

Cd-tolerance markers of *Pfaffia glomerata* (Spreng.) Pedersen plants: anatomical and physiological features

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Received: 16 December 2012; Accepted: 14 February 2013

ABSTRACT

Physiological and anatomical features of Cd-tolerance in *Pfaffia glomerata* were examined by exposing plantlets to nutrient solutions with increasing Cd concentrations (0, 15, 45, and 90 $\mu\text{mol Cd L}^{-1}$), and possible Cd-tolerance markers were established. Cd contents were found to be higher in roots than in shoots. According to the bio-concentration factor data, this species is effectively a Cd-hyperaccumulator, as previously attested. Cd induced the appearance of xeromorphic characteristics in leaves (decreased water potential, increased numbers and decreased stomata size) and increased root endodermis thickness. The enzymatic antioxidant systems of roots and leaves were differently affected by Cd. The coordinated activities of antioxidant enzymes were effective in reducing Cd-induced reactive oxygen species in plants, mainly in leaves. Root endodermis thickness, stomatal size and numbers, root superoxide dismutase, and guaiacol peroxidase, as well as leaf guaiacol peroxidase and catalase activities can all be considered Cd-tolerance markers in *Pfaffia glomerata*. Due to its high root Cd accumulation, *Pfaffia glomerata* may be useful in Cd-phytoextraction programs, however the pharmacological use of plants grown in the presence of Cd must be avoided.

Keywords: adaptation, Cd-oxidative damage, phytoextraction, tolerance.

INTRODUCTION

Water and soil contamination by heavy metals is a growing environmental issue around the world. Although traces of heavy metals are natural soil components, human activities such as mining and agriculture are contributing to toxic ion accumulation in the environment (Kabata-Pendias and Pendias, 2001). The heavy metal contamination of soils in Brazil is directly linked to anthropogenic activities, especially mining and modern agricultural techniques that can leave hazardous metal residues in the environment (Kabata-Pendias and Pendias, 2001), which demand effective remediation. Among the heavy metals, cadmium (Cd) is of particular interest for its universal toxicity even at low concentrations (Das et al., 1997), making the restoration of Cd-contaminated areas very important. This element has high mobility and is not known to have any metabolic role in plants or animals (Barceló et al., 1990).

Phytoremediation is currently one of the most effective environmental reclamation techniques available and has been widely used. This is ecologically friendly and cost-effective, with a potential market in countries such as Brazil, which have numerous metallic ore resources. Although native vegetation types growing on naturally mineralized or metal-contaminated sites have been described in Brazil, relatively few potential phytoremediator species were also identified (Ginocchio and Baker, 2004). Carneiro et al. (2002) reported high growth rates and apparent tolerance of the species of genus *Pfaffia* to soils contaminated with Cd and Zn (90 and 1,450 mg kg⁻¹ respectively), and Skrebsky et al. (2008), Calgaroto et al. (2010), and Gupta et al. (2011) demonstrated reasonable degrees of Cd, Hg, and Pb tolerances in *Pfaffia glomerata* (Spreng.) Pedersen. Authors reported biochemical-antioxidant system activations in the presence of heavy metals that were related to fundamental features of plant survival.

Pfaffia glomerata are perennial subshrubs or shrubs usually found growing at the edges of woods or rivers and in the Brazilian rupestrian field (Nascimento et al., 2007). This species belongs to the family *Amaranthaceae* and has high commercial pharmaceutical value (Montanari Jr., 1999), as its roots are used as aphrodisiacs, stimulants, and to treat diabetes and inflammatory diseases (Oliveira et al., 1980). Several economically important compounds have also been isolated and identified from the roots of *Pfaffia glomerata* (De Paris et al., 2000). Due to the intense predatory exploitation of natural sources of this species, its cultivation has been increasingly stimulated (Montanari Jr., 1999),

so that the use of *Pfaffia glomerata* in environmental recuperation programs could represent an interesting strategy for both environmental recovery and economic development.

Many phytoremediation studies have reported changes in the anatomical and physiological characteristics of plants as a consequence of their adaptation to heavy metal-induced environment stress (Barceló et al., 1990; Monni et al., 2001). Researches into the features associated with heavy metal tolerance are important for our understanding of the processes involved in this phenomenon, and for selecting characteristics that could facilitate the identification of potential phytoremediator species. Therefore, the present study evaluated Cd-tolerance at both physiological and anatomical levels in *Pfaffia glomerata* seedlings grown under increasing Cd concentrations in nutrient solution, and Cd-tolerance markers were established. We also investigated Cd uptake and distribution in this plant, and its classification as a Cd-hyperaccumulator species (Carneiro et al., 2002) was revised.

MATERIAL AND METHODS

Plant growth and physiological analyses: *Pfaffia glomerata* seeds obtained at the Federal University of Mato Grosso do Sul (UFMS) were germinated in Styrofoam trays containing sterile vermiculite in a thermostat-controlled dark chamber (70% relative humidity and 25°C). The seedlings were then transferred to a greenhouse for 45 days (at 15 to 31°C; with 825 μmol m⁻² s⁻¹ average photosynthetically active radiation). Seedlings with similar size and weights were later transferred to plastic beakers (six-liter capacity, two plants per beaker) containing Clark's nutrient solution (Clark, 1975). After an initial growth period of 15 days, the most vigorous seedlings were selected and Cd was added (as CdSO₄) to the nutrient solution at different concentrations: 0, 15, 45, and 90 μmol L⁻¹. The solutions were continuously aerated and renewed weekly. Cd concentrations used in this work were chosen based on Carneiro et al. (2002). The pH of the medium was checked and adjusted on a daily basis to 5.5±0.1. The plants were exposed to Cd for 20 days.

Chlorophylls *a* and *b* were quantified according to Arnon (1949), and the carotenoid content referring to Duke and Kenyon (1986). β-carotene was quantified using the 2,500 L mol⁻¹ cm⁻¹ molar absorptivity coefficient (Sandmann and Böger, 1983). One fully expanded leaf

per experimental unit was used for pigment analysis, with five replicates per treatment; both water status and pigment contents were evaluated after 20 days of treatment with Cd. The water status of fully expanded leaves from the third or fourth nodes was assessed in the early morning by measuring their water potential (Ψ_w maximum), with the aid of a pressure chamber (Soil Moisture Equipment Corp – Model 3005, Santa Barbara, CA, USA).

After 20 days of Cd treatment, the plants were harvested and separated into roots and shoots, stored in paper bags, and dried to a constant weight in a forced-air circulation oven at 70 °C in order to determine biomass production.

Plant cadmium concentration: Cd concentrations in the roots and shoots were determined by atomic absorption spectrophotometry (Perkin-Elmer Analyst 400) after a 0.1 g wet digestion of dried material in 5 mL of a strong acid solution — $\text{HNO}_3/\text{HClO}_4$, 3:1, v v⁻¹ (Van Assche et al., 1990). Cd affecting plant growth was verified by its critical dose solution values of CDS_{10} and CDS_{50} , which promoted decreases of 10 and 50% in plant yield respectively, and Cd-toxicity levels (TCL — Cd-concentrations in shoots that promoted 10% decreases in shoot yields). Cd transport between plant tissues was verified by the primary transport index (PTI) calculated as the ratio of its concentration in the shoots and roots (Moral et al., 1994). In order to study Cd uptake and bioconcentration behavior, the following indices were used: bioconcentration factor (BCF), calculated by dividing the Cd-concentration in plant tissues (ppm) at harvest by the metal one (mg g^{-1} of dry weight – DW) in the solution (Sharma and Agrawal, 2006); total accumulation rate — TAR ($\text{mg plant}^{-1} \text{d}^{-1}$) was determined following Zhu et al. (1999), and Cd uptake ($\text{mg plant}^{-1} \text{d}^{-1}$) following Sharma and Agrawal, (2006). TAR and total Cd uptake were calculated using the following formulas:

$$\text{TAR} = \frac{\text{root Cd content} + \text{shoot Cd content}}{(\text{total dry matter} \times T)} \quad (1)$$

$$\text{Uptake} = \frac{\text{total Cd content}}{T} \quad (2)$$

where,

T: experimental period (20 days).

Light microscopy: Anatomical analyses were performed at the end of the experimental period

(20 days after initiating the Cd-treatment). Root samples were collected and fixed for 48 hours in Karnovsky's fixative solution (2.5% glutaraldehyde and 2.5% paraformaldehyde), dehydrated in a graded ethanol series, and included in butanol/plastic resin (Historesin, Laica). Semi-thin sections (5 to 8 μm thick) were prepared using a Jung AG rotary microtome, stained with toluidine blue (equal volumes of 0.3% of basic toluidine and 1% sodium tetraborat). Fully expanded leaves were collected for leaf anatomical studies, fixed in FAA (formaldehyde + acetic acid + 70% ethyl alcohol 5:5:90 v/v/v) (Johansen, 1940) during 72 hours and then stored in 70% ethyl alcohol. Paradermal sections were manually prepared from the mid third of abaxial epidermis of leaves and stained with a 1% safranin solution. All slides were examined and photographed using a Ken-a-Vision TT18 light microscope equipped with a Canon Power Shot A620 digital camera. Measurements of the anatomical characteristics were made using Sigma Scan Pro 4.0 for Windows (SPSS, Inc., Chicago, IL, USA). The items assessed in the root system were done in the root hair zone and included epidermis and endodermis thickness. Stomatal density was defined as the number of stomata per mm^2 ; the areas of the stomata were calculated using the formula for the area of an ellipse (πab) and are given in μm^2 after measuring the polar (a) and equatorial (b) diameters of each stomata (Lawson et al., 1988). A minimum of five samples was examined for each Cd treatment.

Oxidative damage and antioxidant enzymes: Oxidative damage was estimated in terms of lipid peroxidation based on the production of 2-thiobarbituric acid (TBA)-reactive metabolites, particularly malondialdehyde (MDA), following the methods of Heath and Packer (1968) and Buege and Aust (1978). Measurements of MDA were performed as in Hodges et al. (1999), which takes into account the possible influence of interfering compounds in the assay for the TBA-reactive substances. Readings were taken using a spectrophotometer at 535 and 600 nm, and the MDA concentrations were expressed as nmol g^{-1} of fresh tissue.

Antioxidant enzymes were extracted by macerating 200 mg of fresh leaves and roots in 800 μL of an extraction buffer containing 100 mmol L^{-1} potassium phosphate buffer (pH=7.8), 100 mmol L^{-1} EDTA, and 1 mmol L^{-1} L-ascorbic acid. The protein contents of all of samples were determined using Bradford's method. Superoxide dismutase — SOD (EC 1.15.1.1) activity was measured photometrically at 560 nm, based on the inhibition by SOD of nitrobluetetrazolium (NBT) reduction. One unit of SOD was defined as the

amount needed to bring about a 50% inhibition of the NBT reduction state (Beyer and Fridovich, 1987). Catalase — CAT (EC 1.11.1.6) activity was measured photometrically at 240 nm as described by Kraus et al. (1995), with minor alterations as described by Azevedo et al. (1998), through the determination of the rate of conversion from H_2O_2 to O_2 . Total ascorbate peroxidase — APX (EC 1.11.1.11) activity was measured as the decrease in absorbance at 290 nm, resulting from ascorbate oxidation (Nakano and Asada, 1981). Guaiacol peroxidase — GPX (EC 1.11.1.7) activity was achieved photometrically at 470 nm (Souza and MacAdam, 1998) by measuring tetraguaiacol formation at 28 °C. Concerning the glutathione reductase — GR (EC 1.6.4.2), it was determined as described by Cakmak and Horst (1991) by measuring the decrease in absorbance at 340 nm due to NADPH oxidation.

Statistical analyses: All measurements were performed on five replicate plants per treatment. Data were analyzed for statistical significance using a one-way analysis of variance (ANOVA). When any effects were significant ($p \leq 0.05$), differences between means were evaluated using Scott-Knott's test. Regression analyses were also performed to test for relationships between the variables, using the software Sigma-Plot 11.0 (Systat Software Inc., USA).

RESULTS

Plant growth and visual symptoms: While shoot DW was not significantly affected ($p > 0.05$) by Cd doses administered in solution, root DW decreased in plants at both of the highest Cd concentrations (Figure 1). Reduction of 33.3 and 44.4% were seen in the roots of 45 and 90 $\mu\text{mol Cd L}^{-1}$ treated plants. Total biomass production did not significantly differ ($p > 0.05$) among the different treatments (Figure 1). Plants growing in the highest Cd concentrations showed wilting and yellowing of leaves and blackening of their roots by the end of the experimental period.

Pigment content: Decreases in chlorophyll *a* content and increasing levels of *b* were observed in plants exposed to the highest Cd concentrations ($p < 0.05$), as seen in Table 1. Moreover, between the Cd-treated plants, the β -carotene levels were greater in 90 $\mu\text{mol Cd L}^{-1}$.

Cadmium concentration and plant responses: A regression curve was used to establish the relationship

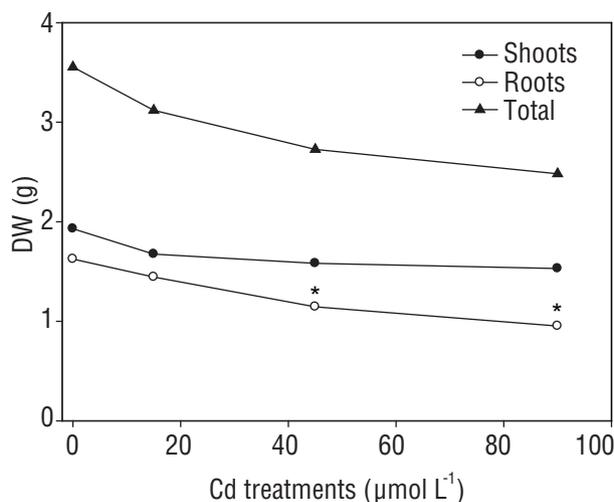


Figure 1. Shoot, root, and total dry weight (DW) of 60-day-old *Pfaffia glomerata* plants cultivated during 20 days in nutritive solution containing 0, 15, 45, and 90 $\mu\text{mol Cd L}^{-1}$. Each point is the mean of five measurements. *significant by the Scott-Knott's test ($p < 0.05$).

Table 1. Pigment content (mg g^{-1} of fresh weight), and water status (MPa) of 60-days-old *Pfaffia glomerata* plants cultivated during 20 days in nutritive solution containing 0, 15, 45, and 90 $\mu\text{mol Cd L}^{-1}$. Values are the mean of five measurements.

	Cd doses (mmol L^{-1})			
	0	15	45	90
Chlorophyll <i>a</i>	1.33 a	1.26 a	1.39 a	0.48 b
Chlorophyll <i>b</i>	0.43 b	0.43 b	0.47 b	0.87 a
Carotenoids	49.67 a	40.80 b	41.01 b	48.72 a
Ψ_w maximum	-0.36 a	-0.46 b	-0.46 b	-0.66 c

Several letters within the same line indicate significant differences (Scott-Knott, $p < 0.05$).

between Cd concentrations in the roots ($r^2=0.99$) and shoots ($r^2=0.93$) and those in the nutrient solutions (Figure 2). The Cd levels of the roots and shoots increased as Cd exposure increased (Figure 2). Increases of 6,829 and 9,266% in Cd concentrations were seen in shoots and roots in relation to the control, respectively, at the highest Cd exposures. These were always higher in roots than in shoots, regardless of the Cd solution concentrations, and the former showed marked increases as Cd exposure increased (Figure 2). Those from the shoots, by contrast, presented only small increases as Cd exposure increased (Figure 2).

The Cd-critical dose (CDS) and the Cd-toxicity level (TCL) were determined from regression curves. The Cd-CDS₁₀ was 20.48 for roots ($y=1.5685-0.6074x$, $r^2=0.97$) and 47.92 mmol L⁻¹ for shoots ($y=1.8912-0.0038x$, $r^2=0.72$), 50% shoot and root yield reductions (Cd-CDS₅₀) were not observed. The TCL *Pfaffia glomerata* was approximately 129 mg g⁻¹ DW ($y=1.8632-0.0015x$; $r^2=0.84$).

The Cd PTI was higher at 45 μmol Cd L⁻¹, and did not differ among the other Cd treatments (Table 2). The BCF of the shoots decreased at the highest Cd concentration (Table 2), and that of the roots had the same result, but was it lower at 45 than at 90 μmol Cd L⁻¹. TAR increased as Cd exposure was superior (Table 2). As expected, Cd uptake was higher in the presence of Cd than in controls, and increased as Cd concentrations in the nutrient solution increased (Table 2).

Root and leaf anatomical responses: Quantitative anatomical changes were observed in the root tissues of *Pfaffia glomerata* in response to Cd levels (Table 3). Root epidermis and endodermis thicknesses were higher in the roots of plants exposed to 90 μmol Cd L⁻¹ ($p<0.05$), but no changes were seen in terms of root diameters (Table 3). The relationships between endodermal thickness/shoot and root Cd contents (Figure 3) suggested a possible role of the endodermis in limiting Cd translocation to shoots at exposure levels above ~42 μmol Cd L⁻¹ (Figure 3); above this exposure the slope of the curve relating shoot Cd content and Cd doses decreased (Figure 2), corroborating this hypothesis.

Stomatal area decreased and stomata density increased as external Cd concentrations increased (Figure 4a).

Water status: The water status of *Pfaffia glomerata* decreased in the presence of Cd and was lower in plants with the highest Cd-exposure levels (Table 1). Decreases in stomatal areas associated with increasing stomatal densities were correlated with decreasing water status of the plants (Figure 4b).

Antioxidant enzyme activities: MDA level increased significantly in roots of plants exposed to Cd and in leaves of plants from 90 μmol Cd L⁻¹ (Table 4). SOD activity increased in the roots, but did not significantly change in the leaves as Cd exposure increased ($p>0.05$), as seen in Table 4. Initial increases in root SOD activity declined with exposure to more than 45 μmol Cd L⁻¹, however they still remained higher than the control.

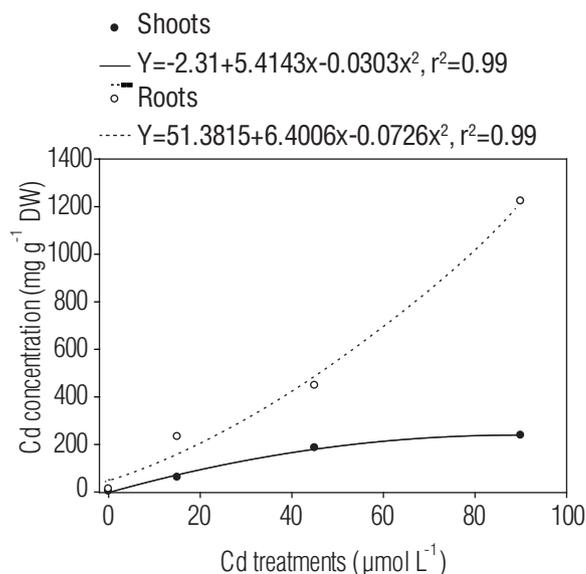


Figure 2. Relationships between the Cd concentrations of the roots and shoots and Cd treatments in 60-day-old *Pfaffia glomerata* plants cultivated during 20 days in nutritive solution containing 0, 15, 45, and 90 μmol Cd L⁻¹. Each point is the mean of five measurements.

Table 2. Primary transport index (PTI), shoot and root bio-concentration factors (BCF), total accumulation rate (TAR), and Cd uptake in 60-days-old *Pfaffia glomerata* plants cultivated during 20 days in nutritive solution containing 0, 15, 45, and 90 μmol Cd L⁻¹. Values are the mean of five measurements.

	Cd doses (mmol L ⁻¹)			
	0	15	45	90
Cd PTI	0.28 b	0.26 b	0.41 a	0.19 b
Shoot BCF	–	36.66 a	36.70 a	23.52 b
Root BCF	–	138.02 a	88.59 c	120.92 b
TAR (mg g ⁻¹ d ⁻¹)	0.39 d	9.25 c	14.91 b	31.23 a
Cd Uptake (mg g ⁻¹ d ⁻¹)	1.39 c	22.08 b	40.32 b	77.87 a

Different letters within the same line indicate significant differences (Scott-Knott, $p<0.05$).

The CAT levels in leaves responded differently to MDA, being lowest at 90 μmol Cd L⁻¹ and highest at 45, but not significantly different from 0 (control) or 15 μmol Cd L⁻¹ (Table 4). Roots, on the other hand, showed the highest CAT levels with the most superior Cd exposure, and lower levels in plants exposed to the 45 μmol Cd L⁻¹ treatment (Table 4).

Low APX levels were also seen in roots in the 45 μmol Cd L⁻¹ treatment, while APX ones in the leaves

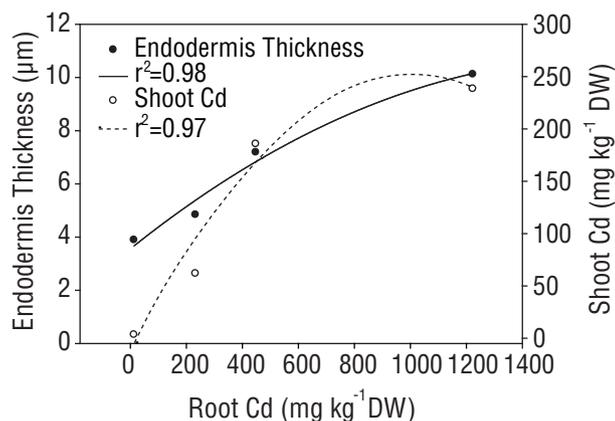


Figure 3. Relationships between endodermal thicknesses and shoot and root Cd concentrations of 60-day-old *Pfaffia glomerata* plants cultivated during 20 days in nutritive solution containing 0, 15, 45, and 90 $\mu\text{mol Cd L}^{-1}$. Each point is the mean of five measurements.

Table 3. Anatomical characteristics of 60-day-old *Pfaffia glomerata* plants cultivated during 20 days in nutritive solution containing 0, 15, 45, and 90 $\mu\text{mol Cd L}^{-1}$. Values are the mean of five measurements.

	Cd doses (mmol L^{-1})			
	0	15	45	90
Root tissues				
Epidermis (mm)	7.63 d	9.21 c	13.28 b	16.80 a
Endodermis (mm)	3.89 c	4.84 c	7.18 b	10.11 a
Root diameter (mm)	147.19	146.19	146.52	147.84 NS
Stomata				
Stomata density (number/ mm^2)	83.75 c	107.25 b	120.50 a	117.25 a
Stomata surface area (mm^2)	2,665.57 a	1,500.86 b	1,067.33 b	1,239.32 b

Different letters within the same line indicate significant differences (Scott-Knott, $p < 0.05$). NS: nonsignificant.

did not statistically differ among the different treatments (Table 4). GPX activity decreased in roots at 15 $\mu\text{mol Cd L}^{-1}$ and increased at 90 $\mu\text{mol Cd L}^{-1}$. GPX levels in the leaves increased in the highest Cd treatments (Table 4). GR activity in the roots decreased in response to higher Cd exposure, while its activity increased in the leaves with the same exposures (Table 4).

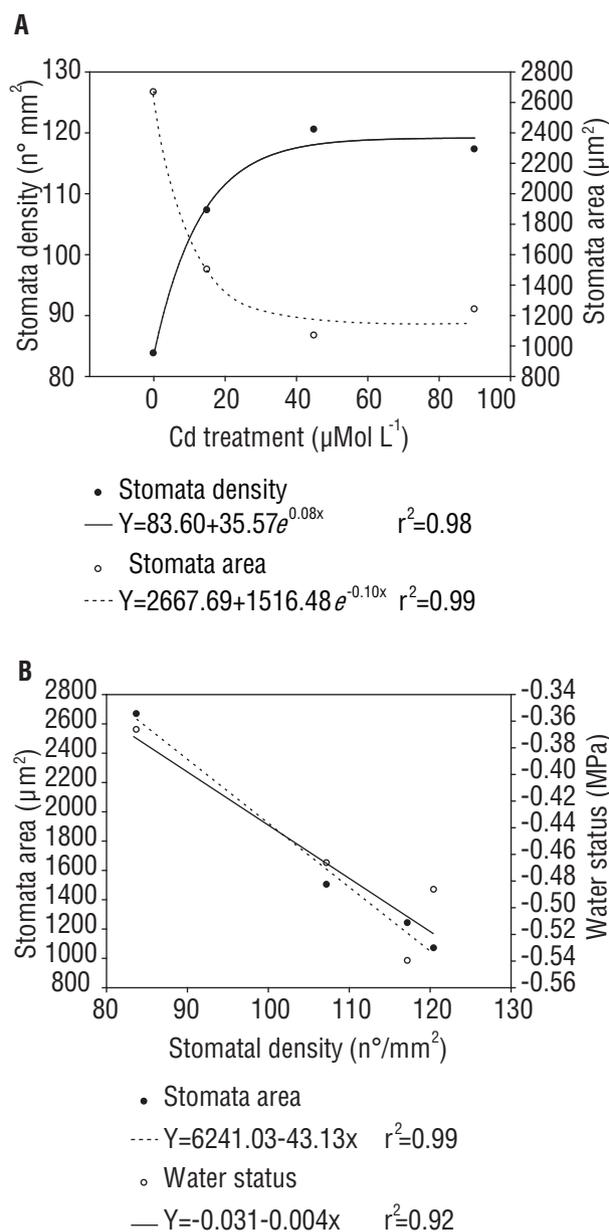


Figure 4. Relationships between stomatal densities and surface areas and Cd exposure (a) and between stomatal surface area, stomatal density, water status and the stomata densities (b) of 60-day-old *Pfaffia glomerata* plants cultivated during 20 days in nutritive solution containing 0, 15, 45, and 90 $\mu\text{mol Cd L}^{-1}$. Each point is the mean of five measurements.

DISCUSSION

Cd tolerance and uptake by *Pfaffia glomerata*:

Deleterious effects of Cd on the growth of *Pfaffia glomerata* plants were only observed at the highest medium Cd concentrations. Roots showed consistently

Table 4. Lipid peroxidation (MDA) and activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR) in the roots and leaves of 60-day-old *Pfaffia glomerata* plants cultivated during 20 days in nutritive solution containing 0, 15, 45, and 90 $\mu\text{mol Cd L}^{-1}$. Values are the mean of five measurements.

Cd (mmol L^{-1})	MDA	SOD	CAT	APX	GPX	GR
	($\text{nmol MDA g}^{-1} \text{FW}$)	($\text{Umg}^{-1} \text{protein}$)	($\text{mmol H}_2\text{O}_2 \text{mg}^{-1} \text{protein min}^{-1}$)	($\text{mmol Ascorbate mg}^{-1} \text{protein min}^{-1}$)	($\text{mmol Guaiacol mg}^{-1} \text{protein min}^{-1}$)	($\text{mmol NADPH mg}^{-1} \text{protein min}^{-1}$)
Roots						
0	27.30 c	1.26 d	0.33 b	7.65 c	9.35 b	1.32 a
15	37.43 b	5.86 a	0.29 b	8.62 b	8.05 c	0.53 b
45	44.62 a	4.75 b	0.20 c	4.94 d	9.57 b	0.44 b
90	48.36 a	3.55 c	0.49 a	9.40 a	10.20 a	0.58 b
Leaves						
0	144.45 b	5.49	0.79 b	2.36	0.82 c	0.50 c
15	137.72 b	8.23	0.84 b	3.94	0.74 c	0.52 c
45	129.07 b	6.33	1.00 a	3.38	1.00 b	0.76 b
90	298.81 a	6.91 NS	0.60 c	3.28 NS	1.60 a	0.95 a

Different letters within the same column indicate significant differences (Skott-Knott, $p < 0.05$). NS: nonsignificant.

higher Cd contents than shoots (Figure 2) as they came into more direct contact with Cd in nutrition solution, and therefore they were presumably more subject to the toxic effects of that heavy metal. Additionally, it is known that roots have a defensive role in protecting aboveground plant organs from heavy metal exposure (Mazhoudi et al., 1997). Nonetheless, the Cd-tolerance of *Pfaffia glomerata* is supported by their high Cd-CDS₁₀ and by the absence of 50% Cd-induced yield reductions. Shoot and root CDS₁₀ values were 47.92 and 20.48 mmol Cd L^{-1} , respectively, and they were much higher than those reported for other plants considered to be Cd-tolerant and used in Cd-phytoremediation programs in Brazil. CDS₁₀ levels of 2.4 and 1.5 mmol Cd L^{-1} in shoots and 9.0 and 6.4 mmol Cd L^{-1} in roots were reported for *Eucalyptus maculate* and *E. urophylla*, respectively (Soares et al., 2005); shoot CDS₁₀ in *Cedrela fissilis* was reported to be 6.7 mmol L^{-1} (Paiva et al., 2000). The CDS₁₀ of *Pfaffia glomerata* was about four to nine times higher than seen in these other species. CDS₁₀ levels similar to *Pfaffia glomerata* were reported in the native Brazilian tree *Tabebuia impetiginosa* (Paiva et al., 2000), indicating the potential value of native species in phytoremediation programs. In addition to relatively small decreases in biomass and its high CDS₁₀ value, the Cd-tolerance of *Pfaffia glomerata* was accompanied by a higher TCL (129 mg g^{-1} shoot DW). In species that are highly sensitive to this heavy metal, such values tend to range from 5 to 10 mg Cd g^{-1} , while tolerant species show those from 50 to 200 mg Cd g^{-1} (Kabata-Pendias and Pendias, 2001).

Visual symptoms like growth and biomass reductions and morphological alterations in roots are commonly seen in plants growing under toxic heavy metal

conditions, and occur even in tolerant or accumulator species (Gomes et al., 2011). The wilting and yellowing of leaves seen in plants at the highest Cd exposures are linked to Cd-induced decreases in both water potential and chlorophyll *a* biosynthesis (Table 1). Water balance disturbances have been reported in plants under heavy metal stress (Barceló et al., 1990; Wójcik et al., 2005), probably due to changes in size and number of xylem vessels resulting from alterations in plant hormonal balances (Poschenrieder and Barceló, 1999). Heavy metal effects on chlorophyll biosynthesis related to changes in δ -ALA-D enzyme activity have been reported (Skrebsky et al., 2008), and decreased water potentials and resulting tissue dehydration were also related to decreased δ -ALA-D activity (Singh et al., 1997), which catalyzes the condensation of two molecules of δ -aminolevulinic acid — ALA to porphobilinogen (Gibson et al., 1955). Nevertheless, at least to the studied time, reductions in plant water status and chlorophyll levels apparently did not provoke exaggerated effects on the photosynthetic system of *Pfaffia glomerata* as those plants did not show any detectable reductions in shoot biomass production. We also observed that between Cd treated plants, carotenoid contents were greater in those of the highest Cd exposure (Table 1), as well as these plants showed increased MDA levels (Table 4). Increases in carotenoid levels have been found to be related to increasing oxidative stress in plants, thus aiding them in avoiding major deleterious effects on photosynthesis (Singh et al., 2006).

Plants growing on contaminated substrates tend to concentrate Cd in their roots, and when Cd concentrations in the growth medium increase, metal concentrations in the roots can exceed shoot contents by more than 100-

fold (Kabata-Pendias and Pendias, 2001), as it was seen in the present study. Indeed, we verified a pronounced increase of Cd concentration in roots than in shoots of Cd treated plants compared to control, mainly from the highest Cd dose (9,266% in roots and 6,829% in shoots — Figure 2). One of the heavy metal-tolerance mechanisms of plants functions by decreasing metal translocation to the shoots, thus avoiding exaggerated negative growth effects by reducing heavy metal interference with photosynthetic processes, such as chlorophyll synthesis, chloroplast organization, and PSII activity (Sandalio et al., 2001).

Cd levels were found to be quite high in the shoots of *Pfaffia glomerata* (with means >200 mg per g of dry shoots). The shoot metal concentrations seen in *Pfaffia glomerata* classified the species as a Cd-hyperaccumulator, as previously reported by Carneiro et al. (2002). Although the BCF is more important than shoot concentrations *per se* when evaluating the phytoremediation potential of a given species (Zhao et al., 2003). The BCF is typically lower than one in excluding species, whereas that of metal-accumulating species is often greater than it (Baker, 1981). Accordingly, *Pfaffia glomerata* did conform to the definition of a metal accumulating species, as both its root and shoot BCF levels were greater than 1 (Table 2). However, it decreased as Cd exposure increased (Table 2). BCF levels generally tend to decrease as substrate metal concentrations increase (Zhao et al., 2003), indicating a diminishing efficiency of heavy metal accumulation with increasing exposure (Zhao et al., 2003). Decreasing BCF levels of both shoots and roots were only seen at doses up to their respective CDS_{10} , indicating that Cd-induced physiological changes (and thus Cd uptake) happen mainly up to these levels. The higher root BCF seen in plants at the highest Cd exposures in relation to that seen at $45 \mu\text{mol Cd L}^{-1}$ might mainly be associated with the greater Cd-PTI shown by plants exposed to $45 \mu\text{mol Cd L}^{-1}$, since Cd-uptake increased as Cd exposure increased. Furthermore, the increased Cd-uptake may explain the increasing TAR of plants as Cd exposure increased, as biomass production was only slightly affected.

According to Pence et al. (2000), decreases in BFC may result from the saturation of metal uptake and/or of root-to-shoot transport when internal metal concentrations are high. However, we observed increases in Cd-uptake that, in association with the lack of difference in the PTI (except in the $45 \mu\text{mol Cd L}^{-1}$ treatment where PTI was higher), indicated that Cd-uptake was not restricted or saturated in *Pfaffia glomerata* at substrate concentrations of up to $90 \mu\text{mol Cd L}^{-1}$. This demonstrates the ability of

this species to acquire, tolerate, and accumulate Cd in its tissues. Additionally, species of the genus *Pfaffia* are characterized by well-developed and bulky tuberous root systems (Marchioretto et al., 2010), and high uptakes and root retentions of Cd that argue for the use of *Pfaffia glomerata* in Cd-phytoextraction programs.

Morphological features associated with Cd tolerance in *Pfaffia glomerata*: Differential Cd tolerance levels observed among several plant species have been attributed to genetic or physiological features, such as the presence of blockers in roots that resulted in Cd allocation to apoplastic spaces (Marchial et al., 1996). Anatomical alterations in plants are related to changes in physiological processes and plant vigor under heavy metal stress (Lux et al., 2004), and may be associated with their water status and with metal distributions within the plants (Gomes et al., 2011). Roots have shown increased epidermal and endodermal thicknesses in the presence of Cd (Table 3), which is typical of exposure to heavy metal contamination (Lux et al., 2004; Gomes et al., 2011).

Epidermal thickening may increase negative charge accumulation and biological filtering of metal ions (Gomes et al., 2011). Increasing endodermal thickness (an apoplastic barrier) may lower Cd translocation to the shoots (Figure 3) and decrease its toxic effects on photosynthetic systems, and heavy metal tolerance may be related to the proportion of the root represented by root epidermal and apoplastic barrier tissues (Lux et al., 2004). The chemical modifications of apoplastic barriers and their thickening are important plant adaptations to stress (Enstone et al., 2003), and a number of studies has reported that the endodermis is the most important site of heavy metal accumulation (Ederli et al., 2004; Lux et al., 2004; Wójcik et al., 2005). According to Enstone et al. (2003), this tissue serves as a physiological barrier protecting the plant from various stress factors. As seen under salt-stress, the development of apoplastic barriers (such as the endodermis) has been correlated with increasing resistance to the radial flow of water and solutes in roots, resulting in reduced ion uptakes (i.e. Na^+) into the shoots and in better survival under subsequent acute stress (Krishnamurthy et al., 2011).

Cd exposure resulted in the appearance of xeromorphic characteristics in leaves (increased stomatal density but reduced stomata size), probably due to water balance disturbances (Poshchenrieder and Barceló, 1999), and the enhanced accumulation of primary metabolites in the leaves (Lunácková et al., 2003/4). Decreases in the water potentials of Cd treated

plants were associated with induced xeromorphic characteristics (Figure 4B). The increase in lipid peroxidation activity in leaves (as seen at the highest Cd doses — Table 4) may have resulted in those in primary metabolites (soluble proteins and lipids) reported by Gupta et al. (2011). Environmental factors have produced a wide variety of adaptations in the leaf anatomies of different taxa (Dickison, 2000) with some features (such as the reduction in stomatal pore area), contributing to reduced water loss through transpiration (Dickison, 2000). Smaller stomata create high resistance, resulting in lower transpiration rates (Abrams et al., 1994), but the fact that phytomass production was not observed to be affected by increasing Cd concentrations in the present study (Figure 1) suggests that assimilation processes were not limited by carbon (CO₂) availability. Increasing stomatal density is often a response to decreasing stomatal surface areas to assure surface gas exchange and, consequently, adequate carbon influx. Increasing stomatal density and decreasing stomatal surface areas may represent anatomical leaf tolerance mechanisms in *Pfaffia glomerata* for controlling transpiration processes, as the regulatory efficiencies of stomata opening mechanisms are associated with stomatal size (Abrams et al., 1994).

Induced xeromorphic characteristics (in addition to the thickness of endodermis) may be important to Cd-tolerance in *Pfaffia glomerata*. The observed reductions in leaf water potential apparently did not constrain biomass production, and may be associated with the increased endodermis thickness and reduced water flow into the xylem vessels (Krishnamurthy et al., 2011). Decreases in transpiration rates, due to heavy metal effects on stomatal characteristics (Yang et al., 2004), and by mechanical blocking of water movements by the endodermis (Krishnamurthy et al., 2011) help preventing heavy metal movement to the upper parts of the plant, thus avoiding deleterious effects on photosynthetic sites. These same characteristics are apparently associated with the higher Cd concentrations encountered in the roots.

Based on significant water potential decreases and induced xeromorphic characteristics observed in leaves under Cd-stress conditions (both indicate lower transpiration rates than seen in control plants), and absence of any differences in total biomass production, *Pfaffia glomerata* plants can apparently accumulate biomass at rates similar to control plants while using lower water volumes. Similar results were reported by Rossato et al. (2011) in *Pluchea agittalis* plants exposed to toxic lead concentrations.

Physiological features associated with Cd tolerance in *Pfaffia glomerata*: Plants are able to increase their antioxidant enzyme activities in order to reduce oxidative stress, thus increasing their Cd tolerance (Singh et al., 2006). Antioxidative defenses fall into two general classes: low molecular weight antioxidants, composed of lipid-soluble membrane-associated antioxidants (α -tocopherol and β -carotene) and water-soluble reductants (GSH and ascorbate), and enzymatic antioxidants (SOD, CAT, GPX, and APX) (Cao et al., 2004). The role of SOD in antioxidative defenses is to eliminate reactive oxygen species (ROS) that generate H₂O₂, with the resulting H₂O₂ being removed by CAT, APX, and GPX (Cao et al., 2004). High activity levels of these enzymes have commonly been reported in plants exposed to heavy metals (Calgaroto et al., 2010; Gupta et al., 2011).

Significant increases in lipid degradation products (MDA) were observed in the roots and leaves of Cd treated plants (Table 4). The increased accumulation of lipid peroxides in seedlings was indicative of enhanced production of toxic oxygen species, and lipid peroxidation may be a consequence of ROS generation. Both redox (Cu and Fe) and non-redox actives (Zn and Cd) metal ions have been reported to increase lipid peroxidation via ROS generation in plants (Gallego et al., 1996). Cd-induced oxidative stress in sunflower leaves was observed to be mediated by ROS, as low levels of lipid peroxidation, the maintenance of GSH content, and increases in lipoxygenase activity were observed in the presence of free radical scavengers (Gallego et al., 1996).

Lipid peroxidation in roots may be a consequence of ROS generation, as was indicated by increasing SOD activity in Cd-treated plants (Dixit et al., 2001). Increases in SOD activity have been related to higher values in superoxide radical concentrations due to the synthesis of enzymes induced by superoxide-mediated signal transduction (Rossato et al., 2011). SOD represents the first line of antioxidant defense system (Mitller, 2002) and has a marked role in ROS scavenging in *Pfaffia glomerata* roots under Cd stress. We observed similar Cd effects on root CAT and APX activities: while increased activities were seen in roots at the highest Cd concentrations, both decreased at 45 $\mu\text{mol Cd L}^{-1}$. Leaves exposed to 45 $\mu\text{mol Cd L}^{-1}$ resulted in moderate oxidative damage (the lowest MDA levels among the Cd-treated plants), which would seem to be related to its increased Cd-PTI. Under these conditions, the low responses of APX and CAT would be compensated by increased GPX activity in this plant organ, and GPX was seen to show higher sensitivity to

increasing ROS levels in the roots of *Pfaffia glomerata*. CAT and APX, in turn, appear to be activated under conditions of higher oxidative stress, since the highest activities of these enzymes were seen in roots under the biggest ROS stress.

Unlike in roots, the SOD activity of leaves did not differ in the several Cd treatments (Table 4), and its role in scavenging excess ROS (seen by increased MDA levels in leaves at the highest Cd exposures) may be performed by other ROS-scavenging enzymes found there. GPX activity once again showed responses to increased ROS levels, with its activity increasing in leaves at higher Cd treatments, even though the MDA level in the 45 $\mu\text{mol Cd L}^{-1}$ treatment was not statistically different from control plants (Table 4). APX was insensitive to increasing ROS levels (the highest MDA activity was seen with the highest Cd treatments) and remained stable; CAT activity was not able at the same time (Table 4). The different affinities of CAT (mM range) and APX (μM range) for H_2O_2 from ROS suggest that APX might be responsible for fine-tuning ROS for signaling, while CAT may be responsible for removing excess ROS during stress (Mittler, 2002).

In regards to GR, its activity decreased in roots in response to increasing Cd exposure, while increasing in the leaves (Table 4). GR catalyses the NADPH-dependent reduction of oxidized glutathione, and GSH (the product of this reaction) is an essential antioxidant and a substrate for phytochelatin synthesis (Dixit et al., 2001). This enzyme is responsive to different types of stress (Foyer et al., 1991), and both increases (Dixit et al., 2001) and decreases (Gallego et al., 1996) in its activity have been observed in response to Cd exposure. The higher GR activities seen in leaves (as observed in the present study) has been associated with its crucial role in combating oxidative stress in these tissues (Foyer et al., 1991).

In conclusion, our results demonstrated that *Pfaffia glomerata* has great potential for use in Cd-phytoextraction programs, being a Cd-hyperaccumulator species, as previous proposed by Carneiro et al. (2002). The high Cd-tolerance of this species is closely related to anatomical and physiological features, including endodermal thickness, stomatal size and number, root SOD and GPX activities, and leaf GPX and CAT activities can serve as Cd-tolerance markers for this species. Due to its high Cd-uptake and root accumulation growing *Pfaffia glomerata* plants in Cd-contaminated areas for subsequent medical use must be strictly avoided.

ACKNOWLEDGEMENTS

Authors are grateful to the *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* (FAPEMIG) for their financial support.

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