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Tolerance to Cd of Soybean (*Glycine max*) and Eucalyptus (*Eucalyptus globulus*) Inoculated with Arbuscular Mycorrhizal and Saprobe Fungi

C.A. ARRIAGADA[1,3], M.A. HERRERA[1], I. GARCIA-ROMERA[2], and J.A. OCAMPO[2*]
[1]*Departamento Ingeniería Rural, Escuela Técnica Superior de Ingenieros Agrónomos y de Montes, Universidad de Córdoba, Apartado 3048, 14080 Córdoba, Spain, Email. ir2aresc@uco.es;*
[2]*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, 18008 Granada, Spain, Tel. +34-58-181600, Fax. +34-58-129600, Email. jocampo@eez.csic.es;*
[3]*Present address: Facultad de Ciencias Silvoagropecuarias, Escuela de Ingenieria Forestal, Camino la Piramide 5750, Santiago, Chile*

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Abstract

The application of Cd inhibited the development of mycelia of the saprobe fungi *Fusarium concolor* and *Trichoderma koningii* and the hyphal length of the arbuscular mycorrhizal fungi *Glomus mosseae* and *G. deserticola in vitro*. The application to soil of 25 and 50 mg l^{-1} of Cd decreased the shoot dry weight, the percentage of AM root length colonization and the succinate dehydrogenase activity of AM mycelia of soybean (*Glycine max* L.) and eucalyptus (*Eucalyptus globulus* Labill.), respectively. However, *G. deserticola* increased the shoot dry weight of eucalyptus, compared with non AM inoculated control, in presence of 50 mg l^{-1} of Cd. When 50 mg l^{-1} of Cd was applied, *T. koningii* increased the effect of *G. deserticola* on shoot dry weight of eucalyptus. There was higher Cd accumulation in the plants when this metal was applied at low doses, and the AM and saprobe fungi increased the accumulation of

*The author to whom correspondence should be sent.

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the metal in the plants. The highest Cd uptake was observed in the shoot of eucalyptus inoculated with *G. deserticola* plus *T. koningii* in presence of 50 mg l^{-1} of Cd. In eucalyptus Cd was accumulated in the stem more than in the leaves. In presence of 50 mg l^{-1} of Cd the highest accumulation of this metal in the stem took place when eucalyptus was inoculated with *G. deserticola* together with *T. koningii*.

Keywords: Arbuscular mycorrhizal fungi, Cd, phytoextraction, saprobe fungi

1. Introduction

The heavy metals contamination of soil is a serious environmental problem (Galli et al., 1994). Cadmium (Cd) is one of the most important environmental pollutants. The level of 0.1–0.5 mg Cd kg^{-1} soil that usually found in unpolluted soil can rise to 150 mg Cd kg^{-1} soil in heavy polluted soil (Jackson and Alloway, 1991). Sources of soil contamination of Cd are the mining, atmospheric pollution from metallurgical industries and disposal of wastes (Alloway, 1995). Cd contaminations of soils cause loss of vegetation and toxicity in plants, animals and humans (Alloway, 1995). The use of plants to remove large amounts of bioavailable Cd from Cd-contaminated soils has been proposed (Khan et al., 2000). There is evidences indicating that various plant species have the ability to absorb Cd by roots and translocate its to the shoots (Baker y Walker, 1989; Huang y Cunningham, 1996). Soybean has been used to Cd sequestration from contaminated soils and eucalyptus is considered a hyper accumulator of heavy metals (Alloway, 1995).

Cd pollution not only limits plant establishment, but also decrease the numbers of soil microorganisms, fungi among them, and their activity (Gaad, 1993; Smylla and Mroczkowska-Badner, 1991; Weissenhorn et al., 1993). Soil microorganisms are important in the recovery of disturbed and toxic environment because they produce plant growing substances, immobilize heavy metals in the soil, bind soil particles into stable aggregates which improve soil structure, reduce erosion potential and can contribute to nutrient availability to plants (Gadd, 1993).

The arbuscular mycorrhizal (AM) fungi are a great component of the soil microbial biomass. This symbiosis benefits plant growth, particularly through enhanced phosphorus, water and mineral nutrient uptake (Li et al., 1991; Pearson and Jackobsen, 1993; Smith and Read, 1997). AM fungi protect plants against the toxic effects of excessive concentrations of heavy metals (Fabig, 1982; Gildon and Tinker, 1983; Haselwandter and Berreck, 1994; Heggo et al., 1990; Rivera-Becerril et al., 2002). However, previous studies have shown conflicting results as to whether AM fungi increase or decrease Cd uptake by plants (Fabig, 1982; Heggo et al., 1990; Karagiannidis and Nickolaou, 2000).

Cd accumulation in AM plants seems to depend on Cd soil concentration and on the kind of AM fungi (Karagiannidis and Nickolaou, 2000).

On the other hand, it is known that soil microorganisms affect AM symbiosis. Saprobe fungi are important and common components of rhizosphere soil where they obtain greater nutritional benefit from organics and inorganic compounds released from living roots together with sloughed cells (Alexander, 1977; Dix and Webster, 1995). Their importance lies in the large microbial biomass they supply to soil and in their capacity to degrade toxic substances (Madrid et al., 1996; Wainwright, 1992). Some experimental results confirm the existence of synergistic effects of saprobe fungi on plant root colonization by AM fungi (Fracchia et al., 1998; Garcìa-Romera et al., 1998; McAllister et al., 1996).

Because soil contamination with Cd reduces the size of the microbial population, the role of AM and saprobe fungi in restoration of these soils may be important.

The aim of this work is to know if the interaction between AM and saprobe fungi increases the tolerance of soybean and eucalyptus to high doses of Cd in soil.

2. Material and Methods

In vitro experiments

The effect of Cd on spore germination and hyphal length of *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (BEG no. 12) from Rothamsted Experimental Station was tested in 9 cm diameter plastic Petri dishes. Sporocarps of *G. mosseae* were isolated by wet sieving the soil (Gerdemann, 1955) from alfalfa plant pot cultures and were stored in water at 4°C. The spores of *G. mosseae*, obtained by dissecting the sporocarps, were surface-sterilized as described by Mosse (1962). Ten surface-sterilized spores per plate were placed 1 cm from the edge of a Petri dish with 10 ml of 10 mM 2-(N-morpholin) ethane sulphonic acid (MES) buffer (pH 7) plus 0.04 g of Gel-Gro (ICN Biochemicals, Aurora, OH, USA). $CdSO_4$ was added to Petri dishes to a final concentration of 0, 5, 15, 20, 25, 30 and 35 mg l^{-1}. Five replicates were used. The plates were incubated at 25°C in the dark for 21 days, and were sealed to reduce dehydratation and contamination. Hyphal length of the germinated *G. mosseae* spores was determined under a binocular microscope at 40× magnification at the end of the experiment using the gridline intersect method (Marsh, 1971). All the fungal mycelia were measured.

The saprobe fungi *Fusarium concolor* Schlecht. BAFC Cult. No. 2183 (Booth, 1977) and *Trichoderma koningii* Rifai (BAFC Cult. no. F8844; Rifai, 1969) are present in the rhizosphere soil and roots of maize cultivated in the province of

Buenos Aires, Argentina. These fungi were isolated by the particle washing method using a multichamber washing apparatus (Widden and Bisset, 1972). Strains are kept at the fungal culture collection of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires in Buenos Aires, Argentina. Both saprobe fungi were transferred to tubes of potato dextrose agar (PDA) and 2% malt extract at 4°C as stock culture. An aqueous suspension in sterile distilled water containing approximately 10^6 spores ml^{-1} of each saprobe fungus was prepared from cultures grown in potato dextrose agar (PDA, DIFCO) for 1 wk at 27°C. Two ml of this suspension were inoculated in 250 ml flasks containing 125 ml of sterile AG liquid medium (Galvagno, 1976) in a shaker at 28°C. The AG medium consisted in 1 g glucose, 0.4 g asparagine, 0.05 g $MgSO_2$, 0.05 K PO_2 and 100 ml distilled water. $CdSO_4$ was added to AG medium to a final concentration of 0, 25, 50 and 100 mg l^{-1} Cd.

After 2 weeks the number of spores per ml of culture medium was evaluated by using a Neubauer chamber (McAllister, 1992). The culture medium was filtered through a disk of filter paper, dried at 80°C for 72 h and the dry mycelium of the saprobe fungi was weighted (McAllister, 1992). In 50 mg l^{-1} Cd treatment the concentration of Cd was analysed in the AG medium after 2 weeks culture of *F. concolor* and *T. koningii* (Mingorance, 2002). AG medium with 50 mg l^{-1} Cd but without fungal culture was used as control. Five replicates were used in these experiments.

Greenhouse experiments

The experiments were carried out using eucalyptus (*Eucalyptus globulus* Labill.) and soybean (*Glycine max* L.) as test plants. Seeds were surface-sterilised with $HgCl_2$ for 10 min and thoroughly rinsed with sterilised water and sown in moistened sand. After germination, uniform seedlings were planted in 0.3 l pots filled with a mixture of sterilized sand:vermiculite:sepiolite (Named substrate pot) at a proportion of 1:1:1 (V:V:V). Plants were grown in a greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 E m^{-2} s^{-1}, 400–700 nm, with a 16/8 h day/night cycle at 25/19°C and 50% relative humidity. Plants were watered from below and fed every week with 10 ml of a nutrient solution plus 50 mg l^{-1} of P (Hewitt, 1952).

Glomus deserticola (Trappe, Bloss and Menge) from the Instituto de Investigaciones Agrobiològicas de Galicia (CSIC) and *G. mosseae* (BEG n°12) were the AM fungi used. The AM fungal inoculum was a root-and-soil inoculum consisting of rhizosphere soil containing spores and colonized root fragments of *Medicago sativa* L. in amounts of 8 g per pot, which were predetermined to have achieved high levels of root colonization. Uninoculated were given a

filtrate (Whatman no. 1 paper) of the inoculum containing the common soil microflora, but free of AM fungal propagules.

An aqueous suspension in sterile distilled water containing approximately 10^8 spores ml^{-1} of *F. concolor* and *T. koningii* was prepared from cultures grown in potato dextrose agar (PDA, DIFCO) for 1 wk at 27°C and 2.5 ml of this suspension were inoculated per pot.

Four treatments were used: (1) Uninoculated controls, (2) substrate pot inoculated with *F. concolor* or *T. koningii*, (3) substrate pot inoculated with *G. mosseae* or *G. deserticola*, and (4) substrate pot inoculated with *F. concolor* or *T. koningii* and either *G. mosseae* or *G. deserticola*. Plants were inoculated at the time of transplanting (after 3 weeks of growth). The saprobe fungi were inoculated at the same time as *G. mosseae* or *G. deserticola*. Five replicate pots per treatment were used.

Cd was applied to soybean pots at the concentration of 0, 25 and 50 mg Cd l^{-1} of substrate pot and to eucalyptus pots at the concentration of 0, 50 and 100 mg Cd l^{-1} of substrate pot.

Plants were harvested after 12 weeks and dry mass was determined. After the harvest two samples of fresh weight were taken from the entire root system at random. One of the samples were cleared and stained (Phillips and Hayman, 1970), and the percentage of root length colonization was measured (Giovannetti and Mosse, 1980). In the second sample succinate dehydrogenase (EC 1.3.99.1) (SDH) activity was measured in fungal mycelia by the reduction of tetrazolium salts at the expense of added succinate (MacDonald and Lewis, 1978); the percentage of AM fungal mycelia with SDH activity was determined under a compound microscope (Ocampo and Barea, 1985).

Cd content in the shoots of soybean and in the leaves and stem of eucalyptus was analysed (Mingorance, 2002).

The percentage values were arcsine transformed for statistical analyses. The data were analysed by one-way analysis of variance (ANOVA) and treatment means were compared using the Tukey's multiple range test (Sokal and Rohlf 1981). Before ANOVA all data are testing with normality test and homogeneous variances. These tests showed no significant differences.

3. Results

The presence of 100 mg Cd l^{-1} of growth medium decreased significantly the dry weight of the mycelium of *F. concolor* and *T. koningii* but no effect on spore numbers was found (Table 1).

Table 1. Dry weight of mycelium and spores number of *Fusarium concolor* and *Trichoderma koningii* in presence of different concentration of Cd in the growth medium.

Saprobe fungus	Concentration of Cd (mg l^{-1})	Dry weight of mycelium (mg)	Number of spores x10^5
F. concolor	0	53.7 b	18.5 a
	25	49.9 b	17.0 a
	50	49.2 b	15.5 a
	100	35.5 a	16.0 a
T. koningii	0	67.2 d	6.5 b
	25	66.3 d	6.6 b
	50	65.9 d	6.7 b
	100	58.3 c	5.9 b

Column values followed by the same letter are not significantly different as determined by Tukey's multiple range test (P=0.05).

Table 2. Effect of cadmium on the hyphal length of *Glomus mosseae* and *G. deserticola* spores.

Cd dose (mg l^{-1})	Hyphal length (mm)	
	G. mosseae	*G. deserticola*
0	23 a	29 a
5	5 b	8 b
15	4 b	6 b
20	2 bc	4 bc
25	1 c	2 c
30	1 c	1 c

Column values followed by the same letter are not significantly different as determined by Tukey's multiple range test (P=0.05).

The hyphal length of *G. mosseae* and *G. deserticola* spores decreased when 5 mg Cd l^{-1} of Gel-gro was applied (Table 2). No hyphal length was observed when higher doses than 30 mg Cd l^{-1} was used.

The concentration of 50 mg l^{-1} Cd in the growth medium decreased until 32 and 30 mg l^{-1} after culture of *F. concolor* for 1 and 2 weeks. *T. koningii* reduced Cd content in the AG medium until 38 and 36 mg l^{-1} after 1 and 2 weeks.

Table 3

Table 4

G. deserticola increased the shoot dry weight of soybean but *G. mosseae* did not affect the shoot dry weight of this plant (Table 3). *T. koningii* increased the shoot dry weight of soybean when inoculated together with *G. deserticola*. The application of 25 and 50 mg Cd l^{-1} decreased the shoot dry weight of soybean in all treatments. Neither the saprobe fungi nor the arbuscular fungi increased the shoot dry weight of plants to which 25 and 50 mg l^{-1} Cd was applied. *T. koningii* increased the percentage of AM root length colonization and the percentage of AM mycelium with SDH activity of *G. deserticola.* However, no effect of both saprobe fungi on the percentage of AM root length colonization and the percentage of AM mycelium with SDH activity of *G. mosseae* were observed. The doses of Cd used in our experiments decreased the percentage of AM root length colonization and the percentage of AM mycelium with SDH activity of soybean either in presence or in absence of saprobe fungi (Table 3).

The application of de 50 and 100 mg Cd l^{-1} decreased the shoot dry weight of eucalyptus (Table 4). The inoculation of *G. mosseae* did not affect the shoot dry weight of eucalyptus. Plants inoculated with *G. deserticola* had higher shoot dry weight than those noninoculated with the AM fungus either in the absence or in the presence of Cd. When 50 mg Cd l^{-1} were applied to eucalyptus, joined inoculation of *G. deserticola* and *T. koningii* had higher shoot dry weights of plants than the other plants grown in presence of this dose of Cd. The percentage of AM root length colonization of eucalyptus decreased in presence of the doses of Cd used. *F. concolor* did not affect the percentage of AM root colonization and SDH activity of eucalyptus in any treatments. However, *T. koningii* increased the percentage of root length colonization and SDH activity of eucalyptus by *G. deserticola* in absence of Cd or when 50 mg Cd l^{-1} was applied, but this effect of the saprobe fungus disappeared when 100 mg Cd l^{-1} was applied.

Table 5 shows that there was higher accumulation of Cd in the shoot dry weight of soybean and eucalyptus when 50 mg Cd l^{-1} than when 100 mg Cd l^{-1} were applied. Higher concentration of Cd in the shoot of soybean and eucalyptus colonized with *G. deserticola* than in non AM inoculated controls were observed. *F. concolor* and *T. koningii* did not affect Cd concentration of soybean and eucalyptus in most of the treatments tested. However, when *T. koningii* was inoculated together with *G. deserticola* in the presence of 50 mg Cd l^{-1} soil an increase of Cd concentration in the shoot of soybean and eucalyptus was observed. Non significant differences in Cd content of the shoot of soybean and eucalyptus inoculated with *G. mosseae* compared with noninoculated controls were observed (data not shown).

The concentration of Cd in eucalyptus inoculated with *G. deserticola* were higher in the stem than in the leaves (Table 6). When 50 mg Cd l^{-1} soil was applied, the concentration of this metal increased in plants inoculated with *G. deserticola* either in the stem or in the leaves compared with non AM

inoculated plants. Eucalyptus inoculated with *G. deserticola* together with *T. koningii* and grown in presence of 50 mg Cd l^{-1} soil showed the highest concentration of Cd in the stem. When 100 mg Cd l^{-1} soil was applied *G. deserticola* increased Cd concentration in the stem of eucalyptus compared with non AM inoculated plants. However, in presence of 100 mg Cd l^{-1} soil similar Cd concentration in the leaves of AM and non AM colonized plants were observed.

Table 5. Cd concentration in the shoot of soybean (*Glycine max*) and *Eucalyptus globulus* inoculated or not with *G. deserticola* or with the saprobe fungi *Fusarium concolor* and *Trichoderma koningii* in presence of different Cd concentrations applied to soil.

Treatments	Cd shoot concentration (mg Cd kg^{-1})			
	Soybean		*E. globulus*	
	A	B	B	C
Control	0.8 b	0.6 a	7.8 e	6.2 d
F. concolor	1.0 b	0.5 a	9.4 e	6.5 d
T. koningii	1.0 b	0.5 a	8.8 e	6.9 d
G. deserticola	1.4 c	0.9 b	19.3 f	9.1 e
G. deserticola + F. concolor	1.5 c	0.8 b	19.8 f	9.8 e
G. deserticola + T. koningii	1.7 c	1.5 c	27.4 g	9.7 e

A = 25 mg Cd l^{-1}; B = 50 mg Cd l^{-1}; C = 100 mg Cd l^{-1}. Values followed by the same letter are not significantly different as determined by Tukey's multiple range test (P=0.05).

Table 6. Cd concentration in leaves and stem of *Eucalyptus globulus* inoculated or not with *G. deserticola* and/or with the saprobe fungi *Fusarium concolor* and *Trichoderma koningii* in presence of different Cd concentrations.

Treatment	Cd in leaves (mg kg^{-1})		Cd in stem (mg kg^{-1})	
	A	B	A	B
Control	1.0 a	3.2 b	7.1 c	3.4 b
F. concolor	1.9 a	3.3 b	7.7 c	3.6 b
T. koningii	1.9 a	3.2 b	7.3 c	3.8 b
G. deserticola	3.5 b	3.1 b	16.8 d	6.5 c
G. deserticola + F. concolor	3.3 b	3.4 b	17.1 d	6.8 c
G. deserticola + T. koningii	3.5 b	3.5 b	24.5 e	6.4 c

A = 50 mg Cd l^{-1}; B = 100 mg Cd l^{-1}. Values followed by the same letter are not significantly different as determined by Tukey's multiple range test (P=0.05).

4. Discussion

Cadmium decreases the population of soil fungi by affecting spore germination and hyphal growth (Gaad, 1993; Weissenhorn et al., 1993). In fact, Cd inhibited the development of mycelia of saprobe fungi but, as happens with other fungi, the tolerance of the spores of *F. concolor* and *T. koningii* was higher than those of mycelia (Smylla and Mroczkowska-Badner, 1991). On the other hand, 5 mg l^{-1} of Cd decreased the hyphal length of *G. mosseae* and *G. deserticola*, which will affect the development of the arbuscular fungi outside the root. However, in spite of the fungitoxic effect of Cd, the saprobe and AM fungi are capable of absorbing and to store heavy metals in its fungal structures (Huang et al., 1990; Joner and Leyval, 1997; Schüepp et al., 1987). In fact 20 and 14 mg l^{-1} of Cd uptake by *F. concolor* and *T. koningii* respectively from the medium of culture was observed. Nevertheless, Cd uptake by saprobe and AM fungi was not sufficient to eliminate the phytotoxicity caused by the application of 25 and 50 mg l^{-1} of Cd on soybean and eucalyptus.

The sensibility of the plants to the phototoxicity of Cd was different in the different plant species (Huang and Cunningham, 1996). In effect, the application to soil of 25 and 50 mg l^{-1} of Cd decreased the shoot dry weight of soybean and eucalyptus, respectively. The percentage of AM root length colonization and the metabolic activity of the arbuscular fungi, measured as SDH activity of the fungal mycelium inside the root, of soybean and eucalyptus also decreased by these doses of Cd. Possibly the negative effect of Cd on the AM symbiosis can be due partly to the toxic effect that has on the plants and partly to the inhibition of the extraradical development of the AM fungi. Nevertheless, due to the decrease of the metabolic activity of the AM fungi, direct effect of Cd on the development of the AM fungi inside the root cannot be discarded.

Synergistic action of saprobe fungi belonging to *Fusarium* and *Trichoderma* genera on the AM colonization of root has been observed (Fracchia et al., 1998; Garcia-Romera et al., 1998). However, the action of the saprobe fungi on the mycorrhization of the plants was very variable depending on the soil, plant and AM fungi (Fracchia et al., 2000). In fact, in our experiments we observed that only *T. koningii* was able to increase the shoot dry weight of soybean and eucalyptus inoculated with *G. deserticola* as well as the percentage of AM root length colonization and the metabolical activity of the AM fungi.

The plant protection by saprobe and AM fungi to toxicity of Cd was dependent on the type of plant, microorganism and Cd concentration (Heggo et al., 1990). In fact, *G. deserticola* increased the shoot dry weight of eucalyptus in presence of 50 mg l^{-1} of Cd whereas did not have effect on the toxicity of Cd in soybean. And *G. mosseae* did not have any effect on the protection of both plants to the doses of Cd used. The presence of *T. koningii* increased the

protective action of *G. deserticola* in eucalyptus when 50 mg l^{-1} of Cd was applied. Cd uptake by saprobe and AM fungi has been described (Joner and Leyval, 1997; Schüepp et al., 1987). The facts that saprobe fungi can absorb heavy metal and that can increase AM colonization of plants may explain that the combined inoculation of *G. deserticola* and *T. koningii* increased the tolerance of eucalyptus to the application of 50 mg l^{-1} of Cd. Nevertheless, when 100 mg l^{-1} of Cd were applied the protective effect of *G. deserticola* and *T. koningii* disappear. Possibly Cd uptake by AM and saprobe fungi was not sufficient to decrease the toxic level of Cd to the plants. On the other hand, the fact that the saprobe fungi, applied individually, did not decrease the toxic action of Cd on soybean and eucalyptus indicates that the beneficial effect of *T. koningii* is attributable to the synergistic effect on AM root colonization of soybean and eucalyptus by *G. deserticola* more than to Cd uptake.

There was higher Cd accumulation in the plants when this metal was applied at low doses. At these doses *G. deserticola* increased the accumulation of the metal in the plants, in spite of the fact that the application of 25 and 50 mg l^{-1} of Cd diminished the AM colonization of soybean and eucalyptus respectively. It is known that the AM fungi not only are able of retaining Cd in its fungal structures but also they are capable of transferring Cd to the plant (Heggo et al., 1990; Joner and Leyval, 1997; Schüepp et al., 1987). The highest Cd uptake was observed in the shoot of eucalyptus inoculated with *G. deserticola* plus *T. koningii* in presence of 50 mg l^{-1} of Cd. The fact that *T. koningii* increased the AM root length colonization and the AM fungal metabolic activity to levels similar to those of plants inoculated with *G. deserticola* in the absence of Cd, allows to the AM fungi to increase plant resistance to its toxic action.

On the other hand, our results show that about 80% of Cd was accumulated in the stem more than in the leaves of eucalyptus especially when 50 mg l^{-1} of Cd were applied. The higher accumulation of Cd in the less metabolically active part of the plant indicates that the damage caused on the physiology of the plant will be minor that when it is accumulated in the metabolically active parts of the plant (Leep and Dickinson, 1998). The AM fungi seem to contribute to the redistribution of Cd inside the plant. In fact, it was higher accumulation of Cd in the stem that in the leaves of eucalyptus colonized with *G. deserticola*, especially when there were applied 50 mg l^{-1} of this metal. In presence of 50 mg l^{-1} of Cd the highest accumulation of this metal in the stem took place when the percentage of AM root length of eucalyptus colonized with *G. deserticola* was increased by the synergistic action of *T. koningii*. The fact that the major Cd concentration takes place in the stem, where the harmful effects on the development of the plant are minor, can explain why *G. deserticola* conferred resistance to eucalyptus to the toxic action of Cd in spite of the accumulation of

high quantity of this metal in the plant. However, when the dose is increased to 100 mg l^{-1} of Cd the quantity of this metal decreased in the stem of mycorrhizal and nonmycorrhizal plants. The doses of 100 mg l^{-1} of Cd decreased the AM root length colonization and metabolic activity of *G. deserticola* and also eliminates the synergistic effect of *T. koningii* on AM root colonization by *G. deserticola*. These results indicates that the presence of 100 mg l^{-1} of Cd decreased the development of the AM fungus inside the root and decreased its contribution to the accumulation of Cd in the plant.

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