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RESEARCH ARTICLE

Mycorrhiza influence on maize development under Cd stress and P supply

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The role of arbuscular mycorrhizal fungi (AMF) on cadmium (Cd) accumulation and on the possible attenuation of Cd stress was studied in maize plants (*Zea mays* L. var. Exceller). Plants inoculated or not with Glomus macrocarpum were exposed to Cd (0-20 μmol L^{-1}), at two P levels (5 and 10 mg L^{-1}) in the nutrient solution. The experiment was conducted in a hydroponic system, using a randomized 2 x 2 x 2 factorial design. The mycorrhiza-Cd interaction on plant growth, nutrients and Cd accumulation, AMF root colonization and on extra-radical mycelium was investigated. Mycorrhiza promoted plant growth whereas Cd addition reduced plant biomass production. No difference in plant Cd concentrations was found between mycorrhizal (M) and non-mycorrhizal (NM) plants, where Cd accumulated mainly in roots. In general, roots showed a slightly higher Cd concentration in the cell wall than in the cytoplasmic fraction, with M roots presenting 26% more Cd in the cell wall fraction than NM roots. Mycorrhizal plants showed higher P/Cd, N/Cd and S/Cd ratios in shoots and roots compared to NM plants. Mycorrhizal colonization and the length of extra-radical mycelium were diminished by Cd addition, the reduction being more pronounced under high-P supply. Addition of Cd induced guaiacol peroxidase (GPOX) activity in roots; however, M plants, in addition to the higher root protein contents, showed no induction of GPOX activity in the presence of Cd, suggesting higher tolerance to Cd. It is concluded that Cd affected mycorrhizal symbiosis by decreasing root colonization and the development of the extra-radical mycelium. Nevertheless, the higher growth and nutrients/Cd ratios observed in M plants indicate an efficient symbiosis capable of alleviating Cd stress.

Key words: arbuscular mycorrhizal fungi, Cd distribution, extra-radical mycelium, heavy metals, peroxidase, phosphorus

Micorriza influencia o desenvolvimento do milho sob estresse de Cd e suprimento de P: A influência dos fungos micorrízicos arbusculares (FMA) no acúmulo e na atenuação potencial do estresse causado pelo Cd foi estudada em plantas de milho (*Zea mays* L. var. Exceller). Plantas associadas ou não com o FMA *Glomus macrocarpum* foram expostas ao Cd (0-20 mmol L^{-1}) em dois níveis de P (5 e 10 mg L^{-1}), em solução nutritiva. O experimento foi conduzido em sistema hidropônico, com delineamento totalmente casualizado, em esquema fatorial 2 x 2 x 2. Avaliou-se o efeito da interação micorriza-Cd sobre o crescimento da planta, o acúmulo de Cd e de nutrientes, a colonização micorrízica da raiz e a produção de micélio extra-radical. A micorriza promoveu o crescimento da planta enquanto a adição de Cd reduziu a produção de biomassa vegetal. Não foram observadas diferenças nas concentrações de Cd de plantas micorrizadas (M) e não-micorrizadas (NM), acumulando-se o Cd principalmente nas raízes. Em geral, as raízes mostraram maior concentração de Cd na fração da parede celular do que na citoplasmática, sendo que raízes de plantas M apresentaram quantidade de Cd 26% maior na fração da parede celular do que raízes de plantas NM. As plantas M mostraram maiores relações P/Cd, N/Cd e S/Cd na parte aérea de que plantas NM. A colonização micorrízica e a quantidade de micélio extra-radical produzido pelo FMA inoculado diminuíram pela adição de Cd, sendo a diminuição mais pronunciada em plantas com maior suprimento de P. A adição de Cd induziu a atividade da guaiacol peroxidase (GPOX) nas raízes; no entanto, plantas M, além de apresentarem maiores conteúdos de proteína nas raízes, não mostraram indução da GPOX na

presença de Cd, sugerindo maior tolerância ao metal. Conclui-se que o Cd afetou a simbiose micorrízica diminuindo a colonização radicular e o desenvolvimento do micélio extra-radical. No entanto, o maior crescimento e relações nutrientes/Cd observados em plantas M indicam uma simbiose eficiente capaz de atenuar o estresse causado pelo Cd.
Palavras-chave: absorção de Cd, fósforo, fungos micorrízicos arbusculares, metais pesados, micélio extra-radical, peroxidase

INTRODUCTION

Cadmium is a potentially toxic metal and thus its transfer from plants to humans is of great concern. This heavy metal is ranked number seven among the top toxins, mainly due to its negative influence on the cell's enzymatic systems (ATSDR, 1999), and it has been estimated that 70% of the Cd intake by humans comes from plant foods (Wagner, 1993). The critical Cd level in nutrient solution for conventional crop plants is reported to be 8 μmol L^{-1} (Yang et al., 1995). Nevertheless, cereals such as maize, rice and barley tolerate Cd as much as 100 μmol L^{-1} (Inouhe et al., 1994). In Cd-enriched soils, plants may accumulate 20 mg Cd kg^{-1} in their shoots. In the case of maize, 23 mg Cd kg^{-1} in the shoot dry weight is considered the limit of phytotoxicity relevant for production (Klein et al., 1981). However, some studies reported the ability of maize plants to accumulate metal ions and showed their relative tolerance to excessive soil metal concentrations (Florijn et al., 1993). Cadmium is taken up through the roots and accumulated mainly in this organ, but it can be also translocated to shoots, grains or fruits (Page et al., 1981). Maize roots have a great ability to retain Cd (Nocito et al., 2002), a feature that is largely desirable in the context of the food chain, thereby avoiding translocation of high quantities of Cd to the aerial part. In plants, Cd may cause a variety of toxic effects, from morphological and physiological disturbances to disorders at the enzymatic and molecular levels (Benavides et al., 2005). It may particularly damage the photosynthetic apparatus and influence mineral nutrition causing nutritional disturbances that may interfere with other metabolic processes. Cadmium also induces the production of reactive oxygen species affecting important macromolecules and modifying the activity of enzymes related to the antioxidant defense system. Among such enzymes, glutathione reductase, catalases and peroxidases play an important role in the control of reactive oxygen species levels in cells (Gratão et al., 2005).

Environmental and health problems caused by excessive accumulation of metals in soils are leading to the search for new technologies for soil remediation (Salt et al., 1998). Screening for heavy metal-tolerant plants and plants able to accumulate metals in their tissues is, nowadays, of great scientific concern. The alleviation of plant stress caused by excessive concentrations of heavy metals by mycorrhizal fungi has been reported (Riveira-Becerril et al., 2002; Shen et al., 2006). Immobilization of metals in the fungal biomass is proposed as a mechanism by which these fungi may increase plant tolerance to heavy metals. Mycorrhizal roots may act as a barrier against metal transport, reducing metal transfer and enhancing root/shoot Cd ratios. This effect is attributed to metal adsorption on hyphal walls, since chitin has an important metal-binding capacity (Joner et al., 2000). Recently, it has been suggested that glomalin, a glycoprotein produced by arbuscular mycorrhizal fungi (AMF), may have a metal chelating function diminishing metal availability for plants (Gonzalez-Chavez et al., 2004). Another possible mechanism of metal tolerance includes dilution of metal concentrations in plant tissues due to the promotion of plant growth by AMF. The AMF associated with metal-tolerant plants may contribute to the accumulation of heavy metals in roots in a non-toxic form inside hyphal cell walls or complexed to phosphate materials inside the cells (Galli et al., 1995). Maize, a universal host for AMF and high biomass-producing plant, shows relative tolerance to excess of metals in soils, features that could make this plant a good option for soil remediation (Jiang et al., 2001; Jurkiewicz et al., 2004).

The aim of this study was to investigate mycorrhiza effects on Cd accumulation and developmental processes in maize plants in response to Cd addition. It is hypothesized that mycorrhization may contribute to Cd stress alleviation by improving nutritional status. The effect of Cd addition on AMF intra and extra-radical development is also discussed.

MATERIAL AND METHODS

Experimental design: A pot experiment was conducted under greenhouse conditions at the Instituto Agronômico, Campinas, southeastern Brazil, using hydroponic culture. The experimental design was completely randomised in a 2 x 2 x 2 factorial scheme, with seven replicates. The treatments were composed of two Cd (0 and 20 μmol L^{-1}) and two P (5 and 10 mg L^{-1}) concentrations in the nutrient solution, and inoculation or not with AMF.

AMF inoculum: The AMF used was *Glomus macrocarpum* (Tul. and Tul.) strain IAC-50 and propagated on stock cultures with *Brachiaria brizantha* Stapf for six months. The original spores arose from a non-contaminated soil. Colonized root fragments, mycelium and a sand-soil mixture containing spores were used as inoculum. Each pot received approximately 1,500 spores, at the time of sowing. The non-mycorrhizal treatments received washings of the soil-inoculum mixture filtered through Whatman n°42 filter paper.

Pot culture experiment: Maize (*Zea mays* L. var. Exceller) seeds were surface-disinfected with 1:3 (v/v) of 2.5% sodium hypochlorite solution for 10 min and then rinsed several times with sterilized water. Seed plants were germinated in trays containing sterilized ground quartz silica (2-3 mm) with and without AMF inoculum. Two liters of sterilized silica sand were placed in 2.70 L plastic pots. After 20 d in the trays, two plants were transplanted to each pot, and a week later, one plant was left per pot. Maize plants were cultivated in a hydroponic system with silica and irrigated with complete nutrient solution (N-NO_3 154.6; N-NH_4 19.5; S-SO_4 18.7; Ca 151.2; K 70.9; Mg 18.8; B 0.53; Fe 1.99; Mn 0.97; Cu 0.076; Zn 0.3; Mo 0.15 mg L^{-1}) (Furlani and Furlani, 1988), with P and Cd concentrations adjusted for each treatment. Cadmium was supplied as $Cd(NO_3)_2$ and P as KH_2PO_4. Speciation calculations using Visual MINTEQ ver. 2.23 (Gustafsson, 2003) indicated that 82 and 75% of the Cd in solution was the free Cd^{2+} ion, for solutions with 5 and 10 mg L^{-1} of P, respectively. The total volume of nutrient solution added to each pot during the experiment was of 2.850 L corresponding to 6.406 mg of Cd added cumulatively in Cd-treated plants, resulting in a concentration of environmental relevance and in the range found in Cd-polluted soils (Sanità di Toppi and Gabrielli, 1999). Plants were harvested after 70 d at the flowering stage. Day and night temperatures ranged between 29°C and 16°C respectively, with a photoperiod of 12h and an irradiance of about 1200 μmol quanta m^{-2} s^{-1}.

Analytical methods: Shoots and roots were separated at harvest. Shoots were washed in distilled water, dried (60°C for 76 h), weighed and ground for chemical analysis. Some of the root material was kept for mycorrhizal colonization determination, some stored in liquid nitrogen until use in the enzymatic assay and some dried and ground. Concentrations of P, K, Ca, Mg, S, Cu, Fe, Mn, Zn, and Cd in plant shoots and roots were determined, after dry-digestion with HNO_3 and $HClO_4$, by inductively coupled plasma-optical emission spectroscopy. Total-N in the digest was determined by Kjeldahl analysis (Bremner, 1965).

Mycorrhizal colonization was evaluated by the grid-line intersected technique (Giovannetti and Mosse, 1980) by first clearing the roots with 25 mg KOH L^{-1}, followed by root acidification in 1% HCl and staining with 0.05% trypan blue. The length of the extra-radical mycelium (ERM) of AMF in the substrate was estimated according to Boddington et al. (1999) and Melloni and Cardoso (1999). The ERM was extracted from 20 g of substrate by flotation in 1 L of tap water, blended, sieved (45 μm) and filtered onto a nitrocelullose membrane filter (0.45 μm). The extracted ERM was stained with 0.05% trypan blue in lactoglycerol and the total length of ERM assessed under light microscope using an ocular at 125x magnifications. Sixty-four fields were counted and the results expressed as m of mycelium in 1 g of dry substrate.

Fractionation of root cytoplasm and cell wall for determination of Cd content in each fraction was performed according to Inouhe et al. (1994) with modifications. Roots frozen in liquid nitrogen (0.5-1.0 g FW) were homogenized using a mortar and pestle in 20 mmol L^{-1} Tris-HCl buffer (pH 7.8). The homogenates were centrifuged twice at 1,500 *g* for 10 min and at 10,000 *g* for another 10 min. The resulting supernatant was collected and represented the cytoplasmic fraction. The precipitate in the tube was collected, dried at 37-40°C and represented the cell wall fraction. Cadmium concentration

in the cytoplasmic fraction was determined directly by flame atomic absorption spectrometry (F-AAS). The dried cell wall fraction was hydrolyzed with 2 mL of 65% HNO_3 for 20 min at 100ºC. Four drops of 70% w/w $HClO_4$ were added to the hydrolysates and further hydrolyzed for 15 min at 100ºC. After this process, three drops of 30% H_2O_2 were added, the mixture maintained at 100ºC for 15 min and finally diluted to 5 mL with deionized water. Cadmium content was determined by F-AAS.

Acid phosphatase (APase) (EC3.1.3.2) activity in leaf was determined "*in vivo*" according to Besford (1980). Sixty days after transplanting, 0.1 g of fresh tissue was taken from the youngest fully-expanded leaves and incubated with *p*-nitrophenylphosphate (p-NP) in 0.1 mol L^{-1} sodium acetate buffer (pH 4.0) for 20 min at 30ºC. The *p*-nitrophenol formed was measured spectrophotometrically at 410 nm. Activity of APase was expressed in µg *p*-NP g^{-1} FW h^{-1}.

Guaiacol peroxidase (GPOX) (EC1.11.1.7) activity was assayed spectrophotometrically using a diode array spectrophotometer (Hewlett Packard, 8452 A Palo Alto, CA, USA) according to Boscolo et al. (2003). Roots frozen in liquid nitrogen (0.1-0.05 g) were washed three times in deionized water and homogenized in 50 mmol L^{1} phosphate buffer (KH_2PO_4/K_2HPO_4, pH 6.8). Homogenates were centrifuged at 10,000 *g* for 8 min and the supernatant used immediately to determine peroxidase activity. The reaction mixture contained 500 µmol L^{-1} phosphate buffer, 8 mmol L^{-1} guaiacol, 8 mmol L^{-1} H_2O_2 and protein extract. The increase in absorbance was recorded at 470 nm (extinction coefficient, $\varepsilon = 26.6$ mmol L^{-1} cm^{-1}). Total soluble proteins in each extract were determined using the Bio-Rad protein assay (Bradford, 1976). Determinations of enzyme activity were performed in duplicate using five roots per treatment.

The nutrient and Cd translocation indexes (TI) were calculated as the percentage of the total amount of nutrient/Cd absorbed that was translocated to the shoots. Cadmium transfer index (TF) was calculated as the ratio of Cd concentration in the plant tissues (shoot or root) to that in the solution. All data were processed by an analysis of variance (three-way ANOVA). Significant treatment effects were determined by the Tukey test (α= 0.05). Data expressed as a percentage were transformed to arcsin-square root values prior to statistical analysis.

RESULTS

Cadmium addition decreased shoot and root biomass production. In contrast, AMF inoculation and the higher P supply in the nutrient solution favored plant growth (Table 1). Mycorrhizal (M) plants showed 6.5% and 22% higher shoot and root biomass production, respectively, than non-mycorrhizal (NM) plants. Plants under high P solution showed 1.6 and 1.3 times more shoot dry mass and root fresh mass, respectively, than plants under low-P solution (Table 1). Root colonization and AMF extra-radical mycelium (ERM) length decreased dramatically by Cd addition and P supply (Table 1).

No significant difference was found between M and NM plants for tissue Cd accumulation, Cd translocation index (TI) or Cd transfer factor (TF) (Table 2). Cadmium-treated plants showed less shoot and root accumulation of Cd at the higher P supply in the nutrient solution and M plants had less Cd in the tissues. Distribution of Cd in root cells showed differences between cell wall and cytoplasmic fractions, with 56% of Cd in the cell wall and 44% in the cytoplasmic fraction. In general, M roots accumulated 26% more Cd in the cell wall fraction than NM roots, reaching 32% more under low P (Figure 1). Concentration of P in the solution did not influence Cd distribution in root cells, except for non-mycorrhizal plants, which showed lower Cd concentrations in both cell fractions. Cadmium retained in each of the root fractions showed a high positive correlation with total Cd concentration in shoots ($r = 0.872$, $P < 0.001$) and roots ($r = 0.889$, $P < 0.001$) and a negative correlation with biomass production ($r = -0.402$, $P < 0.01$).

Mycorrhizal plants showed higher concentrations of Mg and S in shoots and S, Cu and Fe in roots than NM plants, but they had a lower K and Mn concentrations in shoots and roots (Table 3). Cadmium addition decreased shoot N, Cu and Mn and root N, Mg and Zn concentrations, but increased shoot P, S and Zn and root K, S and Mn concentrations. Cadmium addition increased shoot S and Cd concentrations, but decreased the shoot levels of all other nutrients (Table 4). Mycorrhizal plants showed higher concentrations of N, P, S, Ca, Mg, Fe and Zn in shoots and lower Mn concentration. At high P level there were higher shoot nutrient concentrations. Mycorrhizal plants also showed higher P/Cd, N/Cd and S/Cd ratios in shoots and roots than the NM counterparts

Table 1. Shoot dry weight (SDW), root fresh weight (RFW), root mycorrhizal colonization (Myc. Col.) and arbuscular mycorrhizal fungi extra-radical mycelium length (ERM) in maize plants, inoculated (M) or not (NM) with *G. macrocarpum*, and treated with 0 and 20 µM Cd L^{-1} at two (5 and 10 mg L^{-1}) P concentrations in the nutrient solution. Means with the same letter are not significantly different ($P > 0.05$) by the Tukey test. Lower case letters are used to compare treatments with and without mycorrhizal inoculation for each Cd and P concentration, and upper case letters to compare between Cd treatments at each P concentration. * Significant difference between P levels for each Cd and mycorrhizal treatments.

Treatments		**SDW** (g)		**RFW** (g)		**Myc. Col.** (%)	**ERM** (mg^{-1})
Cd	P	NM	M	NM	M	M	M
0	**5**	7.3 a A	7.7 a A	25.8 a A	27.8 a A	44.5 A	5.11 A *
0	**10**	12.3 b A *	13.4 a A *	31.6 b A *	35.2 a A *	42.8 A	2.27 A
20	**5**	6.2 a B	6.4 a B	18.5 b B	22.3 a B	26.6 B *	2.23 B
20	**10**	9.3 b B *	10.3 a B *	27.3 a B *	30.5 a B *	13.1 B	1.89 A

Table 2. Cadmium concentration in shoots and roots, total Cd absorbed, Cd translocation index (TI) and transfer factor ($TF_{sol\text{-}shoot}$) in maize plants inoculated (M) or not (NM) with *G. macrocarpum*, and treated with 0 and 20 µmol Cd L^{-1} at two (5 and 10 mg L^{-1}) P concentrations in the nutrient solution. Statistics as in Table 1.

Treatments		**Cd shoots** mg kg^{-1}		**Cd roots** mg kg^{-1}		**Total Cd** mg kg^{-1}		**TI**		**$TF_{SOL\text{-}SHOOT}$**	
Cd	P	NM	M	NM	M	NM	M	NM	M	NM	M
0	**5**	0.14 aB	0.14 aB	3.30 aB	3.02 aB	3.4 a	3.1 a	—	—	—	—
0	**10**	0.24 aB	0.24 aB	1.78 aB	1.76 aB	2.0 a	2.0 a	—	—	—	—
20	**5**	29.5 aA*	30.5 aA*	369 aA*	355 aA*	399 a	385 b	7.2	7.1	10.5	11.3
20	**10**	20.1 aA	16.9 aA	275 aA	253 aA	295 a	269 b	—	—	—	—

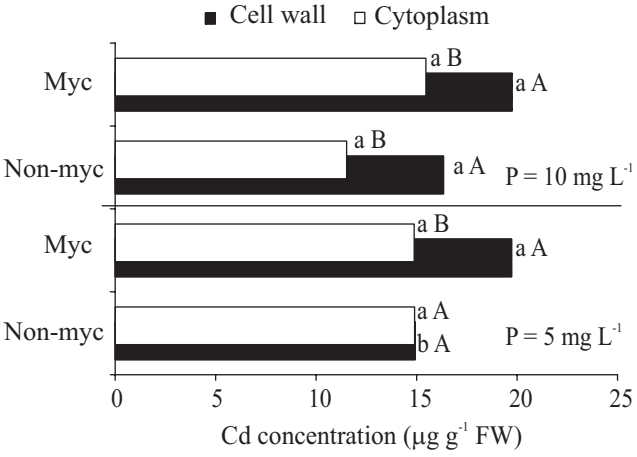

Figure 1. Cadmium distribution in cytoplasm and cell wall fractions of mycorrhizal and non-mycorrhizal maize roots treated with 20 µmol Cd L^{-1}, at two P concentrations in the nutrient solution. Means with the same letter are not significantly different ($P > 0.05$) by the Tukey test. Lower case letters are used for comparisons of mycorrhizal treatments in each cell fraction and P concentration, and upper case letters for comparison between cell fractions for each mycorrhizal treatment and for each P concentration.

(Table 5) in both P concentrations, but they were higher at the higher P level in the nutrient solution.

The APase activity in leaves was influenced by the presence of mycorrhiza since M plants had lower APase activity than NM plants (Figure 2) without Cd stress. Plants at the low P level showed higher APase activity than plants under high P. Cadmium addition increased the foliar APase activity only in plants under low P (Figure 2).

In general, Cd addition increased GPOX activity by 14% in roots in relation to non-Cd-treated plants (Figure 3). However, the increase of GPOX activity was statistically significant in NM roots at low P concentration, where it was 53% higher in relation to M plants in response to Cd addition (Figure 3). In the presence of Cd at high P concentration, M and NM plants showed a different GPOX activity pattern, being 30% higher in M plants. The GPOX activity was positively correlated with Cd concentration in roots ($r = 0.626$, $P < 0.001$) and shoots ($r = 0.671$, $P < 0.001$).

Mycorrhization increased root protein concentration by 30% but the total soluble protein in M roots decreased 24% due to Cd addition under low P (Figure 3). Protein

Table 3. Shoot and root nutrient concentrations of mycorrhizal (M) and non-mycorrhizal (NM) maize plants treated with 0 and 20 µmol Cd L^{-1} at two (5 and 10 mg L^{-1}) P concentrations in the nutrient solution (* *F* test, ns = non-significant).

Treatments		AMF	N	P	K	S	Ca	Mg	Cu	Fe	Mn	Zn
Cd	P		g kg^{-1}						mg kg^{-1}			
	Shoot											
0	5	NM	16.92	0.62	18.63	2.76	5.34	2.50	6.70	107	48.2	21.1
0	5	M	16.24	0.58	15.40	3.68	6.0	3.12	4.46	130	39.8	21.6
0	10	NM	15.38	0.7	12.60	4.06	4.24	2.88	4.02	119	36.0	18.7
0	10	M	14.92	0.7	12.80	4.66	4.86	3.06	3.18	120	31.4	16.2
20	5	NM	13.88	0.66	17.65	5.62	5.08	2.32	3.16	129	44.2	21.7
20	5	M	14.47	0.66	16.18	6.78	5.04	2.64	3.10	132	28.4	25.8
20	10	NM	14.28	0.74	14.20	6.70	4.56	2.68	3.42	103	40.4	20.4
20	10	M	14.46	0.78	13.60	6.50	4.62	3.04	3.50	129	25.2	19.2
	Significance*											
	Cd		0.001	0.05	ns	0.001	ns	ns	0.001	ns	0.05	0.05
	P		0.05	0.001	0.001	0.001	0.01	0.05	0.05	ns	0.01	0.01
	AMF		Ns	ns	0.09	0.001	ns	0.01	0.05	ns	0.001	ns
	Cd x AMF		Ns	ns	ns	ns	ns	ns	0.05	ns	0.05	ns
	Cd x P		0.05	ns	ns	0.01	ns	ns	0.01	ns	0.05	ns
	P x AMF		Ns	ns	ns	0.01	ns	ns	ns	ns	ns	ns
	Cd x AMF x P		Ns	ns	ns	0.05	ns	ns	ns	ns	ns	ns
	Root											
0	5	NM	17.4	0.46	8.32	2.60	7.08	2.27	15.7	481	90.4	35.8
0	5	M	18.9	0.66	7.10	3.18	10.22	1.83	18.2	832	54.2	31.8
0	10	NM	15.5	0.56	5.50	2.40	8.23	2.42	10.0	572	53.2	21.1
0	10	M	16.4	0.62	3.64	2.64	7.69	3.20	10.9	628	37.2	19.3
20	5	NM	13.4	0.50	8.90	3.22	7.58	1.76	12.0	777	166	14.7
20	5	M	15.4	0.56	8.72	3.44	9.18	1.66	14.5	710	54.4	18.5
20	10	NM	14.4	0.58	6.30	2.92	11.23	2.84	11.8	619	93.2	15.5
20	10	M	16.0	0.58	4.50	3.04	7.94	2.84	13.1	595	25.6	15.8
	Significance											
	Cd		0.001	ns	0.01	0.01	ns	0.01	ns	ns	0.01	0.001
	P		Ns	ns	0.001	0.05	ns	0.001	0.01	0.01	0.001	0.001
	AMF		0.01	0.03	0.001	0.05	ns	ns	ns	0.001	0.001	ns
	Cd x AMF		Ns	ns	ns	ns	ns	0.01	ns	0.05	0.001	ns
	Cd x P		0.01	ns	ns	ns	ns	0.01	0.01	ns	ns	0.001
	P x AMF		Ns	ns	0.05	ns	0.01	0.001	ns	0.01	0.05	ns
	Cd x AMF x P		Ns	ns	ns	ns	ns	ns	ns	0.05	ns	ns

concentration in roots was reduced 14% in plants under high P (Figure 3). Protein concentration was negatively correlated with root Cd concentration ($r^2 = -0.440$, $P < 0.05$) and positively with root P concentration ($r^2=0.402$ $p<0.05$), AMF colonization ($r^2 = -0.440$, $P < 0.01$) and mycorrhizal ERM amount ($r^2 = 0.440$, $P < 0.05$).

DISCUSSION

Cadmium caused a decrease in plant growth due to various effects on plant metabolism; this is an expected response since Cd is a relatively mobile metal in plants that may cause a general decrease in plant growth (Hernandez and Cooke, 1997). Inoculation with AMF alleviated this effect and enhanced plant growth even in the presence of Cd, confirming the important potential of AMF in revegetation of metal contaminated soils and remediation practices. High plant growth is a key factor for phytoremediation success of metal contaminated sites (Gorhe and Paszkowski, 2006). The stimulatory effect of AMF inoculation on the development of metal-treated plants was also observed for maize, soybean, pea

Table 4. Shoot nutrients contents (concentration x dry mass) of mycorrhizal (M) and non-mycorrhizal (NM) maize plants treated with 0 and 20 µmol Cd L^{-1} at two (5 and 10 mg L^{-1}) P concentrations in the nutrient solution. (*F test; ns = non significant).

Treatments		FMA	N	P	K	S	Ca	Mg	Cd	Cu	Fe	Mn	Zn
Cd	P		(mg plant^{-1})							(µg plant^{-1})			
0	5	NM	120.5	4.44	131	19.6	37.8	17.8	0.99	38.4	768	316	150
0	5	M	128.5	4.78	124	29.1	47.4	24.5	1.12	35.3	1031	383	171
0	10	NM	188.5	8.26	153	49.9	59.3	35.5	3.04	46.6	1475	443	228
0	10	M	199.1	9.34	171	62.2	64.6	40.9	3.17	42.5	1600	418	236
20	5	NM	85.75	3.92	109	34.8	31.3	14.3	197	18.9	717	274	134
20	5	M	88.94	4.16	110	41.8	31.1	16.3	183	19.1	812	212	158
20	10	NM	137.1	6.60	135	60.1	43.6	25.5	193	33.1	978	393	192
20	10	M	153.2	8.28	143	68.8	48.8	32.1	178	37.0	1377	267	203
Significance*													
Cd			0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.01	0.001	0.01
P			0.001	0.001	0.001	0.001	0.001	0.001	N.S	0.001	0.001	0.01	0.001
FMA			0.01	0.01	N.S	0.001	0.05	0.001	N.S	N.S	0.01	0.01	0.08
Cd x FMA			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cd x P			ns	ns	ns	0.05	ns	ns	ns	0.05	0.07	ns	ns
P x FMA			ns	0.05	ns	ns	ns	ns	ns	ns	ns	ns	ns
Cd x FMA x P			ns	ns	ns	ns	ns	ns	ns	ns	0.08	ns	ns

Table 5. P-, S- and N-to-Cd ratios in shoots and roots of Cd-treated mycorrhizal (M) and non-mycorrhizal (NM) maize plants at two (5 and 10 mg P L^{-1}) P concentrations in the nutrient solution. Means with the same letter are not significantly different ($P > 0.05$) by the Tukey test. Lower case letters are used to compare M and NM plants for each P concentration, and upper case letters to compare P treatments for each mycorrhizal status.

Treatment		Nutrient/Cd ratio in shoots			Nutrient/Cd ratio in roots		
AMF	P	P/Cd	S/Cd	N/Cd	P/Cd	S/Cd	N/Cd
NM	5	21.6 a A	193 b A	478 a A	1.3 b A	8.63 a B	34 b A
M	5	23.8 a A	246 a A	510 a A	1.56 a A	9.65 a A	44 a A
NM	10	33.8 b B	312 b B	718 b B	2.06 b B	10.32 b B	53 b B
M	10	46.9 a B	391 a B	870 a B	2.30 a B	12.34 a B	73 a B

and sunflowers plants (Riveira-Becerril et al., 2002; Andrade et al., 2004, 2008; Jurkiewicz et al., 2004).

High heavy metal concentrations may reduce intra-radical mycorrhizal colonization (Liao et al., 2003; Andrade et al., 2004) as observed in the present investigation in which Cd severely reduced mycorrhizal colonization of maize, especially in plants with high-P supply. In addition, the lower amount of mycorrhizal ERM in Cd-treated plants showed the deleterious effect of this metal in the development of extra-radical mycelium. Nevertheless, mycorrhizal colonized plants and metal-tolerant fungal ecotypes have been found in metal contaminated soils (Hildebrandt et al., 2007), and so they may have higher potential for phytoremediation purposes than non-metal adapted ecotypes. Recently, Nogueira and Cardoso (2007) observed a relationship between intra-radical colonization, extra-radical mycelium length and mycorrhizal effectiveness which may be mutually affected by P availability. Our data corroborate these findings since we observed a positive correlation ($r^2 = 0.571$, $P < 0.003$) between root colonization and the amount of extra-radical mycelium, showing the relation between fungal intra-radical colonization rate and its extra-radical development.

The ability of maize roots to retain Cd has been already documented (Nocito et al., 2002). Maize plants accumulated Cd mainly in root and less than 10% of absorbed Cd was translocated to the shoots, showing the

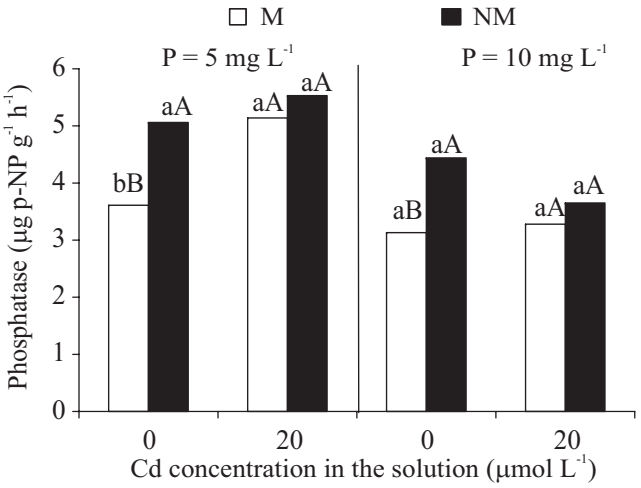

Figure 2. Acid phosphatase activity [mg *p*-nitrophenylphosphate (p-NP) g^{-1} h^{-1}] in maize leaves, inoculated or not with *G. macrocarpum*, and at two Cd and P concentrations in the nutrient solution. Means with the same letter are not significantly different ($P > 0.05$) by the Tukey test. Lower case letters are used for comparisons of treatments between mycorrhizal (M) and non-mycorrhizal (NM) plants for each Cd and P concentration, and upper case letters for comparison between Cd treatments for each mycorrhizal treatment and P concentration.

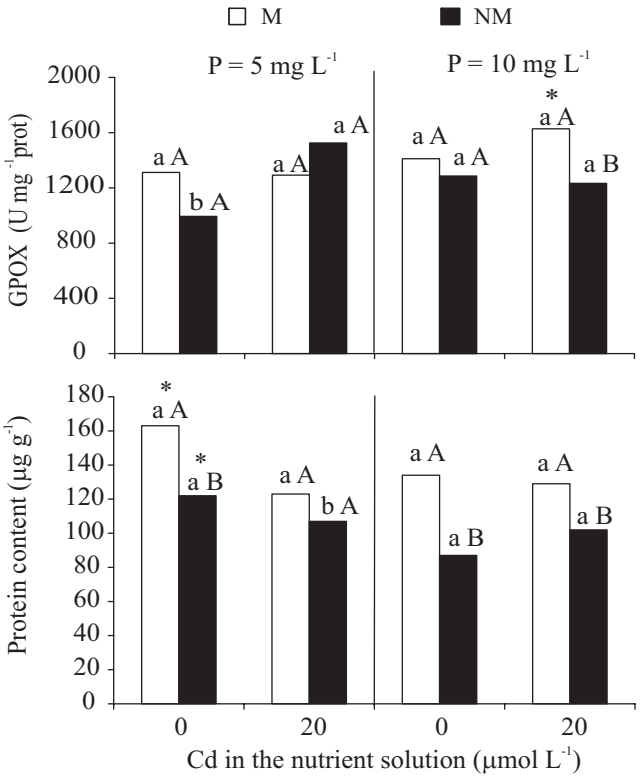

Figure 3. Guaiacol peroxidase (GPOX) activity and total soluble protein concentrations in mycorrhizal (M) and non-mycorrhizal (NM) maize roots in two Cd and P concentrations in the nutrient solution. Means with the same letter are not significantly different ($P > 0.05$) by the Tukey test. Upper case letters are used for comparing M and NM plants for each Cd and P concentrations, and lower case letters for comparing Cd treatments for each P level and mycorrhizal status; * Significant difference between P levels for each Cd and mycorrhizal treatments.

high Cd-retention capability in both M and NM roots. This feature is of interest from the point of view of the food chain, avoiding Cd accumulation in shoots, but undesirable for phytoextraction practices, where the aim is for higher metal concentration in shoots than in roots.

Plant roots may accumulate Cd in the apoplast by ionic interactions with cell wall components (Cohen et al., 1998). Maize roots accumulated similar Cd^{2+} concentrations in cell wall and cytoplasm fractions independently of the mycorrhizal condition, with a slightly higher proportion in the cell wall fraction. These results are comparable to those observed by Inouhe et al. (1994), who reported that cereal roots accumulated Cd almost equally in both fractions. The AMF extra-radical hyphae can transport Cd from soils to host roots (Joner and Leyval, 1997), modifying metal uptake by plants and influencing plant responses towards excessive metal concentrations (Joner and Leyval, 2001). Mycorrhizal maize roots showed, in general, higher Cd levels bound to the cell wall fraction. This Cd-binding to cell wall components may be seen as a barrier mechanism whereby plants may reduce Cd concentration in shoots (Lozano-Rodriguez et al., 1997). In addition, M plants may increase root/shoot Cd ratios protecting shoots from excessive metal concentrations (Joner et al., 2000), suggesting that AMF hyphae act as a barrier in metal transport by adsorption of the metal on hyphal walls. M plants had a lower Cd transfer factor value from the nutrient solution to the shoots and a lower total Cd concentration in the plant, indicating that AMF could somehow be efficient in avoiding Cd uptake from the solution. Nevertheless, since there were no differences in shoot Cd concentrations between M and NM plants, it appears that extra-radical mycelium of *G. macrocarpum* does not alter the lower Cd translocation (IT) to the shoots.

Excessive Cd concentrations can induce deficiencies and imbalances of plant mineral nutrients (Greger and Lindberg, 1987). Cd reduced shoot N, P, K, Ca and Mg contents as well as some essential micronutrient contents. In contrast, Cd addition caused an increase in shoot and root S concentrations. Increases in sulphate uptake as a direct effect of Cd accumulation in roots, due to a higher sulphate influx in Cd-treated roots could be an adaptive response to support S demand for phytochelatin biosynthesis, an important group of Cd-chelating peptides involved in cell Cd detoxification (Nocito et al., 2002).

Mycorrhizal plants showed higher N, P, Ca, Mg and S contents in shoots than NM plants but they had a lower K concentration in shoot, probably due to a dilution effect caused by a higher biomass production. The AMF inoculation had a great influence on the ratio of P, N and S to Cd, in shoots and roots of Cd-treated plants, where M plants showed higher P/Cd, N/Cd and S/Cd ratios, in both shoots and roots, than NM plants. Higher ratios of P/metal in M plants have also been observed elsewhere for other plant species (Andrade et al., 2004) suggesting that the higher P status of these plants may alleviate metal stress by phosphate complexation with metal ions inside the cells. The higher shoot N and S contents in M plants may indicate a higher production of thiol-rich proteins which, in addition to P complexation, play an important role in Cd detoxification in vascular plants.

Acid phosphatases (APase) are related to the intracellular hydrolysis of phosphate reserves and its expression is regulated by several environmental and physiological factors (Duff et al., 1994). Thus, APase activity can be considered an indicator to evaluate plant P-nutritional status, since its activity increases with P deficiency level in addition to being related to P-use efficiency in shoots (Besford, 1980) and to leaf senescence (Snapp and Lynch, 1996). In the present study, foliar APase activity was decreased by the M condition, with NM plants showing higher enzyme activity, possibly as a result of the higher P supply and the better P nutrition of M plants. Interestingly, in M plants under low P, Cd addition affected foliar APase activity, resulting in an increase in relation to non Cd-treated plants. Increases in APase activity were observed in radish (*Raphanus sativus*) leaves submitted to excessive amounts of Cr (Dube et al., 2004) and in Mexican sunflower (*Tithonia diversifolia)* plants under excess Pb (Olivares, 2003). Hence, it is suggested that Cd may be interfering in P uptake and inducing APase activity to counteract a possible extra demand for P in the intracellular environment by forming a non-toxic metal complex with P compounds.

Cadmium stress can interfere with the activity of the antioxidant defence system (Gratão et al., 2005). Therefore, changes in enzyme activities such as peroxidases, in response to the presence of Cd in the growth solution, may be useful in detecting signs of metabolic stress in plants. The root system, as the plant organ where the mycorrhizal association occurs and being in direct contact with the contaminated solution, may be particularly affected by excess metal concentrations and also by mycorrhization. Thus, GPOX activity in maize roots was influenced by Cd, as evidenced by the positive correlation found with Cd concentration in plant tissues and root cellular fractions, and also by mycorrhization, since M and NM plants showed different activity patterns, as observed in mycorrhizal jackbean by Andrade et al. (2005). Cadmium addition induced the activity of the enzyme indicating its involvement in the defence system against antioxidative stress caused by Cd ions in maize roots. During stress, reactive oxygen species may accumulate in cells and, as a consequence, induction of enzymes such peroxidases, superoxide dismutases and catalases is to be expected (Gratão et al., 2005).

The total soluble protein concentrations in AMF colonized roots were higher than in non-colonized ones, confirming the results of other authors (e.g., Gianinazzi-Pearson and Gianinazzi, 1995). Root protein concentration was positively correlated with mycorrhizal colonization and with the amount of mycorrhizal ERM, confirming the influence of mycorrhiza on root physiology. Stimulation of *de novo* protein synthesis by mycorrhization has already been observed (Barker et al., 1998). These as well as other expressed proteins, resulting from a marked alteration of gene expression, occur in M plants (Wyss et al., 1990), and may be contributing to the higher protein concentrations found in M maize roots. Distinct induction of genes encoding for proteins potentially involved in heavy metal tolerance was observed in M and NM roots, which may contribute to enhanced metal tolerance in AM plants (Hildebrandt et

al., 2007). The lower protein concentration observed in roots of plants under high P solution was, possibly, related to the dilution effect caused by the greater growth. Increased protein expression in roots has already been observed as a plant reaction to Al toxicity (Souza et al., 2002), and so, a similar phenomenon could occur in the case of Cd stress in maize roots. In this case, M plants, in addition to the higher root protein concentrations showed no induction of GPOX activity in the presence of Cd. These findings might suggest higher tolerance to excess Cd in M plants.

CONCLUSIONS

The results evidenced the different physiological response of M and NM plants in response to excess Cd. Addition of Cd seriously affected mycorrhizal development diminishing intra-radical root colonization and especially extra-radical development. Nevertheless, the higher growth and nutrient/Cd ratios observed in M plants indicate an efficient symbiosis that somehow alleviated nutritional and Cd stress. Possibly, the higher protein concentrations and the lack of GPOX induction in the presence of Cd in M plants underlie the higher Cd tolerance under the specific conditions of this study.

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