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# Lead Induced Responses of *Pfaffia glomerata*, an Economically Important Brazilian Medicinal Plant, Under In Vitro Culture Conditions

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**Abstract** Plantlets of *Pfaffia glomerata* (Spreng.) were exposed in vitro for 30 days to five lead levels (0–400 μM) to analyze the effects on growth and oxidative stress and responses of various antioxidants vis-à-vis lead accumulation. The plantlets showed significant lead accumulation in roots (1,532 μg $g^{-1}$ DW) with a low root to shoot lead translocation (ca. 3.6%). The growth of plantlets was negatively affected by various lead treatments, although the level of photosynthetic pigments did not alter significantly in response to any lead treatment. However, plantlets suffered from oxidative stress as suggested by the significant increase in malondialdehyde levels in root (8.48 μmol $g^{-1}$ FW) and shoot (3.20 μmol $g^{-1}$ FW) tissues with increasing lead treatments. In response to the imposed toxicity, increases in the activities of catalase in root (4.14 ΔE $min^{-1}$ $mg^{-1}$ protein) and shoot (3.46 ΔE $min^{-1}$ $mg^{-1}$ protein) and superoxide dismutase in root (345.32 units $mg^{-1}$ protein) and shoot (75.26 units $mg^{-1}$ protein), respectively, were observed, while the levels of non-protein thiols and ascorbic acid were not affected significantly in either roots or shoots.

**Keywords** Lead · In vitro culture · Oxidative stress · *Pfaffia glomerata*

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Lead (Pb) is the most common metal contaminant in the environment (Watanabe 1997; Tripathi et al. 2006). Industrial activities, such as mining, smelting, burning of fossil fuels, dumping of municipal sewage sludge, and the manufacturing of pesticides and fertilizers have been the main cause of its spread in the environment (Pendias and Pendias 1992; Chaney and Ryan 1994; Huang et al. 1997). Average yearly emissions of Pb were 332,350 × 103 kg $year^{-1}$ in the atmosphere, 138 × 106 kg $year^{-1}$ in the aquatic ecosystems and 796 × 106 kg $year^{-1}$ in the land during 1983–1984 (Nriagu and Pacyna 1988). Pb is a non-essential element in the metabolic process and is a potential carcinogen in humans (IARC 2006). It has limited solubility in soil and generally has a low plant uptake due to complexation with organic matter, sorption on oxides and clays or precipitation as carbonates, hydroxides and phosphates (Lim et al. 2004). Nevertheless, even low level of Pb accumulation may negatively affect crop yields (Foder et al. 1998; Tripathi et al. 2006) by upsetting processes germination (Wierzbicka and Obidzinska 1998), cell division (Liu et al. 1994), photosynthesis (Foder et al. 1996), nitrogen assimilation (Singh et al. 1997) and activities of several enzymes (Mishra et al. 2006; Gupta et al. 2009). Since an increased generation of reactive

oxygen species (ROS) is commonly observed in plants as a response to many stresses including Pb (Clijsters et al. 1999), cells possess multi dimensional ways to regulate their levels. These include various enzymatic and non-enzymatic antioxidants. A few important enzymatic antioxidants include catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX; Mittler 2002), while non-enzymatic ones include ascorbic acid (AsA), glutathione (GSH) or non-protein thiols (NP-SH), carotenoids and α- tocopherol (Gratao et al. 2005). These antioxidants agents efficiently maintain ROS under normal circumstances. However, this regulation gets disturbed under conditions of increased ROS production by adverse environmental conditions and results into a state of oxidative stress leading to the oxidation of biomolecules or even cell death (Buckner et al. 2000).

In Central and South America, around 90 species of *Pfaffia* are well known as a folk medicine, roots of *Pfaffia* are used as aphrodisiac, stimulant and in the treatment of diabetes and inflammatory diseases (Oliveira et al. 1980). Extracts from roots of *Pfaffia glomerata* possess a central nervous system depressant activity (De Paris et al. 2000). Several economically important compounds have been isolated and identified from roots of *P. glomerata*, such as ecdysterone, saponins, glomeric acid, pfameric acid, rubrosterone, oleanolic acid and beta-glucopyranosyl oleanolate (Shiobara et al. 1993). Carneiro et al. (2002) showed that an unclassified species of the genus *Pfaffia* exhibited high tolerance to soil contamination, growing quite abundantly in soil containing 90 and 1,450 mg $kg^{-1}$ of Cd and Zn, respectively. The species *P. glomerata*, also shows potential to grow on Pb contaminated sites. In this context, we designed the present experiments to evaluate the Pb induced responses at the level of antioxidant mechanisms to see whether these contribute towards Pb tolerance in *P. glomerata*.

## Materials and Methods

*Pfaffia glomerata* (Spreng.) Pedersen, identified as BRA, collected from the field of Querência do Norte, Parana, Brazil, was used in this study. A voucher specimen is kept in the Herbarium at Department of Biology, University Federal de Santa Maria, Brazil (SMDB 7606). Nodal segments (1.0 cm long) without leaves from 35 day old plantlets in in vitro culture were inoculated in MS medium (Murashige and Skoog 1962), supplemented with 30 g $L^{-1}$ of sucrose, 0.1 g $L^{-1}$ of myo-inositol, 6 g $L^{-1}$ of agar. For the treatment of plantlets, we used $Pb(NO3)_2$, and treatments of Pb were 0 (control), 50, 100, 200, 400 μM. To each cultivation flask (8.0 cm in diameter, 13.0 cm of height with a volume of 653.12 $cm^3$), 100 mL of MS medium was added (pH adjusted to 5.6) and autoclaved. The in vitro cultured plants were grown in a growth chamber at 25 ± 1°C on a 16/8 h light/dark cycle with ~120 μmol $m^{-2}$ $s^{-1}$ of irradiance by cold fluorescent lamps. After 30 d of Pb exposure, 60 plantlets per replicate were randomly harvested.

Lead concentration was estimated in both roots and shoot of plantlets. The Pb concentration was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), using a Perkin Elmer Optima 4300 DV (Shelton, USA) equipped with a cyclonic spray chamber and a concentric nebulizer. The emission line selected was 220.353 nm.

At harvest, the plants were divided into root and shoots. Roots were rinsed twice with distilled water. Subsequently, growth and biochemical parameters were determined. Plant biomass was measured on fresh and dry weight basis. For fresh weight, excess water from the root was dried using tissue paper and weighed (expressed as g $plant^{-1}$). To obtain dry weight, roots and shoots were dried at 65°C to constant weight and weighed. Root length was determined according to Tennant (1975) and shoot length was measured with Vernier callipers, both expressed in cm $plant^{-1}$. The concentration of total chlorophyll was measured by the method of Arnon (1949) and total carotenoid concentration was determined by using the formula given by Duxbury and Yentsch (1956). Total protein concentration was measured following the method of Lowry et al. (1951) using bovine serum albumin as the standard. Non-protein thiol concentration in the plant tissues was measured spectrophotometrically with Ellman's reagent (Ellman 1959). Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). The degree of lipid peroxidation was measured following the method of El-moshaty et al. (1993). Catalase activity was assayed following the modified method of Aebi (1984). The activity of superoxide dismutase was assayed according to Misra and Fridovich (1972).

The experiment was done as randomized block design. Two-way analysis of variance (ANOVA) was done with all the data to confirm the variability of data and validity of results, and Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments (Gomez and Gomez 1984).

## Results and Discussion

The experiments were carried out thrice in in vitro culture and gave reproducible results. When *P. glomerata* plantlets were raised under increasing concentrations of Pb, plants accumulated significant amounts of Pb in a concentration dependent manner (Fig. 1) showing positive linear

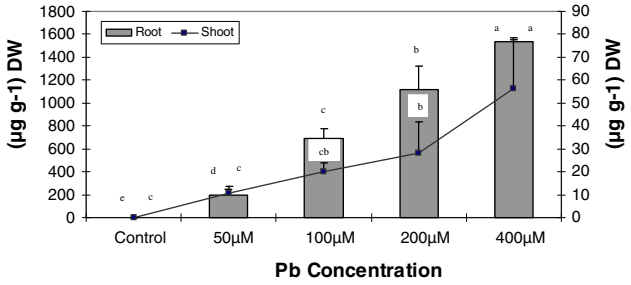


**Fig. 1** Lead concentration in *Pfaffia glomerata* plantlets treated with different levels of Pb for 30 days. All the values are mean of triplicates ± SD. ANOVA significant at $p < 0.01$. *Different letters* indicate significant different values at a particular duration (DMRT, $p < 0.05$)

relationships with the Pb concentration in the MS medium. The maximum Pb accumulation was found to be 1,532 µg $g^{-1}$ DW in roots and 56.50 µg $g^{-1}$ DW in shoots at 400 µM after 30 d of Pb treatment. The root to shoot Pb translocation averaged ca. 3.6%. *Pfaffia glomerata* plantlets showed significant toxicity with increasing Pb concentration (Table 1). Plantlets seemed to grow well in all treatments, however, the leaves turned light yellow and were stunted and the roots became bunched. Both fresh and dry weights of root and shoot tissues decreased in a Pb concentration dependent manner (Table 1) with the observed decrease at 400 µM Pb being more than 50% in all conditions. Plant length also decreased on Pb concentration basis with the maximum decrease at 400 µM being about 74% for root and 31% for shoot (Table 1). Photosynthetic pigments did not exhibit any significant response to Pb exposure (Table 2). However, total soluble proteins decreased with increased Pb concentration. The decline in protein level in roots was significant even at 50 µM, whereas in shoots, a significant decline occurred only above 100 µM. The maximum decline in protein levels was about 66% in roots and 11% in shoots at 400 µM Pb.

Table 3 shows the effects of Pb on the levels of NP-SH, AsA and lipid peroxidation. In both roots and shoots, the level of NP-SH, in general, did not show any significant alteration to any Pb treatment except the significant increase in shoot NP-SH at 50 µM Pb. The concentration of AsA also was not altered except a significant increase in root AsA at 50 µM of Pb, while a significant decline in shoot AsA occurred at of 400 µM of Pb. Root lipid peroxidation increased linearly with increasing Pb levels, whereas it increased in shoots only upon addition of Pb level exceeding 100 µM. The maximum increase in MDA concentration was recorded at 400 µM of Pb in both roots (178%) and shoots (44%). The activity of both the

**Table 1** Effect of Pb concentrations on fresh weight, dry weight and plant length in *Pfaffia glomerata* after 30 days

| Pb conc. | Fresh weight (mg) | | Dry weight (mg) | | Plant length (cm) | |
|---|---|---|---|---|---|---|
| | Root | Shoot | Root | Shoot | Root | Shoot |
| Control | 247 ± 80a | 527 ± 160a | 109 ± 10a | 42 ± 7a | 115 ± 25.6a | 9.75 ± 1.5a |
| 50 µM | 136 ± 30b | 500 ± 70a | 65 ± 10ab | 35 ± 6ab | 71.3 ± 17.1b | 7.96 ± 2.2ab |
| 100 µM | 126 ± 30b | 352 ± 90ab | 57 ± 10ab | 26 ± 8bc | 70.31 ± 17.4b | 7.90 ± 0.8ab |
| 200 µM | 125 ± 30b | 360 ± 90ab | 55 ± 8b | 21 ± 5c | 60.18 ± 6.4cb | 6.75 ± 0.9b |
| 400 µM | 94 ± 10b | 241 ± 90b | 51 ± 7b | 21 ± 5c | 30.31 ± 17.9c | 6.70 ± 1.5b |

All the values are mean of triplicates ± SD. ANOVA significant at $p < 0.01$. Different letters indicate significant different values at a particular duration (DMRT, $p < 0.05$)

**Table 2** Effect of Pb concentrations on total chlorophyll, total carotenoids and on total soluble protein concentrations in *Pfaffia glomerata* after 30 days

| Pb conc. | Total chlorophyll (mg $g^{-1}$) FW | Total carotenoids (mg $g^{-1}$) FW | Total soluble protein (mg $g^{-1}$) FW | |
|---|---|---|---|---|
| | | | Root | Shoot |
| Control | 1.55 ± 0.19a | 0.378 ± 0.06a | 1.45 ± 0.16a | 2.17 ± 0.08a |
| 50 µM | 1.50 ± 0.13a | 0.389 ± 0.06a | 0.76 ± 0.03b | 2.15 ± 0.08a |
| 100 µM | 1.45 ± 0.29a | 0.344 ± 0.04a | 0.71 ± 0.04b | 2.12 ± 0.04a |
| 200 µM | 1.37 ± 0.22a | 0.312 ± 0.07[a] | 0.55 ± 0.07c | 2.01 ± 0.05b |
| 400 µM | 1.35 ± 0.06a | 0.297 ± 0.07[a] | 0.49 ± 0.03d | 1.93 ± 0.04b |

All the values are mean of triplicates ± SD. ANOVA significant at $p < 0.01$. Different letters indicate significant different values at a particular duration (DMRT, $p < 0.05$)

[a] Represents significant value of DMRT

**Table 3** Effect of Pb concentrations on non-protein thiol, ascorbic acid and on lipid peroxidation levels in *Pfaffia glomerata* after 30 days

| Pb conc. | Non protein thiol (μmol $g^{-1}$) FW | | Ascorbic acid (μg $g^{-1}$) FW | | Lipid peroxidation (μmol $g^{-1}$) FW | |
|---|---|---|---|---|---|---|
| | Root | Shoot | Root | Shoot | Root | Shoot |
| Control | 0.872 ± 0.1a | 1.375 ± 0.1b | 212.14 ± 13.7b | 487.44 ± 65.4a | 3.05 ± 0.67c | 2.22 ± 0.12b |
| 50 μM | 0.929 ± 0.0a | 1.834 ± 0.1a | 251.28 ± 18.5a | 505.71 ± 47.5a | 4.45 ± 0.9cb | 2.40 ± 0.13b |
| 100 μM | 0.916 ± 0.0a | 1.513 ± 0.0b | 215.27 ± 16.9b | 486.92 ± 7.9[a] | 6.19 ± 0.11b | 2.59 ± 0.12b |
| 200 μM | 0.914 ± 0.0a | 1.508 ± 0.0b | 211.10 ± 10.9b | 450.39 ± 14.8a | 8.38 ± 0.09a | 2.98 ± 0.29a |
| 400 μM | 0.818 ± 0.1a | 1.429 ± 0.1b | 200.92 ± 2.4b | 370.54 ± 18.3b | 8.48 ± 2.23a | 3.20 ± 0.24a |

All the values are mean of triplicates ± SD. ANOVA significant at $p < 0.01$. Different letters indicate significant different values at a particular duration (DMRT, $p < 0.05$)

[a] Represents significant value of DMRT

**Table 4** Effect of Pb concentrations on catalase and on superoxide dismutase activities in *Pfaffia glomerata* after 30 days

| Pb conc. | Catalase (ΔE $min^{-1}$ $mg^{-1}$ protein) | | Superoxide dismutase (units $mg^{-1}$ protein) | |
|---|---|---|---|---|
| | Root | Shoot | Root | Shoot |
| Control | 2.96 ± 0.06d | 2.03 ± 0.05e | 100.96 ± 1.14e | 65.70 ± 0.71c |
| 50 μM | 3.30 ± 0.15c | 2.26 ± 0.11d | 178.50 ± 4.55d | 66.01 ± 0.07c |
| 100 μM | 3.50 ± 0.08c | 2.53 ± 0.06c | 221.23 ± 1.66c | 66.77 ± 0.47c |
| 200 μM | 3.73 ± 0.11b | 3.08 ± 0.08b | 301.15 ± 3.60b | 71.59 ± 0.85b |
| 400 μM | 4.14 ± 0.12a | 3.46 ± 0.07a | 345.32 ± 1.80a | 75.26 ± 1.88a |

All the values are mean of triplicates ± SD. ANOVA significant at $p < 0.01$. Different letters indicate significant different values at a particular duration (DMRT, $p < 0.05$)

enzymes, CAT and SOD demonstrated significant increases in both roots and shoots in response to Pb treatment (Table 4). The maximum increases in the activities of CAT (40% in roots and 70% in shoots) and SOD (242% in roots and 15% in shoots) were observed at 400 μM Pb.

Lead (Pb) is one of the most frequently encountered metals in polluted environments and is also one of most toxic elements to the plants (Piechalak et al. 2002). In our present study, we analyzed responses of an economically important medicinal plant, *Pfaffia glomerata*, to Pb exposure to understand its potential to tolerate Pb stress. We noticed an increase in Pb concentration in the plant tissues with an increase in the Pb levels in the medium (Fig. 1); however the level of translocation from root to shoot was low. Based on comparative studies of metal concentration in plant parts, Baker and Walker (1990) suggested that uptake, translocation and accumulation mechanisms differed for various metals and by species. It is known that the root system partially defends the above ground parts from Pb (Broyer et al. 1972; Mazhoudi et al. 1997), as shown in the present study. Mostly, the plants with highest tolerance take up the smallest proportion of the total metal and have the lowest shoot metal concentrations (Sudhakar et al. 1992; Liu et al. 2004a). Our study also indicated a significant uptake of Pb by roots with a small translocation too. Such a restriction in root-to-shoot translocation might have been due to complexation of Pb ions in roots, for example, by non-protein thiols (glutathione and phytochelatins; Mishra et al. 2006).

Although plantlets accumulated a very small amount of Pb in shoots, we found that the fresh weight and dry weight of both roots and shoots as well as the height of plantlets decreased significantly (Table 1). Similar results of significant decline in biomass were observed by Tandy et al. (2005) for *Helianthus annuus* growing in nutrient solution with Pb. Lead is known to adversely affect morphology, growth and photosynthetic processes of plants (Singh et al. 1997). Hence, it may appear that the negative impact to plant metabolism might have resulted in the observed decline in plant growth. However, the concentration of total chlorophyll and carotenoids did not alter upon Pb treatment (Table 2). This was in contrast to the previous observations of decreases in photosynthetic pigments in tomato (Cho and Park 2000) and in *Bacopa* (Mishra et al. 2006). Therefore, it seems that the decline in plant growth was not related to decreased chlorophyll pigment status but rather than other components viz., the level of total soluble proteins and lipids were affected, which would in turn cause decreased in photosynthetic activities. This hypothesis was found to be true as we observed significant

declines in total soluble protein concentration at higher concentration of Pb (Table 2) as well as a significant increase in the lipid degradation product, MDA (Table 3). Lipids and proteins are common targets for oxidative damage in tissues under environmental stress (Prassad 1996), suggesting that these changes were due to oxidative stress generation due to Pb accumulation (Mishra et al. 2006). To measure the counter-responses of plants to Pb-induced oxidative stress, we analyzed their antioxidant status. Although the enzymatic antioxidants viz., CAT and SOD demonstrated significant increases upon Pb exposure, the level of the non-enzymatic antioxidant, AsA did not change significantly. Increases in SOD and CAT activities may be linked to an increase in their substrates as well as to de novo synthesis of enzyme protein (Chongpraditnum et al. 1992; Verma and Dubey 2003; Reddy et al. 2005), which in turn may be associated with an induction of their genes (Fátima and Ahmad 2005). Similar results of increases in enzymatic activities were found with two cultivars of horse gram (*Macrotyloma uniflorum*) and Bengal gram (*Cicer arietinum*) (Reddy et al. 2005) and *Rumex dentatus* (Liu et al. 2004b). Hence, plants positively respond to the oxidative stress being imposed by Pb accumulation. SOD is the first enzyme of the antioxidant defense system dismutating superoxide radicals to produce hydrogen peroxide, whereas CAT is one of the major enzymes involved in degradation of hydrogen peroxide (Mittler 2002). In organelles, such as chloroplasts which contain high concentrations of AsA, direct reduction of superoxide radicals by AsA is also rapid. Thus, the two major enzymes of antioxidant metabolism were increased suggesting that plants tolerate Pb toxicity to some extent. However, the lack of a significant response by AsA might have led to a reduced potential for the plants to respond to oxidative stress and hence they experienced toxicity. Tolerance to Pb is not one way phenomenon and requires support from other pathways. According to a proposed mechanism, metal detoxification occurs at two levels. The primary means of detoxification is complexation of the metal via some ligand to render it non-toxic and the secondary mechanism is control of oxidative metabolism (Mishra et al. 2006). The level of NP-SH also was not altered significantly at most of the Pb exposures. This result is not consistent with other investigations, which have shown increased NP-SH in plants exposed to Pb (Mishra et al. 2006; Gupta et al. 2009). Non-protein thiols (NP-SH) are an inconclusive term including thiolic constituents like glutathione and phytochelatins. These components are essential for detoxification of a metal like Pb (Gupta et al. 1995).

Therefore, from our present investigation, we conclude that *P. glomerata* plantlets were unable to coordinate the various secondary response mechanisms needed to achieve desired tolerance of Pb-induced oxidative stress, and that primary complexation of Pb ions presumably does not provide enough support to significantly reduce Pb toxicity.

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