

Role of Vesicular Arbuscular Mycorrhizal Inoculation of *Zea mays* on Heavy Metals Tolerance.

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THE STUDY evaluated the physiological responses of maize plant inoculated with vesicular arbuscular mycorrhiza (VAM) isolated from polluted site and growing in heavy metal (Cd or Pb) polluted soil. Chlorophyll, soluble carbohydrate, soluble protein, nutrient elements (P and N) content and phosphatase activity were analyzed. The phosphatase activity in inoculated plants was significantly increased in comparison to the non-inoculated ones. In addition, the contents of N, P, chlorophyll, total soluble carbohydrates and protein were significantly increased in plants inoculated with mycorrhiza grown in heavy metal (Cd or Pb) contaminated soil in comparing with the non-inoculated ones. Therefore, it can be concluded that the VAM inoculation is capable to alleviate the damage caused by heavy metal (Cd or Pb) on maize plants that maintain the growth and this could be related to nutrient status offered by VAM. Isolation of heavy metal tolerant VAM fungi can be a potential biotechnical tool for inoculation of plant to enhance its heavy metal tolerance.

Keywords: Heavy metals, VAM, *Zea mays*, Phosphatase.

Heavy metal pollution has disastrous effect on plant productivity and threatens human and animal health (Adriano, 2001). Heavy metals accumulate in the soil and water tables and many reach to toxic levels. Contamination with heavy metals resulted from urban activity, agricultural practices and industry (Clemens, 2001). However, elevated concentrations of both essential and nonessential heavy metals in the soil can lead to toxicity symptoms and growth inhibition in most plants (Hall, 2002).

Toxic level of heavy metals can affect adversely on the number, diversity and activity of soil organisms, resulting in the selection of the most metal-resistant microorganisms. Therefore, autochthonous microorganisms isolated from polluted environments are likely to play an important role in the metal tolerance exhibited by the plants growing in contaminated soils. Fungi are known tolerate and detoxify metals by several mechanisms, including extra and intracellular precipitations, biosorption to the cell wall and sequestration (Zafar *et al.*, 2007).

Mycorrhiza is the mutualistic symbiotic association of a specific group of soil-borne fungi with the roots of higher plants (Sieverding, 1991). Plant receives support from mycorrhizal fungi, with the help of its symbiotic association, in the

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aspect of the uptake of phosphorus and other nutrients. Moreover, the mycorrhiza enhances the plant growth by increasing growth hormones, protein, and lipid and carbohydrate contents. Generally, endomycorrhizal plants are often more competitive and able to tolerate environmental stress than non-mycorrhizal plants (Tamboli and Vyas, 2012). However, the mechanisms by which VAM symbiosis influences the metabolism of host plants under heavy metal stress are not clear. Symbiotic interaction between VAM and host plants (especially during the growth) need to be studied under heavy metal stress conditions. The objective of this study was to determine the physiological responses of maize plant inoculated with VAM isolated from polluted site, grown in heavy metals (Cd or Pb) polluted soil. The alkaline phosphatase and acid phosphatase activities, biomass, photosynthetic pigments, the protein, carbohydrates, phosphorus and nitrogen contents were concerned.

Material and Methods

Soil Preparation

The soil, used throughout the present study, was collected from Zagazig, Sharkia Governorate, Egypt. Soil samples were sieved (2-mm mesh size) to discard the impurities and gravels. Then, soil was sterilized by 5% formalin for 7 days to eliminate native arbuscular mycorrhizal fungi, after which soil was aerated.

Plant material and growth condition

Grains of maize (*Zea mays* L.), were obtained from Field Crop Research Institute, surface sterilized with 3% sodium hypochlorite for 5 min, then thoroughly washed with sterilized distilled water. Five sterilized maize grains were sown in sterilized pots that filled with 8 kg sterilized soil. Pots were placed randomly in the greenhouse under natural photoperiods (12-13 h) and temperature of $28\pm 4^{\circ}\text{C}$. The inoculum used was collected from sludge-amended agricultural soil in Bahr El-Baqar, Sharkia, Egypt. It was extracted and then maintained as described by Gerdemann and Nicolson (1963). Isolate contained two different species of vesicular arbuscular mycorrhizal fungi, *Glomus mosseae* and *G. clarum* (Rabie *et al.*, 2013). The inoculum consisted of crude soil, mycelia with spores, and colonized root segments from stock cultures of the corresponding VAM fungi. It was added at the rate of 80 g per pot at sowing. Known concentration of heavy metal solutions of Cd and Pb were prepared using soluble compounds of CdSO_4 and $(\text{CH}_3\text{COO})_2\text{Pb}\cdot 3\text{H}_2\text{O}$ and applied at the levels: Cd; 25 and 100 mg/kg soil and Pb; 100 and 1000 mg /kg soil. Pots were placed randomly in the greenhouse under natural conations.

Each treatment was replicated three times in completely randomized design. Plants were harvested after 3, 6, 9 weeks from sowing. Dry weight (DW) of shoots and roots were obtained by oven-drying at 80°C until constant weight.

Mycorrhizal Dependency (MD)

Mycorrhizal dependency was calculated according to Gerdemann (1975) as the following formula: $\text{MD \%} = \frac{\text{DW of inoculated plant at particular level of heavy metal}}{\text{DW of non-inoculated plant at the same level of heavy metal}} \times 100$.

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Biochemical and physiological parameters

Chlorophyll a, b and carotenoid contents were extracted from fresh leaf on aqueous acetone solution (85%), and then determined according to Metzner *et al.* (1965). Total soluble carbohydrate content was determined according to Said and Naguib (1964). Total soluble protein was determined according Lowery *et al.* (1951). Nitrogen was determined by the Kjeldal method as described by Jackson (1973). Total phosphorus in maize dry leaves was determined by the molybdovanado phosphate colorimetric method (Jakson, 1958).

Acid Phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNP, 0.115 M) as substrate. Enzyme extract was prepared in extraction buffer containing 50 mM citrate buffer (pH 5.3) for acid and 50 mM Glycine NaOH buffer (pH 10.3) for alkaline phosphatase by grinding 0.5 g fresh root samples in 10 ml extraction buffer. Extracts were centrifuged at 10000 rpm for 10 min and the supernatant was used as source of enzyme. Phosphatase activity was determined by Tabatabai and Bermner (1969) method and was expressed in μ moles PNP released /h/ g fresh weight.

Statistical analysis

Statistical analyses were carried out using MSTAT-C program version 2.10 (1991). Least significant difference (LSD) was employed to test for significant difference at $P \leq 0.05$ (Gomez and Gomez, 1984).

Results and Discussion*Mycorrhizal dependency (MD)*

Mycorrhizal symbiosis is key component in helping the plants to cope with adverse environmental conditions. Under, heavy metals stress, biomass declined in non-mycorrhizal plants compared with the control. On the other hand, VAM plants grown in non-polluted and polluted soil showed a higher shoot and root dry weight than non-mycorrhizal plants (Table 1).

In this study, the value of mycorrhizal dependency clearly not affected by heavy metals stress (Table 1). This finding agree with Burleigh, *et al.* (2003) and Wang *et al.*, (2006), who suggested that microsymbioses application may play an intelligible main role in the synergistic interactions. This may be a sign showing the ecological importance of VAM association for plant survival and growth under heavy metals stress. We suggest that improved growth and nutrient uptake by mycorrhizal plants are an indication of enhance the tolerance to heavy metals, compared with the non-mycorrhizal plants.

Photosynthetic pigments

As shown in Table 2, the chl.a, chl. b and carotenoid contents was significantly increased in mycorrhizal maize plants compared with non mycorrhizal ones. The results also showed a progressive decrease in chl. a, chl. b and carotenoid contents in maize plants grown under heavy metals. These results agree with the results recorded by Elahi *et al.*, (2010).

TABLE 1. Effect of heavy metals (Cd & Pb) on biomass and Mycorrhizal dependency (MD) of maize plants inoculated with VAM fungal, grown at different concentration of Cd or Pb.

Treatments (mg/kg)		Shoot dry Wt. (g)	Root dry Wt. (g)	MD%
Control	NM	2.27b	5.52b	106.9
	M	2.45a	5.88a	
Cd 25	NM	1.99bc	4.46c	114.57
	M	2.09b	5.03bc	
Cd 100	NM	1.29e	3.75d	107.14
	M	1.52d	3.88d	
Pb 100	NM	1.62d	4.01d	111.19
	M	1.72c	4.54c	
Pb 1000	NM	1.13e	3.59e	111.01
	M	1.46d	3.78d	
LSD 0.05		0.18	0.36	

Different letters within the same column indicate a significant difference. n=3.

TABLE 2. Photosynthetic pigment, total soluble protein and total soluble carbohydrates contents, in the leaves of non-inoculated or inoculated maize plants with VAM fungal grown at different concentrations of Cd or Pb.

Treatments (mg/kg)		Chl.a mg/g fw	Chl. b mg/g fw	Carotenoid mg/g f w	Soluble carbohydrates content ($\mu\text{g/g dw}$)	Soluble protein content (mg/g dw)
Control	NM	2.378b	0.960b	1.457b	779.65b	19.45b
	M	2.599a	1.438a	1.892a	879.31a	21.09a
Cd 25	NM	1.898d	0.831d	1.143d	643.45d	15.20e
	M	1.982c	0.870c	1.200e	694.45c	17.07c
Cd 100	NM	1.603f	0.731g	0.990f	484.83g	11.79g
	M	1.721g	0.784f	1.030g	504.02f	13.31f
Pb 100	NM	1.624g	0.725g	1.035c	566.21e	13.89e
	M	1.754c	0.804c	1.148d	641.42d	16.06d
Pb 1000	NM	1.344j	0.598i	0.839h	413.79i	8.78i
	M	1.561h	0.663h	0.891g	438.62h	11.16h
LSD 0.05		0.003	0.006	0.008	6.67	0.51

Different letters within the same column indicate a significant difference, n=3.

In addition the decline in chl. content in plants due to Cd and Pb stress a result of inhibition of important enzymes, such as δ -aminolevulinic acid dehydratase and impairment in the supply of Mg^{2+} , Fe^{2+} and Zn^{2+} (Van Assche and Clijsters, 1990). The carotenoids act as light-harvesting pigments, and can protect chlorophyll and chloroplast membrane destruction by quenching triplet Chlorophyll and removing oxygen from excited Chl. oxygen complex (Young, 1991). It seemed that the enhancement of photosynthetic pigments contents in mycorrhizal plants can be attributed to the increase of absorption and translocation of essential ions which in turn accelerate the metabolic rates related to the biosynthesis of such contents.

Total soluble carbohydrates

Results recorded significant increase in soluble carbohydrate content in inoculated plants compared to non-inoculated ones (Table 2). This was in line with the results of Meenakshisundaram and Santhagura (2010) who's stated that VAM fungal inoculation induced an increase in soluble carbohydrate in *Sorghum bicolor* compared to non-inoculated control. This increment was correlated with the increase in Chl. contents; ameliorate the photosynthetic rate and nutrient uptake.

Total soluble protein

In this investigation, the total soluble protein content was significantly decreased with increasing the heavy metal (Cd or Pb) concentrations (Table 2). VAM inoculation increased significantly the total soluble protein content regardless of heavy metal levels. At low Cd concentration, the total protein increase by 18.88%, while at high concentration increased by 12.89% compared with non mycorrhizal plant grown at the same level. Same trends were recorded with Pb application. This could be due to their interference with the enzymes activities, which essential for normal metabolic and developmental processes as well as due to their direct interactions with proteins, pigments and carbohydrates (Bertrand and Guary, 2002). Based on these results and other results was early published for our group, the mechanisms related to physiological interactions between VAM fungi and maize plants involved in the increase of protein synthesis as well as induction of antioxidant enzymes to avoid heavy metal mediated oxidative stress (Rabie *et al.*, 2013). Additionally, VAM fungi may be can detoxify the heavy metals via exudation of metal-binding proteins (Howe *et al.*, 1997).

Phosphorus and nitrogen contents

Generally, the results recorded a marked increase in P and N contents in VAM inoculated maize plants compared to the non-inoculated ones (Table 3). Although the results revealed that heavy metals (Pb or Cd) reduce the uptake and transportation of both nutrients in plants as also found by Godbold and Kettner, (1991). This increase in P content in inoculated maize plant may be due to the fast movement of this nutrient element into mycorrhizal hyphae and to increase the phosphatase activities in inoculated maize, which directly affect its content in leaves of mycorrhizal inoculated plants. This coincides with a finding by Ghani, (2010).

The results revealed also an increase of N content in inoculated plants was concurred with Olesniewicz and Thomas, (1999), who's reported that VAM fungi enhanced N₂-fixation by increasing root biomass, root-N absorption rates and finally the plant N contents. It seemed that the foliar N and P contents of inoculated maize plants reaffirms the key role of VAM fungi sustaining the plant cover in nutrient under heavy metals stress (Carrasco *et al.*, 2011).

TABLE 3. Phosphorus and nitrogen contents in the leaves of non-inoculated or inoculated maize plants with VAM fungal grown at different concentrations of Cd or Pb.

Treatments (mg/kg)	P content (ppm)			N content (ppm)			
	3 Weeks	6 weeks	9 weeks	3 Weeks	6 weeks	9 Weeks	
Control	NM	63.10b	100.6b	141.0b	1.640b	2.35b	2.230b
	M	65.60a	108.8a	150.2a	1.750a	2.50a	4.18a
Cd25	NM	54.80d	86.70d	126.0e	1.360e	1.93e	2.31e
	M	59.20c	97.60c	139.0c	1.57c	2.29c	2.50c
Cd100	NM	40.00f	65.90g	97.00i	1.00h	1.64h	2.03g
	M	45.80e	86.40d	108.4g	1.140g	1.860f	2.290e
Pb100	NM	46.40e	78.40e	116.4f	1.290f	1.860f	2.150f
	M	55.20d	86.80d	128.4d	1.430d	2.07d	2.430d
Pb1000	NM	18.20h	62.40h	84.40j	0.930i	1.570i	1.860h
	M	25.60g	69.10f	98.80h	1.00h	1.79g	2.05g
LSD 0.05		1.148	1.02	0.78	0.066	0.044	0.084

Different letters within the same column indicate a significant difference, n=3.

Phosphatase activity

In this study it was observed that the activities of alkaline (ALP) and acid phosphatase (ACP) were significantly increased in inoculated maize plants grown in both contaminated and non-contaminated soil in compared to the non-inoculated plants (Table 4). Concerning to mycorrhizal plants grown at high Cd concentration, ACP activity was 0.120, 0.278 and 0.398 μ mole PNP after the three growth periods (3, 6 and 9 weeks) respectively. While at low Cd concentration activity was 0.165, 0.350 and 0.470 μ mole PNP. Similar trend was obtained with Pb application. Although the results recorded significant increase in the activity of ALP in inoculated plants grown at low Cd concentration was 27.14%, while at high Cd concentration was 42.08% in comparison with non-inoculated plants grown at the same concentrations after 9 weeks from planting. In addition, it has been observed that the inhibition of phosphatase activity depends on these concentrations (Zheng, *et al.*, 2009). This increment in phosphatase activities with the time of growth in inoculated plants led to increase in phosphate translocation to leaves (Selvaraj, 1998; Ezawa *et al.*, 2001). Fries *et al.*, (1998) showed the considerable increase in ACP and ALP activities in maize roots were closely correlated with the levels of VAM colonization in roots. Such response has also been observed by Tschkova and Galabova, (2003) in other inoculated plants under heavy metals stress and they suggested the inoculation as a possible process of detoxification and resistance which in divergent with the present results.

TABLE 4. Acid (ACP) and alkaline phosphatase (ALP) activities of non-inoculated (NM) or inoculated (M) maize plants with VAM grown on heavy metals (Cd or Pb) concentrations.

Treatments (mg/kg)	Micro-symbiotic	3 week		6 weeks		9 weeks	
		ACP (μmole PNP/h/g fw)	ALP (μmole PNP/h/g fw)	ACP (μmole PNP/h/g fw)	ALP (μmole PNP/h/g fw)	ACP (μmole PNP/h/g fw)	ALP (μmole PNP/h/g fw)
Control	NM	0.140c	0.105c	0.288d	0.218c	0.396d	0.344c
	M	0.223a	0.134a	0.396a	0.307a	0.537a	0.424a
Cd 25	NM	0.118e	0.087e	0.259f	0.157g	0.357e	0.291e
	M	0.165b	0.124b	0.350b	0.263b	0.470b	0.370b
Cd100	NM	0.109f	0.056h	0.239h	0.115i	0.309g	0.221h
	M	0.120e	0.092de	0.278e	0.208d	0.398d	0.314d
Pb100	NM	0.108f	0.064g	0.218i	0.131h	0.338f	0.251g
	M	0.133d	0.096d	0.306c	0.199e	0.426c	0.352c
Pb1000	NM	0.096g	0.034i	0.216j	0.110i	0.312g	0.216h
	M	0.108f	0.077f	0.247g	0.173f	0.386d	0.277f
LSD 0.05		0.005	0.005	0.008	0.008	0.011	0.009

Different letters within the same column indicate a significant difference, n=3.

Conclusion

In conclusion, the results recorded that the vesicular-arbuscular mycorrhiza (VAM) alleviates the detrimental effect of heavy metals through improved the phosphatase activities and the nutrients uptake include P. This result indicates that the effect of VAM fungi on phosphorus uptake constitutes one of the main mechanisms for increasing plant adaptation to heavy metals. These physiological changes may be correlated to the enhancement of the rate of biosynthesis of some molecules in mycorrhizal plants like photosynthetic pigments, protein and carbohydrates.

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(Received 5 /1/2015;
accepted 12/4/2015)

دور فطريات الميكوريزا في تحسين تحمل نبات الذرة الشامية للعناصر الثقيلة.

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مركز البحوث الزراعية.

تهدف هذه الدراسة الي تقييم التغيرات الفسيولوجية الناتجة عن تلقیح نبات الذرة بفطر الميكوريزا المعزول من تربة ملوثة بالعناصر الثقيلة والمنزوع بتربة ملوثة بالكادميوم أو الرصاص. ولقد سجلت النتائج زيادة معنوية في السكريات الذائبة والمحتوي البروتيني والكلورفيل في النبات الملحق بفطر الميكوريزا النامي في تربة ملوثة بالكادميوم أو الرصاص بالمقارنة بالنباتات غير الملحقة والنامية في نفس التركيزات. وكذلك سجلت النتائج زيادة معنوية في نشاط انزيم الفوسفاتيز الحامضي والقلوي في نبات الذرة الملحق بفطر الميكوريزا النامي في تربة ملوثة مقارنة غير الملحقة تحت نفس الظروف. ولقد أظهرت هذه الدراسة مدي فاعليت فطر الميكوريزا علي زيادة قدرة نبات الذرة التغلب علي التأثير الضار للعناصر الثقيلة وهذا راجع تحسين امتصاص العناصر الهامه لنمو النبات مثل الفسفور والنتروجين. إذا استخدام فطر الميكوريزا المعزول من تربة ملوثة بالعناصر الثقيلة يعتبر تكنيك ناجح يعمل علي تحسين تحمل النبات لهذه العناصر.