

CADMIUM EFFECT ON THE ASSOCIATION OF JACKBEAN (*Canavalia ensiformis*) AND ARBUSCULAR MYCORRHIZAL FUNGI

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ABSTRACT: The effect of cadmium (Cd) on mycorrhizal association and on shoot and root Cd concentration was investigated in jackbean plants under hydroponic conditions. The treatments consisted of the inoculation of three different species of arbuscular mycorrhizal fungi (AMF), *Glomus etunicatum*, *G. intraradices* and *G. macrocarpum*, and a non-inoculated control, two Cd (0 and 5 $\mu\text{mol L}^{-1}$) and two P (1 and 10 mg L^{-1}) levels in the nutrient solution. Mycorrhizal colonization, length of AMF extraradical mycelium, guaiacol peroxidase activity in roots, plant growth and root and shoot Cd and P concentrations were determined. Mycorrhizal status did not promote jackbean growth but in most of the cases mycorrhization increased root and shoot Cd concentrations. Cd ions were accumulated mainly in roots and only small amounts were translocated to the shoot. Cd addition did not affect root colonization by AMF but the AM extraradical mycelium (ERM) was sensitive to the added Cd. ERM length was reduced by 25% in the presence of Cd. This reduction was more pronounced under conditions of low P concentration. Also at this P concentration, Cd addition decreased guaiacol peroxidase activity in non-mycorrhizal roots and in roots colonized by *G. macrocarpum*. However, mycorrhizal roots maintained lower values of peroxidase activity. *G. etunicatum* showed the best performance when associated to jackbean plants and it could be a promising association for phytoremediation of Cd-contaminated soil.

Key words: peroxidase, Cd accumulation, extraradical mycelium, phytoremediation, hydroponics

EFEITO DO CÁDMIO NA ASSOCIAÇÃO DE FEIJÃO DE PORCO (*Canavalia ensiformis*) E FUNGOS MICORRÍZICOS ARBUSCULARES

RESUMO: O efeito do cádmio na associação micorrízica e no teor e acúmulo de Cd na raiz e parte aérea de feijão de porco foi avaliado em condição de hidroponia. Os tratamentos consistiram da inoculação ou não de três espécies de fungos micorrízicos arbusculares (FMAs), *Glomus etunicatum*, *G. intraradices* e *G. macrocarpum*, e uma testemunha (ausência de FMA), duas concentrações de Cd (0 e 5 $\mu\text{mol L}^{-1}$) e de P (1 e 10 mg L^{-1}) na solução nutritiva. Foram determinados a colonização micorrízica, o comprimento do micélio extraradical, atividade da peroxidase nas raízes, crescimento das plantas e teor e acúmulo de Cd e P na raiz e na parte aérea das plantas. A associação micorrízica não promoveu crescimento das plantas mas aumentou a concentração foliar e radicular de Cd. O metal pesado foi acumulado principalmente nas raízes e somente uma pequena quantidade foi translocada para a parte aérea. A colonização micorrízica não foi influenciada pelo Cd adicionado, mas o micélio extrarradicular mostrou-se sensível ao metal, tendo sido reduzido em 25%, principalmente na menor concentração de P adicionado. Nesta mesma concentração de P, a adição de Cd reduziu a atividade da peroxidase nas raízes das plantas não colonizadas e nas colonizadas por *G. macrocarpum*. Entretanto, as raízes micorrizadas mostraram valores menores de atividade da enzima. Melhor desempenho da associação micorrízica foi constatado nas plantas colonizadas por *G. etunicatum*, o qual mostrou-se promissor na fitorremediação de solos contaminados por Cd quando em associação ao feijão de porco.

Palavras-chave: peroxidase, acúmulo de Cádmio, micélio extrarradicular, fitorremediação, hidroponia

INTRODUCTION

Cadmium accumulation in biotic systems due to anthropogenic activities is becoming a growing environmental problem. Atmospheric deposition of industrial emissions, sludge, phosphate fertilizers and mining are

some of the sources of Cd in soils (Jackson & Alloway, 1992). The main symptoms of Cd toxicity in plants are leaf chlorosis, leaf and root necrosis and general decrease in growth (Hernandez & Cooke, 1997).

Little is known about the interactions between Cd and arbuscular mycorrhiza. Some reports showed that

arbuscular mycorrhizal fungi (AMF) decreased or did not affect foliar Cd concentrations in soils with high amounts of this metal (Gildon & Tinker, 1983; Heggo et al., 1990; Tonin et al., 2001). However, AMF attenuated the negative effects of Cd in *Pisum sativum*, in spite of higher, or similar, concentrations of Cd in mycorrhizal than in non-mycorrhizal plants (Rivera-Becerril et al., 2002). Some authors suggest that negatively charged surfaces on AMF mycelium adsorbed Cd decreasing its transfer to root cells (Joner et al., 2000), or that AMF hyphae do in fact transfer Cd from the soil to the roots but with restricted translocation to the shoot (Joner & Leyval, 1997). Thus, the interaction between plants, AMF and Cd involves a variety of arranged factors, which influence plant and fungal development and Cd availability. The enhanced nutrient supply, mainly phosphorus, to the host plant by the AMF may attenuate the effect of physiological stress caused by Cd (Meharg & Cairney, 2000). Some scarce data suggest that the antioxidative system is affected by mycorrhizal fungi in conditions of high metal concentrations (Schutzendubel & Polle, 2002).

The aim of this study was to investigate the effects of Cd on the association of jackbean and different species of AMF, the participation of AMF in Cd uptake and the relation between the activity of root guaiacol peroxidase and mycorrhization.

MATERIAL AND METHODS

Experimental design

A pot experiment was carried out under greenhouse conditions in Campinas, SP, Brazil, from April to May 2002, using a hydroponic sand culture. Greenhouse conditions were 28/16°C day/night temperature, about 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity and 12h photoperiod. The experiment consisted of a $2 \times 2 \times 4$ factorial scheme and completely randomised design, with five replications. Treatments were two Cd concentrations, 0 and 5 $\mu\text{mol L}^{-1}$, two P concentrations, 1 and 10 mg L^{-1} in the nutrient solution and the inoculation or not of three AMFs.

AMF inoculum

The mycorrhizal fungi were *Glomus etunicatum* (Becker & Gerdemann) (IAC-42), *G. intraradices* (Schenck & Smith) (IAC-43) and *G. macrocarpum* (Tul. & Tul.) (IAC-50). Spores used as inoculum arose from a non-contaminated soil. AMF inoculum was obtained from stock -cultures with *Brachiaria brizantha* Stapf and belong to the collection maintained by IAC, in Campinas, Brazil. The inoculum consisted of sand-soil mixture containing spores, mycelium and colonized root fragments, with approximately 1,500 spores cm^{-3} of soil-inoculum. Non-mycorrhizal treatments received washings (20 mL) of the soil-inoculum mixture filtered through Whatman n°42 filter paper.

Pot culture experiment

Two liters of sterilised quartz sand (0.045 to 1 mm of size) were used to fill in 2.79 L plastic pots. Jackbean (*Canavalia ensiformis* L. D.C) seeds were disinfected with 1:3 (v/v) of 2.5% sodium hypochlorite solution for 10 minutes and then rinsed several times with sterilized water. Three seeds were sown per pot and after emergence thinned to one plant per pot. Jackbeans were cultivated in hydroponics system with sand, and irrigated with complete nutrient solution (N-NO₃ 154.6; N-NH₄ 19.5; S-SO₄ 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 & Furlani, 1988), with P and Cd concentrations adjusted for each treatment. Cd was supplied as Cd(NO₃)₂. Cd-nutrient solutions had a pH of 5.58. Speciation calculations using Visual MINTEQ ver. 2.23 (Gustafsson, 2003) indicated that 92 and 90% of the Cd in solution was free Cd²⁺ ion, for solutions with 1 and 10 mg L^{-1} of phosphorus, respectively. The total volume added to each pot during the experiment was of 5 L corresponding to 0.554 mg of Cd added cumulatively. The plants were harvested after 45 days at the flowering stage.

Analytical methods

Shoots and roots were separated at harvest. The shoots were washed in distilled water, dried at 60°C, weighed and the leaves ground. Root subsamples were: 1) washed, dried and ground, 2) stored in ethanol 50% in order to evaluate mycorrhizal colonization and 3) stored in liquid nitrogen until enzyme analysis. Plant Cd and P concentrations were determined after dry-digesting with HNO₃ and HClO₄ by ICP-AES (Inductively-Coupled Plasma Atomic Emission Spectrometry). Mycorrhizal colonization was evaluated by the grid-line intersected method (Giovannetti & Mosse, 1980) by first clearing the roots with 25 g L^{-1} KOH, followed by root acidification in 1% HCl and staining with 0.05% trypan blue. The length of the extraradical mycelium (ERM) of AMF in the substrate was estimated according to Melloni & Cardoso (1999). The ERM was extracted by wet-sieving from 10 g of substrate, which was mixed in a blender with 1.5 L of tap water. A subsample of 11 mL was vacuum-filtered onto nitro-cellulose membrane (0.40 μm pore size). The extracted ERM was stained by 0.05% trypan blue in lactoglycerol and the total length of ERM assessed under compound microscope (125x magnification). Sixty four fields were counted and the results expressed as cm of mycelium in 1g of dry soil.

Guaiacol peroxidase (EC 1.11.1.7) (GPX) was assayed spectrophotometrically using a diode array spectrophotometer (8452.A, Hewlett Packard, USA), according to Boscolo et al. (2003), based on the method of Calmak & Horst (1991). Liquid nitrogen frozen roots were washed three times in deionized water and about 0.05 g were homogenized in a phosphate buffer (KH₂PO₄/

K₂HPO₄, pH 6.8). The homogenates were centrifuged at 10,000 x g for 8 minutes and in the supernatant was immediately determined the peroxidase activity. The reaction mixture contained 500 mmol L⁻¹ phosphate buffer, 8 mmol L⁻¹ guaiacol, 8 mmol L⁻¹ H₂O₂ and protein extract. The increase in absorbance due to tetraguaiacol formation was recorded at 470 nm (ε = extinction coefficient of 26.6 mmol L⁻¹ cm⁻¹). Proteins in each extract were assayed according to Bradford (1976). Determinations of enzyme activity were performed with three parallels in three replicates per treatment.

All data were processed by analysis of variance and Tukey test at 5% for mean comparisons. Data expressed as percentage were arcsin-square root transformed prior to statistical analysis.

RESULTS AND DISCUSSION

Cadmium addition to the nutrient solution did not affect jackbean growth and no chlorosis symptoms were observed in plants. As expected, high P concentration in the nutrient solution (10 mg L⁻¹) promoted plant growth (Figure 1). AMF did not influence plant growth (Figure 1), because in sand culture with salts as nutrient supply, the mycorrhizal growth effect, which is usually related to an enhanced nutrient absorption, is less relevant than in the soil (Weissenhorn & Leyval, 1995). The similar growth between mycorrhizal and non-mycorrhizal plants allowed to confront plants with comparable biomass production, avoiding dilution or concentration effects on nutrients and Cd contents (Jarrell & Beverly, 1981).

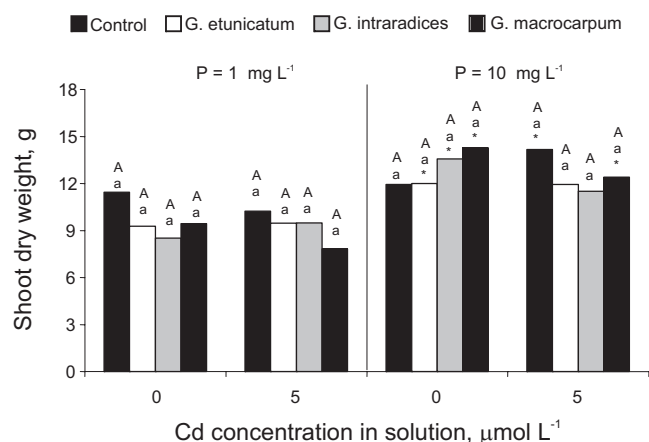


Figure 1 - Shoot dry weight of *Canavalia ensiformis* in association or not with the arbuscular mycorrhizal fungi *Glomus etunicatum*, *G. intraradices* and *G. macrocarpum* with different Cd and P concentrations in the nutrient solution. Means with the same letter are not different ($P \leq 0.05$). A, B compare between arbuscular mycorrhizal fungi (AMF) treatments in each P and Cd treatments. a, b compare between Cd treatments in each of the AMF and P treatments. *difference between P doses in each Cd and AMF treatment.

Although the amount of Cd added cumulatively during the experiment (0.554 mg pot⁻¹) was not very high, it can be considered of environmental relevance. The added Cd concentration may simulate a chronic Cd stress condition (Sanita di Toppi & Gabbrielli, 1999) since soil solutions with moderate level of Cd pollution have concentrations varying from 0.32 to 1 μmol Cd L⁻¹ (Wagner, 1993). Most of the publications regarding Cd toxicity in plants are related to acute stress with non-realistic concentrations of Cd (Sanita di Toppi & Gabbrielli, 1999).

Cd was taken up by jackbean roots and translocated to leaves, however its concentration was more than 20 times higher in roots than in leaves (Figure 2). On the average, 96% of the absorbed Cd was retained in the roots, which was already observed for bean, pea and maize plants (Weigel & Jäger, 1980; Rivera-Becerril et al., 2002; Rauser, 2000). Mycorrhizal roots accumulated higher amounts of Cd (16% more) than non-mycorrhizal

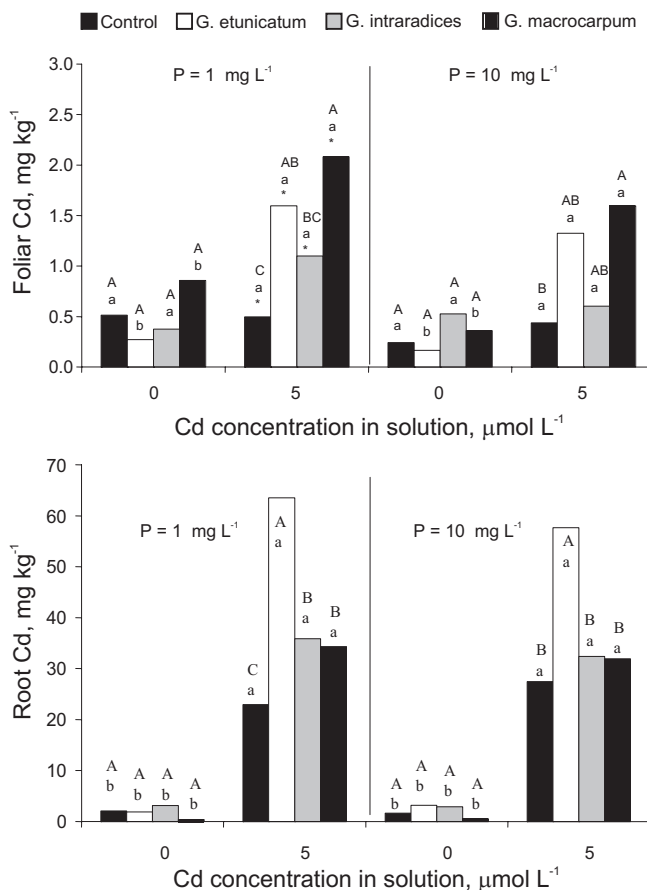


Figure 2 - Foliar and root Cd concentrations of *Canavalia ensiformis* in association or not with the arbuscular mycorrhizal fungi *G. etunicatum*, *G. intraradices* and *G. macrocarpum* with different Cd and P concentrations in the nutrient solution. Means with the same letter are not different ($P \leq 0.05$). A, B compare between arbuscular mycorrhizal fungi (AMF) treatments in each P and Cd treatments. a, b compare between Cd treatments in each of the AMF and P treatments. *difference between P doses in each Cd and AMF treatment.

roots. The association of *C. ensiformis* and *G. etunicatum* lead to higher Cd accumulation in both, roots and leaves, and in addition, only this AMF promoted a higher foliar P concentration (Figure 3). Therefore, the association *C. ensiformis*-*G. etunicatum* might be a promising symbiosis for Cd polluted soils.

Root AMF colonization did not change by Cd addition, but P concentration in the nutrient solution had an inhibitory effect, being 24% lower in plants cultivated under 10 mg P L⁻¹ (Figure 4). Several authors showed that high heavy metal concentrations in soils can reduce mycorrhizal colonization (Gildon & Tinker, 1983; Liao et al., 2003; Andrade et al., 2004) and some reported even an elimination of root colonization due to the presence of Cd in the nutrient solution (Weissenhorn & Leyval, 1995), in spite of these concentrations being higher than that added in the present study. The colonization rates were different among the inoculated AMF. The highest rate of root colo-

nization was observed for *G. etunicatum*, followed by *G. intraradices* and *G. macrocarpum* (Figure 4). However, the amount of ERM was reduced by Cd and was also influenced by P concentration in the nutrient solution. In general, the ERM length was 25% lower in the presence of Cd and the reduction was more pronounced at low P concentration (Figure 4). However, the length of ERM was about 1.5 times higher at low P than at high P concentration (Figure 2). *G. intraradices* presented the highest amounts of external mycelia followed by *G. etunicatum* and *G. macrocarpum* which had low amounts of ERM (Figure 2). Root colonization and amount of ERM were highly correlated ($R^2=0.908$, $P < 0.001$) and both were positively correlated with root P concentration, possibly due to phosphorus transfer to the roots by AMF hyphae and P accumulation inside intraradical hyphae. Despite the importance of the ERM on mycorrhizal symbiosis functioning, studies on this subject have been usually neglected.

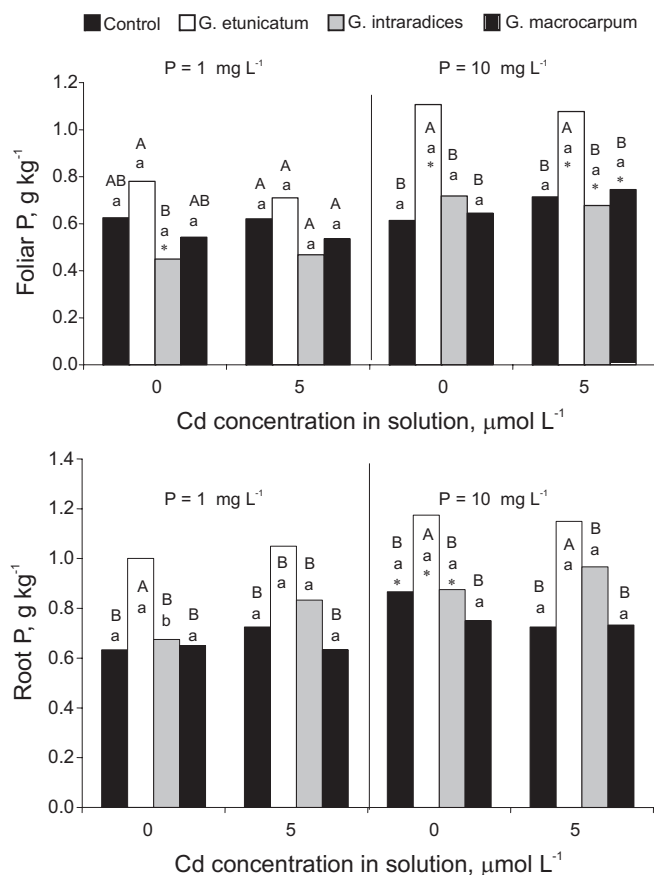


Figure 3 - Foliar and root P concentrations of *Canavalia ensiformis* in association or not with the arbuscular mycorrhizal fungi *G. etunicatum*, *G. intraradices* and *G. macrocarpum* with different Cd and P concentrations in the nutrient solution. Means with the same letter are not different ($P \leq 0.05$). A,B compare between arbuscular mycorrhizal fungi (AMF) treatments in each P and Cd treatments. a, b compare between Cd treatments in each of the AMF and P treatments. *difference between P doses in each Cd and AMF treatment.

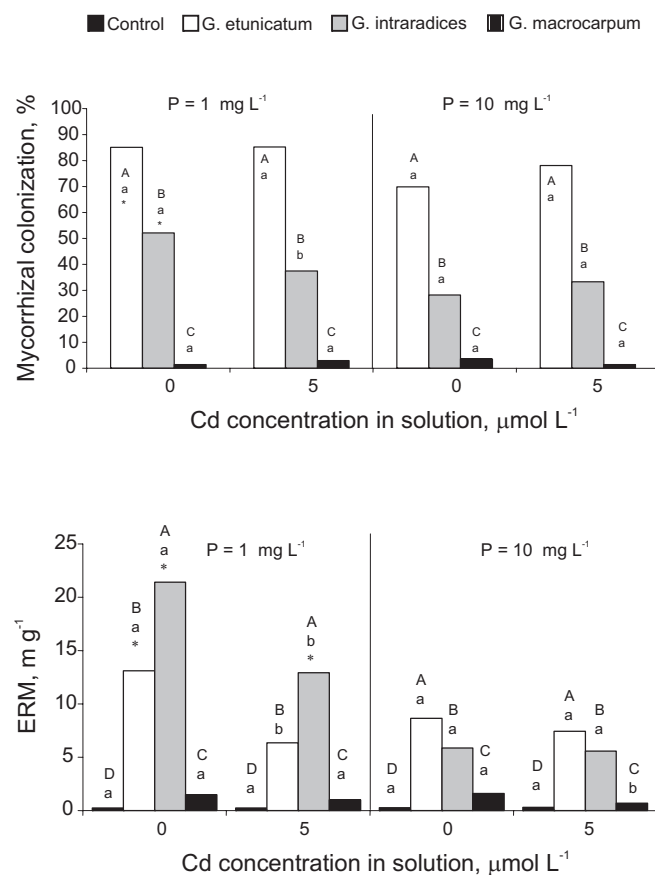


Figure 4 - Mycorrhizal colonization and length of extraradical mycelium (ERM) of *Glomus etunicatum*, *G. intraradices* and *G. macrocarpum* with different Cd and P concentrations in the nutrient solution. Means with the same letter are not different ($P \leq 0.05$). A,B compare between arbuscular mycorrhizal fungi (AMF) treatments in each P and Cd treatments. a, b compare between Cd treatments in each of the AMF and P treatments. *difference between P doses in each Cd and AMF treatment.

Table 1 - Guaiacol peroxidase (GPX) activity in roots of *Canavalia ensiformis* grown hydroponically and associated or not to arbuscular mycorrhizal fungi (AMF) in different Cd (0 and 5 $\mu\text{mol L}^{-1}$) and P concentrations (1 and 10 mg L^{-1}) in the nutrient solution. (U = unit of enzymatic activity)

Treatment		Control	<i>G. etunicatum</i>	<i>G. intraradices</i>	<i>G. macrocarpum</i>
Cd	P				
$\mu\text{mol L}^{-1}$	mg L^{-1}	----- U mg protein ⁻¹ -----			
0	1	44.93 A	16.76 A	23.47 A	39.28 A
5	1	31.14 B	10.70 A	20.57 A	19.90 B
Average		38.03 a	13.68 a	22.02 a	29.59 a
0	10	28.36 A	12.38 A	16.00 A	25.13 A
5	10	27.03 A	11.76 A	15.59 A	18.89 A
Average		27.70 b	12.07 a	15.79 b	22.02 b

A,B compare between Cd rates in each of the P and AMF treatments and a, b compare between P concentrations independently of Cd concentrations by the Tukey test. Means with the same letter are not different ($P \leq 0.05$)

Extraradical hyphae of mycorrhizal fungi are able to transport Cd from the soil solution to the plant and even restrict metal transfer to the shoot due to fungal immobilization in the roots (Joner & Leyval, 1997; Joner et al., 2000).

Among the antioxidant enzymes, which play an important role in the cellular defence against oxidative stress, peroxidases can transform peroxides into non-reactive species (Chaoui et al., 1997). An induction in the activity of antioxidant enzymes is expected as a strategy to overcome oxidative stress due to excess of Cd. However, the addition of 5 $\mu\text{mol Cd L}^{-1}$ reduced guaiacol peroxidase (GPX) activity only in non-mycorrhizal roots and in roots colonized by *G. macrocarpum* (Table 1), which had a very low infection rates (Figure 4), at low P condition. Since increase and decrease of antioxidant activity in response to Cd have been reported in different plant species and Cd concentrations, it is possible that, besides the detoxification function, enzyme molecules may also be sensitive targets of Cd toxicity in plant roots (Gallego et al., 1996). P concentration in the nutrient solution modified GPX activity in roots, which was 25% lower in plants at high P concentration (Table 1). This suggests that higher P concentration in the solution could be buffering the effect of added Cd.

The response of AM symbiosis under heavy metal stress conditions depends on plant and fungi species. In the present experiment, *G. etunicatum* associated to jackbean plants had the best performance and, so, this could be a promising association for phytoremediation of Cd-contaminated soils.

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