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The ability of two Leguminous Plants to increase Zinc metal tolerance by Arbuscular mycorrhizal fungi colonization

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Abstract

Pot experiment was carried out in green house at Agriculture Faculty (Saba bacha), Alexandria University. The experiment was done to investigate the role of arbuscular mycorrhizae (AMF) to increase uptake of zinc (Zn) by some crops. Two species of leguminous soybean (*Glycine max*. L) and lentil (*Lens culinaris*. L) were grown in sandy soil . Zinc was applied as $ZnSO_4.7HO_2$, in four concentrations (0, 30, 50 and 70 mg/ kg soil). The plants were collected after 60 day from sowing. The results indicating that AMF colonization increased the tested plant resistance to Zinc metal. It also significantly stimulated the formation of root nodules, either increased the P uptake in all treatments, which might be one of the tolerance mechanisms conferred by AMF. All treatments were compared with the control, Mycorrhizal plants inoculated by *G. intraradices* shown more accumulation of zinc in roots and large reductions in shoots of the two legumes, indicating that the decreased Zinc metal uptake and growth dilution were induced by AMF treatment, thereby reducing the Zinc metal toxicity to the plants.

Keyword: Arbuscular Mycorrhizal fungi, Zinc tolerance, Leguminous plants, MD-mycorrhizal dependency.

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Introduction

Health hazards posed by the accumulation of toxic metals in the environment accompanied by the high cost of removal and replacement of metal-polluted soil have prompted efforts to develop phytoremediation strategies that would utilize plants to extract excessive soil metals. However, plant establishment and growth in metal-contaminated sites are inhibited by adverse soil factors, such as pH, poor physical structure, toxic metals, and nutrient deficiencies (Pichtel and Salt 1998; Wong 2003). Nitrogen (N) is one of the most important nutrients for plant growth. The lack of N and inhibition of N uptake induced by heavy metals have a strong impact on plant growth. Therefore, plants that can accumulate more N may grow better in contaminated soil. Leguminous plants have advantages in N_2 –fixing by formation of root nodules (Ma *et al*. 2006). However, their root nodules may be inhibited by heavy metals in the soil, and thus the N_2 - fixing ability of leguminous plants may be hindered. leguminous plants may be hindered. *Medicago sativa* (Alfalfa) was found to grow in soils contaminated by heavy metals since it can accumulate metal concentrations above the tolerance levels of most plants (Tiemann *et al*., 1999). AMF can enhance plants' tolerance to metal and are often used in the restoration of metal-contaminated sites (Vogel-Mikun *et al*., 2005). A number of studies have indicated that mycorrhizas can assist pioneer plant species to colonize metal-contaminated sites by improving the plants, phosphorus (P) uptake and subsequently enhancing their growth (Smith and Read 1997; Karandashov and Bucher 2005; Vogel-Mikun *et al*. 2005). The objective of this study was to investigate whether AMF colonization could enhance the formation of nodules, increase P and N uptake, and increase metals tolerance of leguminous plants. [including soybean (*Glycine max*. L) and lentil (*Lens culinaris*)].

Materials and methods

Inocula preparation

Rhizobium bacteria are common soil inhabitants, distinguished from other genera of soil bacteria by their ability to nodulate leguminous plants and fix nitrogen in the nodules. Arbuscular mycorrhizal (AM) fungi species belonging to the genus *Glomus* was used in this study. It was obtained from Hanover University (Germany) and was propagated several times on maize plants grown in a sandy soil for 10 weeks.

Plant growth conditions

Pot experiment was conducted during summer 2010 under greenhouse conditions at Agriculture faculty (Saba bacha), Alexandria University. Plastic pots 25 cm depth and 12 cm inside diameter with holes in their bottom, were filling with 900 kg of sandy soil, leaving the upper 5cm without soil. Four concentrations (0, 30, 50 and 70 mg/ kg) of Zinc ($ZnSO_4.7HO_2$) were applied. Soybean (*Glycine max*. L) and lentil (*Lens culinaris*. L) seeds were surface-sterilized with 0.05% NaOCl solution and subsequently washed many times with distilled water. After being immersed in distilled water for 24 h, and allowed to germinate on moist filter paper. When the radicals appeared, uniform seedlings were selected for the experiment. The seedlings were grown in 900 g plastic pots containing 860 g of soil plus 40 g of inoculum for the +AMF treatment, or 860 g soil plus 40 g of sterilized inoculum for the -AMF treatment. The soil moisture was maintained at 70% of field water holding capacity by adding appropriate amounts of distilled water by regular weighing of pots. Three replicates were used in each treatment. After two weeks, plants were thinned to 2 plants per pot. Soil of each pot was fertilized with 150 mg N kg^{-1} soil in the form of NH_4NO_3, 170 mg kg^{-1} soil in the form of K_2SO_4 and 30 mg P kg^{-1} soil in the form of Ca $(H_2PO_4)_2$. The containers were arranged using completely randomized design.

Plant harvest

Treated plants were harvested after 60 days. After the shoots were cut off, the roots were carefully washed free of soil with tap water, fully rinsed in distilled water, and then washed with 1 mmol/L $CaCl_2$ solution for 30 min. to remove all the particles may adhered to the root surface and to desorbed metals associated with cell walls. The root nodules were visually counted.

Root colonization

The cleaned roots were cut into segments about 1 cm in length. A randomly selected fresh root subsample about 1 g was taken for the assessment of root colonization. The roots were cleaned in 10% KOH, stained with 0.05% trypan blue (Phillips and Hayman 1970). The percent of colonization was done .The gridlines intersect method of Giovannetti and Mosse (1980) was used to estimate the mycorrhizae infection percentage using the following equation:

$$\textbf{AMF infection \%} = \frac{\text{Number of segments containing AMF}}{\text{Total number of examined segments}} \times 100$$

Also, the mycorrhizal dependency (MD) of plant growth was calculated according to the following formula ((Plenchette *et al*., 1983) :

$$\mathrm{MD} = \frac{(\text{Dry mass mycorrhizal plant}) - (\text{Dry mass non} - \text{mycorrhizal plant})}{\text{Dry mass mycorrhizal plant}} \times 100$$

The relative increase or decrease of zinc uptake of mycorrhizal plants relative to the non-mycorrhizal plants was calculated based on the following formula (Wang *et al*., 2005):

The relative Increase/ or decrease of Zn uptake =

$$\frac{\text{Zn uptake of mycorrhizal plants} - \text{Zn uptake of non} - \text{mycorrhizal plants}}{\text{Zn uptake of mycorrhizal plants}} \times 100$$

Also three aspects of plant Zn efficiency were assessed. according to Harper *et al*. (1997) were Zn uptake efficiency was calculated based on the ability of the root to take up Zn from the soil and Zn translocation efficiency was computed as the ability of the plant to transport Zn to the shoot.

$$\text{Uptake efficiency } (\mu\text{g g} - 1) = \text{Zn uptake of the plants/root dry weight}$$

$$\text{Translocation efficiency} = \text{shoot Zn uptake/root Zn uptake}$$

Another aspect was zinc phytoextraction efficiency which is calculated based on the ability of the root to transport Zn to shoot according to the following equation:

$$\text{Phytoextraction efficiency } \mu\text{g g} - 1 = \text{shoot uptake/root dry weight}$$

Plant biomass determination and Heavy metals analysis

After samples of plants were oven dried at 70 C° for 48 hours, ground and dry weights of shoots and roots were recorded. Half g of the oven dried plant material was digested with H2SO4-H2O2 mixture according to Lowther (1980). In the digested solution Zn was determined in the digested solutions (Jackson, 1967) using the atomic absorption spectrophotometer (A Analyst 400). In addition, the N concentration was determined by the Kjeldahl nitrogen method (Lowther, 1980). The vanadomolybdate calorimetric method (Jackson, 1967) was used to measure total P in the digested plant samples using spectrophotometer. The yellow colored phosphovanadomolybdate complex was measured at 480 nm Wavelength.

Statistical analysis

Pots were arranged on greenhouse bench in a randomized complete design with three replications per treatment. Data were subjected to analysis of variance according to Snedecor and Cochran (1972) and L.S.D test at 0.05 level of probability was used to compare between means.

Results and Discussion

Root colonization and root nodule number of different leguminous plants

Table. (1) showed that no root colonization by *G. intraradices* was detected in non-inoculated plants with AMF, and under the inoculation with AMF soybean plants had the highest root colonization among the two legumes. Root nodules were only detected in soybean plant under the non-inoculated treatments. The inoculated treatments significantly enhanced the development of root nodules compared with the non-inoculated treatments, and soybean plant developed more nodules than Lentil plants (Table 1).

On the other hand, The highest rate of Zn (Table 1) decreased colonization with 36.63% under roots of soybean plants and degree of colonization was sharply decreased only 14.74% by Zn additions in the case of Lentil plants. These findings are consistent with Marques *et al*. (2006) who reported that High levels of zinc can reduce and completely eliminate AMF colonization and AM fungal spore germination in the soil.

Plant biomass

Compared with the non-inoculated treatments, plants inoculated with mycorrhiza significantly enhanced shoots and roots biomass of all plants. Shoots and roots biomasses of soybean plant were the highest among the two plants especially with inoculation by *G. intraradices*, which might be attributed to the higher capacity of N accumulation. On one hand, the capacity for N2 -fixation of soybean plant was higher and the root nodules might be efficient in N2 –fixation (Ma *et al*., 2006).

Table 1. The effect of Zn rates on shoot and root dry weights, root colonization (%) and nodule numbers of two leguminous plants with and without AMF.

Treatment	Zn rate mg/kg soil	AMF infection (%)	Nodule number	Roots Dry weight	shoot Dry weight	Whole plant	MD (%)	Shoot / Root
soybean plant without (-AMF) inoculation	0	0	1	1.18	3.11	4.29	-	2.64
	30	0	2	1.38	3.26	4.64	-	2.36
	50	0	3	1.40	2.34	3.74	-	1.67
	70	0	4	1.23	2.11	3.34	-	1.71
Mean		0		1.29	2.71			
soybean plant with (+AMF) inoculation	0	62.30	1	1.21	3.22	4.43	3.16	2.66
	30	71.33	12	1.55	4.39	5.94	21.89	2.83
	50	48.60	10	1.72	3.92	5.64	33.69	2.28
	70	36.63	6	1.29	2.77	4.06	17.73	2.15
Mean				1.44	3.58			
L.S.D 0.05				1.23	0.97			
Lentil plant without (-AMF) inoculation	0	0	0	0.98	2.33	3.31	-	1.42
	30	0	0	1.3	2.56	3.86	-	1.97
	50	0	0	1.21	2.32	3.53	-	1.52
	70	0	0	0.85	1.15	2.00	-	1.35
Mean				1.09	2.09			
Lentil plant without (+AMF) inoculation	0	52.20	3	1.27	2.57	3.84	13.80	2.02
	30	68.32	8	1.45	4.12	5.57	30.70	2.84
	50	16.70	6	1.42	3.20	4.62	23.60	2.25
	70	14.74	2	0.94	1.54	2.48	19.35	1.64
Mean				1.27	2.86			
L.S.D 0.05				0.96	0.58			

The growth of two plants in the present study was increased by AMF colonization, indicating that AMF colonization increased the plants' tolerance to metals. In addition to the increased P accumulation, AMF colonization stimulated the formation of root nodules, especially in, soybean plant indicating that better P nutrition in soybean plant could also contribute to better N nutrition by stimulating the development of root nodules of soybean plant (Table 4). This is similar to previous results reported by (Toro *et al*., 1998) who reported that better P nutrition in *Sesbania rostrata* could also contribute to better N nutrition by stimulating the development of root nodules. The higher numbers of root nodules in plant treated with AMF treatments led to higher N accumulation in the plants compared with the -AMF treatments. Andrade *et al*. (2004) also reported that inoculation by *Glomus macrocarpum* increased root nodule numbers of soybean plants, which then attributed to better P nutrition in mycorrhizal plants.

Phosphorus is a key element in forming root nodules, and therefore the AMF colonization is beneficial to the development of root nodules (Barea *et al*., 2005). It was also reported by Chen *et al*. (1999) that AMF could increase N accumulation in mycorrhizal plants because of higher nitrogenase-fixation activity in mycorrhizal plants and consequently increased efficiency of N2 -fixation. On the other hand, high levels of Zn concentration in soil could inhibit the development of root nodules and plants biomasses. These findings are consistent with the results of Diaz *et al*. (1996) who found that increasing doses of Zn or Pb reduced plant biomass.

(Table 1) shown that Mycorrhizal dependency(MD) increased plant biomass by 3.16% , 21.89%, 33.69% and 17.73%, respectively .Similarly, In comparison with Lentil plant plants,(MD) with G. intraradices increased plant biomass by 13.80%, 30.70%,23.60% and 19.35%, respectively.

Zinc concentrations

In general, the two leguminous plants showed significant differences in heavy metal concentrations in their tissue (Table 2). The concentrations of Zn in the roots were significantly higher than those in the shoots of all two plants. In the non-inoculated plants, the concentrations of Zn in the shoots of soybean plants were significantly higher than those of Lentil plant. Compared with the non-inoculated treatments, colonization by *G. intraradices* decreased the Zn concentrations in the shoots of two species tested and significantly increased the Zn concentrations in the roots of soybean plant. AMF might change the components of root cell walls, thus increasing the adsorption capacity to metals. This strategy may be very important for mycorrhizal plants surviving on metal contaminated soils.

Table 2. The effects of AMF on the concentrations (mg kg^{-1}) of Zn in two leguminous plants.

Treatment	Zn rate mg/kg soil	Zinc content mg/kg		Roots/Shoots (R/S)
		Roots	Shoots	
Soybean plant without (-AMF) inoculation	0	0.00	0.00	0.00
	30	2.39	0.92	2.60
	50	4.41	1.23	3.59
	70	9.85	2.65	3.72
Mean		4.16	1.20	
Soybean plant with (+AMF) inoculation	0	0.00	0.00	0.00
	30	5.22	0.54	9.67
	50	8.93	0.92	9.71
	70	14.35	1.54	9.32
Mean		7.13	0.75	
L.S.D 0.05		2.06	0.54	
Lentil plant without (-AMF) inoculation	0	0.00	0.00	0.00
	30	1.85	0.62	2.98
	50	2.95	1.13	2.61
	70	6.93	1.92	3.61
Mean		2.93	0.92	
Lentil plant without (+AMF) inoculation	0	0.00	0.00	0.00
	30	3.45	0.52	6.63
	50	6.18	0.77	8.03
	70	10.85	1.40	7.75
Mean		5.12	0.67	
L.S.D 0.05		1.61	0.20	

Mycorrhizal plants have various detoxification mechanisms including the retention of toxic metals in roots and the subsequent reduction of translocation to shoots (Tullio *et al*., 2003; Christie *et al*., 2004). The higher R/S Zn- ratio (Table 2) meaning the ability of root tissue to accumulate Zn and consequently low Zn translocation from root to shoot. The results showed that Zn R/S ratio was high in the inoculated two leguminous plants. In soybean plant, Root to shoot ratio of Zn concentration of root-Zn with inoculation *G. intraradices* were between 9-10 folds higher than those in shoots at all Zn application rates to soil. Similarly trend was observed in Lentil plant, and reached between 7-8 folds higher than in shoots.

Zinc uptake

The Zn uptake reflects the effects both plant biomass and Zn concentrations in soil and calculated according to the data in Tables 1 and 2. Under both, the non-inoculated and inoculated treatments, the accumulation of Zn in the shoots of soybean plants was significantly higher than that in the shoots of Lentil plants (Table 3). The AMF colonization increased the uptakes of Zn in the roots of the two plants compared with non-inoculated treatments. Not only were the legumes nutrient uptakes affected by the AMF colonization, but their metal accumulations were also affected, which changed the plants tolerance to metal contamination. In addition to the better nutrient uptake, Ibekwe *et al*. (1995) suggested that the tolerance of Sesbania

Table 3. The effects of AMF on uptake (µg/plant) of Zn by two leguminous plants

Treatment	Zn rate mg/kg soil	Zinc uptake µg/plant		Total Zn uptake (µg /plant)
		Roots	Shoots	
soybean plant without (-AMF) inoculation	0	0	0	0.00
	30	3.30	3.00	6..30
	50	6.17	2.88	9.05
	70	12.12	5.59	17.71
Mean		5.40	2.87	
soybean plant with (+AMF) inoculation	0	0	0	0
	30	8.09	2.37	10.46
	50	15.36	3.61	18.97
	70	18.51	4.27	22.78
Mean		10.48	2.56	
L.S.D 0.05		2.31	0.89	
Lentil plant without (-AMF) inoculation	0	0	0	0
	30	2.41	1.59	4
	50	3.57	2.62	6.19
	70	5.89	2.21	8.1
Mean		2.97	1.61	
Lentil plant without (+AMF) inoculation	0	0	0	0
	30	5.00	2.14	10.70
	50	8.78	2.46	21.60
	70	10.20	2.16	22.03
Mean		6.00	1.69	
L.S.D 0.05		1.81	0.35	

rostrata to heavy metals was significantly enhanced via development of a symbiotic relationship between the roots and Rhizobium since Rhizobium showed less sensitivity to Zn and Cd than plants. In the present experiment, the biomasses of the two legumes and root nodules developed were increased by AMF colonization, possibly due to the fact that the microbial cooperation in the rhizosphere could increase the resistance of the host plant to the toxicity of heavy metals. Rhizobium infected the cells and adsorbed heavy metals, decreasing the influx of heavy metals into protoplasts of roots and thereby enhancing the tolerance of the host plant to metals (Kotrba *et al*., 1999).

Zinc accumulation was different among the legume species, and inoculation by *G. intraradices* yielded different effects on the accumulations of Zn by the two legumes in the present study. Compared with the non-inoculated treatment, the inoculated treatments significantly decreased the Zinc accumulation in the shoots of two leguminous plants.

Zinc uptake efficiency and phytoextraction efficiency were increased with increasing the amounts of added Zn to the soils added, while translocation efficiency showed the opposite trend (Table 4). Compared with the non-inoculated plants, Zn uptake efficiency of inoculated plants was higher with all Zn addition levels but phytoextraction efficiency of inoculated treatment was lower with any Zn additions.

Table 4. Zinc Uptake efficiency (U.e), Phytoextraction efficiency (P.e) and Translocation efficiency (T.e) of two leguminous plants as affected by Zn rate b and arbuscular mycorrhizal fungi

Treatments	Zn rate mg/kg soil	soybean plant (*Glycine max*)			Lentil plant (*Lens culinaris*)		
		U.e	P.e	T.e	U.e	P.e	T.e
plant without (-AMF) inoculation	0	0	0	0	0	0	0
	30	4.57	2.17	0.91	2.76	0	0.66
	50	6.46	2.06	0.47	4.36	1.22	0.73
	70	14.40	4.54	0.46	9.53	2.17	0.38
plant with (+AMF) inoculation	0	0	0	0	0	0	0
	30	6.75	1.53	0.29	7.38	1.51	0.43
	50	11.03	2.1	0.24	15.21	1.73	0.28
	70	17.66	3.31	0.23	23.44	2.30	0.21

Zn translocation efficiency was lower in inoculated treatment plants than in non inoculated ones at all Zn addition levels.

In Conclusion, the protective effect of AMF and rhizobium increased plant growth in soil contaminated by Zinc metal by increasing both the plant nutrient uptake and tolerance to Zn-metal. AMF colonization enabled the plants to accumulate more P, consequently stimulating root nodules and increasing N accumulation. Besides plant nutrient improvement, AMF increased plant tolerance to metals via such mechanisms as dilution effects, increased tolerance to metals, decreased metal uptake, and translocation from root to shoot. These mechanisms could be utilized in phytoremediation or ecological restoration.

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الملخص العربي

أجريت تجربة أصص في صوبة زجاجية باستخدام ارض الرملية (تم تجميعها من منطقة النوبارية تبعد عن الإسكندرية حوالي 50 كم)، لمعرفة دور فطر الميكورهيزا فى امتصاص عنصر الزنك لبعض المحاصيل، حيث لوثت التربة الرملية بـ4 مستويات من الزنك وهى صفر 30 –50 –70 مليغرام/ كجم تربة فى صورة كبريتات الزنك $ZnSO_4.7H_2O$ مع استخدام نباتين من النباتات البقولية وهما فول الصويا والعدس النامية في تربة رملية تعانى من نقص في العناصر مثل الفوسفور والنتروجين بالإضافة الى عناصر صغرى كالزنك والحديد والنحاس والمنجنيز. وتم إضافة لقاح من فطر الميكورهيز *Glomus intraradiaces* في صورة رمل وجراثيم الفطر الى النباتين، وقد تم توزيع المعاملات في تصميم عشوائي كامل بثلاث مكررات. وقد تم اخذ العينات النباتية بعد 60 يوم من الزراعة وتحليلها لمعرفة نسبة معدلات الإصابة بالفطر والوزن الجاف لمجموع الجذري والخضري ،تركيز وامتصاص الزنك في كلاهما. يتبع النتائج أن التلقيح بهذا الفطر أدى إلى تحفيز تكوين العقد الجذرية و زيادة محتوى النبات من عنصر الزنك بل وحدث تراكم معنوي للزنك في المجموع الجذري وتراكم اقل في المجموع الخضري في النباتين وبذلك قلل من سمية عنصر الزنك في النباتات.

مفتاح الكلمات : فطريات الميكوريزا، مقاومة الزنك، نباتات بقولية، اعتمادية الميكوريزا.