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EFEITOS FISIOLÓGICOS DO MERCÚRIO EM PLANTAS DE *Pfaffia glomerata* (Spreng.) Pedersen

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
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EFEITOS FISIOLÓGICOS DO MERCÚRIO EM PLANTAS DE *Pfaffia glomerata* (Spreng.) Pedersen

por

Nicéia Spanholi Calgaroto

Dissertação apresentada ao Programa de Pós-Graduação em Agronomia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Agronomia.**

Orientador: Prof. Fernando Teixeira Nicoloso

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elaborada por
Nicéia Spanholi Calgaroto

como requisito parcial para a obtenção do grau de
Mestre em Agronomia

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Santa Maria, 26 de fevereiro de 2009.

DEDICATÓRIA

Aos meus pais, Nice e Deonildo, e ao meu irmão,
Julian, pela compreensão e pelo apoio durante mais
esta etapa de minha vida.
Ao Marcelo, meu namorado,
pelo carinho e incentivo que me deste
em todos os momentos.

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Agronomia
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EFEITOS FISIOLÓGICOS DO MERCÚRIO EM PLANTAS DE *Pfaffia glomerata* (Spreng.) Pedersen

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ORIENTADOR: FERNANDO TEIXEIRA NICOLOSO
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O mercúrio (Hg) é um metal pesado não-essencial altamente tóxico aos organismos. A contaminação dos solos, ar e água por este metal leva a sua acumulação em peixes, aves e mamíferos e a intoxicação humana, através da alimentação. Devido aos efeitos danosos do Hg ao ecossistema, é necessário que estas áreas sejam reabilitadas. A fitorremediação é uma técnica de baixo custo que pode ser utilizada com tal objetivo. Para que a planta seja utilizada na fitorremediação, no entanto, é necessário haver conhecimento sobre o comportamento vegetal quando exposto ao contaminante. Portanto, o objetivo deste trabalho foi caracterizar aspectos fisiológicos e bioquímicos da toxidez do Hg e o papel do Zn em aliviar estes efeitos em plantas de *P. glomerata*. Foram realizados dois experimentos, cujas plantas foram cultivadas *in vitro* e aclimatizadas *ex vitro*, quando foram aplicados os tratamentos. Após nove dias de exposição aos tratamentos, foram avaliados parâmetros de crescimento e parâmetros bioquímicos ligados ao estresse oxidativo. No primeiro experimento, as plantas foram expostas a quatro concentrações de Hg (0,0, 1,0, 25 e 50 µM). Já no segundo experimento, o Hg e o Zn foram adicionados à solução nutritiva de modo a formar quatro tratamentos (sem Zn e Hg (controle); 50 µM Zn; 50 µM Hg; 50 µM Zn + 50 µM Hg). A concentração de Hg foi maior nas raízes do que na parte aérea das plantas expostas ao Hg e sua presença induziu o estresse oxidativo, bem como causou danos aos tecidos vegetais nas concentrações de 25 e 50 µM Hg. A concentração de Hg foi maior na parte aérea do que nas raízes apenas nos tratamentos em que o Hg não foi adicionado à solução nutritiva (controle e 50 µM Zn). A massa fresca e seca das raízes e da parte aérea diminuiu e foi observado aumento da concentração de malondialdeído (MDA) nos tecidos em relação ao controle, quando expostos a 50 µM Hg, indicando que houve dano aos tecidos. A atividade das enzimas antioxidantes e a concentração de antioxidantes não enzimáticos aumentaram com a presença de Hg e com a adição de Zn. O tratamento de Zn diminuiu a massa fresca e seca das plantas, por outro lado não houve alteração na peroxidação lipídica. Houve interação entre os elementos Zn e Hg quando fornecidos simultaneamente na solução nutritiva. O sistema antioxidante de *P. glomerata* foi importante na regulação do dano oxidativo. No entanto, em 50 µM Hg, estes mecanismos não foram suficientes para reverter o dano causado por este elemento. O Zn parcialmente evitou os danos causados pelo Hg, observados pela significante diminuição da peroxidação lipídica e pela maior porcentagem de sobrevivência de plantas neste tratamento.

Palavras-chave: *Pfaffia glomerata;* estresse oxidativo; interação Zn-Hg; metal pesado; antagonismo iônico

ABSTRACT

Master Dissertation
Graduate Program in Agronomy
Universidade Federal de Santa Maria

PHYSIOLOGICAL EFFECTS OF MERCURY IN PLANTS OF *Pfaffia glomerata* (Spreng.) Pedersen

AUTHOR: NICÉIA SPANHOLI CALGAROTO
ADVISER: FERNANDO TEIXEIRA NICOLOSO

Mercury (Hg) is a highly toxic non-essential heavy metal. Soil, water and air contamination by Hg leads to its accumulation in fish, birds and mammals, thus entering human food. Due to the hazardous effects to the ecosystem, it is necessary that contaminated soils be rehabilitated. Phytoremediation is a cheap technique that can be utilizated with this objective, however, it is necessary to be knowledgeable about plant behavior during metal exposure. Therefore, the aim of this study was to characterize biological and biochemical aspects of Hg toxicity and the role of Zn to alleviate these effects in *P. glomerata* plants. For both experiments, plants were grown *in vitro* and acclimated *ex vitro*, for the application of treatments. Nine days later, growth and biochemical parameters of oxidative stress were evaluated. In the first experiment, plants were exposed to four concentrations (0.0, 1.0, 25 and 50 µM Hg). In the second experiment, four treatments of Hg and Zn were added to the nutrient solution (without Zn or Hg (control); 50 µM Zn; 50 µM Hg and; 50 µM Zn + 50 µM Hg). The Hg concentration was higher in shoot than in roots only in treatments without Hg (control and 50 µM Zn). Fresh and dry weight of roots and shoot decreased and a high malondialdeide (MDA) concentration was observed at 50 µM Hg, indicating that tissue damage occurred. The antioxidant enzyme activity and non-enzymatic concentration increased with the presence of Zn+Hg. Plants exposed to Zn showed a decrease in fresh and dry weight, but there was no significant increase in MDA production. There was an interaction between Zn and Hg. The antioxidant system of *P. glomerata* plants was important in oxidative damage regulation, however, at 50 µM Hg these mechanisms were not able to revert the damage caused by Hg. Zinc addition parcially prevented Hg damage, observed by the significant decrease in lipid peroxidation and higher survival percentage.

Keywords: *Pfaffia glomerata*; oxidative stress; mercury, Zn-Hg interaction, heavy metal, ionic antagonism

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LISTA DE ABREVIATURAS

ALA - ácido 5-aminolevulínico

APX - ascorbato peroxidase

AsA - ácido ascórbico

ATP - adenosina trifosfato

CAT - catalase

Cd - cádmio

CETESB - Centro Tecnológico de Saneamento Básico

CH_3Hg^+ - metilmercúrio

cDNA - ácido desoxirribonucléico complementar

Cu - cobre

$CuSO_4$ - sulfato de cobre

CuZnSOD - superóxido dismutase dependente de cobre e zinco

DMSO - dimetilsulfóxido

DNA - ácido desoxirribonucléico

DNPH - dinitrofenilidrazina

DTNB - ácido 5-5´-ditio-bis-(nitrobenzóico)

EDTA - ácido etilenodiaminotetracético

EROs - espécies reativas de oxigênio

Fe - ferro

FeSOD - superóxido dismutase dependente de ferro

GPX - glutationa peroxidase

GSH - glutationa reduzida

GSSG - glutationa oxidada

HCl - ácido clorídrico

Hg - mercúrio

Hg^{2+} - íon mercúrio

$HgCl_2$ - cloreto de mercúrio

HO• - radical hidroxila

H_2O_2 - peróxido de hidrogênio

HNO_3 - ácido nítrico

H_2SO_4 - ácido sulfúrico

K - potássio

KI - iodeto de potássio

K_2HPO_4 - fosfato de potássio

MDA - malondialdeído

Mn - manganês

MnSOD - superóxido dismutase dependente de manganês

NADPH - nicotinamida adenina dinucleotídeo fosfato

NPSH - tióis não-protéicos

O_2 - oxigênio molecular

O_2^- - radical superóxido

$^1O_2$ - radical oxigênio singleto

PBG - porfobilinogênio

pH - potencial de hidrogênio

PVP - polivinilpirrolidona

SOD - superóxido dismutase

TBA - ácido tiobarbitúrico

TCA - ácido tricloroacético

Zn - zinco

Zn^{2+} - íon zinco

$ZnCl_2$ - cloreto de zinco

δ-ALA-D - delta-aminolevulinato desidratase

-SH - grupo sulfidril

SUMÁRIO

1 INTRODUÇÃO

Devido às atividades antropogênicas tais como a mineração, a aplicação de fertilizantes, de lodo de esgoto e de fungicidas contendo mercúrio (Hg) no solo, tem aumentando significativamente a disponibilidade desse metal pesado nas áreas agricultáveis e outros ecossistemas (PATRA & SHARMA, 2000). No ano 2000, o nível médio de Hg colocado em terras aráveis foi de 39 kg km^{-2} (HAN et al., 2002). Esta grande quantidade de Hg adicionada no solo resultou na contaminação da cadeia alimentar (ZHOU et al., 2008). Estudos demonstraram que o Hg pode acumular-se nos tecidos de plantas superiores (WANG & GREGER, 2004; ISRAR et al., 2006), invertebrados aquáticos e peixes (BOENING, 2000).

Diferente de outros metais, tais como Cu, Zn ou Mn, o Hg não é um elemento essencial às plantas e aos organismos em geral, e a exposição a concentrações relativamente baixas de Hg no solo resulta em séria toxidez (SALT et al., 1995).

O aumento dos níveis de Hg no solo pode induzir a uma série de efeitos adversos no crescimento e no metabismo das plantas (VERMA & DUBEY, 2003; PATRA et al., 2004), tais como no processo fotossintético e na transpiração (KRUPA & BASZYNSKI, 1995), redução da absorção de água e nutrientes minerais (CHO & PARK, 2000; PATRA & SHARMA, 2000), inibição da síntese de clorofila (GODBOLD & HUTTERMANN, 1986; CARGNELUTTI et al., 2006) e aumento da peroxidação lipídica (CHO & PARK, 2000).

Diversos estudos mostraram que a toxidez induzida por Hg nas plantas resulta da ligação de sua forma iônica Hg^{2+} aos grupos sulfidril (-SH) das proteínas, com consequente quebra de sua estrutura e substituição de elementos essenciais (VAN ASSCHE & CLIJSTERS, 1990; HALL, 2002; SCHÜTZENDÜBEL & POLLE, 2002). A interferência do Hg na atividade mitocondrial também foi observada (MESSER et al., 2005), bem como a formação de ligações covalentes dos íons Hg^{2+} com o DNA (CHAOUI et al., 1997) e a indução da troca de cromátides irmãs no núcleo (BEAUFORD et al., 1977). Deste modo, o Hg altera o desenvolvimento normal da plantas, levando à inibição do crescimento da raiz e da parte aérea (SUSZCYNSKY & SHANN, 1995).

A redução da taxa fotossintética na presença de Hg pode ser uma consequência da inibição da síntese de clorofilas, devido à inibição da atividade da enzima delta-aminolevulinato desidratase (δ-ALA-D). A δ-ALA-D é sensível aos metais pesados porque contém grupos -SH em sua estrutura (MORSCH et al., 2002). Esta enzima catalisa a

condensação assimétrica de duas moléculas de ácido δ-aminolevulínico (ALA) originando o porfobilinogênio (PBG) (GIBSON et al., 1955). A síntese de PBG promove a formação de porfirinas, hemes e clorofila, que são essenciais para o metabolismo da clorofila e da fotossíntese (JAFFE et al., 2000).

Outro efeito do Hg é induzir o estresse oxidativo (ALI et al., 2000; CHO & PARK, 2000; ORTEGA-VILLASANTE et al., 2005; CARGNELUTTI et al., 2006; ZHOU et al., 2008) através da produção acelerada de espécies reativas de oxigênio (EROs), tais como o ânion superóxido (O_2^-), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($OH^\cdot$) (PATRA & SHARMA, 2000; ISRAR & SAHI, 2006).

As plantas desenvolveram uma variedade de estratégias para prevenir a acumulação excessiva de metais não-essenciais nas células e/ou transformar estes metais em formas menos tóxicas (COBBETT, 2000). Algumas plantas produzem metabólitos que se ligam aos metais pesados no citosol, tais como glutationa (GSH), peptídeos (metalotioneínas) e prolina (HALL, 2002). No entanto, quando estes mecanismos de defesa não são suficientes e a superprodução de EROs ocorre, causando o estresse oxidativo, outros mecanismos antioxidantes são acionados (PATRA et al., 2004). Enzimas antioxidantes, tais como a superóxido dismutase (SOD, E.C. 1.15.1.1), a ascorbato peroxidase (APX, E.C. 1.11.1.11) e a catalase (CAT, E.C. 1.11.1.6), bem como o sistema de defesa não-enzimático, que inclui os tióis não-protéicos (NPSH), o ácido ascórbico (AsA) e os carotenóides, têm suas atividades ou produção aumentadas. Estas alterações metabólicas podem ser utilizadas como um indicativo do grau de estresse oxidativo (ZHOU et al., 2006).

Em vista da crescente contaminação dos solos por Hg e da expansão da contaminação a outros ecossistemas, é importante estabelecer metodologias para a reabilitação destas áreas contaminadas. A idéia de se utilizar plantas para remover e reciclar seletivamente metais em excesso no solo surgiu com a descoberta de plantas, geralmente endêmicas de solos naturalmente mineralizados, que acumulam altas concentrações de metais em sua folhagem (GARBISU & ALKORTA, 2001). A utilização de plantas hiperacumuladoras de metais pesados na remediação de ambientes contaminados tem sido atribuída como uma tecnologia prática e com maior custo benefício do que as técnicas convencionais (CHANEY et al., 1997).

Uma espécie não identificada pertencente ao gênero *Pfaffia* foi encontrada em uma área contaminada por minério de calamina, da Companhia Mineira de Metais, no estado de Minas Gerais. O conteúdo de metais pesados presente nos tecidos desta espécie foi estudado por Carneiro et al. (2002). Esses autores constataram que as plantas acumularam quantidades

bastante significativas de Cd e Zn na parte aérea, o que pode indicar um potencial genético à tolerância a metais pesados.

Para que uma espécie vegetal seja utilizada na reabilitação de áreas contaminadas por metais pesados é necessário estudar os aspectos fisiológicos e bioquímicos da planta quando exposta ao contaminante. No entanto, não há disponibilidade de informações acerca da toxicologia do Hg e sobre os mecanismos pelo qual esse elemento produz estresse oxidativo nas plantas de *Pfaffia glomerata*. Deste modo, os objetivos do presente trabalho foram:

1.1 Objetivos:

1.1.2. Objetivos Gerais

Caracterizar os efeitos do Hg em aspectos morfológicos, fisiológicos e bioquímicos de plantas de *Pfaffia glomerata*.

Caracterizar os efeitos fisiológicos da suplementação de zinco em plantas de *P. glomerata* submetidas ao estresse por Hg.

1.1.3. Objetivos Específicos

Avaliar a atividade de enzimas antioxidantes (catalase, ascorbato peroxidase e superóxido dismutase) e os níveis de antioxidantes não-enzimáticos (carotenóides, ácido ascórbico e tióis não-protéicos) em plântulas de *P. glomerata* após exposição ao Hg e ao Zn.

Determinar os níveis de peroxidação lipídica e a concentração de peróxido de hidrogênio após exposição ao Hg e ao Zn.

Avaliar a atividade da enzima δ-ALA-D e as concentrações de clorofila e de prolina em plântulas de *P. glomerata* após exposição ao Hg e ao Zn.

Determinar o conteúdo de Hg absorvido pelas plântulas de *P. glomerata* após exposição ao Hg e ao Zn.

2 REVISÃO DE LITERATURA

2.1 Mercúrio - escala global

O Hg ocorre naturalmente no ambiente associado a outros elementos, principalmente com o enxofre, com quem forma o minério cinábrio (HgS), composto de cor vermelha ou preta, atóxico, cujas maiores reservas encontram-se na Espanha (Almadén) e na Itália (CANELA, 1995). O Hg metálico é obtido por aquecimento do cinábrio seguido de condensação. Outras fontes naturais de Hg são a degradação da crosta terrestre através das erupções vulcânicas, a evaporação natural e as minas de Hg, as quais são responsáveis por emissões deste elemento na ordem de 2700-6000 toneladas/ano (CLARKSON, 1992).

O aporte antrópico do Hg ocorre através de indústrias que queimam combustíveis fósseis, da produção eletrolítica de cloro-soda, produção de acetaldeído, incineradores de lixo, polpa de papel, tintas, pesticidas, fungicidas, lâmpadas de vapor de Hg, baterias, produtos odontológicos, amalgamação de Hg em extração de ouro, entre outros. Estimativas indicam que 200.000 toneladas de Hg foram emitidas para a atmosfera desde 1890, sendo que aproximadamente 95% permanecem no solo terrestre, 3% nas águas oceânicas superficiais e 2% na atmosfera (CANELA, 1995; OSA, 1994).

A concentração de Hg normalmente encontrada no solo varia de 20–625 ppb (ATSDR, 1999). O Hg está presente no solo em várias formas e estados de oxidação. Assim como o efeito de um metal é determinado sinergisticamente ou antagonisticamente por outros cátions metálicos e seus ânions associados presentes no solo (MUNZUROGLU & GECKIL, 2002), alguns elementos podem formar complexos estáveis com o Hg (CATHUM et al., 2005) reduzindo tanto a quantidade de Hg absorvida pelas plantas quanto a sua disponibilidade na solução do solo, além de reduzir a toxicidade dos solos contaminados. Além disso, há uma forte afinidade do Hg^{2+} e seus compostos inorgânicos às substâncias que contêm enxofre (grupos –SH e cisteína). O Hg se liga a esses compostos formando um complexo que limita grandemente a sua mobilidade nos solos (USEPA, 1997).

Muitos compostos inorgânicos de Hg, quando adicionados à matéria orgânica e a outros fatores redutores do solo, decompõem-se à forma não-iônica (Hg^0), bastante volátil e

facilmente absorvida pela planta através dos estômatos (FREAR & DILLS, 1967). As formas iônicas Hg^{+2} e CH_3Hg^+ (metilmercúrio) são fortemente complexadas por ácidos húmicos, fúlvicos e outras moléculas orgânicas presentes nos ecossistemas naturais (MIRETSCKI et al., 2005). No solo, esses complexos organo-mercuriais são adsorvidos na superfície das argilas e na matriz sólida, que consiste principalmente de óxidos de ferro, alumínio, manganês e substâncias húmicas (ROULET et al., 1998). Os solos argilosos, portanto, têm alta capacidade de retenção de Hg. Nos solos bem oxigenados, as moléculas de Hg predominantes são os compostos solúveis $HgCl_2$, Hg(OH)Cl e $Hg(OH)_2$. O Hg orgânico pode ser convertido a CH_3Hg^+ ou $(CH_3)_2Hg$ (dimetilmercúrio), suas formas mais tóxicas, pela ação de microrganismos (bactérias metanogênicas), processo conhecido como biotransformação (FARREL et al., 1990; DAUGHNEY et al., 2002). A biotransformação do Hg inorgânico em CH_3Hg^+ representa um sério risco ambiental, visto que se acumula na cadeia alimentar aquática. Por ter capacidade de permanecer por longos períodos nos tecidos dos organismos, o Hg pode ser encontrado nos peixes predadores da extremidade da cadeia em concentrações elevadas, podendo culminar no regime alimentar da população humana (BOENING, 2000).

2.2 Mercúrio no Brasil

No Brasil, a maior parte dos estudos sobre a contaminação dos solos com Hg está relacionada à região da Amazônia, onde ocorreu grande parte da extração de ouro em décadas passadas (DOREA et al., 2003; BRABO et al., 2000; CASTILHOS & BIDONE, 2000) e cuja contaminação têm levado à acumulação de Hg em peixes de água doce, bem como à contaminação das populações humanas ribeirinhas por utilizarem o peixe como fonte de alimentação.

Existem estudos relacionados à contaminação por Hg na região sul do Brasil, tal como nos sedimentos do rio Camaquã, devido à mineração do ouro e prata durante o período colonial, até a década de 1990 (PESTANA & FORMOSO, 2003; PESTANA et al., 2000). Também há a descrição da contaminação por Hg de solos do município de Lavras do Sul - RS, e sedimentos do rio Camaquã que passam pela cidade, devido à exploração de ouro no século XX. Nestes locais foram encontrados *hot spots* de concentração de Hg, demonstrando a persistência da contaminação, que iniciou em 1900 (PESTANA & FORMOSO, 2003).

O CETESB (Centro Tecnológico de Saneamento Básico) publicou em 2001 o "Relatório de Estabelecimento de Valores Orientadores para Solos e Águas Subterrâneas no Estado de São Paulo", no qual apresentou uma lista preliminar de valores orientadores para proteção da qualidade de solos e das águas subterrâneas. Estes valores foram reformulados em 2005 e apresentados pela DECISÃO DE DIRETORIA Nº 195-2005-E (CETESB, 2005) (Tabela 1). Os valores orientadores são divididos em:

Tabela 1 - Valores orientadores para solo e água subterrânea para detecção de contaminação por mercúrio.

Solo ($mg.kg^{-1}$ de peso seco)					Água subterrânea ($\mu g.L^{-1}$)
Referência de Qualidade	Prevenção	Intervenção			Intervenção
		Agrícola (APMax)	Residencial	Industrial	
0,05	0,5	12	36	70	1

Fonte: CETESB (2005).

- Valor de Referência de Qualidade (VRQ): é a concentração de determinada substância no solo ou na água subterrânea, que define um solo como limpo ou a qualidade natural da água subterrânea, devendo ser utilizado em ações de prevenção da poluição do solo e das águas subterrâneas e no controle de áreas contaminadas.
- Valor de Prevenção (VP): é a concentração de determinada substância, acima da qual podem ocorrer alterações prejudiciais à qualidade do solo e da água subterrânea. Este valor indica a qualidade de um solo capaz de sustentar as suas funções primárias, protegendo-se os receptores ecológicos e a qualidade das águas subterrâneas. Deve ser utilizada para disciplinar a introdução de substâncias no solo e, quando ultrapassado, a continuidade da atividade será submetida à nova avaliação, devendo os responsáveis legais pela introdução das cargas poluentes procederem ao monitoramento dos impactos decorrentes.
- Valor de Intervenção (VI): é a concentração de determinada substância no solo ou na água subterrânea acima da qual existem riscos potenciais, diretos ou indiretos, à saúde humana. Para o solo, foi calculado utilizando-se procedimento de avaliação de risco à saúde humana

para cenários de exposição Agrícola - Área de Proteção Máxima (APMax), Residencial e Industrial.

Grazia & Pestana (2006) constataram que as amostras de solo analisadas de vários locais no município de Lavras do Sul estão contaminadas em níveis superiores aos de intervenção para Hg, conforme dados obtidos da CETESB (2001; 2005) para o estado de São Paulo.

2.3 Efeitos fisiológicos do mercúrio e do zinco em plantas

Stefanov et al. (1995) relataram que as espécies de plantas diferem na sua sensibilidade aos metais. As plantas que crescem em habitats com altas concentrações de metais provavelmente têm a habilidade para inativar estes elementos. Este processo acontece devido a formação de complexos entre o íon metálico e os grupos –SH produzidos pelas plantas. Também, as plantas que crescem em habitats metalíferos mudam a composição química e a organização física das suas membranas celulares, impedindo que os íons sejam absorvidos pelas células. Portanto, a absorção do Hg orgânico e inorgânico do solo pelas plantas é pequena (LODENIUS, 1990), e há provavelmente uma barreira para a translocação desse elemento das raízes para a parte aérea das plantas (PATRA et al., 2004). Os fatores que afetam a disponibilidade do Hg presente na solução do solo às plantas incluem a sua concentração externa e o tempo de exposição, o conteúdo de matéria orgânica presente no solo, a capacidade de troca de cátions, o conteúdo de óxidos e de carbonatos e o potencial redox (CHO & PARK, 1999).

As diferenças no conteúdo de Hg variam entre os diferentes tipos e idade dos tecidos e entre as árvores de mesma espécie (RASMUSSEN et al., 1991). O Hg inorgânico na forma Hg^{2+} tem alta afinidade pelos grupos -SH e, consequentemente, podem interromper funções onde proteínas estão envolvidas (CLARKSON, 1972). Um íon de Hg pode se ligar a dois locais em uma molécula de proteína sem deformar a cadeia, mas uma concentração suficientemente alta deste elemento pode levar a precipitação protéica (PATRA & SHARMA, 2000). A ação tóxica do Hg pode estar relacionada a uma inibição não-específica de uma variedade de enzimas e a várias enzimas respiratórias contendo tióis *in vitro* (PATRA et al., 1994).

O Hg afeta tanto as reações fotoquímicas como as de carboxilação da fotossíntese. Ele inibe fortemente a cadeia de transporte de elétrons fotossintética, sendo que o fotossistema II é o alvo mais sensível. Íons Hg^{2+} interagem com Zn^{2+} situados nas proteínas D1 e D2 e com o grupo de íons Mn do complexo evoluidor de oxigênio, ambos localizados no lado doador do fotossistema II, e também com o dímero de clorofila a no centro do fotossistema I. Os íons Hg^{2+} formam complexos organometálicos com aminoácidos das proteínas do cloroplasto e também causam a depleção de um polipeptídeo da submembrana do fotossistema II (BERNIER & CARPENTIER, 1995; BERNIER et al., 1993; SERSEN et al., 1998; YOU et al., 1999). O Hg, quando adicionado na solução nutritiva na forma de $HgCl_2$, apresentou efeito oxidante em plântulas de pepino (*Cucumis sativus*), caracterizado por significante decrescimento no conteúdo de clorofila e danos à membrana, consequentes dos altos níveis de peroxidação de lipídios e oxidação de proteínas (CARGNELUTTI et al., 2006).

Nas raízes, o Hg interage fortemente com grupos sulfidril de enzimas e proteínas vitais (VAN ASSCHE & CLIJSTERS, 1990). O íon Hg^{+2} liga-se com as proteínas dos canais de água, causando uma obstrução física do fluxo de água (MAGGIO & JOLY, 1995) e, consequentemente, afetando a transpiração das plantas. Zhang e Tyerman (1999) relataram que o Hg inibe a captação de água via aquaporinas da membrana plasmática de vegetais.

Outro sintoma da toxidez de Hg em plantas é a redução do crescimento (DU et al. 2005), indicando que a acumulação de Hg nas raízes bloqueia a captação e o transporte de nutrientes (BOENING, 2000), e induz ao excesso de produção de etileno (GOREN & SIEGEL, 1976).

Sendo um metal de transição, o Hg pode induzir ao estresse oxidativo em plantas, resultando na peroxidação de lipídios, no vazamento de K^+, na alteração da atividade de enzimas antioxidantes e na indução de compostos contendo tióis (ALI et al, 2000). Os danos oxidativos induzidos por Hg nas células vegetais têm sido vinculados ao excesso de produção de espécies reativas de oxigênio (EROs) (ALI, 2000).

Estudos recentes têm demonstrado que o Hg pode induzir à expressão de genes codificadores das enzimas superóxido dismutase, peroxidase e catalase (SÄVENSTRAND & STRID, 2004), enzimas antioxidantes responsáveis pela defesa da planta contra as EROs. Zhou et al. (2006, 2008) constataram que, em concentrações de 10, 20 e 40 µM Hg, as plantas de alfafa (*Medicago sativa*) apresentaram aumento na peroxidação de lipídios, alteração na atividade de várias enzimas antioxidantes, tais como as enzimas ascorbato peroxidase,

glutationa, superóxido dismutase (SOD) e peroxidase (POD), o que evidencia a indução do estresse oxidativo pelo Hg.

O Zn, diferente do Hg, é um elemento essencial para o crescimento e o desenvolvimento vegetal (CAKMAK & MARSCHNER, 1993). As funções do Zn estão ligadas à atividade da enzima delta-aminolevulinato desidratase (δ-ALA-D, enzima dependente de Mg ou de Zn para que sua atividade seja máxima) (JAFFE, 1995); à atividade da enzima superóxido dismutase dependente de Cu e Zn (CuZnSOD) (GRENE, 2002); à indução da síntese de fitoquelatinas (HIRATA et al., 2001), à proteção dos grupos –SH de enzimas (CAKMAK, 2000) e à proteção das membranas celulares da oxidação pelas EROs (ARAVIND & PRASAD, 2005). Além disso, o Zn tem um papel importante na regulação da absorção de vários íons (CAKMAK & MARSCHNER 1988). Há relatos da interação entre Zn e Cd e do efeito protetor do Zn em plantas, efeito decorrente da ativação do sistema de defesa antioxidante pelo Zn, bem como por evitar a absorção e/ou translocação do Cd para a parte aérea (CATALDO et al., 1983; THYS et al., 1991; TSUJI et al., 2002; ARAVIND & PRASAD, 2003).

2.4 Fitorremediação

Solo contaminado é aquele que apresenta concentrações de determinado elemento químico acima do esperado em condições naturais. A contaminação por metais pesados tem origem da atividade antrópica ou da acumulação resultante de processos biogeoquímicos ocorridos na natureza (McBRIDE, 1994). As principais rotas antrópicas de entrada de metais pesados no solo são a deposição de rejeitos industriais, fertilizantes e pesticidas e resíduos urbanos como compostos de lixo e lodo de esgoto. No solo, estes rejeitos sofrem transformações químicas que podem liberar metais pesados para a solução do solo e causar toxidez às plantas e organismos, ou ainda serem adsorvidos às argilas ou complexados à matéria orgânica, representando uma fonte poluidora potencial e importante via de exposição dos metais poluentes.

A destruição da cobertura vegetal em áreas contaminadas agrava a degradação do solo, promovendo erosão hídrica e eólica e a lixiviação dos contaminantes para o lençol freático,

desencadeando progressivo grau de contaminação de outras áreas, animais e plantas (Fig. 1). Essas áreas precisam ser reabilitadas, e para tal exigem estudos diversificados sobre o solo, a vegetação e a água (CUNNINGHAM et al., 1996). A presença de metais pesados no solo é perigosa porque, além dos desequilíbrios ambientais causados, os contaminantes podem entrar na cadeia alimentar e colocar em risco à saúde humana (LANTSY & MACKENZIE, 1979; ANGELONE & BINI, 1992).

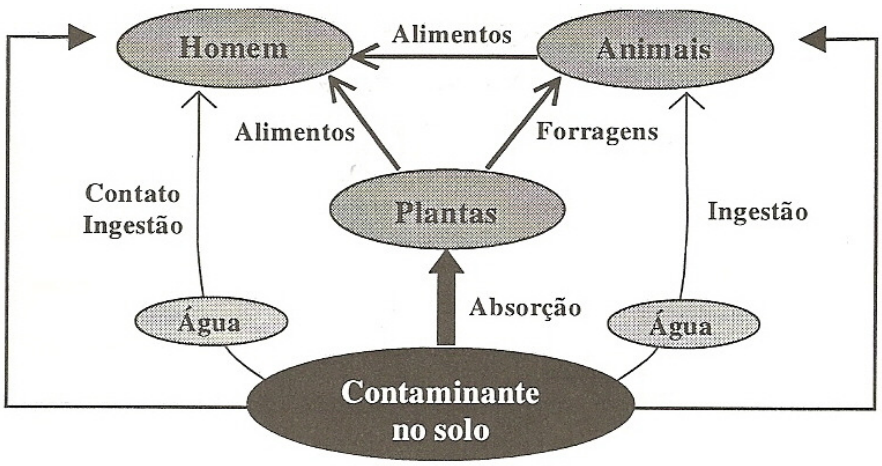

Figura 1 - Principais vias de exposição pelas quais contaminantes do solo atingem plantas, animais e homem. Fonte: Accioly & Siqueira (2000).

A recuperação de áreas contaminadas por metais pesados, tal como o Hg, pode ser feita através de vários métodos, tais como escavação, incineração, extração com solvente, oxidorredução e outros que são bastante dispendiosos. Alguns processos deslocam a matéria contaminada para locais distantes, causando riscos de contaminação secundária e aumentando ainda mais os custos com tratamento (CUNNINGHAM et al, 1996). Por isso, em anos recentes passou-se a dar preferência por métodos *in situ* que perturbem menos o ambiente e sejam mais econômicos. Dentro deste contexto, a biotecnologia oferece a fitorremediação como alternativa capaz de empregar sistemas vegetais fotossintetizantes e sua microbiota com o fim de desintoxicar ambientes degradados ou poluídos (CUNNINGHAM et al, 1996).

A fitorremediação envolve técnicas biológicas e químicas, como o uso de plantas e sua microbiota associada, de amenizantes de solo e de práticas agronômicas que, aplicadas em conjunto, removem, imobilizam ou tornam os contaminantes menos disponíveis ao ecossistema (ACCIOLY & SIQUEIRA 2000). Na Alemanha, a remediação de locais contaminados com o auxílio de plantas é utilizada para o tratamento de esgoto municipal a

pelo menos 300 anos (CUNNINGHAM et al., 1996). O tipo de poluente, a concentração e a presença de toxinas no solo ou na água devem estar dentro dos limites de tolerância da planta. Portanto, é fundamental conhecer o sistema do metabolismo vegetal a ser empregado e os fatores determinantes da técnica para posterior avaliação de sua eficiência.

A fitorremediação oferece várias vantagens que devem ser levadas em conta: (a) permite a reciclagem de metais e produção de madeira; (b) pode ser uma solução permanente; (c) aplicação *in situ*, evitando escavação; (d) usa energia solar para realizar os processos; (e) aplicável a grande variedade de contaminantes, e (f) tem ótima resposta social, melhor estética, razão pelo qual é mais aceitável publicamente do que outras tecnologias. Grandes áreas podem ser tratadas de diversas maneiras, a baixo custo, com possibilidades de remediar águas contaminadas, o solo e subsolo e ao mesmo tempo embelezar o ambiente. Entretanto, o tempo para se obter resultados satisfatórios pode ser longo (CUNNINGHAM et al., 1996). Riscos tais como a possibilidade dos vegetais entrarem na cadeia alimentar, devem ser considerados quando empregar esta tecnologia (CUNNINGHAM et al., 1996). Em contraste com outras tecnologias que são apropriadas para pequenas áreas com altos níveis de contaminação, a fitorremediação é ideal para grandes áreas cuja contaminação do solo seja média ou baixa ou quando se empregam amenizantes (WATANABE, 1997). Além disso, a vegetação reduz a erosão eólica ou hídrica, contribuindo para minimizar a disseminação dos contaminantes para outras áreas, enquanto o processo de remediação está em curso (ACCIOLY & SIQUEIRA, 2000).

A fitorremediação pode ser classificada, dependendo da técnica a ser empregada, da natureza química ou da propriedade do poluente e baseada nos processos fisiológicos da planta em fitoestabilização e fitodescontaminação (Fig. 2).

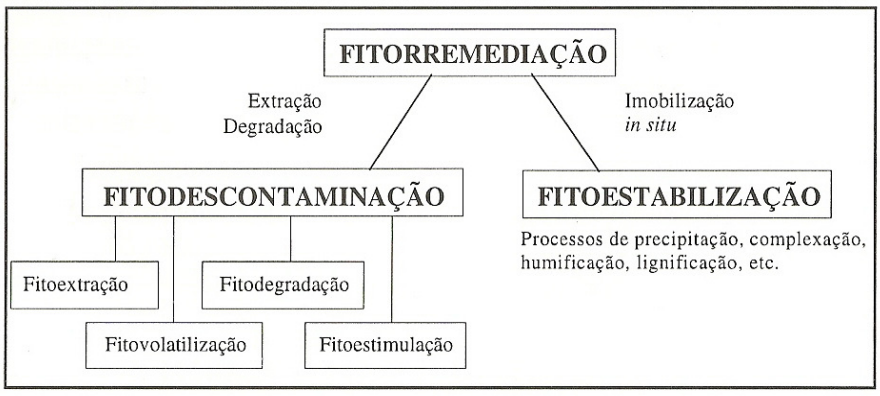

Figura 2 - Processos da fitorremediação do solo. Fonte: Accioly & Siqueira (2000).

A fitodescontaminação visa reduzir a concentração dos contaminantes do solo e da água a um nível aceitável, através da ação direta das plantas, da degradação do contaminante pela microflora e/ou da associação destes. Os processos que estão incluídos na descontaminação do solo incluem a fitodegradação (os contaminantes orgânicos são mineralizados dentro das células vegetais por enzimas específicas); a fitoextração (absorção dos contaminantes pelas raízes, os quais são nelas armazenados ou são transportados e acumulados nas partes aéreas) a fitovolatilização (os íons são absorvidos pelas raízes, convertidos em formas não tóxicas e depois liberados na atmosfera) (BROOKS, 1998) e a fitoestimulação, na qual, em razão da liberação de exsudatos radiculares, há o estímulo à atividade microbiana, que atua degradando o composto contaminante no solo (SANTOS et al., 2007).

A fitoestabilização visa reduzir o potencial de dano ao meio ambiente, pela redução da mobilidade e disponibilidade do contaminante no solo. Consiste no uso de plantas para imobilizar contaminantes no sistema solo-planta, visando reduzir a biodisponibilidade destes e prevenir sua entrada nas águas subterrâneas ou na cadeia alimentar. Portanto, não há a remoção do contaminante do solo, mas sim sua imobilização, humificação e lignificação nos tecidos vegetais (ACCIOLY & SIQUEIRA, 2000).

A tolerância, definida como a capacidade da planta em conviver com o excesso de contaminantes acumulados em seus tecidos, é um aspecto fundamental na fitorremediação, envolvendo inúmeros mecanismos. Esses resultam do impedimento na absorção (SHAW, 1989), ou de mecanismos bioquímicos de tolerância ao contaminante (BARCELÓ & POSCHENRIEDER, 1992). As plantas que apresentem impedimento à absorção são interessantes para os processos de fitoestimulação e fitoestabilização, porque, na fitoextração, é imprescindível que haja absorção do contaminante (ACCIOLY & SIQUEIRA, 2000). Segundo Schat & Kalff (1992), os mecanismos bioquímicos envolvidos na tolerância podem ser através de: (a) produção intercelular de compostos ligantes, tais como aminoácidos, ácidos orgânicos e fitoquelatinas; (b) alterações nas formas de compartimentalização; (c) alterações no metabolismo celular, e (d) alterações na estrutura da membrana.

De acordo com Antosiewics (1992), espécies herbáceas coletadas em áreas contaminadas são fontes potenciais para programas de fitorremediação, uma vez que as mesmas apresentam adaptações a ambientes estressantes. A maioria das plantas fitorremediadoras conhecidas são de clima temperado e pertence a família Brassicaceae (ACCIOLY & SIQUEIRA, 2000).

2.5 *Pfaffia*

O gênero *Pfaffia* é composto por cerca de 90 espécies distribuídas na América Central e do Sul. No Brasil, 27 espécies foram descritas (TANIGUCHI et al., 1997), sendo que a *Pfaffia glomerata* é a espécie do gênero de maior importância medicinal e comercial (VIGO et al., 2003; FIGUEIREDO et al., 2004; ZIMMER et al., 2006). Em condições naturais, a *Pfaffia glomerata* ocorre principalmente à beira de rios e nas orlas das matas de galeria, onde pode receber bastante luz, e por isso é tida como uma espécie higrófita e heliófita (SMITH & DOWNS, 1972). Entretanto, esta espécie se desenvolve bem em solos drenados, tanto argilosos quanto arenosos (RIBEIRO & PEREIRA, 1994; MONTANARI et al., 1999; BENTES et al., 2000), em altitudes de até 1000 m e em regiões com precipitação pluviométrica entre 1200 – 1500 mm anuais (CORREA JÚNIOR et al., 2002). Caracteriza-se por apresentar plantas perenes, subarbustivas ou arbustivas, com caules eretos ou semi-eretos, geralmente ocos, glabros ou levemente pilosos, com altura de 0,5 a 2,5 metros. A raiz é tuberosa e geralmente bifurcada. Floresce nos meses de novembro a junho, e suas inflorescências são do tipo espiga subglobosa. As flores são hermafroditas, bracteadas, com cinco sépalas livres iguais, cinco estames, anteras monotecas e ovário contendo apenas um óvulo; estilete curto ou ausente, estigma capitado ou bilobado. Apresenta folhas de pecíolo curto, ovado-lanceoladas, com 5-12 cm de comprimento e 1-2cm de largura (SMITH & DOWNS, 1972).

A *P. glomerata* é conhecida como Ginseng Brasileiro, devido ao formato de suas raízes, muito semelhante às do Ginseng Coreano (*Panax ginseng* C.A. Meyer) e às suas propriedades tônicas e estimulantes, as quais são amplamente empregadas na medicina popular. Portanto, o interesse comercial da espécie está nas raízes tuberosas, que são utilizadas como anti-reumáticas, anti-inflamatórias, analgésicas (NETO et al., 2005), anti-tumorais, antidiabetes e tônico afrodisíaco (MAGALHÃES, 2000; CORREA JÚNIOR et al., 2002), anticancerígeno (LAZZARINI, 2001), para tratar de distúrbios gástricos (FREITAS et al., 2004) e em doenças relacionadas a memória, estresse e envelhecimento (DIAS et al., 1996; MARQUES, 1998; GALVÃO, 1996; TASCHETTO & PAGLIARINI, 2001).

Vários compostos já foram identificados em extratos de raízes destas plantas. Takemoto et al. (1983), em pesquisa realizada com *Pfaffia paniculata* (Martius) Kuntze, descrevem a presença de saponinas com atividade sobre certos tipos de tumores malignos.

Shiobara et al. (1993) isolaram ácidos fáficos até então desconhecidos de *Pfaffia glomerata*: o ácido glomérico (triterpenóide) e o ácido famérico (nortriterpenóide).

O extrato de *P. glomerata*, quando administrado na dose de 1000 mg kg^{-1} induziu maior taxa de natalidade e espermatogênese vigorosa, histologicamente comprovada, em hamsters machos (MICHIHIRO et al., 1998). Efeito semelhante foi observado em ratos, que apresentaram aumento de síntese de DNA nas espermatogônias. Estes resultados indicam uma possível atividade estênica de *P. glomerata.*

Além destas propriedades, a maioria das espécies de *Pfaffia* já citadas forneceu quantidades variáveis de ecdisteróides. A ecdisterona é um hormônio precursor da ecdisona, indutor das mudas nos insetos (ecdises). Rações compostas a base de *P. glomerata* foram administradas em criações de bichos-da-seda, no Japão, prolongando seu estágio larval, a fim de obterem maior rendimento na sericultura (NINAGI & MARUYAMA, 1996). À ecdisona (20-hidroxiecdierona) é também atribuído um efeito antioxidante, o qual confere mais este uso medicinal para espécies de Ginseng Brasileiro (DANIEL et. al, 2006).

O amplo emprego dos metabólitos secundários das espécies de *Pfaffia* na medicina popular e como matéria-prima na indústria de fitoterápicos e de cosméticos tem afetado as reservas naturais devido à exploração predatória, levando à sua colocação como espécies em risco de extinção. Segundo Montanari (2001) e Ferreira (1998), a exportação de biomassa de *Pfaffia* para, principalmente, o Japão e os Estados Unidos, põem em risco a variabilidade genética das populações naturais.

Recentemente, uma nova utilidade tem sido associada ao gênero *Pfaffia*. Carneiro et al. (2002), ao avaliar um total de 31 espécies herbáceas visando a escolha destas para emprego em programas de fitorremediação, encontrou em área de mineração de hemimorfita ($Zn_4S_2O_7(OH)_2.2H_2O$) uma planta do gênero *Pfaffia* sp., conhecida vulgarmente como calaminacia, que se mostrou bastante tolerante ao excesso de metais pesados no solo em comparação a outras espécies estudadas. Neste estudo, *Pfaffia* apresentou grande potencial de acúmulo de metais na parte aérea, uma vez que acumulou teores de até 133 e 272 mg kg^{-1} de Cd e Zn, respectivamente. Os teores de Cd encontrados na *Pfaffia* sp. foram superiores ao valor mínimo de 100 mg kg^{-1} para definir espécies hiperacumuladoras (BAKER, 1981), sendo este o primeiro relato de espécie tropical considerada hiperacumuladora.

2.6 Espécies Reativas de Oxigênio (EROs) e estresse oxidativo

As EROs são formas parcialmente reduzidas do oxigênio atmosférico, resultantes da excitação do O_2 para formar oxigênio singleto (O_2^1) ou da transferência de um, dois ou três elétrons para o O_2 para formar, respectivamente, o radical superóxido (O_2^-), peróxido de hidrogênio (H_2O_2) ou o radical hidroxila (OH^-) (MITTLER, 2002). Elas são geradas endogenamente durante transições do desenvolvimento, tais como na maturação de sementes, e como um resultado normal do metabolismo respiratório e do processo fotossintético.

No entanto, uma ampla variedade de estresses ambientais (tais como temperaturas extremas, seca, salinidade, UV, metais pesados e infecção por patógenos) é potencialmente danosa às plantas (VAN BREUSEGEM et al., 2001), uma vez que sob tais condições ambientais adversas a homeostase redox da célula é interrompida (FOYER et al., 1994), porque cessam o transporte de elétrons na cadeia transportadora de elétrons (CTE) (VAN BREUSEGEM et al., 2001). Com o rompimento da homeostase celular ocorre a aceleração da produção de EROs (LAMB & DIXON, 1997), o que pode causar estresse oxidativo (ASADA, 1994). A produção aumentada de EROs durante o estresse pode ser uma ameaça às células, mas sua produção pode ter função de sinalização para a ativação dos processos de defesa e resposta ao estresse (DESIKIN et al., 2001; KNIGHT & KNIGHT, 2001). A ação das EROs como danosas ou como sinalizadoras e protetoras depende do equilíbrio delicado entre a produção de EROs e sua limpeza em local e tempo apropriado, através da ação do sistema de defesa antioxidante (Fig. 3). A toxicidade do oxigênio pode resultar em dano tecidual e até morte celular se houver produção descontrolada ou limpeza ineficiente das EROs (EDREVA, 2005).

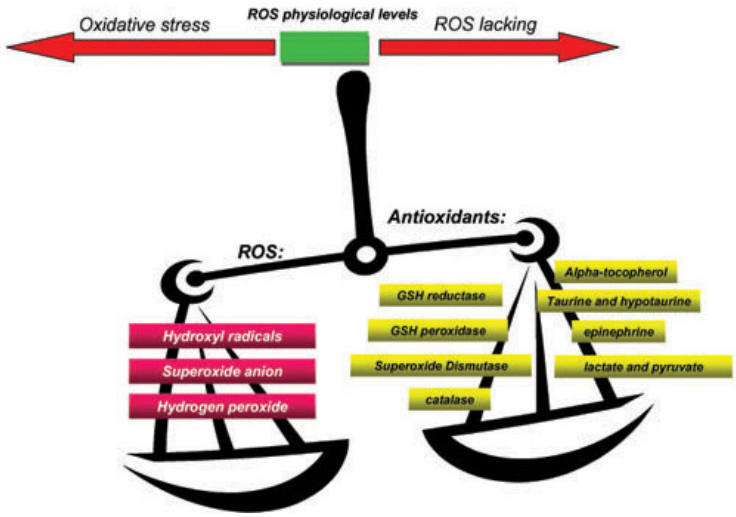

Figura 3 - Relação entre a produção de espécies reativas de oxigênio (ROS = *Reactive Oxigen Species*) e a ação do sistema antioxidante. Disponível em <http://www.asiaandro.com/1008-682X/6/59.htm>.

As células vegetais respondem defensivamente ao estresse oxidativo pela remoção das EROs e através da manutenção dos compostos de defesa antioxidante em níveis que reflitam as condições ambientais (SCANDALIOS, 1997). Quando as EROs são incompletamente reduzidas, elas podem ser extremamente reativas e oxidar as moléculas biológicas, tais como o DNA, as proteínas e os lipídios (RICHTER & SCHWEIZER, 1997) (Fig. 4). Logo, os papéis funcionais da resposta antioxidante incluem a proteção de processos enzimáticos sensíveis ao estado redox, a preservação da integridade das membranas e a proteção do DNA e proteínas (SCANDALIOS, 1997).

O O_2 molecular é reduzido através de quatro etapas, gerando várias outras EROs (Eq. (1); (HIPPELI et al., 1999)):

$$O_2 \rightarrow (H)O_2^- \rightarrow H_2O_2 \rightarrow OH^{\cdot} + H_2O \rightarrow 2H_2O \qquad (1)$$

Um produto oxidante inicial, o radical superóxido (O_2^-), sob posteriores reações dentro da célula, pode formar outras EROs, tais como o oxigênio singleto (O_2^1), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($OH^{\cdot}$).

O radical O_2^- é altamente reativo, formando hidroperóxidos com enes e dienes (SALIN, 1987). Os aminoácidos específicos, tais como histidina, metionina e triptofano, podem ser oxidados pelo O_2^- (KNOX & DODGE, 1985), causando peroxidação lipídica e enfraquecendo as membranas celulares (HALLIWELL & GUTTERIDGE, 1989). O O_2^1 não está sujeito ao processamento enzimático. Os vegetais desenvolveram mecanismos para evitar a sua formação, minimizando a produção de clorofila tripleto, a fonte que gera o O_2^1. Se a

formação desta espécie excitada não pode ser completamente abolida, a destruição da clorofila tripleto e do O_2^1, uma vez produzidos, deve ser necessariamente acelerada (EDREVA, 2005). Os carotenóides são os principais compostos envolvidos em ambas as estratégias, prevenindo assim o dano do sistema fotossintético. Eles absorvem a energia em excesso das moléculas de clorofila excitadas e dissipam-na como calor (EDGE et al., 1997; MITTLER, 2002).

Uma posterior redução do O_2^- gera o H_2O_2. Diferente de outras EROs, o H_2O_2 é capaz de se difundir através das membranas devido a sua alta estabilidade e permeabilidade. A toxicidade do H_2O_2 é pequena quando comparada com outros radicais de oxigênio, mas na presença de metais de transição, H_2O_2 produz o radical hidroxila ($OH^{\cdot}$), o mais reativo oxidante (VAN BREUSEGEM et al., 2001). Logo, a toxicidade do H_2O_2 através da oxidação dos grupos -SH é promovida pela presença de metais catalíticos através das reações Haber-Weiss ou Fenton:

$$O_2^{\cdot -} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

$$\text{Total: } H_2O_2 + O_2^{\cdot} \rightarrow OH^- + OH^{\cdot} + O_2$$

Portanto, limpar o H_2O_2 é essencial para evitar o dano oxidativo nas células vegetais (SAKIHAMA et al., 2002). Alguns metais que se auto-oxidam através das reações de Fenton são o Fe^{2+} e o Cu^+. No entanto, este tipo de reação não tem sido descrito ocorrer para os metais de transição Cd^{2+} e Hg^{2+} em plantas (SCHÜTZENDÜBEL & POLLE, 2002).

A última espécie a ser reduzida é o radical hidroxila ($OH^{\cdot}$), que tem uma alta afinidade pelas moléculas biológicas, e pode modificar proteínas de maneira a torná-las mais suscetíveis ao ataque proteolítico (Fig. 4). Devido ao fato de os radicais $OH^{\cdot}$ serem muito reativos para serem controlados diretamente, organismos aeróbicos tentam eliminar as formas precursoras menos reativas, O_2^- e H_2O_2 (VAN BREUSEGEM et al., 2001).

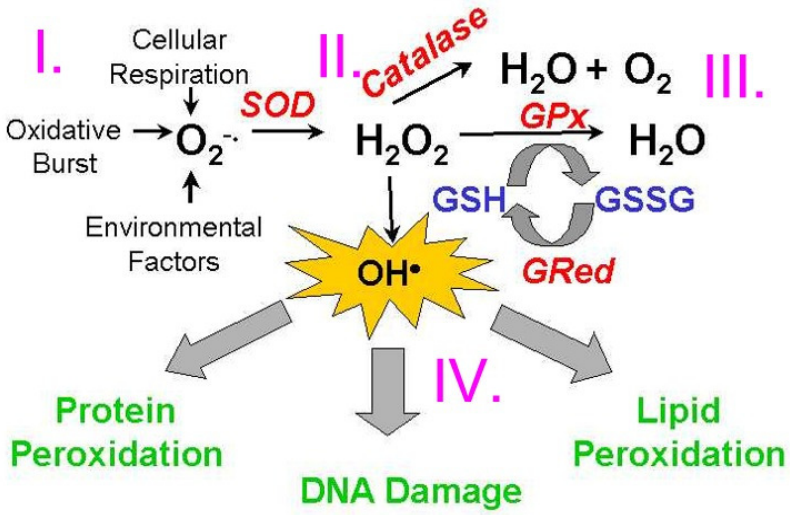

Figura 4 - Formação de espécies reativas de oxigênio (EROs). SOD: superóxido dismutase; O_2^-: radical superóxido; GSSG: glutationa oxidada; GSH: glutationa reduzida; $OH^{\bullet}$: radical hidroxila. Fonte: disponível em <http://www.biozentrum.uni-frankfurt.de/Pharmakologie/EU-Web/Bilder/OxidativestressLPO.jpg>

Os principais mecanismos enzimáticos de limpeza de EROs nas plantas incluem a superóxido dismutase (SOD), ascorbato peroxidase (APX) e catalase (CAT) (ASADA & TAKAHASHI, 1987; WILLEKENS et al., 1997; BOWLER et al, 1992).

Dentro da célula, as SODs constituem a primeira linha de defesa contra as EROs (Fig. 5A). O O_2^- é produzido em qualquer local onde uma cadeia transportadora de elétrons esteja presente, e a ativação do O_2 pode ocorrer em diferentes compartimentos celulares (ELSTNER, 1991). Sendo assim, não é surpreendente encontrar que as SODs estejam presentes em todos estes locais subcelulares, principalmente nos cloroplastos e nas mitocôndrias (FRIDOVICH, 1986).

Diferentes isoformas da enzima SOD são distinguidas com base no seu metal como co-fator. Em geral, as plantas contêm uma MnSOD mitocondrial, tão bem como uma citosólica e cloroplástica Cu/ZnSOD (BOWLER et al., 1994), e uma isoforma FeSOD localizada nos cloroplastos (BOWLER et al., 1994; VAN CAMP et al., 1990). Portanto, as superóxido dismutases são enzimas que contém metais em sua estrutura e que eliminam os radicais superóxidos conforme a reação a seguir:

$$2O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$

A eliminação do H_2O_2 acontece pela ação das enzimas CAT e APX (Fig. 5). As catalases, presentes apenas nos peroxissomos, removem o H_2O_2 quando este está presente em altas concentrações na célula (mM), enquanto que a APX remove o H_2O_2 que é inacessível à

CAT, devido à sua alta afinidade ao H_2O_2 (μM) e à sua presença em diferentes compartimentos celulares (SCANDALIOS, 1994). Assim, a APX é responsável pela modulação fina das EROs por sinalização, enquanto que a CAT é responsável pela remoção de EROs durante estresse (WILLEKENS et al., 1997).

As APXs são as mais importantes destruidoras de H_2O_2, operando tanto no citosol quanto nos cloroplastos. Elas usam o ácido ascórbico (AsA) como substrato redutor e formam parte de um ciclo, conhecido como ciclo ascorbato-glutationa ou ciclo Halliwell-Asada (FOYER et al., 1994) (Fig. 5b). Neste ciclo, o H_2O_2 é destruído em H_2O e O_2, sem a produção de novas formas de espécies reativas. Outras enzimas envolvidas neste ciclo são a monodehidroascorbato redutase (MDAR), dehidroascorbato redutase (DHAR) e a glutationa redutase (GR), além da presença do ascorbato e da GSH como oxirredutores, do H_2O_2 como aceptor de elétrons, e do NADPH como doador de H, que são estreitamente compartimentalizados e agem de maneira altamente coordenada (EDREVA, 2005).

A manutenção do ascorbato na sua forma reduzida é devido à ação do MDAR e do NAD(P)H ou ferredoxina como redutor. O último processo de redução do dehidroascorbato está acoplado a oxidação da glutationa (GSH), que é reduzida pela GSHR através da oxidação do NADPH (FOYER & HALLIWELL, 1976).

O fato de o ciclo ascorbato-glutationa ser encontrado em quase todos os compartimentos celulares, tão bem como a alta afinidade da APX pelo seu substrato, o H_2O_2, sugere que este ciclo tem papel crucial no controle dos níveis de EROs nestes compartimentos (MITTLER, 2002).

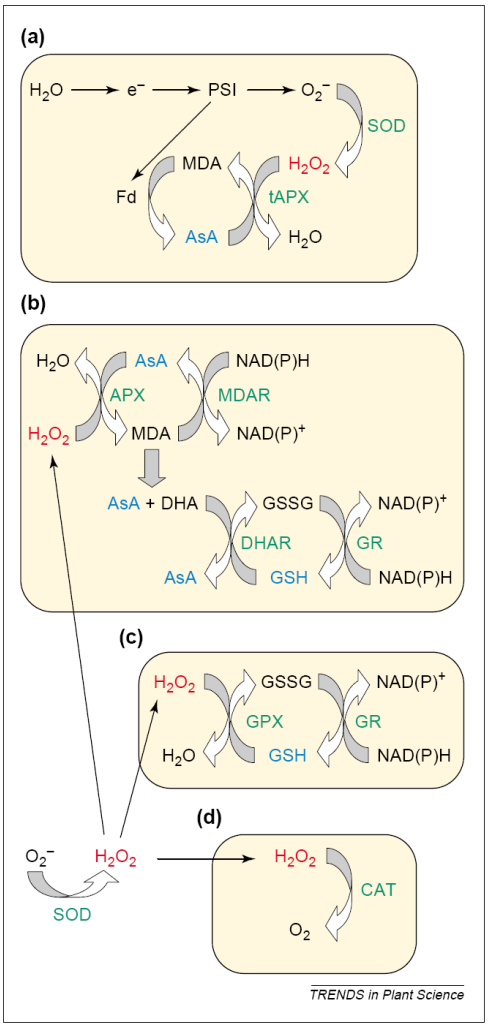

Figura 5 - Mecanismos de remoção de espécies reativas de oxigênio em plantas: (a) Ciclo água-água. (b) Via Halliwell-Asada ou ciclo ascorbato-glutationa. (c) Ciclo glutationa peroxidase. (d) CAT: catalase. APX: ascorbato peroxidase; MDHAR, monodehidroascorbato redutase; DHAR, dehidroascorbato redutase; GR, glutationa redutase (Adaptado de Mittler, 2002).

Outra resposta celular observada após a exposição ao estresse são mudanças no metabolismo dos peptídeos tióis (RAUSER, 1991). Existem várias formas de tióis não-protéicos (i) o tripeptídeo GSH (γ-glutamilcisteinil glicina), (ii) homoglutationa hGSH (γ-L-glutamil-cisteinil-β-alanina) e (iii) fitoquelatinas ou homofitoquelatinas, sintetizadas pela polimerização enzimática da GSH ou da hGSH (GEKELER et al., 1989). Estes compostos têm um papel chave na regulação do balanço redox e podem ser usados como um indicador de estresse oxidativo (RIJSTENBIL & WIJNHOLDS, 1996), na destoxificação de xenobióticos (MARRS, 1996) e metais pesados (COBBETT, 2000).

A influência dos metais pesados sobre os tióis não-protéicos é devido a extrema alta afinidade dos metais aos resíduos -SH (VIARENGO & NOTT, 1993). Os grupos -SH podem formar complexos com formas de Hg em condições *in vitro* e *in vivo* (RABENSTEIN et al., 1985). Sob estresse oxidativo, o pool de GSH é convertido a GSH oxidada (GSSG) e a biossíntese de GSH é estimulada (MAY & LEAVER, 1993; MADAMANCHI et al., 1994).

As fitoquelatinas são rapidamente induzidas por uma ampla faixa de espécies vegetais através da metilação com metais pesados, tais como Cd^{2+} (Fig. 6B), As^{5+}, Cu^{2+}, Ag^{+}, Hg^{2+} e Pb^{2+} (RAUSER, 1999; COBBETT, 2000; GOLDSBROUGH, 2000; HALL, 2002; RAAB et al., 2004) e têm a função de imobilizar os íons metais e os direcionar aos vacúolos, prevenindo assim a sua interferência com o metabolismo celular (VÖGELI-LANGE & WAGNER, 1990; ORTIZ et al., 1995) (Fig. 6A).

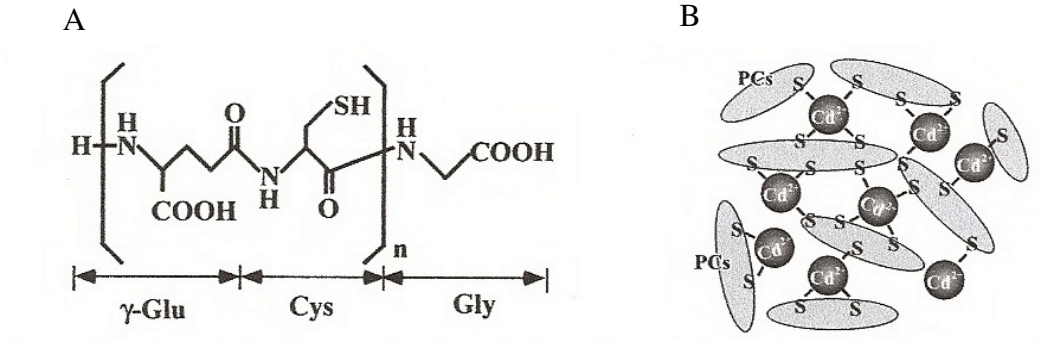

Figura 6 - Estruturas primárias das fitoquelatinas (PCs) (A) e complexos PC-Cd (B). A estrutura das PCs é geralmente indicada como (γ-Glu-Cys)n-Gly, onde n=2 a 11. Os íons de metais pesados, tais como o cádmio (Cd), ligam um, dois, três ou, no máximo, quatro átomos de enxofre de uma única ou de múltiplas moléculas de PCs, resultando em complexos amorfos. Fonte: Hirata et al. (2005).

Os radicais $OH^{\cdot}$ podem ser destruídos pela ação do ascorbato, dos tocoferóis e da GSH, se tais antioxidantes estiverem presentes em quantidades suficientes nos locais de liberação deste radical (NOCTOR & FOYER, 1998; NIYOGI, 1999).

Os metais pesados, tal como o Hg, interferem na fotossíntese por afetar a síntese de pigmentos fotossintéticos, as clorofilas e os carotenóides, e têm portanto, um importante papel no desenvolvimento dos cloroplastos em folhas jovens (SANITÀ DI TROPPI & GABRIELLI, 1999). A enzima responsável pela síntese de porfirinas, hemes e clorofilas é a δ-aminolevulínico ácido desidratase (δ-ALA-D), que é sensível ao Hg devido à sua natureza

sulfidrílica (MORSCH et al., 2002). A δ-ALA-D catalisa a condensação assimétrica de duas moléculas de δ-aminolevulínico ácido (ALA) a porfobilinogênio (PBG) (GIBSON et al., 1955). A atividade diminuída da δ-ALA-D simultaneamente com a redução do conteúdo de clorofila tem sido observada em plantas de *Cucumis sativus* expostas ao Hg (CARGNELUTTI et al., 2006).

A acumulação de prolina livre também foi observada em resposta a uma ampla variedade de estresses abióticos e bióticos em plantas (BACKOR et al., 2004). Possivelmente um osmótico, a prolina é considerada ser uma das primeiras respostas metabólicas ao estresse, e é provável que seja um mensageiro secundário (DELAUNEY et al., 1993; HARE & CRESS, 1997). A acumulação de prolina é mediada pela síntese aumentada de glutamato ou ornitina e menos pela degradação oxidativa diminuída de seus aminoácidos (BACKOR et al., 2004).

Os mecanismos da ação da prolina não são bem conhecidos, mas foi sugerido que sua acumulação permite ajustamento osmótico e a proteção das enzimas (SHARMA et al., 1998; BASAK et al., 2001), membranas biológicas e polirribossomos. A prolina é capaz de destoxificar os radicais livres e EROs (HASEGAWA et al., 2000; HONG et al., 2000; OKUMA et al., 2004; CHEN & DICKMAN, 2005) através da formação de um complexo estável com eles, mantendo assim a taxa $NAD(P)^+$/NAD(P)H durante estresse em valores similares aos da condição normal (DALLAVALLE et al., 1998; HOYAU & OHANESSIAN, 1998; SOUGANDI et al., 2002). A prolina também inibe a perda de íons potássio durante o estresse por metais pesados (WU et al., 1995a; MEHTA & GAUR, 1999).

A acumulação de prolina em plantas tem sido observada em resposta ao excesso de Cu (LIAO et al., 2000; CHEN et al., 2001). Os tratamentos com Cu e Cd promoveram a sua acumulação em algas verdes (*Chlorella* sp., *Pediastrum duplex*), em diatomáceas (*Nitzschia palea*) e na cianobactéria *Anacystis nidulans* (WU et al, 1995a e 1995b; WU et al., 1998).

3 RESULTADOS

3.1 Manuscrito 1

Antioxidant system activation by mercury in *Pfaffia glomerata* plantlets

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Antioxidant system activation by mercury in *Pfaffia glomerata* plantlets

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Abstract

The oxidative stress caused by mercury (Hg) was investigated in *Pfaffia glomerata* plantlets grown in nutrient solution using sand as substrate. Thirty-day-old acclimated plants were treated with four Hg levels (0, 1, 25 and 50 µM). Plants were harvested 9 days after Hg exposure and divided into roots and shoot to analyze growth and several biochemical parameters. Hg concentration increased with Hg levels. Roots and shoot fresh weight and δ-ALA-D activity were significantly decreased at 50 µM Hg, and chlorophyll and carotenoid concentration were not affected. Shoot H_2O_2 concentration increased curvilinearly with Hg levels, whereas lipid peroxidation increased at 25 and 1 µM Hg, respectively. SOD activity showed a straight correlation with H_2O_2 concentration, whereas CAT activity increased in shoots at 1 and 50 µM Hg. APX activity decreased at 1 µM Hg or increased at 50 µM Hg in shoot, whereas it increased at 1 µM in roots. In general, AsA, NPSH and proline increased upon addition of Hg, with the exception of proline in roots, which decreased. These results

contribute to a deeper understanding of Hg effects on oxidative stress in order to assess the role of antioxidants in protecting plants from Hg stress.

Keywords: *Pfaffia glomerata*; Mercury; Oxidative stress; Lipid peroxidation

Introduction

Amongst the metal pollutants, mercury (Hg) is both the best known and the most hazardous metal in the environment. Considerable amounts of Hg are added to agricultural land with the application of sludge, fertilizers, pesticides, lime and manures (Boening 2000).

Being a transition metal, Hg^{2+} is able to induce oxidative stress in plants, resulting in lipid peroxidation, alteration of antioxidant enzyme activities and induction of thiol-containing compounds (Ali et al. 2000). Hg-induced oxidative damage in plant cells has been linked to the excess production of reactive oxygen species (ROS) (Cargnelutti et al. 2006; Zhou et al. 2008). Mercury is able to bind with water channel proteins of root cells causing a physical obstruction to the water flow (Maggio and Joly 1995), which affects photosynthesis, mineral nutrient uptake and transpiration (Patra and Sharma 2000). Another toxic symptom of Hg accumulation in plants is the decreased levels of chlorophyll and proteins (Cargnelutti et al. 2006).

Meanwhile, generation of ROS, particularly H_2O_2 has been proposed as part of the signaling cascade leading to protection from stresses (Neill et al. 2002) that can be caused by abiotic factors, such as drought, chilling, heavy metals, or biotic factors, such as pathogen invasions. In order to protect tissues from oxidative stress, plant cells contain both oxygen radical detoxifying (antioxidant) enzymes such as catalase (CAT, E.C. 1.11.1.6), ascorbate peroxidase (APX, E.C. 1.11.1.11) and superoxide dismutase (SOD, E.C. 1.15.1.1), and non-enzymatic antioxidants such as ascorbate and glutathione (Asada 1996; Rio et al. 1998). Altered antioxidant enzyme activities are frequently used as indicators of stress (Koricheva et al. 1997). However, changes in ROS metabolism and enzyme activities involved in scavenging ROS in plants exposed to Hg have not been investigated in detail.

The genus of *Pfaffia* belongs to the Amaranthaceae family and has about 90 species distributed throughout Central and South America. In Brazil, 27 species have been described (Taniguchi et al. 1997). Carneiro et al. (2002) showed that an undetermined species of the

genus *Pfaffia* exhibited high tolerance to soil contamination, growing quite abundantly in soil mixtures with 90 and 1,450 mg kg^{-1} of Cd and Zn, respectively. In a recent study, Skrebsky et al. (2008) showed that *Pfaffia glomerata* (Spreng.) Pedersen plantlets grown hydroponically seemed to have some degree of Cd tolerance. In line with this and taking into account the high commercial value of *P. glomerata* to the pharmaceutical industries (Montanari et al. 1999), it is important to verify whether this species accumulates other metals, shows Hg tolerance and, if so, which mechanisms are involved in Hg tolerance.

Under this context, the present study was designed to analyze the importance of enzymatic and non-enzymatic antioxidants in both roots and shoots of *P. glomerata* plantlets during a 9-day period of exposure to different mercury concentrations, ranging from those observed in moderately contaminated (1 µM) to highly contaminated soils (25 and 50 µM).

Materials and methods

Plant material and growth conditions

Pfaffia glomerata (Spreng.) Pedersen plantlets for tissue culture were obtained from the Brazilian Ginseng Germplasm Program, Universidade Federal de Santa Maria, RS, Brasil. Nodal segments (1.0 cm long) without leaves were micropropagated in MS medium (Murashigue and Skoog 1962), supplemented with 30 g L^{-1} of sucrose, 0.1 g L^{-1} of myo-inositol and 6 g L^{-1} of agar according to Nicoloso et al. (2001). Thirty-day-old plantlets grown *in vitro* were transferred into pots containing washed sand. These plantlets were supplemented daily with nutrient solution containing the following composition: 65.1 mg L^{-1} NH_4Cl, 76.2 mg L^{-1} $MgSO_4.7H_2O$, 135.2 mg L^{-1} $MgCl_2.6H_2O$, 33.1 mg L^{-1} KH_2PO_4, 181.5 mg L^{-1} KCl, 575.3 mg L^{-1} $Ca(NO_3)_2.4H_2O$, 0.11 mg L^{-1} $CuSO_4.5H_2O$, 0.39 mg L^{-1} $MnCl_2.4H_2O$, 0.57 mg L^{-1} $ZnSO_4.7H_2O$, 0.04 mg L^{-1} $NiSO_4$, 1.54 mg L^{-1} H_3BO_3, 0.09 mg L^{-1} $H_2MoO_4.H_2O$ and 13.34 mg L^{-1} $FeSO_4.7H_2O$. After one month of plantlet acclimation, Hg was added to the nutrient solution as $HgCl_2$ at concentrations of 0 (control), 1, 25 and 50 µM. After nine days of Hg exposure, 3 plantlets per replicate (each treatment consisted of three replicates) were harvested randomly. The plantlets were divided into roots and shoot for evaluation of fresh biomass. Three independent and representative tissue samples were used for Hg

determination. For measurements of H_2O_2, MDA, chlorophyll concentrations, antioxidant enzyme activities and non-enzymatic antioxidant concentrations, fresh samples were used. Both *in vitro* and *ex vitro* cultured plantlets were grown in a growth chamber at 25±1°C on a 16/8 h light/dark cycle with 35 µmol m^{-2} s^{-1} of irradiance by cold fluorescent lamps.

Tissue Hg concentration

To metal determination plantlets were oven-dried at 65°C to constant mass. Dried shoot and roots (0.07–0.1 g) were ground and digested with 5 mL HNO_3 and 0.2 mL H_2O in closed Teflon vessels, which were heated at 100 °C for 3 h in a digester block (Tecnal TE 007D). The samples were then diluted to 50 mL with high-purity water. Hg concentrations were determined using a Varian Atomic Absorption Spectrophotometer (Spectr AA 600, Australia) equipped with a vapor generative accessory (Varian VGA-76). The content absorbed was expressed as µg g^{-1} dry weight.

Chlorophyll and carotenoids determination

Fresh biomass (leaves plus stem) were homogenized in 80% ice-cold acetone in dark and then centrifuged at 10,000 rpm for 10 min at 4°C, the supernatant was used for the immediate determination of pigments. Absorbance of the solution was measured at 663, 645, 510 and 480 nm in a Spectrophotometer (Celm E-205D) in order to determine the concentration of carotenoids, chlorophyll a and chlorophyll b, respectively, with the help of Arnon's formulae (Arnon 1949). Chlorophyll and carotenoids content was expressed as µg g^{-1} fresh weight.

Delta-aminolevulinic acid dehydratase (δ-ALA-D; E.C. 4.2.1.24) activity

Shoots were homogenized in 10 mM Tris-HCl buffer, pH 9.0, at the proportion of 1:1 (w/v). The homogenate was centrifuged at 12,000 g at 4°C for 10 min to yield a supernatant (S1) that was used for the enzyme assay. The supernatant was pre-treated with 0.1% Triton X-

100 and 0.5 mM dithiotreithol (DTT). δ-ALA-D activity was assayed as described by Barbosa et al. (1998) by measuring the rate of porphobilinogen (PBG) formation. The incubation medium for the assays contained 100 mM Tris-HCl buffer, pH 9.0 and 3.6 mM ALA. Incubation was started by adding 100 µL of the tissue preparation to a final volume of 400 µL and stopped by adding 350 µL of the mixture containing 10% trichloroacetic acid (TCA) and 10 mM $HgCl_2$. The product of the reaction was determined with the Ehrlich reagent at 555 nm using a molar absorption coefficient of 6.1×10^4 L^{-1} mol^{-1} cm^{-1} (Sassa 1982) for the Ehrlich-porphobilinogen salt. δ-ALA-D activity was expressed as nmol PBG mg^{-1} protein h^{-1}.

Determination of hydrogen peroxide

The H_2O_2 concentration was determined according to Loreto and Velikova (2001). Approximately 0.1 g of both roots and shoot was homogenized at 4°C in 2 mL of 0.1% trichloroacetic acid (TCA) (w/v). The homogenate was centrifuged at 12,000 g for 15 min. Then, 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1M KI. The H_2O_2 concentration of the supernatant was evaluated by comparing its absorbance at 390 nm with a standart calibration curve. Hydrogen peroxide concentration was expressed as µmol g^{-1} fresh weight.

Estimation of lipid peroxidation

The level of lipid peroxidation products was estimated following the method of El-Moshaty et al. (1993) by measuring the concentration of malondialdehyde (MDA) as an end product of lipid peroxidation by reaction with thiobarbituric acid (TBA). Fresh tissue samples (0.1 g fresh weight) were ground in 2 mL of 0.2 M citrate-phosphate buffer (pH 6.5) containing 0.5% Triton X-100, using mortar and pestle. The homogenate was filtered through two layers of paper and centrifuged for 15 min at 20,000 g. One milliliter of the supernatant fraction was added to an equal volume of 20% (w/v) TCA containing 0.5% (w/v) TBA. The mixture was heated at 95°C for 40 min and then quickly cooled in an ice bath for 15 min. After centrifugation at 10,000 g for 15 min, the absorbance of the supernatant was measured at 532 nm. A correction for non-specific turbidity was made by subtracting the absorbance

value taken at 600 nm. The lipid peroxides were expressed as nmol MDA (mg protein)$^{-1}$, by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Catalase (CAT, E.C. 1.11.1.6) assay

Catalase activity was assayed according to the method of Aebi (1984) with some modifications. Fresh samples (1g) were homogenized in 5 mL of 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 10 g L^{-1} PVP, 0.2 mM EDTA and 10 mL L^{-1} Triton X-100. The homogenate was centrifuged at 12,000 g for 20 min at 4°C and then, the supernatant was used for the enzyme assay. Catalase activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm from a reaction mixture containing 2 mL of 15 mM H_2O_2 in KPO_4 buffer (pH 7.0) and 30 µL extract. Activity was expressed as ΔE min^{-1} mg^{-1} protein.

Ascorbate peroxidase (APX, E.C. 1.11.1.11) assay

Ascorbate peroxidase (APX) was measured according to Zhu et al. (2004). The reaction mixture, at a total volume of 2 mL, contained 25 mM (pH 7.0) sodium phosphate buffer, 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H_2O_2 and 100 µL enzyme extract. H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (E = 2.8 L^{-1} mol^{-1} cm^{-1}) and activity was expressed as µmol ascorbate oxidated min^{-1} mg^{-1} protein.

Superoxide dismutase (SOD, E.C. 1.15.1.1) assay

The activity of SOD was assayed according to Misra and Fridovich (1972). About 200 mg of roots and shoots were homogenized in 5 mL of 100 mmol L^{-1} K-phosphate buffer (pH 7.8) containing 0.1 mmol L^{-1} EDTA, 0.1% (v/v) Triton X-100 and 2% PVP (w/v). The extract was filtered and centrifuged at 22,000 g for 10 min at 4ºC, and the supernatant was used for assays. The assay mixture consisted of a total volume of 1 mL, containing glycine buffer (pH

10.5), 1 mmol L^{-1} epinephrine and enzyme material. Epinephrine was the last added component. Adenochrome formation over the next 4 min was spectrophotometrically recorded at 480 nm. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions used. This method is based on the ability of SOD to inhibit the autoxidation of epinephrine at an alkaline pH. Since the oxidation of epinephrine leads to the production of a pink adrenochrome, the rate of increase of absorbance at 480 nm, which represents the rate of autoxidation of epinephrine, can be conveniently followed. The enzyme has been found to inhibit this radical-mediated process.

Ascorbic acid (AsA) concentration

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Both roots and shoot were homogenized in a solution containing 50 mmol L^{-1} Tris-HCl and 10 mL L^{-1} Triton X-100 (pH 7.5), centrifuged at 6,800 g for 10 min. To the supernatant 10% TCA was added at a proportion of 1:1 (v/v) followed by centrifugation (6,800 g for 10 min) to remove protein. An aliquot of the sample (300 µL) was incubated at 37°C in a medium containing 100 µL TCA 13.3%, 100 µL deionized water and 75 µL 2,4-Dinitrophenylhydrazine (DNPH). The DNPH solution contained 2% DNPH, 0.23% thiourea, 0.27% $CuSO_4$ diluted in 49% H_2SO_4. After 3 h, 500 µL of 65% H_2SO_4 was added and samples were read at 520 nm. A standard curve was constructed using L(+) ascorbic acid.

Non-protein thiols groups (NPSH) concentration

Non-protein thiols concentration was measured spectrophotometrically with Ellman's reagent (Ellman 1959). Roots and shoots samples were homogeneized in a solution containing 50 mmol L^{-1} Tris-HCl and 10 mL L^{-1} Triton X-100 (pH 7.5), centrifuged at 6,800 g for 10 min, and NPSH were determined in a fraction obtained after mixing 1 volume of supernatant with 1 volume of 10% TCA followed by centrifugation (6,800 g for 10 min) and neutralization (to pH 7.4) with 1 M Tris-HCl as described by Jacques-Silva et al. (2001). Reaction was read at 412 nm after the addition of 0.05 mL of 10 mM 5-5-dithio-bis (2-

nitrobenzoic acid) (DTNB). A standard curve using cysteine was used to calculate the content of thiol group in samples, and was expressed as µmol SH g^{-1} fresh weight.

Proline concentration

Determination of proline concentration was according to the procedures of Bates et al. (1973). Approximately 0.25g of fresh tissues was homogenized in 5 mL of 10% aqueous sulfosalicylic acid. The homogenate filtered was centrifuged at 5,000 g for 20 min at 4ºC, and the supernatant was used for assays. Two milliliter of filtrate was reacted with 2 mL acid-ninhydrin and 2 mL of glacial acetic acid in a test tube for 1 h at 100°C, and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene, mixed vigorously with a test tube stirrer for 20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520 nm using toluene for a blank. Proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows: [(µg proline/mL × mL toluene) / 115.5 µg/µmole]/[(g sample)/5] = µmol proline/g of fresh weight material.

Protein extraction

In all the enzyme preparations, protein was determined by the method of Bradford (1976) using bovine serum albumin as standard and was expressed in mg L^{-1}.

Statistical analysis

The analyses of variance were computed on statistically significant differences determined based on the appropriate F-tests. The results are the means ± SD of at least three independent replicates. The mean differences were compared utilizing Tukey test with $p< 0.05$.

Results

Effect of Hg concentration and fresh weight

Tissue Hg concentration increased linearly with increasing external Hg concentrations in both shoots and roots (Fig 1A). Root Hg concentration was about 11-fold higher than that found in shoots, with 713 µg g^{-1} dry weight of Hg in roots treated with 50 µM Hg.

Table 1 – Hg concentration and fresh weight of shoot and roots of *Pfaffia glomerata* plantlets exposed to treatment for nine days.

Treatments	*Hg concentration (µg g^{-1} dry weight)*		*Fresh Weight (g $plant^{-1}$)*	
	Shoot	Root	Shoot	Root
Control	2.8 ± 0.4 cA*	0.6 ± 0.015 cB	2.4 ± 0.13 a*	0.7 ± 0.10 a
1 µM Hg	3.1 ± 0.22 cB	4.4 ± 0.09 cA	2.3 ± 0.18 a	0.7 ± 0.07 a
25 µM Hg	25.9 ± 3.9 bB	531 ± 15.4 bA	1.2 ± 0.16 a	0.6 ± 0.04 ab
50 µM Hg	67.7 ± 7.6 aB	713 ± 120 aA	1.5 ± 0.04 b	0.5 ± 0.006 b

Data represent mean values ± SD based on independent determination.
* Mean values followed by the same lower-case letters in the column, and capital letters in the line did not differ significantly by Tukey test of $p<0.05$.

Shoot accumulated higher Hg concentrations in plants not exposed to Hg in the nutrient solution (control). Although shoots accumulated less Hg than roots when Hg was added to nutrient solution, the Hg concentration increased about 24-fold at the highest external concentration of Hg when compared to the control, reaching about 68 µg g^{-1} dry weight in this tissue.

Shoot and root fresh weight decreased only upon addition of 50 µM Hg (Fig 1B). At this level of Hg, the shoot and root fresh weights were about 15.5% and 20% lower than that of the control, respectively.

Effect of Hg on pigments concentration and δ-ALA-D activity of *P. glomerata* plantlets

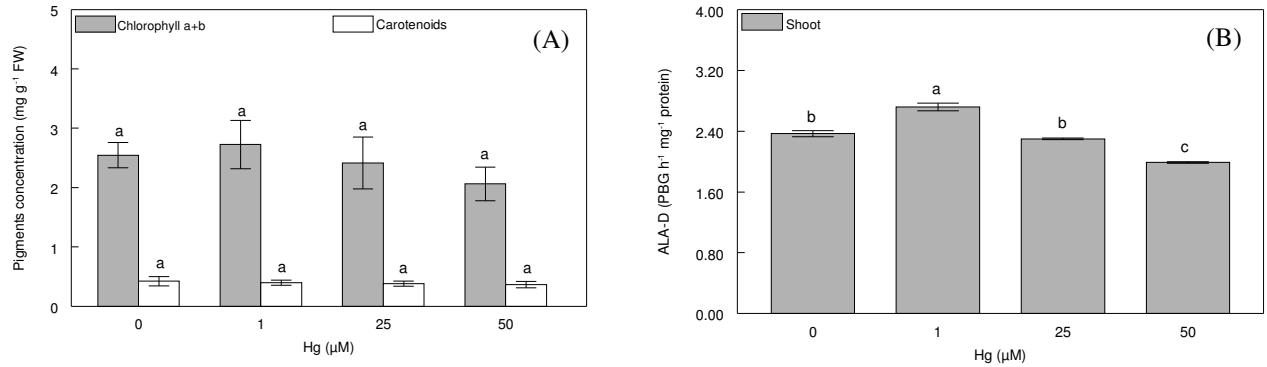

Figure 1 Effect of increasing Hg concentration on the pigment concentration (A) and δ-aminolevulinic acid dehydratase (δ-ALA-D) activity (B) in *P. glomerata* plantlets. Data represent the mean ± SD of three replicates. Identical letters indicate no significant differences among the Hg concentrations ($p<0.05$) according to Tukey´s multiple range test.

There was no effect of Hg on the chlorophyll and carotenoid concentration. Shoot δ-ALA-D activity increased at 1 µM of Hg, whereas it decreased approximately 1.2-fold, when compared to the control, at the highest Hg concentration (50 µM) used.

H_2O_2 concentration and lipid peroxidation (MDA concentration)

The effects of Hg on H_2O_2 formation and lipid peroxidation are shown in Fig 2. Increasing Hg levels in the substrate caused an increase of shoot H_2O_2 concentration, but it peaked at 25µM (Fig 2A). In roots, H_2O_2 concentration either increased at 1µM Hg or decreased at 50 µM Hg, when compared to the control.

A significant increase in malondialdeide (MDA) levels in shoots was only observed at 50 µM Hg, which was about 3-fold higher when compared to the control (Fig 2B). In contrast, the root MDA concentration only increased at 25 µM Hg and significantly decreased at 50 µM Hg.

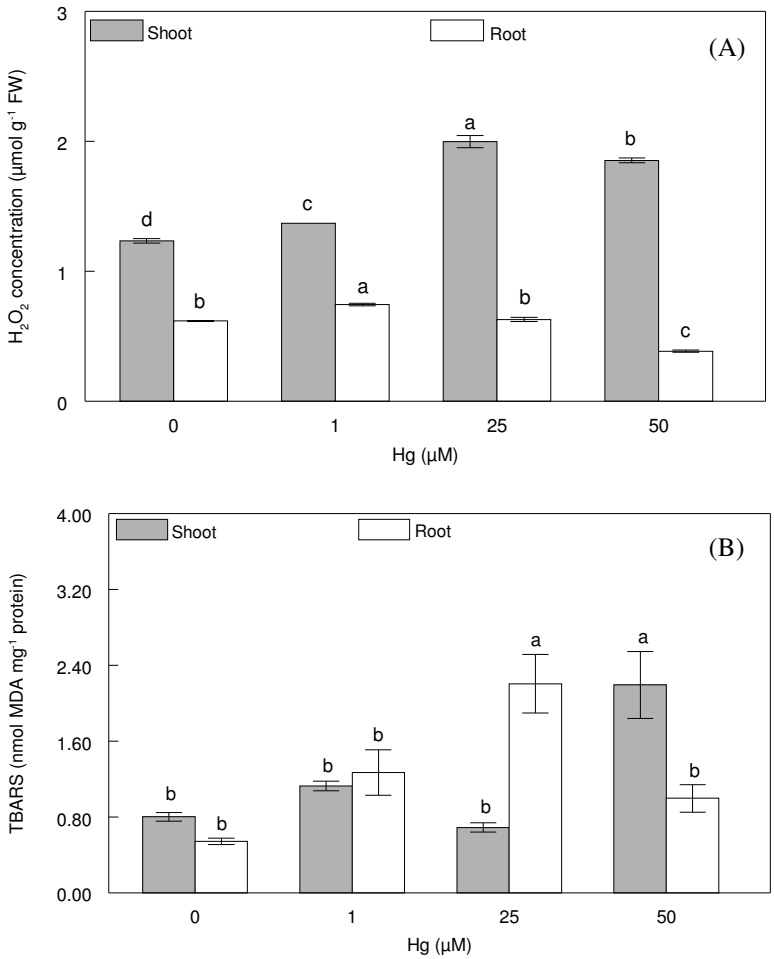

Figure 2 Effect of Hg on H_2O_2 (A) and lipid peroxidation (B) of *P. glomerata* plantlets. Data represent the mean ± SD of three replicates. Identical letters indicate no significant differences among the Hg concentrations ($p<0.05$) according to Tukey´s multiple range test.

CAT, APX and SOD activities

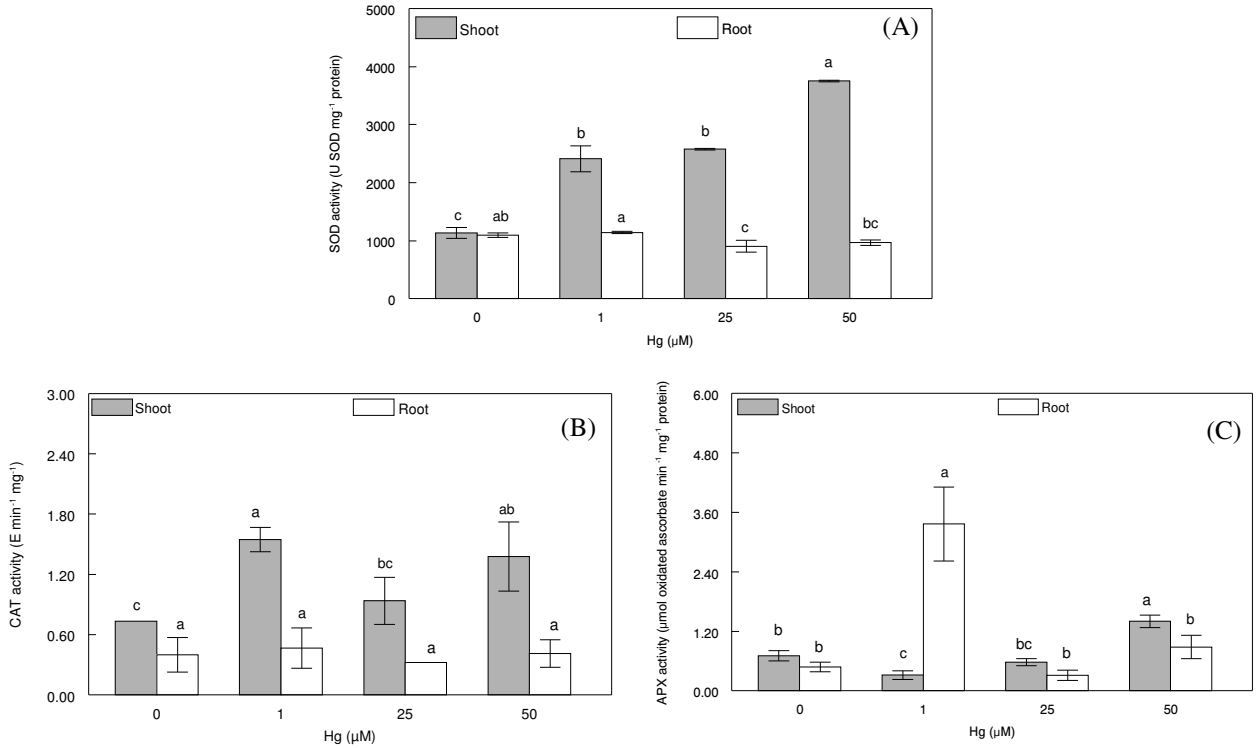

Figure 3 Effect of Hg on superoxide dismutase (SOD) (A), catalase (CAT) (B) and ascorbate peroxidase (APX) (C) activities of *P. glomerata* plantlets. Data represent the mean ± SD of three replicates. Identical letters indicate no significant differences among the Hg concentrations ($p<0.05$) according to Tukey´s multiple range test.

In general, the antioxidative enzyme activities were higher in shoot than in roots. SOD activity increased in shoot with increasing Hg levels in nutrient solution, whereas it decreased in roots (Fig 3A).

CAT activity increased at 1 and 50 µM Hg in shoot, whereas it was not affected in roots (Fig 3B).

APX activity either decreased at 1 µM Hg or increased at 50 µM Hg in shoot, whereas it was altered only at 1 µM Hg in roots, where it increased when compared to the control (Fig 3C).

AsA, NPSH and proline concentration

As shown in Fig 4A, shoot AsA concentration increased only at 1 and 50 µM Hg. On the other hand, AsA concentration in roots either decreased at 1 µM Hg or increased at 25 and 50 µM Hg, when compared to the control.

The non-protein thiol (NPSH) concentration in both shoot and root increased concomitantly with increasing Hg concentration in substrate (Fig 4B). Treatment with 50 µM Hg resulted in the maximal accumulation of NPSH of 1.5-fold higher than that of the shoot control plants.

The shoot proline concentration was slightly, but not significantly, increased by Hg levels up to 25 µM, and at 50 µM Hg level it increased 1.8 fold compared to the control (Fig 4C). In addition, the root proline concentration was significantly altered at 25 µM Hg, where it decreased when compared to the control.

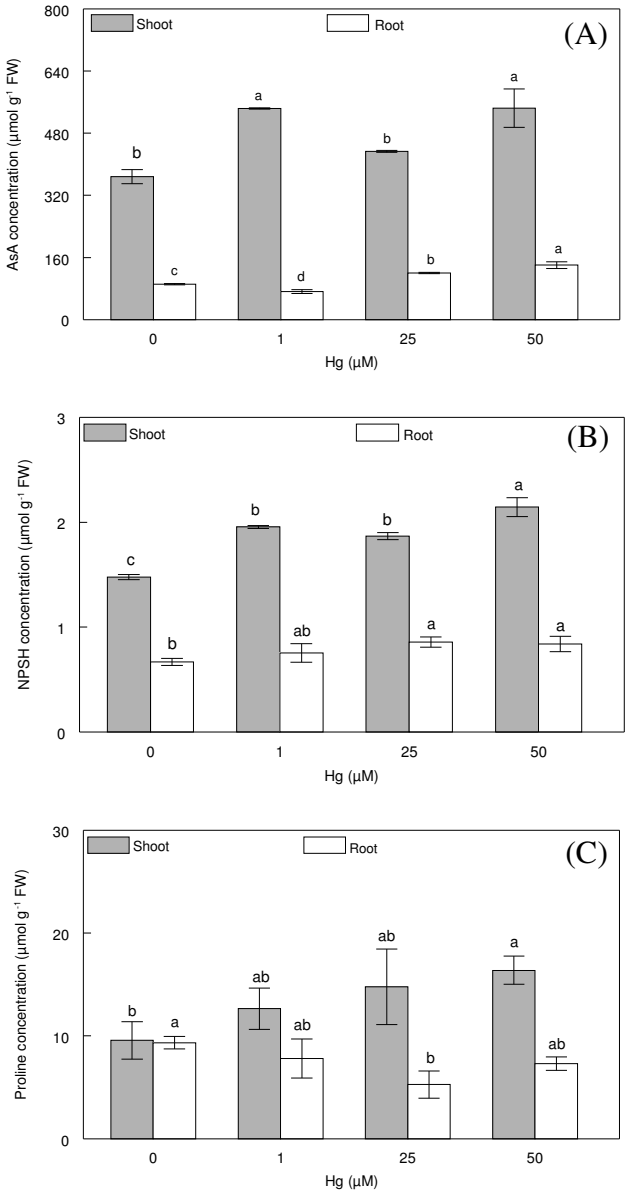

Figure 4 Effect of Hg on (A) ascorbic acid (AsA), (B) non-protein thiol compounds and (C) proline concentration of *Pfaffia glomerata* plantlets. Data represent the mean ± SD of three replicates. Identical letters indicate no significant differences among the Hg concentrations ($p<0.05$) according to Tukey´s multiple range test.

Discussion

Plants may survive in heavy metal-contaminated environments by preventing metals from entering into the cytoplasm and/or detoxifying them inside the cytoplasm. When metals

are initially absorbed by roots, some of them are trapped in the cell wall, which reduces the amount of metals entering the cytoplasm. In the present study, Hg accumulation in the root system (Table 1) indicates that roots serve as a partial barrier to the transport of Hg to shoots (Cavallini et al. 1999). Inversely, shoots accumulate more Hg than roots in plants grown without Hg in the nutrient solution. Uptake of Hg from air via stomata of Hg-untreated plants is a possible reason for the relatively high shoot Hg concentration in these plants. Patra et al. (2004) reported that Hg salts in soil may be reduced by biological and chemical reactions to metallic or methylated compounds, which may volatilize and be taken up through the leaves in plants grown in enclosed spaces. Uptake of gaseous Hg via stomata was also observed in laboratory studies (Cavallini et al. 1999; Iglesia-Turiño et al. 2006).

We also checked the effect of Hg on the growth of nodal segments of *P. glomerata* grown *in vitro*, and observed (data not shown) that as the plant organ used initially did not contain roots, Hg treatment as low as 1 µM had a significant supresive effect on tissue growth. This demonstrates that roots are very important to avoid Hg uptake into the protoplasm.

Hg is known to inhibit water uptake via aquaporins on plasma membranes in higher plants (Zang and Tyerman 1999), which could explain, in the present study, the detrimental effect of high concentrations of Hg on the fresh weight of *P. glomerata* plantlets (Table 1).

Destruction of lipid components of the membrane by lipid peroxidation causes membrane impairment and leakage (Cho and Park 2000). In addition, it has been suggested that the reduction in chlorophyll content in the presence of heavy metals is caused by an inhibition of chlorophyll biosynthesis (Pereira et al. 2006) that, in part, may have been caused by reduction of δ-ALA-D activity. δ-ALA-D is sensitive to metals due to its sulfhydrylic nature (Rocha et al. 1995). This enzyme catalyzes the condensation of two molecules of δ-aminolevulinic acid (ALA) to porphobilinogen (Gibson et al. 1955). The synthesis of porphobilinogen promotes the formation of porphyrins, hemes and chlorophylls, which are essential for adequate aerobic metabolism and for photosynthesis (Jaffe et al. 2000). Altered δ-ALA-D activity concomitant with reduced chlorophyll contents has been reported in many terrestrial plants exposed to various metals (Stobart et al. 1985). In the present study, plantlets of *P. glomerata* exposed to 1µM of Hg had increased δ-ALA-D activity, but did not show any change in the chlorophyll concentration. The stimulating effects of low heavy metal concentrations on several plant growth parameters is normally related to the hormetic effect

that probably represents an overcompensation response to a disruption in the homeostasis of the organism (Calabrese 1999; Lin et al. 2007).

Interestingly, *P. glomerata* plants exposed to 1 µM Hg showed an increase in the H_2O_2 concentration in both shoot and roots (Fig 2A), whereas lipid peroxidation (Fig 2B) did not change, when compared to the control. At the same level of Hg, a significant increase in the activity of SOD and CAT was observed in shoot. Therefore, it seems that a low concentration of Hg in the substrate was enough to activate the antioxidant system which aims to protect important metabolic enzymes, such as δ-ALA-D. However, ALA-D activity decreased at the highest concentration of Hg used, where it was 16% lower than that of the control.

At 25 µM Hg, shoot lipid peroxidation was significantly increased, when compared with the control, indicating that *P. glomerata* could not tolerate the highest Hg concentration. Lipid peroxidation was also higher at 25 µM Hg in roots, even though H_2O_2 concentration did not differ from that of the control. In contrast, SOD activity was inhibited while CAT and APX activities were not affected. Therefore, the significant increase in AsA and NPSH concentrations (Fig 4A and 4B) was not enough to protect roots from lipid peroxidation, hence this caused a relatively depressive effect on fresh biomass (Table 1).

Compared to the control, SOD activity markedly increased in shoots (Fig 3A) exposed to increasing Hg levels, and paralleled the levels of H_2O_2 formed in these tissues. However, at 50µM of Hg the shoot H_2O_2 concentration was lower than that of 25 µM Hg (Fig 2A). This reduction in H_2O_2 levels at 50 µM Hg, surprising in light of the increased SOD activity, might have been due to increased CAT (Fig 3B) and APX (Fig 3C) activities in shoots. In roots, the highest H_2O_2 concentration occurred at 1µM Hg. It is noteworthy that the highest level of APX activity was observed at this Hg concentration, whereas CAT activity did not differ among Hg treatments. In general, the concentration of 1 µM Hg was sufficient to induce alterations in the enzyme activities, and APX showed higher sensitivity, reaching maximal activity at this concentration.

In the present study, concentrations of both NPSH and AsA in shoots and roots increased at 25 and 50 µM Hg (Fig 4A and B). Thiol-based complexing substances are comprised of several acid-soluble sulphydryl-components, such as cysteine, glutathione or GSH and phytochelatins (De Vos et al. 1992). GSH is a key component of the antioxidant network that scavenges ROS either directly or indirectly by participating in the ascorbate-glutathione cycle (Smirnoff 2005). It is also effective in chelating Hg^{2+} (toxic form) to form $Hg(GSH)_2$, a non-toxic form of mercury, reducing the bioavailability of Hg (Gupta et al.

1998). The increase in accumulation of NPSH caused by Hg exposure may indicate that phytochelatins are being constantly produced (Rellán-Alvarez et al. 2005). Ascorbate is another antioxidant that regulates stress-induced peroxidation (Pignocchi and Foyer 2003). According to Smirnoff (2000), L-ascorbic acid (AsA) is involved in the regulation of photosynthesis, cell expansion, root elongation and transmembrane electron transport. AsA is an important component of the plant antioxidant defense system and serves as a reductant for the removal of H_2O_2 among other peroxides (Noctor and Foyer 1998). It has also been shown to detoxify Hg in *Chlorella vulgaris* by donating electrons to free radicals, thus protecting the integrity of –SH groups (Rai 1979).

Proline has several functions during stress: osmotic adjustment (Voetberg and Sharp, 1991), osmoprotection (Kishor et al. 1995, 2005), free radical scavenging and as an antioxidant (Sharma and Dietz 2006), protection of macromolecules from denaturation (Vanrensburg et al. 1993), regulation of cytosolic acidity (Sivakumar et al. 2000) and as a carbon and nitrogen reserve after stress relief (Dıaz et al. 1999). Backor et al. (2004) found that proline treatment produced a significant increase in chlorophyll *a* of the tolerant strain exposed to Cu and a similar response was found for chlorophyll *b*, chlorophyll *a* + *b* and total carotenoids. In wild-type *Trebouxia erici* grown in the presence of supplemental Cu, proline significantly increased the yield of photosystem II. Also, proline improved salt tolerance by up-regulating stress-protective proteins (Khedr et al. 2003; Zhu et al. 2005) and reduced oxidation of lipid membranes (Demiral and Turkan 2004). In the present study, the shoot proline concentration also increased upon addition of Hg (Fig 4C). Interestingly, increasing Hg levels did not alter chlorophyll and carotenoid concentrations. Therefore, our results suggest that the increase in proline content might account for the stability of photosynthetic pigments in *P. glomerata.*

These results present circumstantial evidence for the occurrence of oxidative stress in *P. glomerata* plantlets exposed to Hg, as well as for importance of the antioxidant system in regulating Hg-induced oxidative damage in *P. glomerata*. On the other hand, at 50 µM of Hg, the antioxidant system of the plantlets was not able to reverse Hg-mediated damage.

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3.2 Manuscrito 2

Protective effect of zinc during oxidative stress induced by mercury in *Pfaffia glomerata* plantlets

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Protective effect of zinc during oxidative stress induced by mercury in *Pfaffia glomerata* plantlets

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Abstract

The possible role of zinc (Zn) to reverse oxidative stress caused by mercury (Hg) was investigated in *Pfaffia glomerata* plantlets. Thirty-day-old acclimated plantlets were exposed to four treatments: control, 50 µM Zn, 50 µM Hg and 50 µM Zn + 50 µM Hg for 9 days. Hg concentration was higher in shoot than in roots without Hg in the substrate (control and Zn-alone), whereas it was higher in roots than in shoot when treated with Hg. Fresh and dry weight, as well as δ-aminolevulinic acid dehydratase activity were reduced in Hg-treated plants. Survival percentage, fresh and dry weight and δ-aminolevulinic acid dehydratase (ALA-D) activity of plants treated with 50 µM Zn + 50 µM Hg were greater than in those treated with Hg alone. Moreover, Zn treatment reduced lipid peroxidation caused by Hg, and this effect was related to increased root SOD activity, and shoot CAT and APX activities. In conclusion, the presence of Zn in the substrate caused a significant reduction in Hg-induced oxidative stress.

Keywords: *Pfaffia glomerata*; Mercury; Zn protection; Oxidative stress

Introduction

Mercury (Hg) is extremely toxic to plants and animals; it has a long half-life and is extremely persistent in the environment (Salt et al. 1995). To combat Hg toxicity, plant cells activate the antioxidant system, which includes glutathione (GSH) and ascorbate and antioxidative enzymes, such as superoxide dismutase (SOD, E.C. 1.15.1.1), ascorbate peroxidase (APX, E.C. 1.11.1.11) and catalase (CAT, E.C. 1.11.1.6), that participate in scavenging reactive oxygen species (ROS) such as O_2^-, OH and H_2O_2 (Edreva 2005). Within a cell, SOD constitutes the first line of defense against ROS, and converts O_2 radicals to H_2O_2 (Alscher et al. 2002). Unlike other reactive oxygen species, H_2O_2 is able to diffuse across membranes due to its high stability and membrane-permeability. H_2O_2 toxicity itself is weak compared with other ROS, but in the presence of transition metals, such as Fe^{2+}, H_2O_2 produces the hydroxyl radical (OH), the most reactive active oxygen. Thus, scavenging of H_2O_2 is essential to avoid oxidative damage of plant cells (Sakihama et al. 2002). H_2O_2 is eliminated by CAT and peroxidase enzymes. Catalase removes the bulk of H_2O_2, whereas ascorbate peroxidase can scavenge the H_2O_2 that is inaccessible to catalase because of its higher affinity to H_2O_2 and its presence in different subcellular locations (Scandalios 1994; Creissen et al. 1994).

Reduced glutathione (GSH) plays an important role in the defense against oxidative stress in plant cells and is involved in the complex enzymatic machinery that controls the intracellular levels of H_2O_2 (May et al. 1998). There are several reports showing changes in the levels of GSH and GSSG upon exposure of plants to different heavy metals (Schützendübel and Polle 2002). Therefore, there is evidence pointing towards the implication, at least partially, of GSH in the tolerance to heavy metals (Rellán-Álvarez et al. 2006). Reduced glutathione (GSH) is used to synthesize phytochelatins (PCs). PC accumulation is considered to be the plants' response to addition of heavy metals and, therefore, may play a central role in metal detoxification (Cobbett and Goldsbrough 2002). Moreover, some authors link the drainage of GSH caused by PC synthesis to the impairment of the cellular redox status (Noctor et al. 1998; Xiang and Olivier 1998). Taken together, these reports suggest that maintenance of the GSH pool is important for plant tolerance to metals (Ortega-Villasante et al. 2005).

Studies with animals showed that Zn is an important antioxidant, decreasing ROS production (Fernandez et al. 2003). Some studies have reported the ability of Zn to interact with essential elements such as Cu and Fe, decreasing their content in tissues and retarding the oxidative processes (Santon et al. 2003). It is also involved in cell membrane stabilization, metallothionein (Mt) synthesis (Scheuhammer et al. 1985; Waalkes and Klaassen 1985) and superoxide dismutase (Cu/Zn SOD) structure. Numerous studies with animals have shown that Zn supply may reduce Cd absorption and accumulation, and also prevent or reduce the adverse actions of Cd (Ueda et al. 1987; Sato and Nagai 1989; Dorian and Klaassen 1995) whereas Zn deficiency can intensify Cd accumulation and toxicity (Sarkar et al. 1995). Moreover, Tsuji et al. (2002) reported that Zn can induce PC synthesis much more efficiently than Cd in a marine alga, *Dunaliella tertiolecta*. It was also confirmed that Zn was conjugated by PCs less strongly than other toxic heavy metals (Satofuka et al. 1999). Generally, Zn decreases Cd uptake and accumulation in plants (Honma and Hirata 1978; McLaughlin et al. 1994; Oliver et al. 1997). Hart et al. (2002) attributed the competition for uptake between Cd and Zn to the existence of a common transport system on the plasma membranes. Wu and Zhang (2002) found that an increase of Zn alleviated Cd toxicity stress in barley plants by improving growth and reducing membrane damage.

The genus *Pfaffia* belongs to the Amaranthaceae family and there are about 90 species distributed in Central and South America (Taniguchi et al. 1997). Carneiro et al. (2002) showed that an undetermined species of the genus *Pfaffia* exibited high tolerance to soil contamination, growing quite abundantly in soil mixtures with 90 and 1,450 mg Kg^{-1} of Cd and Zn, respectively. In a recent study developed in our group (Skrebsky et al. 2008) it was shown that *P. glomerata* (Spreng.) Pedersen had some degree of Cd tolerance. Moreover, we also observed (see previous section in this thesis) that the growth and the antioxidant mecanisms of *P. glomerata* plants were significantly affected by high levels of Hg (25 and 50 μM) in the substrate.

Under this context, the present study was designed to analyse the effect of Zn nutrition on the biochemical and physiological alterations caused by Hg in *P. glomerata*.

Materials and methods

Plant material and growth conditions

Plantlets of *Pfaffia glomerata* (Spreng.) Pedersen (JB accession) were grown *in vitro* from nodal segments during thirty days in MS (Murashigue and Skoog 1962) medium, supplemented with 30 g L^{-1} sucrose, 6 g L^{-1} of agar and 0.1 g L^{-1} of myo-inositol, under climatized room conditions according to Nicoloso et al. (2001). Thirty-day-old plantlets were acclimated during three days and transferred to pots containing sand. Both *in vitro* and *ex vitro* plantlets were grown in a growth chamber at 25±1°C on a 16/8 h light/dark cycle with 35 µmol m^{-2} s^{-1} of irradiance by cold fluorescent lamps. Plantlets were supplemented daily with a solution containing the following nutrients: 65.1 mg L^{-1} NH_4Cl, 76.2 mg L^{-1} $MgSO_4.7H_2O$, 135.2 mg L^{-1} $MgCl_2.6H_2O$, 33.1 mg L^{-1} KH_2PO_4, 181.5 mg L^{-1} KCl, 575.3 mg L^{-1} $Ca(NO_3)_2.4H_2O$, 0.11 mg L^{-1} $CuSO_4.5H2O$, 0.39 mg L^{-1} $MnCl_2.4H_2O$, 0.57 mg L^{-1} $ZnSO_4.7H_2O$, 0.04 mg L^{-1} $NiSO_4$, 1.54 mg L^{-1} H_3BO_3, 0.09 mg L^{-1} $H_2MoO_4.H_2O$ and 13.34 mg L^{-1} $FeSO_4.7H_2O$. After one month of acclimation, four treatments (control; 50 µM Zn; 50 µM Hg; and 50 µM Zn + 50 µM Hg) were added to the nutrient solution. Hg and Zn were added as $HgCl_2$ and $ZnCl_2$, respectively. After nine days of treatment, 3 plantlets per replicate (each treatment consisted of five replicates) were randomly harvested. The plantlets were divided into roots and shoot for evaluation of fresh and dry biomass. Three independent and representative tissue samples (shoot and roots) were dried at 65°C to a constant weight, and the material was used for Hg and Zn determination. Fresh samples were used for measurements of H_2O_2, MDA, chlorophyll concentrations, antioxidant enzyme activities and non-enzymatic antioxidant concentrations.

Tissue Hg concentration

To metal determination plantlets were oven-dried at 65°C to constant mass. Between 70 and 100 mg of shoots and roots were digested with 5 mL HNO_3 and 0.2 mL H_2O in closed Teflon vessels, which were heated at 100°C for 3 h in a digester block (Tecnal TE 007D). The samples were then diluted to 50 mL with high-purity water. Hg concentrations were determined using a Varian Atomic Absorption Spectrophotometer (Spectr AA 600, Australia)

equipped with a vapor generative accessory (Varian VGA-76). The content of Hg was expressed as μg g^{-1} dry weight.

Tissue Zn concentration

Zn concentration was determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) using a PerkinElmer *Optima 4300DV* (Shelton, USA) equipped with a cyclonic spray chamber and a concentric nebulizer. The emission lines used were 213.857 nm. Instrumental parameters were adjusted according manufacturer recommendations. Nebulizer, intermediate and principal gas flow rates were set to 0.65, 0.20 and 14 L min^{-1}, respectively. Sample decomposition was carried out in an open digestion system, using a heating block from Velp Scientifica (Milano, Italy) equipped with glass vessels. The content of Zn was expressed as μg g^{-1} dry weight.

Chlorophyll and carotenoids determination

Fresh biomass (leaves plus stem) were determinated following the method of Hiscox and Israelstam (1979). Briefly, 0.1g of shoot was macerated in liquid nitrogen, weighted and placed in a vial containing 7 mL dimethylsulphoxide (DMSO). The photosynthetic pigments were extracted from fluid at 65°C by incubing for 2 h. To liquid extract was added 3 mL of DMSO. A 3 mL sample was transferred to a cuvette and the absorbances at 645, 663 and 470 nm were read in order to determine chlorophyll a, chlorophyll b and carotenoids, respectively, in a Spectrophotometer (Celm E-205D). Pigment concentration was calculated following the equation used by Lichtenthaler (1987). Chlorophyll and carotenoid concentration was expressed as mg g^{-1} fresh weight.

Delta-aminolevulinic acid dehydratase (ALA-D; E.C. 4.2.1.24) activity

Shoots were homogenized in 10 mM Tris-HCl buffer, pH 9.0, at a proportion of 1:1 (w/v). The homogenate was centrifuged at 12,000 g at 4°C for 10 min to yield a supernatant

(S1) that was used for the enzyme assay. The supernatant was pre-treated with 0.1% Triton X-100 and 0.5 mM dithiotreithol (DTT). δ-ALA-D activity was assayed as described by Barbosa et al. (1998) by measuring the rate of porphobilinogen (PBG) formation. The incubation medium for the assays contained 100 mM Tris-HCl buffer, pH 9.0 and 3.6 mM ALA. Incubation was started by adding 100 µL of the tissue preparation to a final volume of 400 µL and stopped by adding 350 µL of the mixture containing 10% trichloroacetic acid (TCA) and 10 mM $HgCl_2$. The product of the reaction was determined with the Ehrlich reagent at 555 nm using a molar absorption coefficient of 6.1×10^4 L^{-1} mol^{-1} cm^{-1} (Sassa 1982) for the Ehrlich-porphobilinogen salt. δ-ALA-D activity was expressed as nmol PBG mg^{-1} protein h^{-1}.

Determination of hydrogen peroxide

The H_2O_2 concentration was determined according to Loreto and Velikova (2001). Approximately 0.1 g of both roots and shoot was homogenized at 4°C in 2 mL of 0.1% trichloroacetic acid (TCA) (w/v). The homogenate was centrifuged at 12000 g for 15 min. Then, 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1M KI. The H_2O_2 concentration of the supernatant was evaluated by comparing its absorbance at 390 nm with a standart calibration curve. Hydrogen peroxide concentration was expressed as µmol g^{-1} fresh weight.

Estimation of lipid peroxidation

The level of lipid peroxidation products was estimated following the method of El-Moshaty et al. (1993) by measuring the concentration of malondialdehyde (MDA) as an end product of lipid peroxidation by reaction with thiobarbituric acid (TBA). Fresh tissue samples (0.1 g fresh weight) were ground in 2 mL of 0.2 M citrate-phosphate buffer (pH 6.5) containing 0.5% Triton X-100, using mortar and pestle. The homogenate was filtered through two layers of paper and centrifuged for 15 min at 20,000 g. One milliliter of the supernatant fraction was added to an equal volume of 20% (w/v) TCA containing 0.5% (w/v) TBA. The mixture was heated at 95°C for 40 min and then quickly cooled in an ice bath for 15 min. After centrifugation at 10,000 g for 15 min, the absorbance of the supernatant was measured

at 532 nm. A correction for non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The lipid peroxides were expressed as nmol MDA (mg protein)$^{-1}$, by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Catalase (CAT, E.C. 1.11.1.6) assay

Catalase activity was assayed according to the method of Aebi (1984) with some modifications. Fresh samples (1g) were homogenized in 5 mL of 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 10 g L^{-1} PVP, 0.2 mM EDTA and 10 mL L^{-1} Triton X-100. The homogenate was centrifuged at 12,000 g for 20 min at 4°C and then, the supernatant was used for the enzyme assay. Catalase activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm from a reaction mixture containing 2 mL of 15 mM H_2O_2 in KPO_4 buffer (pH 7.0) and 30 µL extract. Activity was expressed as ΔE min^{-1} mg^{-1} protein.

Ascorbate peroxidase (APX, E.C. 1.11.1.11) assay

Ascorbate peroxidase (APX) was measured according to Zhu et al. (2004). The reaction mixture, at a total volume of 2 mL, contained 25 mM (pH 7.0) sodium phosphate buffer, 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H_2O_2 and 100 µL enzyme extract. H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (E = 2.8 L^{-1} mol^{-1} cm^{-1}) and activity was expressed as µmol oxidated ascorbate min^{-1} mg^{-1} protein.

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity

The activity of SOD was assayed according to Misra and Fridovich (1972). About 200 mg of roots and shoots were homogenized in 5 mL of 100 mmol L^{-1} K-phosphate buffer (pH 7.8) containing 0.1 mmol L^{-1} EDTA, 0.1% (v/v) Triton X-100 and 2% PVP (w/v). The extract was filtered and centrifuged at 22,000 g for 10 min at 4ºC, and the supernatant was used for

assays. The assay mixture consisted of a total volume of 1 mL, containing glycine buffer (pH 10.5), 1 mmol L^{-1} epinephrine and enzyme material. Epinephrine was the last added component. Adenochrome formation over the next 4 min was spectrophotometrically recorded at 480 nm. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions used. This method is based on the ability of SOD to inhibit the autoxidation of epinephrine at an alkaline pH. Since the oxidation of epinephrine leads to the production of a pink adrenochrome, the rate of increase of absorbance at 480 nm, which represents the rate of autoxidation of epinephrine, can be conveniently followed. The enzyme has been found to inhibit this radical-mediated process.

Ascorbic acid (AsA) concentration

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Both roots and shoot were homogenized in a solution containing 50 mmol L^{-1} Tris-HCl and 10 mL L^{-1} Triton X-100 (pH 7.5), centrifuged at 6,800 g for 10 min. To the supernatant was added 10% TCA in the proportion of 1:1 (v/v) followed by centrifugation (6,800 g for 10 min) to remove protein. An aliquot of the sample (300 µL) was incubated at 37°C in a medium containing 100 µL TCA 13.3%, 100 µL deionized water and 75 µL 2,4-Dinitrophenylhydrazine (DNPH). The DNPH solution contained 2% DNPH, 0.23% thiourea, 0.27% $CuSO_4$ diluted in 49% H_2SO_4. After 3 h, 500 µL of 65% H_2SO_4 was added and samples were read at 520 nm. A standard curve was constructed using L(+) ascorbic acid.

Non-protein thiols groups (NPSH) concentration

Non-protein thiols concentration was measured spectrophotometrically with Ellman's reagent (Ellman 1959). Roots and shoots samples were homogeneized in a solution containing 50 mmol L^{-1} Tris-HCl and 10 mL L^{-1} Triton X-100 (pH 7.5), centrifuged at 6,800 g for 10 min, and NPSH were determined in a fraction obtained after mixing 1 volume of supernatant with 1 volume of 10% TCA followed by centrifugation (6,800 g for 10 min) and neutralization (to pH 7.4) with 1 M Tris-HCl as described by Jacques-Silva et al. (2001).

Reaction was read at 412 nm after the addition of 0.05 mL of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB). A standard curve using cysteine was used to calculate the content of thiol group in samples, and was expressed as μmol SH g^{-1} fresh weight.

Protein extraction

In all the enzyme preparations, protein was determined by the method of Bradford (1976) using bovine serum albumin as standard and was expressed in mg L^{-1}.

Statistical analysis

The analyses of variance were computed on statistically significant differences determined based on the appropriate F-tests. The results are the means ± SD of at least three independent replicates. The mean differences were compared utilizing Tukey test with $p< 0.05$.

Results

Tissue metal concentration

Hg concentrations were higher in roots than in shoot upon addition of Hg in the substrate. Conversely, Hg concentrations were, approximately, 15% and 6.8% higher in shoot when compared with roots, respectively, in control and Zn treatments (Table 1). The addition of Hg and Zn together significantly decreased root and shoot Hg concentrations, when compared to Hg alone. In this treatment, shoot and root Hg levels were 59% and 24% lower than in plants exposed to 50 μM Hg alone. In addition, there was an increase in Zn levels in shoot of plants exposed to Zn + Hg, although in roots, Zn concentration was not altered, when compared to the control.

Table 1 – Hg and Zn concentrations of shoot and roots of *Pfaffia glomerata* plantlets exposed to treatments for 9 days.

Treatments	*Zn concentration ($\mu g\ g^{-1}$ dry weight)*		*Hg concentration ($\mu g\ g^{-1}$ dry weight)*	
	Shoot	Root	Shoot	Root
Control	79.66 ± 4.04 bB*	161.66 ± 10.01 aA	16.86 ± 1.5 cA	2.53 ± 0.25 bB
50 µM Zn	116 ± 4.58 bB	176 ± 37.9 aA	15.5 ± 0.7 cA	1.06 ± 0.52 bB
50 µM Hg	73.66 ± 4.72 bB	143 ± 34 aA	351.5 ± 39.5 aB	797 ± 149 aA
50 µM Zn + 50 µM Hg	216.66 ± 65.57 aA	150.66 ± 23.35 aB	208 ± 94.02 bB	605.33 ± 159.91 aA

Data represent mean values ± SD based on independent determination.

* Mean values followed by the same lower-case letters in the column, and capital letters in the line did not differ significantly by Tukey test of $p<0.05$.

Survival percentage, fresh and dry weight of *P. glomerata* plantlets

In comparison to the control, fresh and dry weight, as well as survival percentage in the Hg-exposed *Pfaffia glomerata* plants decreased significantly, while Zn supplementation partially reversed the negative effect caused by Hg (Fig 1). The survival percentage was about 47% and 20% lower than the control in plants exposed to Hg-alone and Zn+Hg treatments, respectively. In 50 µM Hg treatment, shoot fresh weight decreased about 71.5% when compared to the control, whereas root fresh weight decreased by 59%. On the other hand, in plants treated with Zn+Hg, shoot and root fresh weight decreased about 51.4% and 24.4%, respectively, in relation to the control.

Shoot dry weight in Hg-exposed plants decreased by 69% in relation to the control. In plants exposed to Zn+Hg treatment, the dry weight also decreased, but only by about 26% when compared to the control. Interestingly, root dry weight in Hg-treated plants was slightly but not significantly, decreased by the presence of Hg, when compared to the control (Fig 1C), whereas Zn-treated plants showed greater root dry weight than Hg-treated plants.

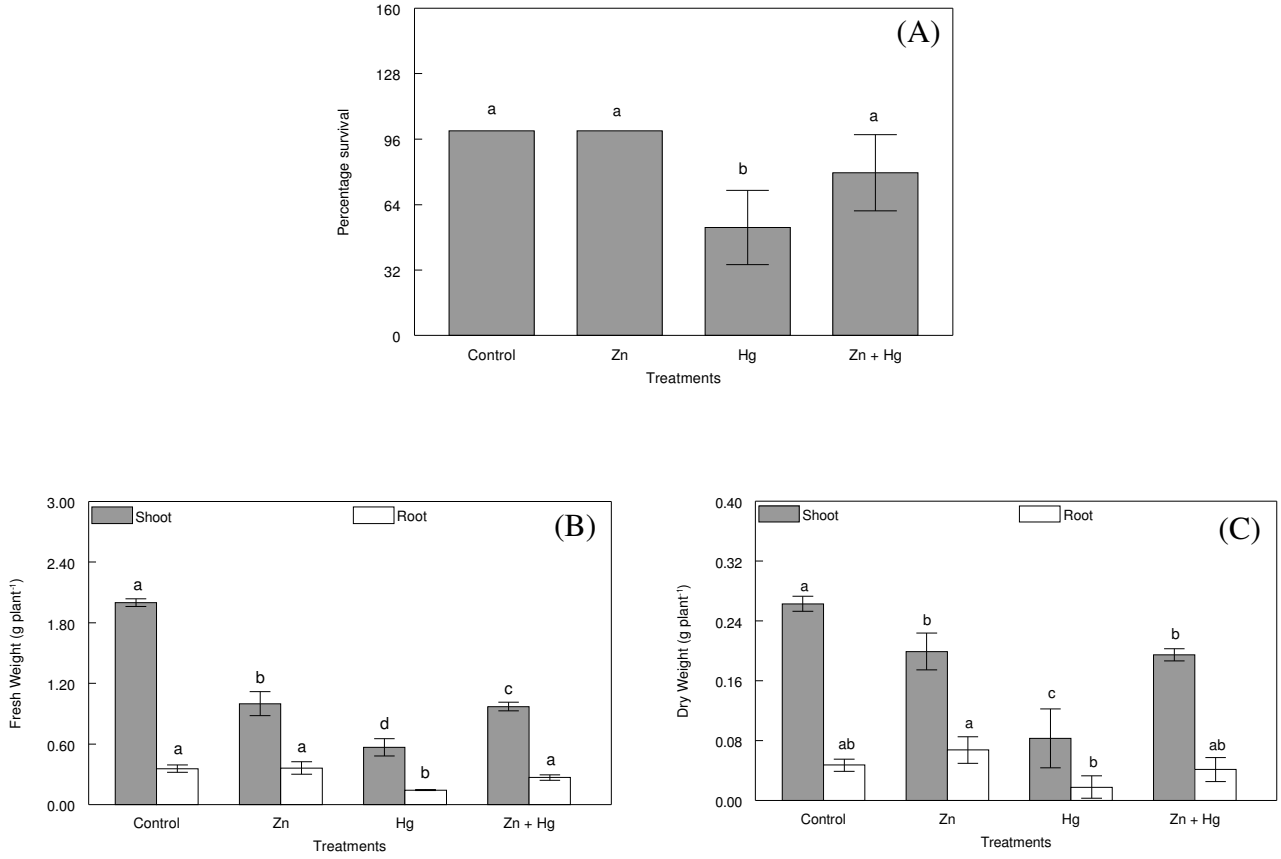

Figure 1 Survival percentage (A), fresh weight (B) and dry weight (C) of *P. glomerata* plantlets exposed to different treatments. Control: without Zn and Hg; Zn: 50 µM Zn as $ZnCl_2$; Hg: 50 µM Hg as $HgCl_2$; Zn + Hg: 50 µM Zn + 50µM Hg. Data represent the mean ± SD of three replicates. Identical letters indicate no significant differences among the treatments according to Tukey´s multiple range test ($p<0.05$).

Delta-aminolevulinic acid dehydratase activity (ALA-D) and chlorophyll and carotenoid concentration

In comparison to the control, chlorophyll a and b concentrations were not significantly altered upon addition of Hg, Zn and Zn+Hg treatments. On the other hand, Hg-treated plants showed higher chlorophyll a concentration than those treated with Zn alone. Carotenoid concentration was increased upon addition of Zn+Hg, when compared to the control.

δ-ALA-D activity decreased upon addition of Hg alone and Zn alone, whereas it was not altered when these two metals were added together, when compared to the control.

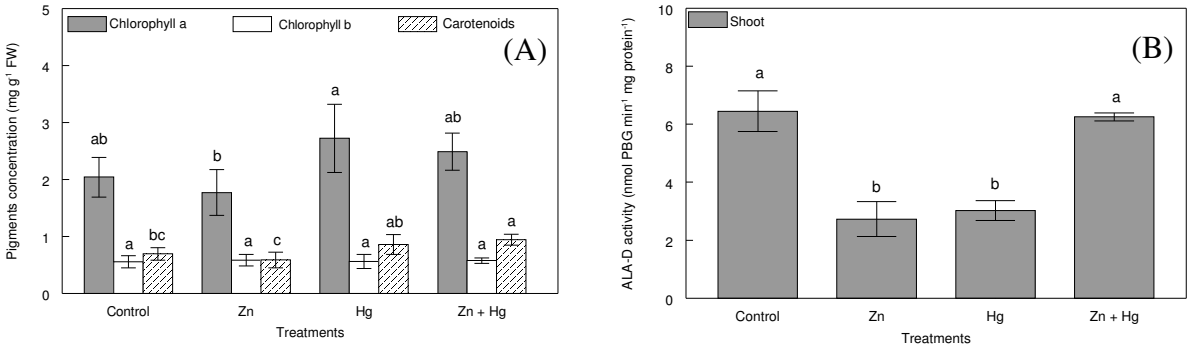

Figure 2 Pigment concentration (A) and δ-aminolevulinic acid (δ-ALA-D) activity (B) of *P. glomerata* plantlets exposed to different treatments. Control: without Zn and Hg; Zn: 50 µM Zn as $ZnCl_2$; Hg: 50 µM Hg as $HgCl_2$; Zn + Hg: 50 µM Zn + 50µM Hg. Data represent the mean ± SD of three replicates. Identical letters indicate no significant differences among the treatments according to Tukey´s multiple range test ($p<0.05$).

Hydrogen peroxide and lipid peroxidation levels

Only treatments containing Hg, applied either alone or supplemented with Zn, had higher tissue H_2O_2 concentrations, when compared to the control.

Shoot lipid peroxidation was significantly increased upon addition of Hg, while it decreased significantly in the treatments with Zn+Hg added together, when compared to the control. On the other hand, lipid peroxidation in roots was not altered in any treatment.

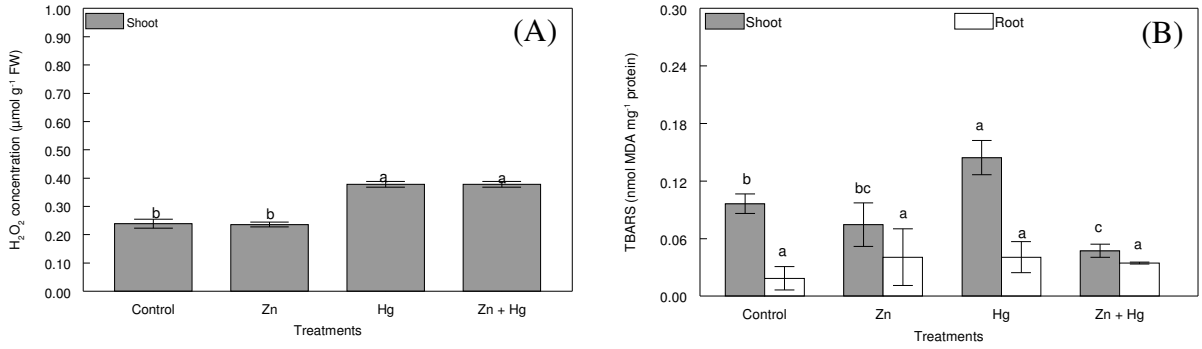

Figure 3 Shoot hydrogen peroxide concentration (A) and lipid peroxidation (B) of *P. glomerata* plantlets exposed to different treatments. Control: without Zn and Hg; Zn: 50 µM Zn as $ZnCl_2$; Hg: 50 µM Hg as $HgCl_2$; Zn + Hg: 50 µM Zn + 50µM Hg. Data represent the mean ± SD of three replicates. Identical letters indicate no significant differences among the treatments ($p<0.05$) according to Tukey´s multiple range test.

Antioxidant enzyme activity

SOD activity was higher in shoot than in roots (Fig 4A). The Hg-treated plants showed 2.3-fold higher shoot SOD activity than the control. Treatments either with Zn alone or together with Hg showed greater shoot SOD activity that the control, but this increase was lower than that observed in plants treated with Hg alone. In the roots, however, the highest SOD activity was found in the control, whereas the minimum SOD activity was found in the Hg-alone treatment, which was 60% lower than the control. Plants exposed to Zn alone and Zn+Hg, showed decreases in SOD activity of approximately 37% and 73%, respectively, when compared to the control.

In comparison to the control, shoot CAT activity decreased upon addition of Hg alone, whereas it was not significantly altered by Zn either alone or together with Hg. In contrast, root CAT activity significantly increased (60%) upon addition of Hg.

Treatment with Zn alone showed greater root APX activity than the control, whereas treatments containing Hg, either alone or together with Zn, showed a significant decrease, when compared to the control. On the other hand, shoot APX activity only increased upon addition of Zn+Hg.

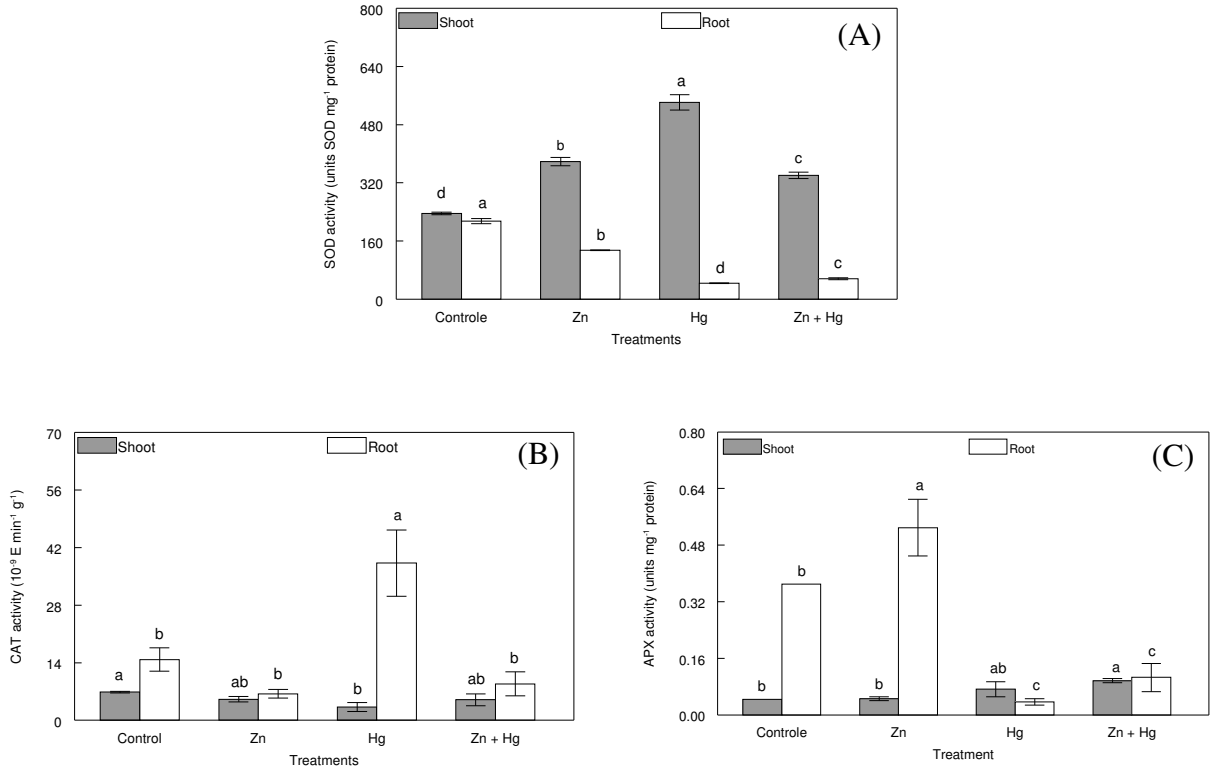

Figure 4 Superoxide dismutase (SOD) activity (A), catalase (CAT) activity (B) and ascorbate peroxidase (APX) activity (C) of *P. glomerata* plantlets exposed to different treatments. Control: without Zn and Hg; Zn: 50 µM Zn as $ZnCl_2$; Hg: 50 µM Hg as $HgCl_2$; Zn+Hg: 50 µM Zn + 50µM Hg. Data represent the mean ± SD of three replicates. Identical letters indicate no significant differences among the treatments according to Tukey´s multiple range test ($p<0.05$).

Antioxidant non-enzymatic concentration

Shoot NPSH concentration was increased upon addition of 50 µM Hg-alone, where NPSH levels were about 1.7-fold higher than the control. No significant effect was observed in plants treated with Hg supplemented with 50 µM of Zn (Fig 5).

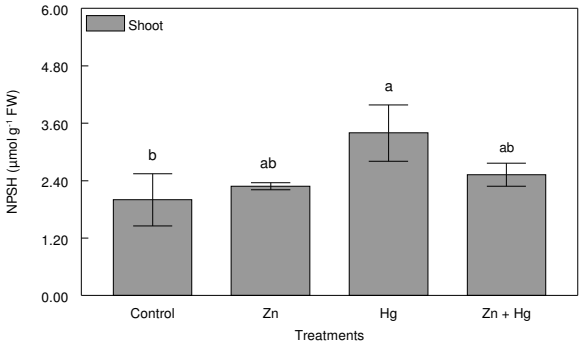

Figure 5 Non-protein thiol (NPSH) concentration of *P. glomerata* plantlets exposed to different treatments. Control: without Zn and Hg; Zn: 50 µM Zn as $ZnCl_2$; Hg: 50 µM Hg as $HgCl_2$ and; Zn+Hg: 50 µM Zn + 50µM Hg. Data represent the mean ± SD of three replicates. Identical letters indicate no significant differences among the treatments according to Tukey´s multiple range test ($p<0.05$).

Discussion

Plants of *Pfaffia glomerata* exposed to Hg alone showed significant oxidative stress symptoms, observed by increases in both shoot H_2O_2 concentration and lipid peroxidation (Fig 3). These alterations were followed by significant changes in the antioxidant system. However, the antioxidant system was not efficient in avoiding damage, resulting in 52.8% of mortality in Hg-treated plants (Fig 1A). Survival percentage was significantly higher in plants exposed to Zn+Hg, when compared to Hg alone, indicating that Zn alleviated the oxidative damage induced by Hg.

Roots accumulated more Hg than shoots in those plants exposed to Hg and Zn+Hg. Conversely, shoots accumulated more Hg than roots in plants grown without Hg. The Hg uptake from air via stomata of Hg-free plants is a possible reason for the relatively high shoot Hg concentration in these treatments. Patra et al. (2004) reported that mercury salts in soil may be reduced by biological and chemical reactions to metallic or methylated compounds, which may volatilize and be taken up through the leaves in plants grown in enclosed spaces. Uptake of gaseous Hg via stomata was also observed in laboratory studies (Cavallini et al. 1999; Iglesia-Turiño et al. 2006).

Our results showed a reduction of shoot Hg concentration together with a significant increase of Zn concentration in plants exposed to Zn+Hg (Table 1), indicating a possible interaction between these metals. Zn, Cd and Hg are divalent metals that constitute group II B of the periodic table of elements. Zn is an essential trace metal while the remaining two are toxic metals. Nevertheless, they share biological responses such as affinity towards thiol groups, antagonism to biological cations, participation in redox reaction and modulation of essential element homeostasis (Peixoto et al. 2003). Hart et al. (2002) attributed the competition for uptake between Cd and Zn to the existence of a common transport system on the plasma membranes. Several other findings (Thys et al. 1991; Cataldo et al. 1983; McKenna et al. 1993; Aravind and Prasad 2003) point to depressed Cd uptake in the presence of Zn as well as an antagonistic effect of Zn under other essential transition metals like Cu and Fe (Powell 2000; Zago and Oteiza 2001). Thus, in our study, Zn may have interacted with Hg.

In the present study, the concentration of Zn in overground tissues (leaves plus stem) of plants exposed to Zn+Hg might have been an antioxidant action that resulted in the significant decrease of lipid peroxidation (Fig 3B) together with the significant increase of APX activity (Fig 5C) in these tissues, when compared to the control.

Shoot and root fresh weight from plants exposed to Hg added alone showed reductions of about 70% and 60%, respectively, when compared to the control. Hg has a high affinity to sulphydryl (-SH) groups generally abundant in proteins, and it might bind to the crucial and functional membrane, activating and inactivating their enzyme functions (Patra et al. 2004). Plant cells contain aquaporins, proteins that facilitate water transport in the vacuolar membrane (tonoplast) and the plasma membrane (Patra and Sharma 2000). Mercury induces conformational changes in aquaporins of higher plants because it is thought to bind to –SH groups of aquaporin proteins, physically blocking the channels and reducing their hydraulic conductivity (Tyermam et al. 1999). Partial recovery of the water flow rate upon addition of $HgCl_2$ was observed in tomato and aspen root systems, implying the presence of aquaporins as regulators of the plant water status (Maggio and Joly 1995; Wan and Zwiazek 1999). In the present study, the symptoms of withering shown by plants treated with Hg may explain the decrease in fresh weight and this data corroborates with others reports (Zang and Tyerman 1999; Cargnelutti et al. 2006).

Zn is an essential component of many vital enzymes and a structural stabilizer for proteins, membrane and DNA-binding proteins (Vallee and Auld 1990). It acts either as a

metal component or as a functional, structural, or regulatory cofactor of a large number of enzymes (Marshner 1986). On the other hand, an excess of Zn in the substrate causes nutritional disorders in plants, hindering development (Soares et al. 2001). In the present study, significant decreases of fresh and dry weight in plants exposed to Zn alone may have been due to Fe deficiency since the shoot Fe concentration was about 53% lower than that of control plants (data not shown). The significant decrease of the intracellular Fe concentration upon Zn addition may have been associated with the significant reduction (50% and 24%, respectively) in shoot fresh and dry weight when compared to the control. Iron is an essential element for plants and plays an important role in several plant biological processes, including the catalytic groups for many redox enzymes and is required for nitrogen fixation and synthesis of chloroplast, DNA, and hormones (Hopkins 1997). In the present study, the lack of bioavailable Fe may have been due to its precipitation in nutrient solution, or by antagonistic interaction between Zn and Fe, which inhibited Fe uptake by roots.

The toxicity of non-essential metals, such as Hg and Cd, is generally accepted to occur by inactivation of enzymes and/or functional proteins by direct binding. However, several studies have also shown that toxicity may be due, at least in part, to oxidative damage caused by generation of reactive oxygen species (ROS) due to the presence of heavy metals (Stohs and Bagchi 1995).

Hg ions can also substitute metal ions in photosynthetic pigments, causing a decrease in photosynthesis rates (Xylander et al. 1996). Van Assche and Clijsters (1990) reported that lipid peroxidation causes membrane impairment and leakage, and suggested that the reduction in chlorophyll content in the presence of metals is caused by an inhibition of chlorophyll biosynthesis. Mercury has been reported to decrease the pigment content in several plants (Ali et al. 2000; Cho and Park 2000; Cargnelutti et al. 2006). However, no effect was observed in pigment concentration in *P. glomerata* plants exposed to Hg, although a significant alteration in δ-ALA-D activity was observed (about 53% when compared to the control). Due to its sulphydryl nature (Barnard et al. 1977), δ-ALA-D is highly sensitive to the presence of heavy metals such as Hg (Rocha et al. 1993, Emanuelli et al. 1996). Thus, the significant decrease in δ-ALA-D activity observed in this study may have resulted from Hg binding with δ-ALA-D –SH groups.

δ-ALA-D is a zinc-dependent enzyme in animals, yeasts, and some bacteria (Jordan 1990). Cysteine residues participate in Zn binding and these enzymes contain a cysteine rich domain that may be involved in metal binding. Plant dehydratases are located in plastids and

are needed for the synthesis of chlorophyll and other cellular tetrapyrroles. They share 35 to 50% identity with non-plant enzymes, but they require Mg to display maximum activity rather than Zn. The peptide region in the plant enzyme corresponding to the Zn domain of animals lacks cysteine and histidine residues and contains aspartate, alanine, or threonine instead (Boese et al. 1991). The reduction in δ-ALA-D activity in Zn treated plants may have been due to Mg utilization for some processes which require Fe during Fe deficiency, as suggested by Oladiji (2003) in a study with rats. Thus, Fe deficiency could have led to Mg unavailability for δ-ALA-D activity. Plants exposed to Zn+Hg, in contrast, showed a sharp increase in δ-ALA-D activity, such that it was equal to the control. The present study indicated that the decrease in δ-ALA-D activity induced by Hg was prevented by the increase of Zn concentration in shoot. Probably, Zn prevented Hg-binding with –SH groups of this enzyme, also preventing its inactivation.

Oxidative stress occurs due to the excess production of free radicals, the inadequate availability of antioxidants or a combination of both. Lipid peroxidation is a basic deteriorating change in unsaturated fatty acids of the cell membranes induced by excess free radicals (Haliwell and Gutteridge 1999). Estimation of malonyldialdehyde continues to be a reliable method to assess the degree of peroxidative damage to cell membranes, as it is the most abundant aldehyde formed as a by-product during this process (Gurer et al. 1998). In the present study, the root MDA concentration was not altered in the presence of Zn or Hg, although other stress symptoms occurred, such as growth reduction and root darkening (data not shown). However, in shoot MDA reached higher concentrations in plants exposed to Hg alone, when compared to the control. This result is in agreement with Cho and Park (2000), who showed that tomato plants exposed to 50 μM $HgCl_2$ had a significant increase in the MDA concentration. The increase of MDA indicates that plants experienced oxidative damage when exposed to Hg alone at 50 μM. In addition, the higher MDA concentration in the shoot indicates that overground tissues are more sensitive to metals than roots, and Zn addition had an important role in ROS detoxification induced by Hg.

The H_2O_2 concentration is, in part, a result of SOD activity, the first antioxidant enzyme involved with ROS detoxification. H_2O_2, although not as reactive as the superoxide radical (O_2^-), can be changed by transition metals, such as Fe and Cu, producing the OH• radical, the most dangerous cellular oxidant (Zago and Oteiza 2001). To avoid a hazardous situation, APX and CAT antioxidant enzymes act to degrade these molecules to H_2O (Fig 4). H_2O_2 and SOD activity were higher in shoot of plants exposed to Hg alone (Fig 3 and 4),

indicating an over-production of superoxide radicals (O_2^-) that resulted in a 2.3-fold increase in SOD activity when compared to the control.

Perhaps Zn addition had a major role in detoxification of superoxide radicals, because its presence allowed the increase of SOD activity. The higher SOD activity observed in shoot of plants exposed to Zn and Zn+Hg may have been due to the presence of Zn, which has a structural role in the Cu-ZnSOD enzyme.

As a result of SOD action in plants exposed to Hg, shoot H_2O_2 was produced, inducing to some extent the scavenging-activity of APX in this tissue. Conversely, in roots exposed to Hg alone, SOD activity was lower, whereas CAT activity was higher. It seems that an excess of ROS production in the presence of Hg in roots would have inactivated antioxidant enzymes such as SOD and APX.

Non-protein-thiol compounds are non-enzymatic antioxidants that protect plant tissue from cellular damage, acting together with the enzymatic antioxidant system. They exist under various forms, such as glutathione tripeptide, homoglutathione and polymerized peptides, phytochelatins or homophytochelatins (Ferrat et al. 2003). These molecules are synthesized by enzymatic polymerization of glutathione or homoglutathione (Gekeler et al. 1989). Glutathione is a well-known antioxidant that plays a key role in the regulation of the redox balance and can be used as an indicator of oxidative stress (Rijstenbil and Wijnholds 1996), in the detoxification of xenobiotics (Marrs 1996) and heavy metals (Cobbett 2000). Hg has high affinity to -SH residues present in non-protein thiols, and can form complexes with Hg forms both *in vivo* and *in vitro* (Rabenstein et al. 1985). In the present study, Hg-treated plants had significant increases in shoot NPSH concentrations (Fig 5), possibly reflecting the a defense mechanism against Hg-triggered oxidative stress. The increase of NPSH concentrations can indicate an increase in GSH concentrations and, consequently, an increase in phytochelatin production. There is strong evidence that PC forms complexes with metals to which they have a high affinity (Mehra et al. 1996) such as Pb, Ag and Hg *in vitro* (Rauser 1999).

In conclusion, the supplementation of Zn to Hg-treated plants alleviated the Hg-toxic effects in *P. glomerata* plants by decreasing Hg uptake and translocation, maintaince of δ-ALA-D activity, decreasing lipid peroxidation and increasing the protective effect of the antioxidant system.

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4 DISCUSSÃO

Em ambos os experimentos foi observado o efeito negativo do Hg no crescimento de plantas de *Pfaffia glomerata*. O crescimento das raízes, a biomassa fresca e seca das raízes e da parte aérea, as atividades enzimáticas e a concentração de antioxidantes não-enzimáticos foram amplamente afetadas na concentração de 50 µM de Hg. Estas plantas experimentaram estresse oxidativo e tiveram seus tecidos danificados.

O primeiro sintoma visível dos efeitos do Hg foi o murchamento das folhas e o escurecimento das raízes. Zhang & Tyerman (1999) relataram que o Hg se liga aos grupos -SH das aquaporinas e obstrui os canais de passagem de água, impedindo assim a sua absorção. O déficit hídrico resultante da obstrução das aquaporinas pelo Hg pode ter interferido em todos os processos metabólicos, incluindo a taxa fotossintética, o que pode ter levado a perda de biomassa fresca e seca das plantas. A adição de 50 µM de Zn na solução nutritiva pode ter evitado a ligação do Hg aos grupos -SH das aquaporinas, um vez que as matérias fresca e seca foram significativamente aumentadas quando comparadas com os valores obtidos de plantas expostas a 50 µM de Hg (Manuscrito 2, Fig. 1B e C).

A concentração de Hg nos tecidos da parte aérea e das raízes aumentou com a presença deste elemento no substrato (Manuscrito 1, Tabela 1; Manuscrito 2, Tabela 1). A concentração de Hg nas raízes foi significativamente maior do que na parte aérea naqueles tratamentos em que o Hg foi adicionado à solução nutritiva. Este fato pode indicar que as raízes funcionaram como uma barreira para o transporte do Hg aos tecidos da parte aérea (CAVALLINI et al., 1999), evitando assim, danos maiores a estes tecidos.

Inversamente, a concentração de Hg foi significativamente maior na parte aérea do que nas raízes de plantas não-tratadas com Hg (Manuscritos 1 e 2, Tabela 1). Este fato pode ter ocorrido devido à facilidade de volatilização do Hg na forma elementar Hg^0, que, conforme relatado por Frear & Dills (1967), pode se difundir facilmente através dos estômatos para o mesofilo foliar e aumentar a sua concentração nos tecidos verdes. Segundo estes autores, a maioria dos compostos inorgânicos de Hg adicionados aos solos é decomposto para produzir Hg^0, quando na presença de matéria orgânica e outros fatores que conduzem para a sua redução. Em geral, as reações do tipo $Hg_2^{2+} = Hg^{2+} + Hg^0$ são comuns na maioria dos solos.

O Hg não causou alteração significativa na concentração das clorofilas e carotenóides (Manuscrito 1, Fig. 1A; Manuscrito 2, Fig. 2A). No entanto, houve significativa diminuição

na atividade da δ-ALA-D de plantas expostas a 25 e 50 μM Hg (Manuscrito 1, Fig. 1B e 2, Fig. 2B). Este resultado confirma o poder inibitório do Hg sobre a atividade da δ-ALA-D (MORSCH et al., 2002). Por outro lado, observou-se aumento significativo da atividade da δ-ALA-D na concentração mais baixa de Hg (1 μM) em relação ao controle. Tal efeito pode ter ocorrido porque a concentração de Hg nos tecidos não foi suficiente para inibir a enzima ou ainda devido ao aumento da síntese de moléculas de enzimas pelo sistema de homeostase das plantas.

Inesperadamente, a atividade da δ-ALA-D foi significativamente diminuída em plantas expostas ao Zn aplicado isoladamente (Manuscrito 2, Fig. 2B). Adicionalmente, não foi observado aumento da concentração de Zn nos tecidos das plantas neste tratamento (Manuscrito 2, Tabela 1), e a concentração de Fe nos tecidos da parte aérea diminuiu significativamente (dados não mostrados). A presença do Zn na solução nutritiva pode ter evitado a translocação do Fe para os tecidos da parte aérea. Em substituição ao Fe, o Mg pode ter sido utilizado por outros processos, como foi sugerido por Oladiji (2003), em seu estudo com ratos. Deste modo, o Mg utilizado em outros processos metabólicos resultou em sua menor disponibilidade para a ativação da enzima δ-ALA-D. A adição de Zn+Hg na solução nutritiva não resultou em diminuição na atividade da δ-ALA-D, quando comparada ao controle. A presença do Zn na parte aérea destas plantas pode ter evitado a ligação do Hg aos grupos –SH da enzima δ-ALA-D, evitando assim sua inativação.

Foi observado haver interação entre os elementos Zn e Hg, quando adicionados simultaneamente à solução nutritiva. A interação Zn-Hg interferiu diretamente nas concentrações destes elementos nos tecidos da planta (Manuscrito 2, Tabela 1) e, consequentemente, na atividade do sistema de defesa antioxidante. A interação Zn-Hg permitiu a reversão do estresse oxidativo induzido pelo Hg, provavelmente porque o Zn competiu com o Hg pelos grupos -SH que constituem várias enzimas e proteínas, o que evitou a inativação de suas funções biológicas. A adição do Zn+Hg na solução nutritiva resultou em aumento significativo da porcentagem de sobrevivência das plantas (Manuscrito 2, Fig. 2A), proporcionada pela significativa diminuição da peroxidação lipídica (Manuscrito 2, Fig. 4B).

Nossos resultados indicam que a exposição ao Hg resulta em aumento na concentração de H_2O_2 nos tecidos das plantas de *P. glomerata* (Manuscritos 1 e 2, Fig. 2A e 3A, respectivamente). Este aumento coincidiu com o aumento nas atividades das enzimas CAT e APX da parte aérea (Manuscrito 1, Fig. 3B e C) e na atividade da CAT nas raízes (Manuscrito 2, Fig. 4B). A concentração aumentada do H_2O_2 pode estar correlacionada ao aumento da

atividade da SOD (Manuscritos 1 e 2, Fig. 3A e 4A, respectivamente) na parte aérea. Este efeito estimulatório está geralmente relacionado ao efeito hormético, que representa uma resposta para compensar o rompimento da homeostase no organismo (CALABRESE, 1999; LIN et al., 2007).

Efeito oposto ocorreu nas raízes, onde a concentração de H_2O_2 foi menor que no controle e a atividade da SOD foi inibida, enquanto as atividades da CAT e da APX não foram afetadas (Manuscrito 1, Fig. 3 e 4). Assim, o significante aumento nas concentrações de AsA e NPSH (Manuscrito 1, Fig. 4A e 4B) não foi suficiente para proteger as raízes da peroxidação lipídica (Fig. 2B).

Em relação aos antioxidantes não-enzimáticos, foi observado aumento na concentração dos grupos –SH com o aumento da concentração de Hg adicionada. O aumento dos grupos –SH leva à inativação da reatividade do Hg por mecanismos de detoxificação citoplasmáticos e representa um mecanismo de defesa contra o dano celular causado pelo metal (PATRA et al., 2004). Os níveis de AsA aumentaram nas concentrações de 25 e 50 µM Hg, o que indica que o mecanismo de remoção das EROs estava sendo ativado. Adicionalmente, a concentração de prolina também aumentou com a concentração de Hg no substrato, mas a concentração de clorofila e carotenóides não foi alterada. O aumento da concentração de prolina pode estar relacionada na manutenção da estabilidade da concentração dos pigmentos fotossintéticos em *P. glomerata*.

Devido à razoável capacidade das plantas de *Pfaffia glomerata* em acumular Hg nas raízes, o uso desta espécie como fitoestabilizadora permitiria a retenção do Hg no solo evitando a expansão do contaminante para áreas adjacentes. Por outro lado, a morfologia tuberosa das raízes também permitiria a retirada do Hg do solo com relativa facilidade. Portanto, a *Pfaffia glomerata* poderia ser utilizada tanto na fitoestabilização como na fitodescontaminação dos solos.

5 CONCLUSÕES

Observou-se estresse oxidativo em plantas de *P. glomerata* em resposta ao Hg, bem como ativação do sistema antioxidante na regulação do dano oxidativo induzido pelo Hg. Na concentração de 50 μM Hg, as plantas não foram capazes de reverter o dano causado por este elemento.

A interação entre Zn e Hg evitou parcialmente os danos causados pelo Hg, observados pela significante diminuição da peroxidação lipídica e pela maior porcentagem de sobrevivência de plantas neste tratamento.

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