized maize rhizosphere. Subramanian et al. (2009) indicated that the acid phosphatase activity in the maize rhizosphere increased by 2-3 times higher than that of uninoculated soil suggesting that this could reduce the pH and stimulate the availability of P besides Zn as a consequence of synergetic interaction. A strong positive correlation has been established between the available P and Zn (Subramanian et al., 2008). In another classic study by Dodd et al. (1987), the onion AMF root had acid phosphatase activity 30-40 times higher than uninoculated roots. There literature in conjunction with the present study, it is quite evident that AMF colonization facilitates enhancement of acid phosphatase activities in the soil as well in host plant roots. Inoculation of arbuscular mycorrhizal (AMF) fungus (*Glomus intraradices*) improved soil available P and Zn contents (Subramanian et al., 2008). Since the soils in all three experimental locations were deficient in available (DTPA-Extractable) Zn besides low to medium status of available P, the response to AMF inoculation was more pronounced than other studies reported in the literature. AMF colonization assists the acidification of rhizosphere and that facilitate solubilization of tightly bound (residual) fraction of zinc (Subramanian et al., 2009). Acidification in combination with hyphal transport of Zn and P may have constituted for improved the availability of nutrients. These favourable changes alter the P and Zn interactions which may have provided greater ability for AMF plants to sustain Zn deficient conditions. In this study, we found that nutrient concentrations in AMF maize plants particularly P and Zn were consistently higher than uninoculated plants. The critical P and Zn concentrations in maize plants were reported as 0.25% and 15 mg kg^{-1} dry matter, respectively, at the tasseling stage of crop growth (Jones and Eck, 1973). In our study, AMF+ plants have registered P and Zn concentrations in the range of 0.42 – 0.76% and 37.3 – 38.3 mg kg^{-1} dry matter while uninoculated plants had 0.36 – 0.52% and 32.0 – 33.9 mg kg^{-1} dry matter, respectively. As a result of improved nutritional status of plants, maize grains harvested from mycorrhizal treatments were higher in P and Zn concentrations by 41.3% and 19.2%, respectively, in comparison to uninoculated treatments. Our data suggest that AMF inoculation assisted the host plants to utilize immobile nutrients such as P and Zn which is rarely available to uninoculated plants. Similar data have been reported by Liu et al. (2000) who have shown that an isolate of *Glomus inraradices* enhanced the Zn content in field grown maize under low Zn soil concentrations. A close relationship between AMF colonization and host plant Zn nutrition status has been well established (Gao et al., 2007). The response to AMF inoculation increased with incremental levels of either P or Zn. The data agree with the findings of Ortas et al. (2002) who have reported that responses to the inoculation of AMF *Glomus clarum* increases with supply of P and Zn in *Citrus aurantium*. Increasing levels of Zn correspondingly enhance tryptophan concentrations of maize grains in the absence or presence of AMF inoculation. Phosphorus application had increased the tryptophan concentrations in grains of both AMF and uninoculated plants. In a radio-tracer study, it has been demonstrated that the external mycelium of the AMF spreads out beyond the rhizosphere and explore larger soil volume and translocate ^{65}Zn to the tune of 9% of the added amount within 25 days to the host plant. Our earlier studies have shown that the AMF colonization has improved the availability of Zn in soil which was efficiently taken up by the host plant leaving lower "A" values in colonized soils (Subramanian et al., 2008). The improved nutritional status of AMF inoculated plants resulted in higher grain yields by 20% in comparison to uninoculated treatments. The increased yields of AMF inoculated plants thus suggest that significant amounts of P and Zn were translocated from the source to the sink to support kernel development and grain yield (Subramanian and Charest, 1997). Nable and Webb (1993) indicated that application of Zn had a beneficial effects on grain yield of wheat as a consequence of improved root function and plant development. Our study supports that response to AMF inoculation was higher in P60 than P30 indicating a need for application of optimal level of P to derive full benefits from the symbiosis. Arbuscular mycorrhizal fungal (AMF)

Table 3. Total leaf area (cm^2 $plant^{-1}$) and total chlorophyll (mg g^{-1} of FM) in the arbuscular mycorrhiza inoculated (AM+) and non-inoculated (AM-) maize plants (n=15) at 45 days after sowing (DAS) under varying P and Zn levels. The levels of significance for ANOVA, * = $P \leq 0.05$; ** = $P \leq 0.01$; NS = Not significant. Statistics was done for each location separately. Means followed by a common letter are not significantly different at the 5% level by DMRT.

Treatments	Leaf area						Total Chlorophyll					
	Coimbatore		Vagarai		Bhavanisagar		Coimbatore		Vagarai		Bhavanisagar	
(kg ha $^{-1}$)	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+
P_{30}												
Zn0	1051.1^{f}	1256.9^{e}	1798.8^{g}	1803.6^{g}	1635.6^{j}	2665.8^{f}	1.85^{h}	1.88^{h}	2.77^{g}	3.23^{e}	3.02^{h}	3.34^{f}
Zn2.5	1396.7^{d}	1483^{d}	1932.2^{f}	2038.5^{e}	1745.4^{i}	2938.2^{e}	2.01^{g}	2.19^{e}	3.48^{c}	3.42^{d}	3.55^{e}	3.64^{d}
Zn5.0	1380.2^{e}	1650.3^{c}	2067.2^{e}	2210.7^{d}	2009.5^{h}	3165.7^{d}	2.07^{f}	2.47^{b}	3.24^{e}	3.47^{c}	3.74^{c}	3.73^{c}
P_{60}												
Zn0	1715.1^{c}	1952.5^{b}	2457.3^{b}	2524.3^{b}	2129.3^{g}	3686.4^{c}	2.10^{f}	2.24^{d}	2.86^{f}	3.39^{d}	3.26^{g}	3.01^{h}
Zn2.5	2678.3^{a}	2768.2^{a}	2544.7^{b}	2941.3^{a}	3173.7^{d}	4059.6^{a}	2.26^{d}	2.38^{c}	3.48^{c}	3.53^{b}	3.66^{d}	3.67^{d}
Zn5.0	1966.9^{b}	1794.7^{c}	2160^{d}	2302.7^{c}	2138.8^{g}	3303.2^{b}	2.53^{a}	2.51^{a}	3.54^{b}	3.61^{a}	3.95^{b}	4.33^{a}
CD (P=0.05)												
M	**		**		**		**		**		**	
P	**		**		**		**		**		NS	
Zn	NS		*		**		**		*		**	
MXP	NS		*		*		*		**		NS	
PXZn	NS		NS		NS		NS		*		NS	
MXZn	NS		NS		NS		NS		NS		NS	
MXPXZn	NS		NS		*		*		NS		NS	

Table 4. Phosphorous (P) concentration (%) examined in the roots and shoots of arbuscular mycorrhiza inoculated (AM+) and non-inoculated (AM-) maize plants at 45 (n=15) days after sowing (DAS) under varying P and Zn levels. The levels of significance for ANOVA, * = $P \leq 0.05$; ** = $P \leq 0.01$; NS = Not significant. Statistics was done for each location separately. Means followed by a common letter are not significantly different at the 5% level by DMRT.

Treatments	P Content											
	Coimbatore				Vagarai				Bhavanisagar			
	Shoot		Root		Shoot		Root		Shoot		Root	
(kg ha $^{-1}$)	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+
P_{30}												
Zn0	0.403^{f}	0.451^{c}	0.443^{k}	0.494^{i}	0.302^{h}	0.344^{g}	0.416^{i}	0.444^{g}	0.398^{i}	0.461^{e}	0.435^{h}	0.433^{h}
Zn2.5	0.420^{e}	0.562^{b}	0.451^{k}	0.569^{e}	0.346^{g}	0.402^{e}	0.423^{h}	0.481^{f}	0.412^{h}	0.548^{c}	0.444^{g}	0.530^{d}
Zn5.0	0.475^{b}	0.671^{a}	0.476^{j}	0.716^{d}	0.391^{f}	0.422^{d}	0.478^{f}	0.518^{e}	0.459^{e}	0.635^{b}	0.461^{f}	0.678^{c}
P_{60}												
Zn0	0.434^{d}	0.569^{b}	0.519^{h}	0.732^{c}	0.418^{d}	0.446^{c}	0.484^{f}	0.531^{d}	0.433^{g}	0.531^{d}	0.503^{e}	0.713^{b}
Zn2.5	0.446^{c}	0.672^{a}	0.525^{g}	0.749^{b}	0.447^{c}	0.487^{b}	0.518^{e}	0.614^{b}	0.434^{g}	0.631^{b}	0.513^{e}	0.725^{b}
Zn5.0	0.451^{c}	0.680^{a}	0.543^{f}	0.811^{a}	0.481^{b}	0.556^{a}	0.562^{c}	0.622^{a}	0.444^{f}	0.647^{a}	0.528^{d}	0.797^{a}
CD (P=0.05)												
M	*		*		*		*		*		*	
P	*		**		*		**		**		*	
Zn	**		**		*		*		*		*	
MXP	**		*		NS		**		*		**	
PXZn	*		*		*		*		*		*	
MXZn	**		*		*		*		*		**	
MXPXZn	NS		**		*		*		NS		*	

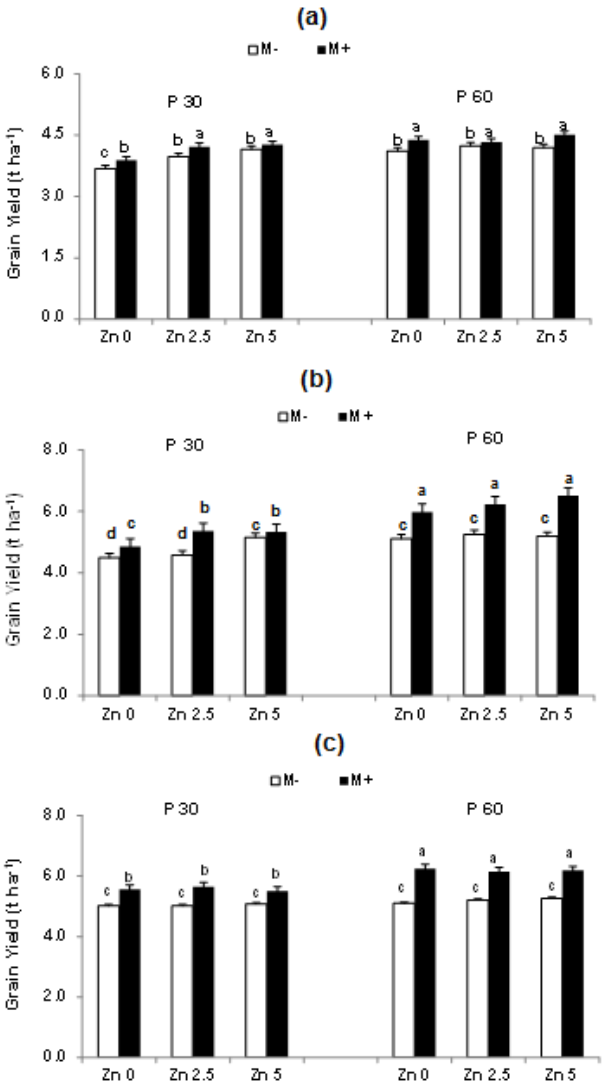

Fig 3. Grain yield (kg ha^{-1}) of arbuscular mycorrhizal fungus inoculated (AMF+) (filled bars) and uninoculated (AMF-) (empty bars) maize plants (n = 3) under two levels of P (30 and 60 kg P ha^{-1}) and three levels of Zn (0, 2.5 and 5.0 kg Zn ha^{-1}) in Coimbatore (a), Vagarai (b) and Bhavanisagar (c) soils. Error bars represent standard errors of three replications.

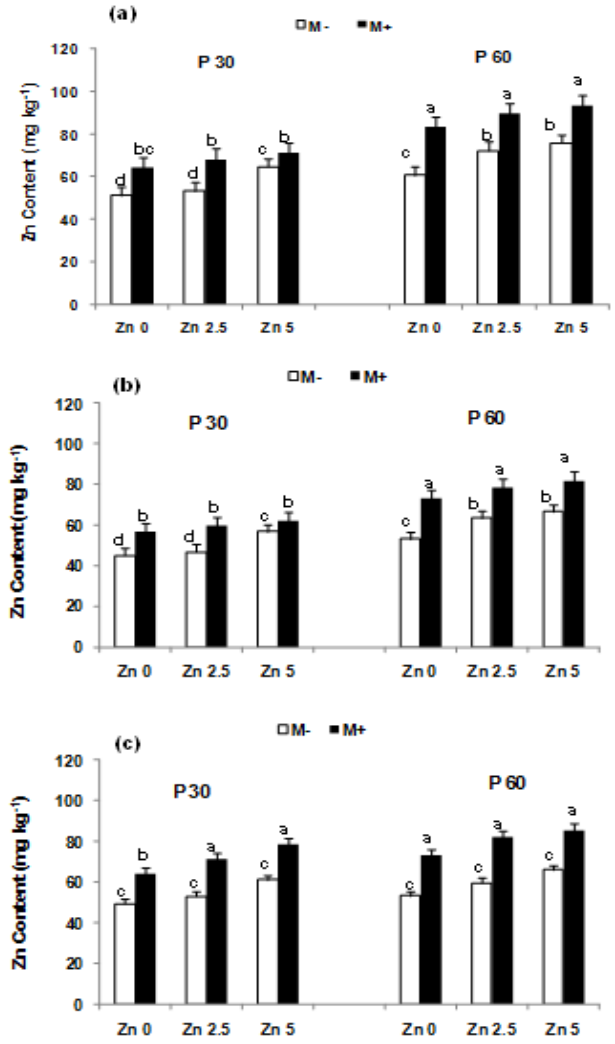

Fig 4. Grain Zn content (mg kg^{-1}) of arbuscular mycorrhizal fungus inoculated (AMF+) (filled bars) and uninoculated (AMF-) (empty bars) maize plants (n = 3) under two levels of P (30 and 60 kg P ha^{-1}) and three levels of Zn (0, 2.5 and 5.0 kg Zn ha^{-1}) in Coimbatore (a), Vagarai (b) and Bhavanisagar (c) soils. Error bars represent standard errors of three replications.

symbiosis enhanced the tryptophan concentration in grains which commensurate with improved Zn status of the inoculated plants as Zn is required for synthesis of tryptophan (Cakmak et al., 1989; Brown et al. 1993). The data are in agreement with the observations of Ryan et al. 2002 who have reported a positive relationship between colonization and grain Zn and P concentrations. The host plant yield response of plants decreased gradually with increased levels of Zn or P suggesting that response of plants to AMF+ inoculation is more pertinent for deficient soils (Fig. 5). Our data are in support of the findings of Ortas et al. (2002) who have reported that mycorrhizal dependency of sour orange strongly decreased by P supply and slightly decreased by Zn supply. Overall, the three set of field experimental data clearly demonstrated that AMF symbiosis facilitates the availability of both P and Zn. The synergistic interaction between these two nutrients may assist in enhanced uptake of zinc which eventually get remobilized into developing grains. Since AMF inoculation improves nutritional qualities of grains, arbuscular mycorrhizal fungal 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.

Materials and Methods

Experimental soil

Characteristics of Experimental soils

All the three experimental soils had slightly alkaline pH and free from salinity and very low in organic carbon status

Table 5. Zinc (Zn) concentration (mg kg^{-1}) examined in the roots and shoots of arbuscular mycorrhiza inoculated (AM+) and non- inoculated (AM-) maize plants (n=15) at 45 days after sowing (DAS) under Varying P and Zn levels. The levels of significance for ANOVA, * = $P \leq 0.05$; ** = $P \leq 0.01$; NS = Not significant. Statistics was done for each location separately. Means followed by a common letter are not significantly different at the 5% level by DMRT.

	Zn Content											
	Coimbatore				Vagarai				Bhavanisagar			
	Shoot		Root		Shoot		Root		Shoot		Root	
Treatments (kg ha $^{-1}$)	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+
P_{30}												
Zn0	24.21^{h}	28.43^{g}	32.61^{i}	35.69^{h}	23.2^{h}	27.4^{g}	33.6^{i}	36.4^{h}	23.19^{i}	27.82^{h}	31.38^{h}	35.58^{f}
Zn2.5	28.69^{g}	34.26^{e}	33.24^{i}	37.19^{g}	27.6^{g}	33.2^{e}	34.3^{i}	38.1^{g}	27.65^{h}	33.88^{f}	32.90^{g}	36.95^{e}
Zn5.0	33.12^{f}	36.21^{d}	38.19^{g}	42.56^{e}	32.1^{f}	35.2^{d}	39.8^{f}	43.8^{e}	32.53^{g}	36.06^{e}	37.60^{e}	41.21^{d}
P_{60}												
Zn0	35.69^{d}	38.69^{c}	40.32^{f}	44.72^{d}	34.8^{d}	37.6^{c}	40.4^{f}	45.1^{d}	35.30^{e}	39.17^{c}	35.99^{f}	43.81^{c}
Zn2.5	38.78^{c}	42.72^{b}	44.78^{d}	52.76^{b}	37.7^{c}	41.7^{b}	43.8^{e}	53.0^{b}	37.84^{d}	42.90^{b}	37.49^{e}	52.08^{b}
Zn5.0	42.69^{b}	49.66^{a}	50.69^{c}	55.81^{a}	41.1^{b}	48.6^{a}	48.2^{c}	54.2^{a}	42.00^{b}	49.71^{a}	41.83^{d}	55.19^{a}
CD (P=0.05)												
M	*		*		*		*		*		*	
P	*		*		*		*		*		*	
Zn	*		*		*		*		*		*	
MXP	NS		NS		NS		*		*		*	
PXZn	NS		*		*		**		*		**	
MXZn	NS		NS		*		*		*		*	
MXPXZn	NS		NS		*		*		*		*	

Table 6. Acid phosphatase (µg of PNP g^{-1} min^{-1}), available zinc (Zn) (mg kg^{-1}) and phosphorus (P) (kg ha^{-1}) concentrations examined in the soils of arbuscular mycorrhiza Inoculated (AM+) and non-inoculated (AM-) maize plants (n=15) at 45 and 75 days after sowing (DAS) under varying P and Zn levels. The levels of significance for ANOVA, * = $P \leq 0.05$; ** = $P \leq 0.01$; NS = Not significant. Statistics was done for each location separately. Means followed by a common letter are not significantly different at the 5% level by DMRT.

	Acid phosphatase						Olsen's P						DTPA Zn					
	Coimbatore		Vagarai		Bhavanisagar		Coimbatore		Vagarai		Bhavanisagar		Coimbatore		Vagarai		Bhavanisagar	
Treatments (kg ha $^{-1}$)	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+	AM-	AM+
P_{30}																		
Zn0	1.25^{d}	1.26^{d}	1.13^{f}	1.16^{e}	1.43^{i}	1.89^{g}	11.14^{i}	13.80^{g}	8.46^{e}	9.70^{b}	12.6^{e}	14.2^{d}	1.15^{g}	1.21^{e}	1.23^{g}	1.28^{f}	1.12^{j}	1.23^{g}
Zn2.5	1.26^{d}	1.27^{d}	1.15^{e}	1.17^{e}	1.61^{h}	1.99^{f}	12.86^{h}	15.24^{f}	8.52^{d}	9.75^{b}	13.0^{e}	13.0^{d}	1.18^{f}	1.25^{d}	1.26^{f}	1.31^{e}	1.23^{g}	1.39^{f}
Zn5.0	1.28^{c}	1.35^{c}	1.27^{d}	1.29^{c}	1.62^{h}	2.03^{e}	15.32^{f}	17.42^{e}	8.59^{d}	9.79^{b}	12.8^{e}	13.5^{d}	1.22^{e}	1.39^{b}	1.30^{e}	1.35^{d}	1.66^{e}	1.85^{d}
P_{60}																		
Zn0	1.29^{c}	1.28^{c}	1.16^{e}	1.28^{d}	1.89^{g}	2.06^{d}	19.72^{d}	21.92^{c}	9.42^{c}	10.45^{a}	21.2^{c}	23.1^{b}	0.99^{i}	1.35^{c}	1.02^{i}	1.39^{c}	1.14^{i}	1.17^{h}
Zn2.5	1.30^{c}	2.03^{b}	1.30^{c}	2.13^{a}	2.11^{c}	2.12^{c}	21.49^{c}	25.14^{b}	9.46^{c}	10.49^{a}	21.8^{c}	24.8^{a}	1.11^{h}	1.42^{b}	1.18^{h}	1.45^{b}	1.86^{d}	1.88^{c}
Zn5.0	1.37^{c}	2.16^{a}	1.38^{b}	2.14^{a}	2.17^{b}	2.24^{a}	21.0^{c}	29.61^{a}	9.52^{c}	10.44^{a}	23.1^{b}	24.5^{a}	1.18^{f}	1.64^{a}	1.25^{f}	1.63^{a}	2.02^{b}	2.13^{a}
CD (P=0.05)																		
M	**		**		**		*		*		*		*		*		*	
P	**		**		*		*		*		*		**		*		*	
Zn	*		*		**		*		*		*		*		**		*	
MXP	*		*		*		*		*		*		*		*		*	
PXZn	*		*		*		NS		NS		*		*		*		**	
MXZn	NS		*		*		*		*		*		*		*		*	
MXPXZn	NS		*		*		*		*		*		*		*		*	

(Table 1). The soil available nitrogen (196 to 272 kg ha^{-1}), phosphorus (12 to 19 kg ha^{-1}), and potassium (222 to 285 kg ha^{-1}) were low, medium and medium status, respectively. The available (DTPA-extractable) Zn status of Coimbatore and Bhavanisagar soils were lower than the optimal available Zn status of 1.2 mg kg^{-1} while Vagarai soil barely maintained that level. The indigenous AMF population in the representative soil samples of Coimbatore, Vagarai, and Bhvanisagar were 6, 5 and 10 per 100g of soil. Since all the three experimental locations had very low spore counts, no attempt was made to fumigate the soil. Despite the experimental locations were 60-70 KMs away from each other, the soils had low fertility status, low indigenous AMF load and free from salinity. Overall, the soil is extremely poor in fertility status that served as an ideal experimental location to assess the response of maize to AMF colonization. It has been proved beyond doubt that mycorrhizal dependency is higher for the low fertility soils (Subramanian et al., 2008).

Field experiments were taken up in three locations viz., Agricultural Research Station, Bhavanisagar, Maize Research Station, Vagarai and Experimental Farms, Tamil Nadu Agricultural University, Coimbatore, to study the effect of AMF on biofortification of grains of maize. All the three experimental sites have been intensely cultivated with a cropping sequence of maize - cowpea – fallow for the past 10 years. The characteristics of the experimental soils are given in Table 1. Besides basic soil characteristics, the experimental soils were evaluated for their indigenous AMF status. Since the indigenous viable spore population was low (< 10 spores 100 g^{-1} soils) in all the three locations no attempt was made to fumigate the soils. The very low level of indigenous population may be attributed to the exclusion of AMF inoculation in all the three experimental locations. It has been clearly demonstrated that the soils exposed to intensive cultivation in conjunction with exclusion of AMF have extremely low numbers of indigenous population (Li and Zhao 2005). In other field studies, it has been shown that AMF colonization in wheat was lower when preceding crop of brassica cover crop in comparison to AMF inoculated cover crop (Ryan and Angus 2003). Further, the low spore counts in the native soil may be due to the slower rate of development in comparison to the inoculums which closely coincides with the observations made in the field tests of Sieverding et al. (1989).

Treatments comprised of two levels of P (30 and 60 kg ha^{-1}) and three levels of Zn (0, 2.5 and 5 kg ha^{-1}) in the presence or absence of AMF inoculation. There were 12 treatments each was replicated three times in a factorial randomized block design (FRBD). Each plot measured 20.16 m^2 (4.8 m length x 4.2 m width) and the plant spacing adopted was 60 x 25 cm and each plot had 112 plants. The AMF inoculum carrying *Glomus intraradices* (2 g) was applied uniformly at the seed hole prior to sowing. Vermiculite based AMF inoculum (*Glomus intraradices* TNAU-03-08) 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 (60 kg ha^{-1}) and full dose of K (50 kg ha^{-1}) were applied in the form of urea and muriate of potash, respectively, as basal at the time of sowing. Basal dose of P as per treatment was applied in the form of single superphosphate. In addition to macronutrients, three levels of Zn (0, 2.5 and 5.0 kg Zn ha^{-1}) as $ZnSO_4$ were applied as per treatment. At each location root and shoot samples (3 replications × 5 plants = 15) were collected at 45 DAS. Mycorrhizal (AMF+) and uninoculated plants were measured for their root mass and root volume. Total leaf area of the AMF+ and AMF- plants was also measured (Chen et al., 1997).

Mycorrhizal colonization

Maize plant roots sampled from AMF+ and AMF- treatments were analyzed for their mycorrhizal colonization at 45 DAS and 75 DAS. Since the trend of response was similar in 75 DAS except 10-15% higher colonization, the data at 45 DAS alone presented. 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 dirts and soil particles. About 100g fresh samples comprising fire and coarse roots drawn from each replication were mixed together and the composite samples were assessed for their AMF colonization. The root segments of 1 cm length in 100 numbers were cut per treatment, and estimated for AMF 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. 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 and colonization percentage was worked out.

Nutrient analysis

Plant material

Fresh leaf samples (fully expanded fourth leaf from the top) were collected at 45 DAS and extracted the chlorophyll content. Besides, 500 mg fresh leaf samples were extracted with 50 ml acetone and the absorbance was read at 663 nm (Bruinsma, 1963). In addition, the shoots and residual roots were dried in hot air oven at 70°C for 48 h. The collected plant samples weighing 0.5 g were digested in triple acid mixture (9:2:1 nitric acid: sulphuric acid: perchloric acid) for nutrient analyses. The colorimetric examination of plant extract to measure the phosphorus (P) level was taken following vanadomolybdo phosphoric acid yellow colour method (Piper, 1966). The plant samples diluted to 50 ml using distilled water were fed to an Atomic Absorption Spectrometer (Varian Spectra AA 220, Australia) to determine Zn concentration. Similarly, Zn level in grains was also analyzed. The powdered grain samples were estimated for their tryptophan concentrations following the protocol described by Sadasivam and Manickkam (1996). The host plant yield response to AMF inoculation was calculated using the formula from Hetrick et al. (1992). The maize cobs were harvested at the full maturity stage when all the leaves got senesced. There were no variations in the date of harvest among treatments. The grain yield response to mycorrhizal colonization was determined using percentage mycorrhizal dependency (MD) which was calculated by the formula furnished below:

$$\text{MD}\ (\%) = \frac{\text{Grain yield (AMF +)} - \text{Grain yield (AMF −)}}{\text{Grain yield (AMF+)}} \times 100$$

Soil

The DTPA (Diethylene Triamine Penta Acetic Acid) extractant (13.1 ml triethanolamine, 1.967 g DTPA and 1.47 g $CaCl_2$ are mixed together, made up to 1 L with pH adjusted to 7.3) was used to extract the available form of Zn from the soil (Lindsay and Norvell, 1978). The extract was filtered through Whatman No.42 filter paper and the absorbance was read in an Atomic Absorption Spectrophotometer (Spectra AA220, Varian, Australia). Available phosphorus in the soils was estimated by extraction with sodium bicarbonate ($NaHCO_3$) following Olsen et al. (1954).

Acid phosphatase

Five grams of soil sample was taken in a boiling tube. 10 mL of distilled water, 0.25 mL of toluene and 1 mL of 10 mM para nitro phenyl phosphate (ρNP) were added and incubated at room temperature for 1 hour. Then 5 mL of 0.5 M $CaCl_2$ and 20 mL of 0.5 M NaOH were added. The content was filtered using Whatman No 42 and volume was made up to 50 mL with distilled water. The intensity of yellow colour developed was read at 420 nm in a spectrophotometer (Varian Cary 50 UV-visible spectrophotometer). The activity of acid phosphatase was calculated using standard graph (Bremner and Tabatabai, 1969).

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, Vagarai and Bhavanisagar) were analyzed separately. Despite the fact that the experimental design (Factorial Randomized Block Design (FRBD)) 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).

Conclusions

Overall, the three set of field experimental data clearly demonstrated that AMF symbiosis facilitates the availability of both P and Zn. The synergistic interaction between these two nutrients may assist in enhanced uptake of zinc which eventually get remobilized into developing grains. Since AMF inoculation improves nutritional qualities of grains, arbuscular mycorrhizal fungal 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.

Acknowledgements

The authors are thankful to the Indian Council of Agricultural Research (ICAR) for providing financial support to the Principal Investigator of the Scheme Dr. K.S. Subramanian under the AMAAS (Application of Microorganisms in Agriculture and Allied Sector) for the past seven years during 2006-2013.

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