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Effects of the ectomycorrhizal fungus *Pisolithus tinctorius* and Cd on physiological properties and Cd uptake by hybrid poplar *Populus alba* × *glandulosa*

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Abstract

The effects of the ectomycorrhizal fungus *Pisolithus tinctorius* and cadmium (Cd) on physiological properties and Cd uptake by *Populus alba* × *glandulosa* was investigated under greenhouse conditions. Cd treatment decreased the photosynthetic rate (P_N) of both non-mycorrhizal (NM) plants (16.3%) and ectomycorrhizal (ECM) plants (11.5%). In addition, the reduction in total dry weight by Cd treatment was greater in ECM plants (24.3%) than that in NM plants (17.6%). Mycorrhizal infection increased the P_N and transpiration rate in both control and Cd-treated plants. Cd treatment increased superoxide dismutase (SOD) activity and decreased glutathione reductase activity, and the increase of SOD activity by Cd treatment was greater in NM plants (40.3%) than that in ECM plants (3.7%). Thiol content increased in both NM and ECM plants treated with Cd solution, and the increase in thiol content in NM plants (43.9%) was greater than that of ECM plants (15.6%). Cd uptake in the leaves, stems, and roots of ECM plants was 69.9%, 167.2% and 72.8%, respectively, higher than in the NM plants. However, the increase in Cd uptake ability of ECM plants resulted in a reduction in dry weight.

Key words: enzyme activity, net photosynthesis, *Pisolithus tinctorius*, *Populus alba* × *glandulosa*, thiol content, transpiration rate

INTRODUCTION

Plants, which naturally hyperaccumulate heavy metals, are the most obvious tools for phytoextraction purposes. Recently, a number of plants with the ability to accumulate specific heavy metals have been identified, and the biochemical mechanisms relevant to accumulation and defense against heavy metal toxicity have been thoroughly assessed (Tong et al. 2004, Xu et al. 2009, Han et al. 2010).

The success of phytoremediation technology is largely dependent on the ability of plants to survive in a deleterious environment and produce a significant amount of dry matter containing elevated levels of contaminants. The contribution of plant–mycorrhiza associations for plant survival in an unfavorable environment is well recognized, particularly in heavy metal polluted soils. The influence of mycorrhizal fungi on plant nutrition is thought to be greater for elements with narrow diffusion zones around plant roots, such as P and heavy metals (Clark and Zeto 2000). Among soil microorganisms, mycorrhizal fungi are the only ones providing a direct link between soil and roots, and can, therefore, be of great importance in phytoremediation-enhancing heavy metal availability and tolerance to plants.

When metals are at toxic concentrations in soil, mycor-

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rhizal rather than non-mycorrhizal host plants are able to colonize these polluted sites (Shetty et al. 1994). Thus, mycorrhizal colonization may be the key to plant survival in contaminated environments by enhancing metal resistance in plants and also by improving essential nutrients uptake (Davies et al. 1996, Filion et al. 1999). Additionally, mycorrhiza enhance the uptake of heavy metals (Gildon and Tinker 1983, Han et al. 2001).

A number of studies have claimed to show that ectomycorrhizas alleviate metal toxicity. In these studies, ectomycorrhizal fungi (ECM) have been demonstrated to alleviate growth depression in tree seedlings due to the toxic effects of heavy metals (Jentschke et al. 1999).

Pisolithus tinctorius (Pers.) Coker and Couch is a widespread ectomycorrhizal basidiomycete forming mycorrhiza with a variety of hosts (Marx 1977) that has great potential in reforestation programs. Subsequently, its tolerance to a wide range of environmental stresses such as high soil temperature, very acidic pH, low P soil, drought, and high concentrations of heavy metals has been demonstrated (Marx 1991) as well as its ability to improve tree growth and survival in the field.

Therefore, the objectives of this research were to determine if *P. tinctorius* could enhance Cd phytoaccumulation and increase plant tolerance as determined by plant development, gas exchange, and antioxidative enzymes in container-grown cuttings.

MATERIALS AND METHODS

Plant and inoculum preparation

Stem cuttings of (1 cm diameter and 10 cm long) hybrid poplar, *Populus alba* × *glandulosa*, were taken from micropropagated stock plants maintained at the Department of Forest Genetic Resources of Korea Forest Research Institute in Korea. A culture of *P. tinctorius* was obtained from Chonnam National University in Korea, which, accordingly, was originally isolated from *Pinus thunbergii* growing in Korea. *P. tinctorius* mycelial cultures were maintained following the method of Marx (1969). The fungal inoculum was prepared using the method of Marx and Bryan (1975). A 1-L flask was filled with a mixture of 60 mL peat and 840 mL vermiculite. Liquid modified Melin-Norkrans (MMN) solution (400 mL) was added with 5 g of glucose to 900 mL of the mixture, mixed thoroughly, and then autoclaved for 1 h at 121°C. The sterilized peat-vermiculite mixture was inoculated with 10 MMN agar plugs (7 mm diameter) taken from the edge of actively growing colonies. Ten MMN agar plugs without mycelia were added to the mixture for the control treatment. The flasks were kept at 20°C in the dark for 3 months. After the 3-month incubation, the inoculum was removed from the flasks, leached to remove non-assimilated nutrients, and kept in a plastic bag at 5°C for 3 days until used for inoculation.

Production of mycorrhizal *P. alba* × *glandulosa*

Ectomycorrhizal associations between *P. alba* × *glandulosa* and *P. tinctorius* were synthesized aseptically in plastic pots. The peat that passed through a 2-mm sieve and vermiculite retained in a 3-mm sieve were mixed in a ratio of 1:1 by volume, then autoclaved for 1 h at 121°C. The fungal inoculum was mixed with the autoclaved clean peat-vermiculite mixture at a ratio of 1:6, by volume, and then placed into plastic pots. Stem cuttings were then inserted into the rooting plastic pots filled with a mixture of fungal inoculum (for the inoculated treatment) or with a mixture of agar only (for the control). The cuttings were grown in a greenhouse for 30 days before Cd treatment.

A subsample of fine roots (0.1 g fresh weight) was cut into 2-3 mm lengths and fixed in 50% ethanol to assess root colonization by mycorrhizal fungi. For the root colonization by ECM fungi, all short root tips that crossed the grid lines (15 lines) were counted and ECM infection was scored on dark blue-colored short root tips. The rate of ECM root colonization was expressed as a percentage of the total number of infected short root tips over the total number of short root tips counted. Root tips colonized by *Pisolithus* were light brown in color, and the initial percent root colonization in plants inoculated with *Pisolithus* was 68%.

Cd treatment

Thirty days after inserting the stem cuttings, Cd was applied every 3 days over a 5-month period. At each Cd application, approximately 200 mL of 0.4 mM $CdSO_4$ solution was applied to the containerized plants. The pots were placed in plastic dishes to retain leached nutrients and the $CdSO_4$ solution. Pots were randomized in the greenhouse and moved about every 2-3 weeks throughout a 5-month experimental period to minimize positional effects. During the experimental period, daily mean temperature and relative humidity were 23.1 ± 2.1°C and 74.3 ± 10.9%, respectively.

Photosynthetic and gas exchange measurements

The photosynthetic parameters, such as net photosynthetic rate (P_N) and transpiration rate (E), were recorded on fully expanded leaves at 5 months after Cd treatment using an infrared gas analyzer (Li-6400; LI-COR, Lincoln, NE, USA) at light saturation (1,100 μmol $m^{-2}s^{-1}$ photon flux density). The CO_2 concentration during measurements was maintained at 400 μmol CO_2/mol air, leaf temperature was 24.0 ± 0.2°C, and relative humidity was 60 ± 5%. Data from two replicate measurements were averaged for each plant.

Biomass and Cd determination

Shoots and roots were carefully removed at harvest (5 months) and thoroughly rinsed twice with distilled water. Leaves, stems, and roots were partitioned and individually placed in paper envelopes. Oven dry weights were obtained after oven drying the tissues at 70°C to constant weight.

Dried leaves, stems, and roots (0.5 g each) were ground in a grinding mill (Retsch MM200; Retsch, Haan, Germany). Cd content in the ground tissue was measured by inductively coupled plasma spectrometer (ICPS-1000IV; Shimadzu, Tokyo, Japan).

Antioxidative enzyme activities

Fresh leaves (0.1 g) were homogenized under ice-cold conditions with 5 mL of 50 mM phosphate buffer (pH 7.0), 10 mM ascorbic acid, and 1.0% (w/v) polyvinylpyrrolidone (Han et al. 2009). The homogenate was centrifuged at 20,000 ×g for 30 min, and the supernatant was collected for enzyme assays.

Superoxide dismutase (SOD) was assayed based on the inhibition of the reduction in nitro-blue tetrazolium in the presence of xanthine at 530 nm according to the method of Beauchamp and Fridovich (1971). Activity of glutathione reductase (GR) was assayed as described by Carlberg and Mannervik (1985). The assay was conducted in a reaction mixture containing 50 mM phosphate buffer (pH 7.8), 0.1 mM NADPH, 0.5 mM oxidized glutathione (GSSH), and 0.1 mL enzyme extract. The change in A_{340} was recorded for 5 min after adding the enzyme extract.

Thiol content

Freshly harvested leaves (0.1 g) were homogenized in 1.5 mL cold buffer containing 5% (w/v) 5-sulfosalicylic acid and 6.3 mM DTPA. The homogenized samples were centrifuged (15,000 ×g, 10 min), and 250 μL of extract was mixed with 50 μL of Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) in 2.5 mL of 0.1 M sodium phosphate buffer (pH 8) containing 1 mM EDTA. Then, the mixtures were incubated at room temperature for 15 min. Absorption was recorded at 412 nm against a blank sample.

Statistical analysis

The data were statistically analyzed using SAS System for Windows ver. 8.01 (SAS Institute, Cary, NC, USA). Mean values per treatment were compared using a general linear model. Tukey's HSD tests were performed when significant differences ($P < 0.05$) were indicated.

RESULTS

Net photosynthetic rate (P_N) and transpiration rate (E)

Both Cd treatment and mycorrhizal infection had effects on P_N or E of hybrid poplar (Fig. 1). Cd treatment decreased P_N of both non-mycorrhizal (NM) and ECM plants, but the mycorrhizal infection did not influence the P_N of hybrid popular. P_N of the NM plants grown in soil with no Cd was higher than those grown in the presence of Cd. Cd treatment decreased the P_N of NM plants by 16.3% and of ECM plants by 11.5%. Similarly, E was reduced in NM plants by 15.4% but slightly increased in ECM plants by 5.8%. In contrast, mycorrhizal infection increased the P_N and E in both control and Cd-treated plants. The increase in P_N was greater in Cd-treated plants (12.2%) than that in control plants (6.1%). Additionally, the increase in E was greater in Cd-treated plants (37.9%) than that in control plants (10.3%).

Dry weight

A significant reduction by the Cd treatment appeared only in leaves of NM plants and in total dry weight of ECM plants (Table 1). The greatest reduction in leaf dry weight was observed in NM plants (34.4%) whereas, the greatest reduction in total dry weight was observed in ECM plants. Cd treatment reduced the total dry weight of NM plants by 17.1% and ECM plants by 18.5%, respectively. However, the root to shoot ratio in Cd-treated ECM plants increased by 21.4% over that in NM plants.

Enzyme activity and thiol content

The enzyme (SOD and GR) activities of plants inoculated with *P. tinctorius* were higher than those of NM plants (Fig. 2). In addition, the increase in enzyme activity due to mycorrhizal infection was greater in control plants than that in Cd-treated plants. More importantly, the increase in GR activity in control plants with a mycorrhizal infec-

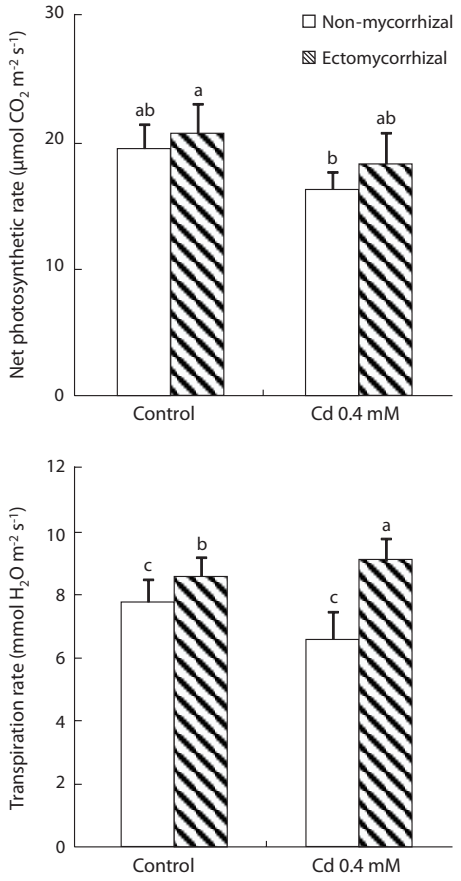

Fig. 1. Changes in net photosynthetic rate and transpiration rate in the leaves of 6-month-old *Populus alba* × *glandulosa* cuttings inoculated with the ectomycorrhizal fungus, *Pisolithus tinctorius,* and grown in medium with or without 0.4 mM $CdSO_4$ solution. Each bar represents the mean of three replicates ± standard deviation. Means with the same letter(s) are not significantly different from each other based on Tukey's test at $P \leq 0.05$.

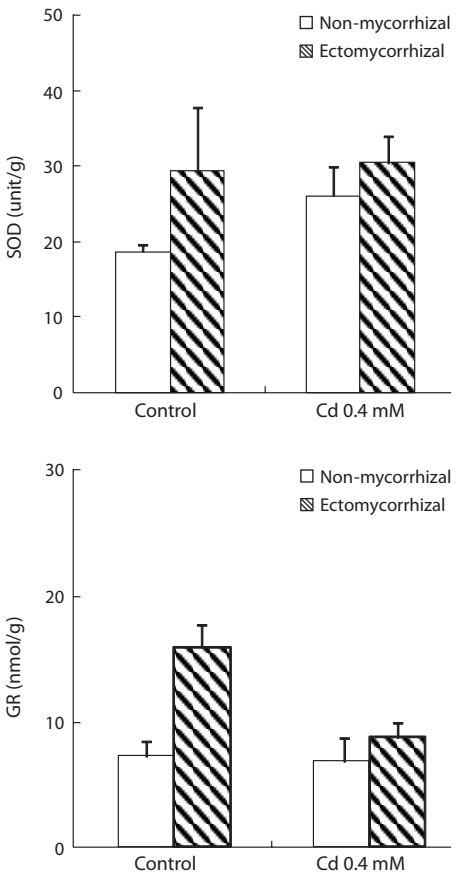

Fig. 2. Changes in superoxide dismutase (SOD) and glutathione reductase (GR) activity in the leaves of 6-month-old *Populus alab* × *glandulosa* cuttings inoculated with the ectomycorrhizal fungus, *Pisolithus tinctorius,* and grown in medium with or without 0.4 mM $CdSO_4$ solution. Each bar represents the mean of three replicates ± standard deviation. Means with the same letter are not significantly different at $P \leq 0.05$.

Table 1. Changes in dry weight of 6-month-old *Populus alba* × *glandulosa* cuttings inoculated with the ectomycorrhizal fungus, *Pisolithus tinctorius*, and grown in medium with or without 0.4 mM $CdSO_4$ solution

Treatment	Leaf	Stem	Root	Total	Shoot/Root
	g (dry weight)				
Control					
Non-mycorrhizal	3.20 ± 0.36^{a}	3.50 ± 0.17^{a}	5.17 ± 0.25^{a}	11.87 ± 0.72^{a}	1.30 ± 0.07^{a}
Ectomycorrhizal	3.10 ± 0.36^{a}	3.33 ± 0.21^{a}	5.03 ± 0.80^{a}	11.47 ± 1.29^{ab}	1.29 ± 0.13^{a}
Cd 0.4 mM					
Non-mycorrhizal	2.10 ± 0.26^{b}	3.20 ± 0.10^{a}	4.53 ± 0.35^{a}	9.83 ± 0.38^{ab}	1.17 ± 0.10^{a}
Ectomycorrhizal	2.63 ± 0.06^{ab}	2.93 ± 0.40^{a}	4.10 ± 0.85^{a}	9.67 ± 0.42^{b}	1.42 ± 0.42^{a}

Each number represents the mean of three replicates ± standard deviation. Means within the column followed by the same letter(s) are not significantly different based on Tukey's test at $P \leq 0.05$.

tion was 120.8% relative to the NM plants. Cd treatment increased SOD activity and decreased GR activity. The increase in SOD activity by Cd treatment was greater in NM plants (40.3%) than that in ECM plants (3.7%). However, the decrease in GR activity by Cd treatment was greater in ECM plants (45.3%) than that in NM plants (5.6%).

Thiol content increased in both NM and ECM plants treated with Cd solution (Fig. 3). The increase in thiol content in NM plants (43.9%) was greater than that of ECM plants (15.6%). No effect of mycorrhizal infection was observed for the control or Cd-treated plants.

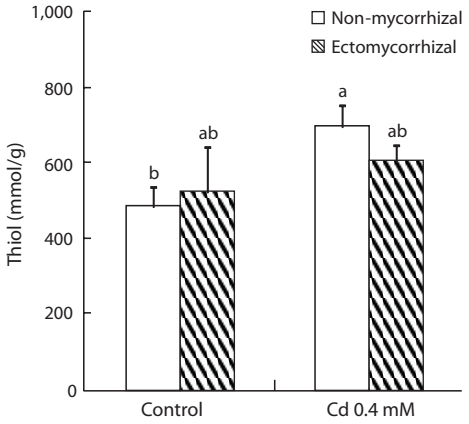

Fig. 3. Changes in thiol content in the leaves of 6-month-old *Populus alba*×*glandulosa* cuttings inoculated with the ectomycorrhizal fungus, *Pisolithus tinctorius,* and grown in medium with or without 0.4 mM $CdSO_4$ solution. Each bar represents the mean of six replicates ± standard deviation. Means with the same letter(s) are not significantly different from each other based on Tukey's test at $P \leq 0.05$.

Cd concentration

Cd concentration in the leaves, stems, and roots of hybrid poplar increased significantly following the ectomycorrhizal inoculation (Fig. 4). Cd concentrations in the leaves, stems, and roots of mycorrhizal plants were 69.9%, 167.2%, and 72.8% higher than in the NM plants, respectively. Lower Cd concentration was obtained in the leaves, stems, and roots of NM plants (156, 67, and 81 mg/g Cd, respectively) than that in the ECM plants. Cd concentration was highest in the leaves of both NM and ECM plants. The shoot to root Cd concentration ratio was higher in ECM plants than that in NM plants.

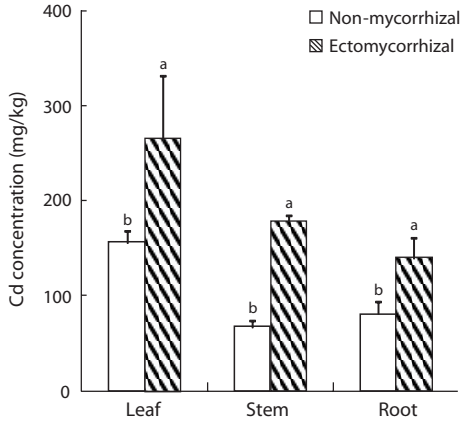

Fig. 4. Changes in Cd uptake in the leaves, stems, and roots of 6-month-old *Populus alab* × *glandulosa* cuttings inoculated with the ectomycorrhizal fungus, *Pisolithus tinctorius,* and grown in medium with or without 0.4 mM $CdSO_4$ solution. Each bar represents the mean of three replicates ± standard deviation. Means with the same letter(s) are not significantly different from each other based on Tukey's test at $P \leq 0.05$.

DISCUSSION

Cd is an effective inhibitor of photosynthesis, and heavy metal accumulation in the leaves in higher plants is associated with a reduction in P_N (Han et al. 2006). In general, *E* is also affected by Cd treatment. The reduction in *E* is probably a result of root damage due to Cd toxicity. Barceló and Poschenrieder (1990) suggested that toxic metals may impair *E* by either interfering with stomatal regulation or reducing water uptake by the root system, or both.

Mycorrhizal infections alleviate Cd toxicity of their hosts by modifying plant physiological processes such as higher photosynthetic activity. Reid et al. (1983) have shown that the presence of vesicular arbuscular mycorrhiza (AM) on root systems of plants is correlated with higher P_N. This enhancement is probably attributable to improved nutrient uptake.

Cd is not a transition metal like Fe and Cu and is not capable of generating reactive oxygen species (ROS) by catalyzing Haber-Weiss or Fenton type reactions (Deckert 2005). Nevertheless, Cd toxicity results from an alteration in the oxidant levels in plants (Foyer and Noctor 2005). Cd accumulation is correlated with ROS generation in sensitive clones of *Holcus lanatus* (Hendry et al. 1992). Cd also elevates lipid peroxidation via ROS formation in plants (Halliwell and Gutteridge 1989).

To scavenge ROS, plants possess a well-organized antioxidative defense system comprising enzymatic and non-enzymatic antioxidants. The cooperative function of these antioxidants plays an important role scavenging ROS and maintaining the physiological redox status of organisms (Cho and Seo 2005). Increased SOD activity may be attributed to the increased production of superoxide, resulting

in the activation of existing enzyme pools or increased expression of genes encoding SOD (Mishra et al. 2006). Increased SOD activity caused by heavy metals has been previously observed in several plant species and is routinely considered to be an adjustment response to stress (Verma and Dubey 2003). However, we did not observe an increase in SOD activity in mycorrhizal plants (Fig. 2). This result might be due to alleviation of Cd toxicity by improving physiological characteristics or a decrease in ROS scavenging ability at high Cd concentrations.

GR is a member of the flavoenzyme family that catalyzes the NADPH-dependent reduction of GSSG to glutathione (GSH). This reaction is crucial for function of the ascorbate-glutathione cycle and for maintaining a proper GSH/GSSG concentration ratio in cells (Mishra et al. 2006). Increases in GR activity as a consequence of Cd exposure have previously been observed in *Brassica juncea* (Qadir et al. 2004), *Phaseolus vulgaris* (Smeets et al. 2005), and *Bacopa monnieri* L. (Mishra et al. 2006).

However, we found that GR activity in the leaves of mycorrhizal plants decreased significantly with Cd treatment, suggesting that the drastic decrease in GR activity resulted from other H_2O_2-scavenging damage by the high concentration of Cd. GR activity in mycorrhizal plants was higher than in NM plants, which may be attributed to an increase in its GSSG substrate similar to that reported by Foyer et al. (1997).

The accumulation of Cd in roots and shoots depends on binding to the extracellular matrix (Horst 1995), complexing inside the cell (Cobbett et al. 1998), and transport efficiency (Marchiol et al. 1996). Furthermore, transport efficiency relies on *E* and thereby on stomatal conductance. Metals may be transported to the shoot via the transpiration stream, and reduced *E* may result in decreased translocation of metals to shoots. As well, the protective effect of AM colonization against Cd toxicity has been explained by the possibility that much Cd is retained in the mycorrhizal roots; thus, translocation to shoots is inhibited (Weissenhorn and Leyval 1995).

In this study, the shoots of mycorrhizal plants showed a higher Cd concentration than the corresponding non-inoculated plants, as shown in a previous study (Kelly et al. 1979, Dixon 1988). This result may be attributed to the high concentration of Cd. Under a high Cd concentration, mycorrhizal plants are likely to transport the excessive metals in the mycorrhizal roots to shoots. Additionally, the increased translocation of metals to shoots may result from the increased *E* and thiol content in mycorrhizal plants. Ding et al. (1994) reported that Cd uptake is proportional to the increase in thiol group content, suggesting that using thiol group content to assess the bioconcentration of heavy metal ions in water hyacinth is a general parameter to monitor heavy metal water pollution (Ding et al. 1994). As a result, the higher Cd accumulation in the shoots of mycorrhizal plants resulted in growth inhibition.

In earlier studies, Davies et al. (2001) and Vivas et al. (2005) demonstrated that mycorrhizal infection under metal-polluted conditions protects against the inhibition of plant growth through enhanced metal tolerance. Several mechanisms may be involved in enhancing plant Cd tolerance by the coinoculation of microorganisms, e.g., the effect of increasing nutrients (P and K) and in decreasing concentration of metals (Cd, Cr, Mn, and Ni). Both mechanisms seem to be responsible for the plant growth effect found in previous studies (Davies et al. 2001, Vivas et al. 2005). However, in the present study, *P. tinctorius* did not increase the growth of hybrid poplar cuttings. A lack of an infection effect by *P. tinctorius* on plant growth might be explained by the continuous transportation of the Cd remaining in the roots to the shoots as a result of transpiration, which contributes to growth inhibition (Russo and Brennan 1979).

In conclusion, although mycorrhizal infection under Cd stress ameliorated the physiological characteristics of the host plants to alleviate Cd toxicity through an increase in photosynthetic activity, growth inhibition of host plant was not protected. This result may be due to a decrease in Cd tolerance by excessive Cd accumulation in tissues.

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