UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA TOXICOLÓGICA

EFEITO DO MERCÚRIO SOBRE PARÂMETROS BIOQUÍMICOS E FISIOLÓGICOS EM PEPINO E MILHO: PAPEL PROTETOR DO ZINCO

TESE DE DOUTORADO

Denise Cargnelutti

Santa Maria, RS, Brasil
2009

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Por

Denise Cargnelutti

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito para a obtenção do grau de **Doutor em Bioquímica Toxicológica**

Orientadora: Profa. Dra. Maria Rosa Chitolina Schetinger
Co-orientadora: Profa. Dra. Vera Maria Morsch

Santa Maria, RS, Brasil

2009

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

EFEITO DO MERCÚRIO SOBRE PARÂMETROS BIOQUÍMICOS E FISIOLÓGICOS EM PEPINO E MILHO: PAPEL PROTETOR DO ZINCO

elaborada por

Denise Cargnelutti

como requisito parcial para a obtenção do grau de
Doutora em Bioquímica Toxicológica

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Santa Maria, 20 de março de 2009.

“Se consegui enxergar mais longe, é porque estava apoiado sobre ombros de gigantes”

Isaac Newton

DEDICATÓRIA

Dedico esta tese aos meus queridos e amados pais, Celita e Selito Cargneluttí, aos meus irmãos do coração, Ademir, Joce, Jocelí e Jocelaine, ao meu namorado Marciel e ao meu cunhado Paulo: Não existem palavras que poderão expressar tudo o que vocês significam para mim, por isso, muito obrigada por fazerem parte da minha vida.

AGRADECIMENTOS

♥ Gostaria de agradecer a Deus e a todos os Santos a qual pedi proteção, saúde e sabedoria. Sem Deus, a minha vida seria uma mera passagem por este mundo, mas com ele, cada minuto “vale à pena”.

♥ À minha família, pelo amor, apoio e carinho em todos os momentos.

♥ Um agradecimento especial vai para as minhas irmãs JOs: Joceli e Jocelaine a qual dividimos a amizade e o apartamento. Gostaria de agradecer pelo apoio incondicional e principalmente por compartilharem das minhas alegrias e angústias.

♥ À Profa. Maria Rosa, minha orientadora, agradeço pela acolhida em seu laboratório quando eu ainda era estudante de Biologia e pela oportunidade da realização do meu trabalho. Agradeço pela serenidade, paciência, confiança e ensinamentos valiosos para alguém que pretende seguir a carreira científica.

♥ À Profa. Vera, minha co-orientadora, agradeço porque você acreditou no meu potencial e me fez acreditar também. Observou e soube valorizar a cada passo do meu crescimento. Obrigada pela disponibilidade, atenção, confiança, dedicação e conhecimentos.

♥ Ao Prof. Fernando, agradeço pela sua persistência e seriedade com que trabalhou e discutiu meus resultados, bem como ajudou a planejar os experimentos. Nunca esquecerei dos momentos de descontração com os colegas no seu laboratório. Admiro a maneira como conduz o seu laboratório e os orientados.

♥ À querida amiga e colaboradora Luciane Tabaldi, que me acolheu como sua IC e fez parte da minha história na bioquímica. Obrigada pela paciência e carinho. Por ter sido firme e prestativa nos momentos cruciais. Por seu conhecimento e amizade.

♥ À minha querida IC, Heloísa, obrigada por toda a ajuda e ensinamentos valiosos sobre o curso da vida.

♥ Às queridas amigas e colaboradoras Liana, Nicéia, sem vocês este trabalho não seria possível ser realizado. Liana, você é inigualável. Nicéia, com sua paciência e serenidade este trabalho pode ser conduzido de maneira mais tranqüila.

♥ À Lú Belmonte, Jamile e Joseila, que também contribuíram para o desenvolvimento deste trabalho, obrigada.

♥ Ao pessoal da fisiologia Rê, Gabriel, Sibi, Vanessa, e a Júlia que sempre me acolheram com afeto, amizade e alegria. Obrigada pela paciência comigo quando pedia ajuda para o TENNANT.

♥ Aos amigos e colegas do Lab. 2208, os antigos Carlos Eduardo, Ahmed, Marga, Vanessa, Rose, Rosi, Gustavo, Maísa, Paula, Cíntia, Lara, Cínthia, Roberta e os novos Jessié, Naiara e Taís, obrigada por terem tornado os anos de convivência e trabalho mais agradáveis no laboratório.

♥ À funcionária do Departamento de Biologia, Marisa Binotto Abbad, agradeço por todo o carinho, ajuda nos momentos difíceis e amizade.

♥ Programa de Pós-Graduação em Bioquímica Toxicológica, e aos professores que me ensinaram com prazer e dedicação parte do que sei e, o que é mais importante, me ensinaram a aprender sozinha. Em especial, gostaria de agradecer ao Prof. João, por sua disponibilidade, paciência e humildade para ensinar.

♥ Aos Professores que aceitaram fazer parte desta banca: Prof. Arthur, Prof. João, Prof. Danilo e a colega Dra. Rosilene, o meu muito obrigado.

♥ À Universidade Federal de Santa Maria (UFSM), à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de estudos, ao Curso e a todos que de alguma forma contribuíram para a realização deste trabalho, obrigada.

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

EFEITO DO MERCÚRIO SOBRE PARÂMETROS BIOQUÍMICOS E FISIOLÓGICOS EM PEPINO E MILHO: PAPEL PROTETOR DO ZINCO

Autora: Denise Cargnelutti
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Co-Orientadora: Vera Maria Morsch
Data e local de Defesa: Santa Maria, 20 de março de 2009.

Neste estudo, foram investigados através da análise de parâmetros bioquímicos e fisiológicos os efeitos do mercúrio (Hg) em plântulas de pepino (*Cucumis sativus* L.) e em híbridos de milho (*Zea mays* L.), e a associação do Hg com o Zn em híbridos de milho. Os parâmetros bioquímicos analisados para *C. sativus* foram: as atividades de enzimas antioxidantes (catalase (CAT), ascorbato peroxidase (APX) e superóxido dismutase (SOD)) e os níveis dos antioxidantes não-enzimáticos (ácido ascórbico (AsA), carotenóides e tióis não-protéicos (NPSH)). Os conteúdos de substância reativas ao ácido tiobarbitúrico (TBARS), clorofila, proteína carbonil, peróxido de hidrogênio (H_2O_2) e a atividade da δ-aminolevulinato desidratase (δ-ALAD) foram também determinados. O crescimento de *C. sativus* foi avaliado baseado na matéria seca (MS) e fresca (MF), e comprimento de raízes (R) e parte aérea (PA). As plântulas de pepino foram expostas de 0 a 500 μM de $HgCl_2$ durante 10 e 15 dias. Os resultados demonstraram que o Hg foi absorvido pelas plântulas de pepino, e seu conteúdo foi maior nas R que na PA. Além disso, uma redução no comprimento das R e da PA foi observada em todas as concentrações e tempos testados. Na concentração de 50 μM $HgCl_2$ a MF das R aos 15 dias aumentou, mas foi reduzida nas outras concentrações. Para as plântulas com 10 dias, foi observada uma redução na MF de R e PA. Em relação à MS das R, houve um aumento na concentração de 500 μM, ambos aos 10 e 15 dias, e também na concentração de 250 μM $HgCl_2$ aos 15 dias. Além disso, uma redução significativa na MS da PA foi observada em todas as concentrações testadas. Os resultados mostraram níveis elevados de TBARS e proteína carbonil, e uma redução no conteúdo de clorofila em plântulas expostas a 250 e 500 μM $HgCl_2$. Um aumento na atividade da CAT e SOD foi observado, respectivamente aos 10 e 15 dias de exposição a 50 μM $HgCl_2$, embora a 500 μM $HgCl_2$, houve uma marcada inibição. Também, tanto aos 10 quanto aos 15 dias, foi observada uma inibição na atividade da enzima APX a 250 e 500 μM $HgCl_2$. Além disso, as plântulas com 10 dias tiveram os níveis de H_2O_2 reduzidos a 250 μM $HgCl_2$ e aumentados a 500 μM $HgCl_2$. Os antioxidantes não-enzimáticos tais como os NPSH, AsA e carotenóides aumentaram em todas as concentrações, exceto os níveis de carotenóides que reduziram em concentrações altas de $HgCl_2$. A atividade da δ-ALA-D aumentou a 50 μM de $HgCl_2$ aos 15 dias, e foi inibida em concentrações altas. Com o propósito de estudar o efeito do metal em três híbridos de milho, BR205, 30F71 e BR205,

em solução nutritiva, os seguintes parâmetros foram analisados para os híbridos após exposição ao Hg (0 – 100 µM Hg): o crescimento, a concentração de Hg nos tecidos e a atividade da δ-ALA-D. Os resultados indicaram uma alta captação do Hg pelos híbridos de milho, principalmente nas R. O crescimento das R e PA foram reduzidos em todas as concentrações testadas. Uma resposta similar também foi observada para a MS e MF das R e PA. Estes híbridos mostraram inibição de maneira dose-dependente na atividade da δ-ALA-D. Contudo, a atividade da δ-ALA-D de 32R21 foi inibida pelo metal apenas em concentrações superiores a 50 µM Hg e a atividade da enzima do híbrido 30F71 não foi afetada pelo mercúrio. Os estudos *in vitro* mostraram que o Hg inibiu a atividade da δ-ALA-D de maneira dependente da concentração e esta inibição foi mista. Com o objetivo de investigar o papel antioxidante do Zn frente ao estresse causado pelo Hg foram estudados os mecanismos de toxicidade do metal em dois híbridos de milho, BR205 e BR205, em solução nutritiva. Os parâmetros analisados para os híbridos de milho após exposição ao Hg (25 µM Hg) e ao Zn (50, 100 e 200 µM Zn) foram: o crescimento, a concentração de Hg e Zn nos tecidos, a atividade de enzimas antioxidantes (CAT, APX e SOD) e os níveis de antioxidantes não-enzimáticos (AsA, NPSH e carotenóides). Os conteúdos de H_2O_2 e proteína carbonil, a atividade da δ-ALA-D e os níveis de clorofila foram avaliados. Os resultados da interação entre Hg e Zn indicaram níveis reduzidos de Hg nos tratamentos com 25 µM Hg + 50 µM Zn. Os tratamentos utilizando 25 µM Hg + Zn foram efetivos em reduzir os níveis de proteína carbonil das R em 32R21 e de H_2O_2 em BR205, aumentados pela exposição ao Hg. Na PA de BR205 o Hg inibiu as atividades da SOD e CAT, enquanto a APX foi ativada. No entanto, a suplementação com Zn aumentou as atividades da CAT e APX. Em 32R21, o Hg reduziu a atividade da APX da PA e o tratamento com 25 µM Hg + 200 µM Zn aumentou a atividade desta enzima. Além disso, os estudos *in vitro* com Hg e/ou Zn mostraram a ativação das enzimas antioxidantes especialmente na PA. Os resultados mostraram que o Zn restaurou o crescimento dos híbridos de milho que haviam sido reduzidos por 25 µM Hg. O Hg reduziu os níveis de clorofila b nos híbridos de milho, mas apenas a concentração de 100 µM Zn foi efetiva em restabelecer os níveis de clorofila b. Os tratamentos com Zn promoveram uma acumulação de NPSH, reduzidos pela exposição ao Hg. O Hg reduziu o crescimento e os níveis de AsA dos híbridos de milho e apenas o comprimento e a massa fresca foram restabelecidos pela suplementação com Zn. Além disso, os tratamentos com Hg inibiram a atividade da δ-ALA-D de BR205, mas a suplementação com Zn ao tratamento com mercúrio restabeleceu a atividade da enzima. No entanto, estudos *in vitro* mostraram que o zinco não preveniu a inibição da atividade da δ-ALA-D causada pelo Hg. Com base no exposto, nossos resultados sugerem que o Hg induz estresse oxidativo em plântulas de pepino e milho. Contudo, o Zn desempenhou um papel importante no combate à toxicidade do mercúrio, atuando na modulação de EROs e indução dos NPSH, possibilitando o crescimento dos híbridos de milho.

Palavras-chave: Antioxidantes; Espécies reativas de oxigênio; Milho; Zinco.

ABSTRACT
Doctoral Thesis
Graduate Program in Toxicological Biochemistry
Universidade Federal de Santa Maria

EFFECT OF MERCURY ON BIOCHEMICAL AND PHYSIOLOGICAL PARAMETERS IN CUCUMBER AND MAIZE: PROTECTIVE ROLE OF ZINC

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Place and date: Santa Maria, March 20, 2009.

In this study, effects of mercury (Hg) in cucumber seedlings (*Cucumis sativus* L.) and maize (*Zea Mays* L.) hybrids, and Hg and Zn association in maize hybrids were investigated through the analysis of biochemical and physiological parameters. The biochemical parameters analyzed in *C. sativus* were: antioxidant enzyme activities (catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD)), and the non-enzymatic antioxidant levels (ascorbic acid (ASA), carotenoids, and non-protein thiol content (NPSH)). The thiobarbituric acid reactive substances (TBARS), chlorophyll, carbonyl protein, hydrogen peroxide (H_2O_2) contents and the δ-aminolevulinic acid dehydratase (δ-ALAD) activity were also determined. The growth of *C. sativus* was evaluated based on dry (DM) and fresh matter (FM), as well as on root and shoot length. Cucumber seedlings were exposed to 0 to 500 μM of $HgCl_2$ for 10 and 15 days. The results showed that Hg was absorbed by the growing cucumber seedlings, and its content was greater in roots than in shoot. A reduction in root and shoot length was observed at all concentrations and time points tested. In the concentration of 50 μM $HgCl_2$ root FM of 15-day-old seedlings increased, but it was reduced at the other concentrations. For 10-day-old seedlings, a reduction in root and shoot FM was observed. Regarding shoot DM, there was an increase at 500 μM on days 10 and 15, and in the concentration of 250 μM $HgCl_2$ on day 15. Furthermore, a significant reduction in shoot DM at all tested concentrations was observed. The results showed higher levels of TBARS and carbonyl protein as well as a chlorophyll content reduction in seedlings exposed to 250 and 500 μM $HgCl_2$. An increase in CAT and SOD activities, on days 10 and 15, respectively, exposed to 50 μM $HgCl_2$ was observed, whereas at 500 μM $HgCl_2$, there was a marked inhibition. An inhibition of APX enzyme at 250 and 500 μM $HgCl_2$ on days 10 and 15 was observed. Moreover, 10-day-old seedlings presented H_2O_2 levels reduced at 250 μM $HgCl_2$ and increased at 500 μM $HgCl_2$. Non-enzymatic antioxidants such as NPSH, AsA and carotenoids were increased at all concentrations, except carotenoid levels, which were reduced at higher concentrations of $HgCl_2$. δ-ALA-D activity increased at 50 μM $HgCl_2$ on day 15, and was inhibited at higher concentrations. The effect of the metal toxicity in three maize hybrids, BR205, 30F71 and 32R21, in nutritive solution, was studied analyzing the following parameters after Hg exposure (0 – 100 μM Hg): growth, tissue Hg concentration and δ-ALAD activity. The results showed a higher uptake of Hg by maize hybrids, mostly in roots. The root and shoot growth was reduced at all tested concentrations. A similar response was

also observed for DM and FM of roots and shoot. These hybrids showed inhibition in a dose-dependent manner in δ-ALA-D activity. However, 32R21 δ-ALA-D activity was inhibited by metal only at concentrations exceeding 50 μM Hg. The enzyme activity from 30F71 was not changed by Hg. *In vitro* studies showed that Hg inhibits the δ-ALA-D activity in a concentration-dependent manner and this inhibition was mixed. In order to investigate the antioxidant role of zinc under stress condition caused by Hg, the mechanisms of metal toxicity in two maize hybrids, BR205 e 32R21, in nutritive solution, were studied. The parameters analyzed in maize hybrids after 25 μM Hg and Zn (50, 100 and 200 μM Zn) exposures were: growth, tissue Hg and Zn concentrations, antioxidant enzymes activities (CAT, APX and SOD) and non-enzymatic antioxidants (AsA, NPSH and carotenoids). The H_2O_2 and carbonyl protein, δ-ALA-D activity and chlorophyll levels were also evaluated. The results of interaction between Hg and Zn indicated reduced Hg levels in treatments with 25 μM Hg + 50 μM Zn. Treatments using 25 μM Hg + Zn were effective in reducing the root carbonyl protein in 32R21, and H_2O_2 in BR205, increased by Hg exposure. Hg inhibited SOD and CAT activities in BR205 shoot, whereas APX activity was increased. However, Zn supplementation increased CAT and APX activities. In the 32R21, Hg reduced shoot APX activity and treatments with 25 μM Hg + 200 μM Zn increased its activity. Moreover, *in vitro* studies with Hg e/or Zn showed activation of antioxidant enzymes especially in the shoot. The results showed that Zn abolished the growth of maize hybrids which had been reduced by 25 μM Hg. The Hg reduced chlorophyll b content in maize hybrids, but only the concentration of 100 μM Zn was effective in restoring chlorophyll b levels. Treatments with Zn induced accumulation of NPSH, which was reduced by Hg exposure. Mercury induced a reduction in the growth and AsA levels from maize hybrids, and only the growth and fresh matter were restored by supplementation with Zn. Treatments with Hg inhibited δ-ALA-D activity from BR205, whereas supplementation with Zn at treatments with Hg restored the enzyme activity. However, *in vitro* studies showed that Zn did not prevent the inhibition of δ-ALA-D activity caused Hg. Our results suggest that Hg induced oxidative stress in cucumber and maize seedlings. However, Zn has played an important role in fighting Hg toxicity, acting in ROS modulation and NPSH induction, allowing the growth of maize hybrids, even in the presence of Hg.

Keywords: Antioxidants; Cucumber; Maize; Reactive oxygen species; Zinc.

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4.1. ARTIGO E MANUSCRITO CIENTÍFICO: CAPÍTULO I

ARTIGO 1:

LISTA DE TABELAS

LISTA DE ABREVIATURAS

ALA – ácido 5-aminolevulínico

ANOVA – análise de variância

ASA – ácido ascórbico

CH_3HgCl - metilmercúrio

CH_3HgOH - hidróxido de metilmercúrio

$CuSO_4$ – sulfato de cobre

DMSO – dimetilsulfóxido

DNPH – dinitrofenilidrazina

DTNB – ácido 5-5' –ditio-bis-(nitrobenzóico), reagente de Ellman

DTT – ditiotreitol

EDTA - ácido etilenodiaminotetracético

ELP – porcentagem de vazamento de eletrólitos

GSH – glutationa reduzida

GSSG - glutationa oxidada

HCl – ácido clorídrico

Hg – mercúrio

Hg^{2+} - íon mercúrico

Hg_2^{++} - íon mercuroso

$HgCl_2$ – cloreto de mercúrio

Hg_2Cl_2 – calomelano

$Hg(CNO)_2$ - fulminato de mercúrio

HgS – sulfeto de mercúrio

$Hg(OH)_2$ – hidróxido de mercúrio

H_2O_2 – peróxido de hidrogênio

HNO_3 – ácido nítrico

H_2SO_4 – ácido sulfúrico

KI – iodeto de potássio

K_2HPO_4 – fosfato de potássio

MDA – malondialdeído

MF – massa fresca

MS – massa seca

NADPH – nicotinamida adenina dinucleotídeo

NPSH – grupos tióis não-protéicos

PA – parte aérea

PBG – porfobilinogênio

PCs – Fitoquelatinas

PVP – polivinilpirrolidona

ROS – espécies reativas de oxigênio

R – raiz

Rpm – rotações por minuto

TBA – ácido tiobarbitúrico

TCA – ácido tricloroácetico

-SH – grupos tiólicos não-protéicos

Zn – zinco

δ-ALA-D – delta-aminolevulinato desidratase

1. INTRODUÇÃO

Um dos principais problemas que o mundo enfrenta é a contaminação e a degradação das suas fontes naturais, tal como o solo. Além de prover a sustentação, o solo é fonte de nutrientes que permite o crescimento e o desenvolvimento das plantas (SIERRA et al., 2008). Contudo, o solo pode servir como local de descarte de resíduos tais como metais pesados. Alguns metais, tais como o cálcio, o cobalto, o cromo, o cobre, o ferro, o potássio, o magnésio, o manganês, o sódio, o níquel e o zinco são nutrientes essenciais em diferentes processos nos organismos vivos. No entanto, outros elementos metálicos, como por exemplo, o cádmio, o chumbo e o mercúrio, não têm nenhuma função biológica conhecida (BRUINS et al., 2000; OLIVEIRA et al., 2003). A similaridade química com elementos essenciais faz com que esses outros elementos sejam potencialmente tóxicos para as células vegetais (CLEMENS, 2006).

Dentre os metais pesados o mercúrio é um dos poluentes mais perigosos do ambiente, causando efeitos tóxicos em plantas (ISRAR et al., 2006; RELLÁN-ÁLVAREZ et al., 2006; CHO & PARK, 2000). Quando presente em altas concentrações em solos, o mercúrio é absorvido pelo sistema radicular das plantas e induz a redução no crescimento (ESTEBAN et al., 2008; ZHOU et al., 2008), no metabolismo (ORTEGA-VILLASANTE et al., 2005; RELLÁN-ÁLVAREZ et al., 2006; ISRAR et al., 2006; ZHOU et al., 2007; ESTEBAN et al., 2008; ZHOU et al., 2008), na fotossíntese (GODBOLD & HUTTERMANN, 1988), na transpiração e na absorção de água, além de induzir o aumento da peroxidação lipídica (CHO & PARCK, 2000; ORTEGA-VILLASANTE et al., 2005; ZHOU et al., 2007; ESTEBAN et al., 2008; ZHOU et al., 2008) alterando assim, o desenvolvimento normal da planta.

O mercúrio causa efeitos tóxicos em plantas devido em parte à inibição de enzimas metabólicas tais como a delta–aminolevulinato desidratase (δ-ALA-D), responsável pelo metabolismo da clorofila. A δ-ALA-D é sensível ao mercúrio, devido a sua natureza sulfidrílica (MORSCH et al., 2002). Consequentemente, a síntese da clorofila bem como a fotossíntese são afetadas pelo mercúrio.

Além disso, o efeito tóxico dos metais pesados em plantas é devido à indução na formação de radicais livres e de espécies reativas de oxigênio (EROs), resultando em estresse oxidativo (DIETZ et al., 1999). As EROs tais como, o ânion superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($OH^{\bullet}$), são produzidas normalmente nas células, mas a sua produção é aumentada quando a célula está em condições de estresse (FOYER et al., 1994; HEGEDÜS et al., 2001). As EROs, causam dano às membranas, aos pigmentos fotossintéticos, proteínas, ácidos nucléicos e lipídios (FOYER et al., 1994). As células das plantas possuem um sistema de defesa antioxidante, formado por componentes enzimáticos e não enzimáticos que normalmente mantêm um equilíbrio de EROs dentro das células (HALLIWELL, 1987; FOYER et al., 1994) e as protegem contra a injúria e a disfunção dos tecidos

Diferentemente do mercúrio, o zinco é um nutriente essencial para os organismos vivos, incluindo as plantas (MARSCHNER, 1995). O zinco apresenta papel fundamental no metabolismo de proteínas, na expressão gênica, na estrutura da cromatina, na integridade estrutural e funcional das biomembranas, no metabolismo fotossintético do carbono e no metabolismo do ácido indol-acético (MARSCHNER, 1995; VALLEE & FALCHUK, 1993; PRASAD, 1995; CAKMAK & BRAUN, 2001). Além de todos os seus efeitos benéficos, o Zn está envolvido na proteção das células contra o estresse oxidativo, através da destoxificação das EROs. Devido ao seu papel fundamental na ativação e expressão de genes (KLUG & RHODES, 1987; VALLEE & FALCHUK, 1993), o Zn pode estar envolvido na expressão de genes induzido pelo estresse oxidativo. Estes genes codificam enzimas que atuam na defesa antioxidante, tais como, a ascorbato peroxidase e a glutationa redutase, que removem o H_2O_2 (GRESSEL & GALUN, 1994; ALLEN, 1995; ALSCHER et al., 1997). Além disso, quando na presença de outros metais pesados tais como o Cd (ARAVIND & PRASAD, 2003, 2004, 2005) e o Hg (TSUJI et al., 2002), o Zn reduz o estresse oxidativo causado por estes metais. Contudo, o efeito protetor do Zn na toxicidade do mercúrio em plantas ainda não foi estudado.

O pepino (*Cucumis sativus* L.) e o milho (*Zea mays* L.) são importantes espécies cultivadas e consumidas no Brasil. O pepino foi selecionado como uma planta teste, devido a sua sensibilidade para uma grande variedade de

contaminantes (GORSUCH et al., 1991, PEREIRA et al., 2006). O milho foi selecionado, pois tem sido usado em muitos estudos de poluição ambiental (WANG et al., 2007). Além disso, há informação disponível insuficiente sobre a toxicologia do mercúrio nestas espécies e sobre os mecanismos pelo qual esse elemento produz estresse oxidativo em plantas.

O estudo da toxicologia do mercúrio no metabolismo das plantas é de grande importância, devido ao aumento crescente da contaminação de solos através do uso de pesticidas agrícolas, despejo do lixo industrial em locais inadequados, utilização do lodo de esgotos e as atividades de mineração. Além do mais, de fundamental importância é o estudo das interações entre nutrientes, tal como o zinco, responsável pelo aumento das defesas defesas antioxidantes das plantas e na produção de maiores concentrações de tióis não-proteícos os quais sequestram os íons mercuriais, reduzindo a sua biodisponibilidade e toxicidade às plantas. Portanto, os objetivos deste trabalho foram:

Objetivos

1.1.2. Objetivo Geral

Avaliar o efeito de diferentes concentrações de mercúrio em parâmetros oxidativos e de crescimento de plântulas de pepino aos 10 e 15 dias de germinação, bem como avaliar o efeito do mercúrio e do zinco e de suas associações nestes parâmetros em plântulas de milho.

1.1.3. Objetivos Específicos

- Avaliar em plântulas de pepino após exposição ao mercúrio: a atividade de enzimas antioxidantes (catalase e ascorbato peroxidase), o conteúdo de clorofila, a peroxidação lipídica e os níveis de proteínas oxidadas, as alterações no crescimento e o conteúdo de mercúrio absorvido pelas plântulas;

- Avaliar em plântulas de pepino após exposição ao mercúrio: os níveis de peróxido de hidrogênio, a atividade das enzimas δ-ALA-D e superóxido dismutase, os níveis de antioxidantes não-enzimáticos (carotenóides, ácido ascórbico e tióis não-protéicos);

- Avaliar em híbridos de milho (BR205, 30F71 e 32R21) após exposição ao mercúrio: o crescimento, o conteúdo de mercúrio absorvido e a atividade da δ-ALA-D in vivo e in vitro;

- Avaliar em híbridos de milho (BR205 e 32R21) após exposição ao mercúrio e ao zinco: o conteúdo de mercúrio e zinco absorvido, a concentração de clorofila, a atividade de enzimas antioxidantes (catalase, ascorbato peroxidase e superóxido dismutase) e a concentração de antioxidantes não-enzimáticos (tióis não-protéicos), o conteúdo de peróxido de hidrogênio e das proteínas oxidadas;

- Avaliar em híbridos de milho (BR205 e 32R21) após exposição ao mercúrio e ao zinco: o crescimento, os níveis de ácido ascórbico e a atividade da δ-ALA-D.

2. REVISÃO DA LITERATURA

2.1. Mercúrio

O mercúrio é um dos metais pesados mais tóxicos encontrado no ambiente (ZHANG & WONG, 2007). Durante os últimos 2500 anos, foi extensivamente usado devido as suas propriedades químicas e físicas únicas. É o único metal encontrado na forma líquida em condições de temperatura ambiente e pressão (1 ATM), formando vapores incolores e inodoros (NASCIMENTO & CHASIN, 2001). No ambiente, ele ocorre associado a outros elementos químicos, formando compostos inorgânicos ou sais. Dentre estes elementos, o mais comum é o enxofre, com o qual forma o sulfeto de mercúrio que é altamente insolúvel (ocorrendo na forma de cinábrio, HgS) que não é considerado tóxico. Este metal pode também ser encontrado na forma de compostos organometálicos. Muitos destes compostos têm importância no uso diário tanto na indústria como na agricultura (BOENING, 2000).

O mercúrio pode ser encontrado nas seguintes formas: mercúrio metálico (Hg^o), mercúrio (I) e mercúrio (II) nos quais os átomos perdem um ou dois elétrons, respectivamente, formando os íons mercuroso (Hg_2^{++}) e mercúrico (Hg^{++}) (NASCIMENTO & CHASIN, 2001). Os sais de mercúrio mais importantes são o $HgCl_2$, um sublimado corrosivo muito tóxico, o Hg_2Cl_2 (calomelano), ocasionalmente ainda usado na medicina, o $Hg(CNO)_2$ (fulminato de mercúrio), detonador usado em explosivos, e o HgS, de cor vermelha, usado como pigmento em tintas (HSDB, 2000). O $HgCl_2$, o $Hg(OH)_2$ e o HgS são as formas de mercúrio inorgânicas prevalentes no ambiente, e o CH_3HgCl (metilmercúrio) e o CH_3HgOH (hidróxido de metilmercúrio) são as formas principais de compostos orgânicos de mercúrio, junto com outros organomercúrios (dimetilmercúrio e fenilmercúrio) existindo em frações pequenas (USEPA, 1997b).

As formas orgânicas do mercúrio (organomercuriais) são aquelas onde o elemento se liga a pelo menos um átomo de carbono. Esses compostos são reconhecidos devido à sua toxicidade, enquanto os que causam maior preocupação são os que contem radicais alquila de cadeia curta, onde o

mercúrio se liga aos grupos metila, etila e propila (WHO, 1989). A tabela 1 apresenta as formas de mercúrio geralmente encontradas no ambiente, e algumas formas de mercúrio geradas através da atividade antropogênica.

Tabela 1- Formas orgânicas e inorgânicas do mercúrio.

Inorgânicas	
- Metálico	Hg^o
- Sais mercurosos	Hg_2Cl_2
- Sais mercúricos	$HgCl_2$
Orgânicas	
- Compostos de alquilmercúrio	CH_3HgCl
- Compostos de arilmercúrio	C_6H_5HgCl
- Compostos de alcoxiarilmercúrio	CH_2OCH_2HgCl

Adaptado de QUEIROZ (1995).

2.1.1. Fontes

Na sua forma natural, o mercúrio surge da degradação da crosta terrestre a partir de vulcões, solos, florestas, lagos e oceanos abertos (MASON et al., 1994). No entanto, as fontes artificiais de mercúrio são mais diversificadas do que as naturais (CARVALHO, 2001), sendo que a quantidade de mercúrio na atmosfera aumentou desde o início da revolução industrial (USEPA, 2003). Por exemplo, o mercúrio é usado em reatores nucleares, na indústria de alvejantes, papel e tecidos, células de níquel-cádmio em baterias, na odontologia e na medicina (GARCIA-GUINEA & HARFFY, 1997), e faz parte de formulações de fungicidas destinados à agricultura (MEAGHER & RUGH, 1996). Em adição, outras fontes artificiais, como as indústrias de mineração, a queima de combustíveis fósseis, a incineração de materiais, as descargas urbanas e as industriais (DEPLEDGE et al., 1994; SEIGNEUR et al., 2004) contribuem de forma significativa para a poluição do ambiente com mercúrio. Embora o uso industrial do mercúrio tenha sofrido reduções (ANVISA, 2001), devido a um controle mais efetivo e a busca por alternativas viáveis,

concentrações altas ainda estão presentes em produtos industriais (BOENING, 2000).

Patra & Sharma (2000) relataram que dois terços dos compostos de mercúrio no ambiente são originados de fontes naturais, e um terço é resultado de atividades humanas, principalmente pelo uso de fertilizantes em solos. A grande poluição do ambiente com mercúrio resultou, principalmente, no aumento da contaminação das espécies vegetais e animais ao longo das cadeias alimentares. De acordo com Chow et al. (1995), a concentração média do mercúrio na crosta terrestre é 0,5 ppm ($\mu g\ g^{-1}$).

2.1.2. Ciclo do mercúrio

Como outros elementos, o mercúrio não é degradado e não pode ser destruído através de combustão ou eliminado do ambiente. Sendo assim, o ciclo de permanência deste elemento no ambiente é tal que os seus compostos são transferidos entre o solo, a atmosfera e as águas superficiais. Através de uma série de transformações químicas complexas é possível obter os três estados de oxidação do mercúrio, como um ciclo no ambiente (LIN & PEHKONEN, 1999; BISINOTI & JARDIM, 2004).

Um agravante para o problema da poluição é que o mercúrio inorgânico pode ser convertido a metilmercúrio e a dimetilmercúrio pela ação de microorganismos (bactérias metanogênicas), processo conhecido como biotransformação (FARRELL et al., 1990; DAUGHNEY et al., 2002). Este processo representa um sério risco ambiental, visto que, o mercúrio se acumula na cadeia alimentar aquática, sendo que a sua concentração aumenta à medida que este metal avança nos níveis tróficos (BOENING, 2000; BAHIA, 1997). O mercúrio pode também ser liberado no ar na forma de Hg^o (forma elementar) que é formado através de processos bioquímicos na presença de solos e de plantas (DU & FANG, 1982; GODBOLD & HÜTTERMANN, 1988; BOUDOU et al., 1991). A maioria dos compostos inorgânicos de Hg adicionados aos solos são decompostos 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 (FREAR & DILLS, 1967).

2.1.3. Mercúrio nos solos

Patra et al. (2004) relataram que as concentrações de mercúrio encontradas normalmente em solos são baixas e não são tóxicas. Contudo, um conteúdo de mercúrio alto foi encontrado em plantas que cresceram em áreas altamente industrializadas (WOJCIECHOWSKA-MAZUREK et al., 1995) e em solos com aplicação do lodo de esgoto. Chang et al. (2002), relataram que o limite máximo de mercúrio permitido para esta prática é no máximo 7 mg Kg^{-1}. Além disso, em solos próximos à cidade de Lavras do Sul (RS) contaminados por mercúrio e outros metais pesados, os níveis de mercúrio atingiram valores de intervenção agrícola e residencial (GRAZIA & PESTANA, 2005). De acordo com a Companhia de Tecnologia de Saneamento Ambiental (CETESB, 2001), os valores norteadores para mercúrio em solos estão apresentados na tabela 2.

Tabela 2- Valores norteadores do mercúrio em solos (Fração total).

	Mercúrio (ppb)
Referência	50
Alerta	500
Intervenção agrícola	2500
Intervenção residencial	5000

Adaptado de CETESB (2001)

A especiação do mercúrio na solução do solo e entre os componentes da fase sólida controla fortemente a solubilidade, a mobilidade e a disponibilidade deste metal em ambos os ecossistemas terrestres e aquáticos (REVIS et al., 1989). Na solução do solo, o mercúrio pode estar complexado em formas inorgânica e orgânica (Tabela 1), que têm diferentes disponibilidade/fitodisponibilidade (YIN et al., 1996; RAVICHANDRAN, 2004). Em solos altamente poluídos com sais de mercúrio solúvel, há um risco ambiental alto (FENGXIANG et al., 2006). O mercúrio é fortemente adsorvido aos constituintes do solo. O Hg^{2+} ou as espécies hidrolisadas são praticamente imóveis no solo, mas quando combinadas com grupos orgânicos passam a ser móveis. A adsorção do mercúrio depende de inúmeros fatores tais como a

forma de mercúrio aplicada, a natureza dos constituintes do solo (orgânico e inorgânico), o pH do solo, os tipos de cátions no complexo de troca, o potencial redox e a classe textural (MORENO et al., 2004). O mercúrio presente em solos pode ser facilmente transferido para o topo da cadeia alimentar, das plantas para os herbívoros e desses para os carnívoros (GNAMUS et al., 2000) colocando em risco o ambiente.

2.1.4. Toxicidade

O mercúrio causa toxicidade em plantas mesmo em concentrações baixas (SALT el al., 1995) devido em parte à alta solubilidade das diversas formas do mercúrio em água, tal como o Hg^{2+} (HEATON et al., 2005). Os íons mercuriais acumulam-se em plantas (PATRA & SHARMA, 2000; DU et al., 2005) e interagem fortemente com os grupamentos sulfidrílicos de enzimas e proteínas no apoplasto das células (ASSCHE & CLIJSTERS, 1990; WOOLHOUSE, 1983). Por exemplo, o Hg^{2+} pode ligar-se às proteínas dos canais de água das células da raiz causando uma obstrução física do fluxo de água (MAGGIO & JOLY, 1995) afetando, por conseqüência, a transpiração em plantas (MAUREL, 1997; ZHANG & TYERMAN, 1999). O outro sintoma tóxico de acumulação de mercúrio em plantas é o crescimento anormal (GODBOLD, 1991; COCKING et al., 1995; DU et al., 2005) bem como os níveis reduzidos de clorofila e proteínas em plantas (CHO & PARK, 2000; LENTI et al., 2002). Também, a acumulação do mercúrio em raízes bloqueia a captação e o transporte dos nutrientes (BOENING, 2000) e induz à produção de etileno em excesso (GOREN & SIEGEL, 1976). Apesar de os mecanismos bioquímicos e moleculares da fitotoxicidade do mercúrio ainda serem desconhecidos (CHO & PARK, 2000), foi sugerido que um dos mecanismos pelo qual o mercúrio induz efeitos tóxicos em plantas é devido à produção de espécies reativas de oxigênio (EROs) e nitrogênio (ERN) em excesso (CHO & PARCK, 2000), ocasionando o estresse oxidativo (DIETZ et al., 1999). O mercúrio atua como um pró-oxidante, reduzindo as defesas antioxidantes e aumentando as espécies oxidantes que atuam junto às reações de Haber-Weiss e de Fenton e assim propiciam a formação de radicais hidroxil (HALLIWELL & GUTTERIDGE,

1990) (Figura 1), que iniciam o processo de peroxidação lipídica e de oxidação protéica.

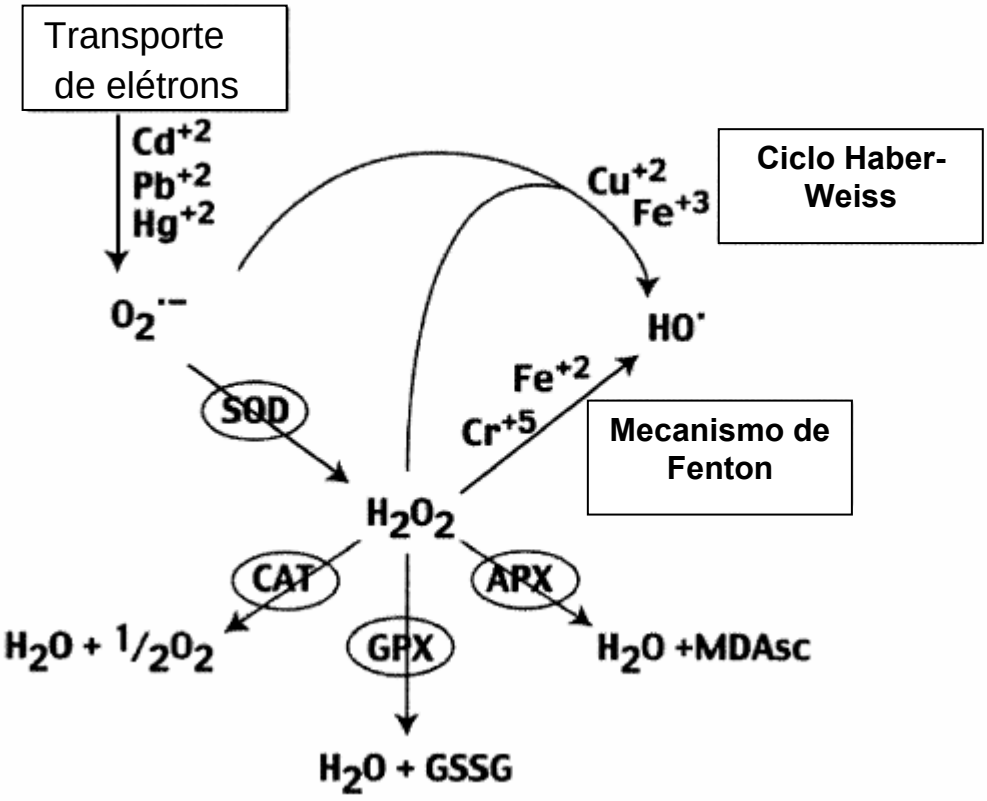

Figura 1. Mercúrio e outros metais pesados induzem estresse celular através da geração de espécies reativas de oxigênio. (Adaptado de Pinto et al., 2003)

A geração de EROs, tais como o ânion superóxido ($O_2^{\bullet -}$), o oxigênio singlete ($^1O_2$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxil ($OH^{\bullet}$) tem demonstrado ser um dos agentes causadores da injúria nos tecidos depois da exposição das plantas aos metais pesados, tal como o mercúrio (CHO & PARCK, 2000; ORTEGA-VILLASANTE et al., 2005; RELLÁN-ÁLVAREZ et al., 2006; ZHOU et al., 2007).

As EROs possuem potencial para interagir de forma não específica com muitos componentes celulares, desencadeando reações peroxidativas e causando um dano significante às membranas e a outras macromoléculas essenciais, tais como os pigmentos fotossintéticos, as proteínas, os ácidos nucléicos e os lipídios (SHALATA & TAL, 1998; OLMOS et al., 1994; FOYER et al., 1994) (Figura 2).

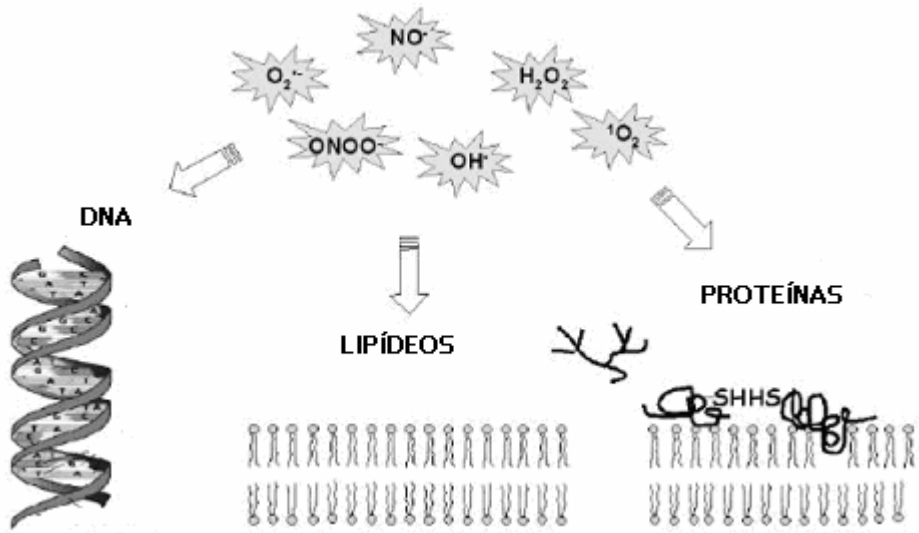

Figura 2. Dano oxidativo às macromoléculas biológicas. (Adaptado de Torres, 2003)

Além disso, a alta afinidade de ligação do mercúrio aos compostos contendo enxofre, nitrogênio e grupos funcionais contendo oxigênio, nas moléculas biológicas, pode induzir à inativação e ao dano dessas moléculas (NELSON, 1999; CLEMENS, 2001).

Sendo assim, para solucionar o problema da contaminação dos solos com mercúrio, estudos tem focalizado na utilização de plantas biorremediadoras. Esta tecnologia faz uso de plantas tolerantes ao mercúrio, que absorvem o metal e descontaminam os solos (CHANG & YEN, 2006). Esta tolerância tem sido conseguida através da inserção de genes em plantas tal como em *Arabidopsis*. A transformação de *Arabidopsis*, inserindo rota bacteriana para conversão de mercúrio, resultou em plantas transgênicas altamente tolerantes a Hg^{2+} e capazes de volatilizar mercúrio (MEAGHER et al., 2000).

2.2. Zinco

O zinco (Zn) é um elemento de transição pertencente ao grupo IIB da tabela periódica, classificado como um micronutriente essencial possuindo várias funções biológicas comprovadas. Apresenta papel fundamental no metabolismo de proteínas, expressão gênica, na estrutura da cromatina, na integridade estrutural e funcional das biomembranas, no metabolismo

fotossintético do carbono e no metabolismo do ácido indol-acético (IAA) (MARSCHNER, 1995; VALLEE & FALCHUCK, 1993; PRASAD, 1995; CAKMAK & BRAUN, 2001). O Zn é requerido como co-fator na função de mais de 300 enzimas diferentes incluindo representantes de todas as seis maiores classes de enzimas funcionais (VALLE & AULD, 1990). Além disso, o Zn é um importante co-fator estrutural para muitas proteínas tais como as proteínas de ligação ao ácido desoxirribonucléico (DNA) chamadas de dedo de zinco (zinc-fingers) (RHODES & KLUG, 1993). A maior parte das funções do Zn nas células é atribuída a sua habilidade para formar ligações de coordenação tetraédrica em diferentes constituintes celulares (CAKMAK, 2000). Os aminoácidos cisteína, histidina e aspartato ou glutamato são os maiores ligantes celulares do Zn, os quais formam coordenação tetraedral (WILLIANS, 1988; VALLEE & AULD, 1990; VALLEE & FALCHUK, 1993). Estes aminoácidos (especialmente a cisteína e a histidina) ligam o Zn com afinidade alta e com estabilidade maior que o Fe (BERG & SHI, 1996). Assim, a formação de radicais livres, através das reações entre o Fe e os resíduos de cisteína e histidina é bloqueada na presença de concentrações adequadas de Zn (BRAY & BETTGER, 1990). Nos sistemas biológicos, são encontradas concentrações altas de Zn, principalmente nas biomembranas. Em raízes de plântulas de aveia, a concentração citoplasmática total do Zn foi estimada em aproximadamente 0,4 mM (SANTA MARIA & COGLIATTI, 1988). Existem muitos locais para a ligação do Zn dentro das membranas, principalmente nos sítios localizados na face citosólica das membranas. A capacidade máxima de ligação deste metal às membranas é de aproximadamente 400 nmol Zn^{2+} mg^{-1} de proteína (PRASAD et al., 1996). Contudo, as concentrações altas de Zn podem causar efeitos tóxicos em plantas (CHANEY, 1993; EBBS & KOCHIAN, 1997) e em animais (STEFANIDOU et al., 2006). O mecanismo pelo qual o Zn causa efeitos tóxicos em plantas é pouco conhecido. Contudo, Gaither & Eide (2001) e Tabaldi et al. (2007) relataram que o Zn pode competir com outros íons metálicos (por exemplo, cálcio e magnésio) pelos sítios ativos de enzimas e proteínas de transporte.

2.2.1. Absorção e transporte

Devido ao seu caráter hidrofílico e divalente, o Zn não pode atravessar as membranas biológicas por difusão passiva, mas deve fazer o uso de transportadores. Esses sistemas de captação usam proteínas de transporte integrais de membrana para mover o Zn através da bicamada lipídica da plasmalema (GAITHER & EIDE, 2001). O Zn é o substrato para os facilitadores de difusão de cátions (CDFs) (WILLIAMS et al., 2000) e para a família ZIPs (ZIPs: ZRT, IRT-like Proteins). As ZIPs estão envolvidas no transporte de Fe, Zn, Mn e Cd (GUERINOT, 2000). Os CDF estão envolvidos no transporte de metais pesados especialmente Zn, Cd e Co (PAULSEN & SAIER, 1997; EIDE, 1998; van der ZAAL et al., 1999).

O zinco é absorvido da solução do solo como um cátion divalente (MARSCHNER, 1995) o qual não pode ser oxidado ou reduzido. Assim, o papel do Zn nas células está baseado no seu comportamento como um cátion divalente que possui uma forte tendência para formar complexos tetraédricos (BERG & SHI, 1996).

2.2.2. Essencialidade

Além de todos os seus efeitos benéficos, o Zn está envolvido na proteção das células contra o estresse oxidativo. O Zn é requerido para a detoxificação das EROs tais como o radical superóxido ($O_2^{\bullet-}$) e o peróxido de hidrogênio (H_2O_2). Devido ao seu papel fundamental na ativação e expressão de genes (KLUG & RHODES, 1987; VALLEE & FALCHUK, 1993), o Zn pode estar envolvido na expressão de genes induzidos pelo estresse oxidativo. Estes genes codificam enzimas que atuam na defesa antioxidante tais como a ascorbato peroxidase e a glutationa redutase, que removem o H_2O_2 (GRESSEL & GALUN, 1994; ALLEN, 1995; ALSCHER et al., 1997). Além disso, estudos relataram que concentrações baixas de Zn nas células das plantas podem aumentar a produção de $O_2^{\bullet-}$ durante o transporte de elétrons no aparato fotossintético (MARSCHNER & CAKMAK, 1989; CAKMAK et al., 1995; CAKMAK & ENGELS, 1999) e induz a geração de $O_2^{\bullet-}$ pela NADPH oxidase ligada a membrana (CAKMAK & MARSCHNER, 1988; PINTON et al., 1994). Em parte, esta resposta a concentrações baixas de Zn foi demonstrada em estudos com animais. Estes estudos mostraram que o Zn interage com a

ligação do Fe às membranas e reduz a produção de radicais hidroxila induzida pelo Fe (GIROTTI et al., 1985; POWELL et al., 1994) (Reações 1 e 2).

Willson (1988) relatou que a proteção das biomembranas e a manutenção da integridade celular pelo Zn está predominantemente controlada pela ligação do Zn aos compostos contendo grupos –SH, particularmente em proteínas de membrana. De acordo com o mesmo autor, sob condições de deficiência de Zn os grupos –SH das membranas podem ser ocupados pelo Fe, com uma concomitante geração de $O_2^{\bullet-}$ e consequente dano às membranas (Figura 3, reação 1). Por competição com o Fe, ou outros metais com atividade redox, tal como o Cu, que se ligam aos grupos –SH das proteínas de membrana, o Zn inibe a geração do $O_2^{\bullet-}$ induzida pelo Fe^{2+} e evita o dano às membranas (Willson, 1988). Quando ligado aos grupos –SH ou a outros sítios de ligação para o Fe nas membranas, o Zn não pode sofrer um ciclo de redução e reoxidação para produzir radicais livres pois é um elemento sem atividade redox nos sistemas biológicos (Figura 3, reação 2).

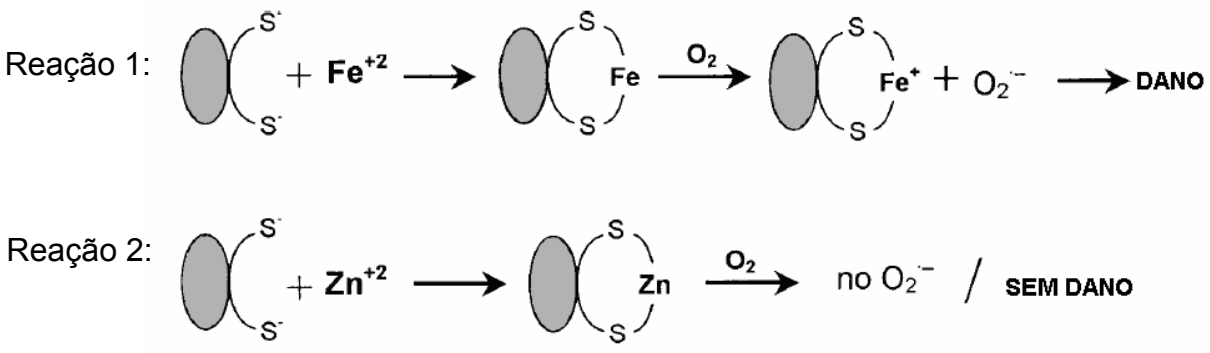

Figura 3. Mecanismo de proteção das biomembranas e manutenção da integridade celular promovida pelo zinco. Ligação do ferro (reação 1) ou zinco (reação 2) aos compostos contendo grupos –SH. (Adaptado de Cakmak, 2000)

2.3. Sistema de defesa antioxidante

Para o combate da toxicidade do mercúrio e proteção das membranas celulares e organelas dos efeitos danosos das EROs, as células das plantas possuem um sistema de defesa antioxidante, formado por componentes

enzimáticos e não enzimáticos que normalmente mantêm um balanço de EROs dentro das células. Dentre os antioxidantes enzimáticos estão a superóxido dismutase (SOD, E.C. 1.15.1.1), a catalase (CAT, E.C. 1.11.1.6) e a ascorbato peroxidase (APX, E.C. 1.11.1.11), bem como antioxidantes de baixo peso molecular, não enzimáticos, como o ácido ascórbico, a glutationa reduzida (GSH) e outros grupos tiólicos não protéicos que removem tipos diferentes de EROs (FOYER et al., 1994) e protegem a célula contra a injúria e a disfunção dos tecidos (MIQUEL, 1989). Além disso, em plantas, os carotenóides e a vitamina E também possuem importante efeito antioxidante no sistema fotossintético (HALLIWELL, 1987) (figura 5).

2.3.1. Enzimas antioxidantes

A SOD participa ativamente na remoção do $O_2^{\bullet-}$ gerado através de diferentes processos do metabolismo celular, tais como o transporte de elétrons na mitocôndria e cloroplastos (ELSTNER, 1991). O Zn se encontra associado com o cobre (Cu) na isoenzima superóxido dismutase dependente de Cu e Zn (Cu/Zn SOD). A Cu/Zn SOD está localizada nos cloroplastos, citosol e possivelmente no espaço extracelular. Muito provavelmente o átomo de cobre é o componente catalítico e o zinco é o componente estrutural da Cu/Zn SOD. Contudo, na deficiência do Zn, a atividade da Cu/Zn SOD é drasticamente reduzida, mas pode ser restabelecida *in vitro* pelo suprimento de Zn (MARSCHNER, 1995). Portanto, o átomo de Zn é um componente estrutural essencial para o funcionamento normal da Cu/Zn SOD. A redução na atividade da SOD ocorre com um aumento simultâneo da acumulação de $O_2^{\bullet-}$. O radical superóxido é um dos principais oxidantes responsáveis pela peroxidação de lipídios e conseqüente aumento na permeabiliadade das membranas (CAKMAK & MARSCHNER, 1988).

A catalase, presente nos peroxissomos, remove o H_2O_2 gerado durante a fotorrespiração e a β–oxidação dos ácidos graxos. É uma das enzimas chave envolvida na remoção de peróxidos tóxicos nas células quando estes estão em concentrações altas, pois apresenta baixa afinidade pelo H_2O_2 (MITTLER, 2002). A CAT pertence à família das oxirredutases presente universalmente

nos organismos que decompõe H_2O_2 em água e oxigênio molecular (MORITA et al., 1994). A APX, outra importante enzima do sistema de defesa antioxidante, é chave no ciclo da glutationa-ascorbato que reduz o H_2O_2 (quando em baixas concentrações na célula) até água usando ascorbato como doador de elétrons, resultando na formação de dehidroascorbato (Figura 1b). Este é reciclado a ascorbato usando a GSH como doadora de elétrons, e a glutationa oxidada (GSSG) é convertida pela enzima glutationa redutase, dependente de NADPH (ASADA & TAKAHASHI, 1987). Deste modo, a SOD age como primeira linha de defesa convertendo o O^{-}_2 a H_2O_2. A APX, a GPX e a CAT então detoxificam o H_2O_2. Em contraste com a CAT (Figura 1d), a APX e a GPX requerem um ciclo regenerador de ascorbato e/ou glutationa (Figura 1a–c). Esse ciclo usa elétrons diretamente do aparato fotossintético (Figura 1-a) ou NAD(P)H (Figura 1b,c) como poder redutor.

Essas enzimas reduzem de forma eficiente as EROs sob circunstâncias normais, mas se a redução completa não ocorrer, como em condições de produção aumentada ou de inibição das defesas antioxidantes, o resultado pode ser um estado de estresse oxidativo levando a oxidação de biomoléculas, tais como, lipídios, proteínas e DNA (RICHTER & SCHWEITZER, 1997). Além disso, a oxidação e a inativação dos componentes celulares podem desencadear o processo de morte celular (BUCKNER et al., 2000).

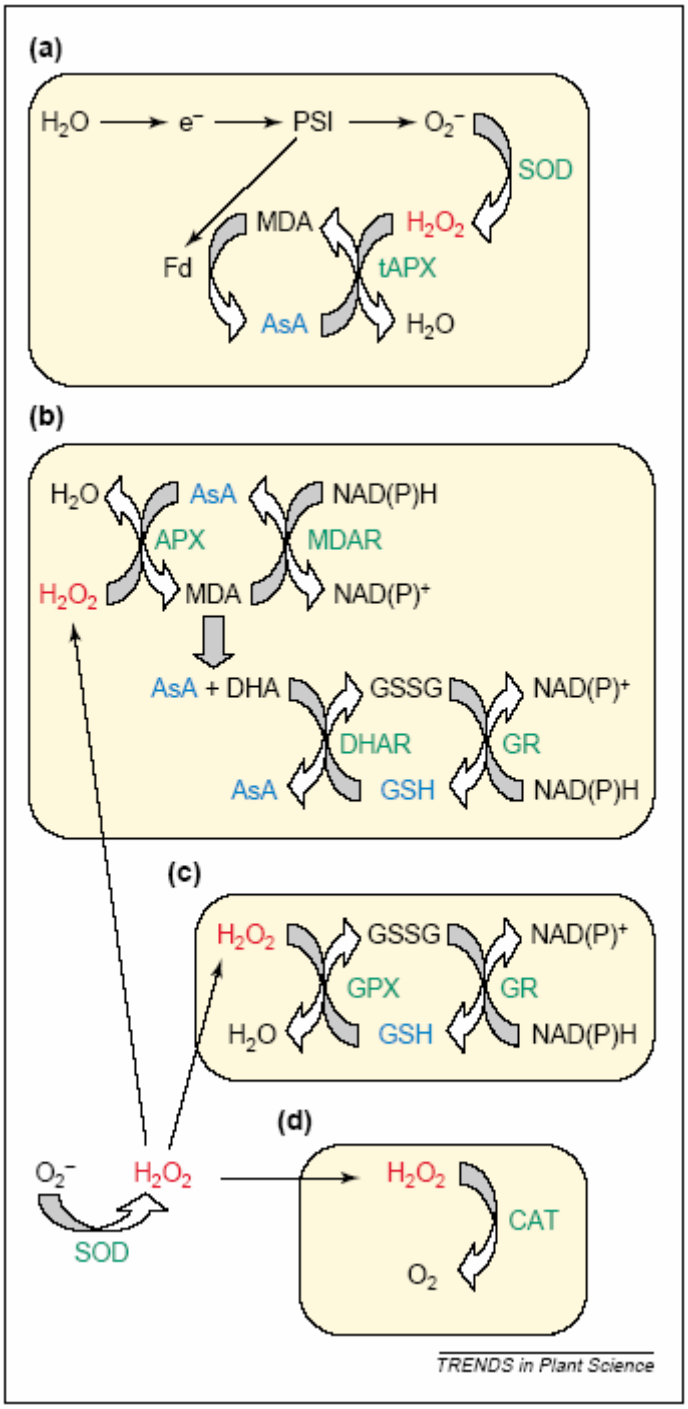

Figura 4 - Caminho das espécies reativas de oxigênio e sua remoção nas plantas (a) Ciclo água-água. (b). Ciclo ascorbato glutationa (c). Ciclo glutationa peroxidase (d). ROS estão indicadas em vermelho, antioxidantes em azul e enzimas removedoras de ROS em verde. (Adaptado de Mittler, 2002)

2.3.2. Antioxidantes não-enzimáticos

Além do sistema de defesa antioxidante enzimático, as defesas antioxidantes não-enzimáticas são de fundamental importância para as células. O ácido L-ascórbico desempenha um importante papel na tolerância das plantas ao estresse como um componente do sistema antioxidante (NOCTOR & FOYER, 1998). Está envolvido na regulação da fotossíntese, na expansão celular, no elongamento das raízes e no transporte de elétrons transmembrana (NOCTOR & FOYER, 1998; SMIRNOFF, 2000). Também é importante na remoção dos radicais livres de oxigênio (SINHA et al., 2005). Os radicais livres de oxigênio estão envolvidos na oxidação do ácido ascórbico para formar ácido deidroascórbico, o qual é regenerado posteriormente até ácido ascórbico (Figura 4) (FRIDOVICH & HANDLER, 1961). Os antioxidantes, tais como, o ácido ascórbico e a glutationa, que são encontrados em concentrações altas (5 – 20 mM ácido ascórbico e 1 – 5 mM glutationa) nos cloroplastos e outros compartimentos celulares, são importantes para a defesa das plantas contra o estresse oxidativo (ZENK, 1996).

Os grupos tióis não protéicos, entre estes a glutationa, são conhecidos por possuírem um papel central nos mecanismos de resposta aos metais traços em plantas terrestres (ZENK, 1996; RAUSER, 1999). A GSH, um tripeptídeo contendo enxofre, é um antioxidante muito importante envolvido na defesa celular contra agentes tóxicos (SCOT et al., 1993). A GSH reduz diretamente a maioria das espécies reativas de oxigênio, enquanto que a enzima glutationa redutase (GR) usa NADPH para reduzir GSSG a GSH (GRANT et al., 1997). Vários radicais livres e oxidantes são capazes de oxidar GSH a GSSG (NOCTOR & FOYER, 1998), atuando como removedor de radicais livres. Estudos mostram que níveis elevados de GSH celular estão associados à tolerância a metais pesados em plantas (CHEN & GOLDSBROUGH, 1994) e a exposição aos metais pesados leva a uma síntese acelerada de GSH em raízes e em culturas de células (SCHNEIDER & BERMANN, 1995). É também o precursor das fitoquelatinas que complexam metais pesados em plantas (ROSEN, 2002). Os níveis de GSH em tecidos de plantas são modificados na presença de metais (KOVIDEVA et al., 1997). Embora seja conhecido o papel da GSH como um importante antioxidante celular, vários aspectos sobre a função de seus componentes precisam ser detalhados (BARTOSZ, 1996).

Também, os carotenóides possuem um papel importante na proteção do pigmento clorofila sob condições de estresse e são conhecidos por manter as reações fotodinâmicas, protegendo a clorofila da peroxidação lipídica e impedindo o colapso da membrana dos cloroplastos (KNOX & DODGE, 1985). O alfa-tocoferol ou vitamina E atua na varredura de radical peroxil, e provavelmente é um dos inibidores mais importantes na reação em cadeia da peroxidação lipídica (HALLIWELL & GUTTERIDGE, 2000). É um antioxidante lipossolúvel que atua bloqueando a etapa de propagação da peroxidação lipídica dos ácidos graxos poliinsaturados das membranas e lipoproteínas. Intercepta o radical peroxila ($RO_2^{\bullet}$), resultante com formação do radical tocoferila, que será regenerado por ascorbato, glutationa ou ubiquinol a tocoferol (BARREIROS et al., 2006; BUETTNER, 1993) (Figura 5).

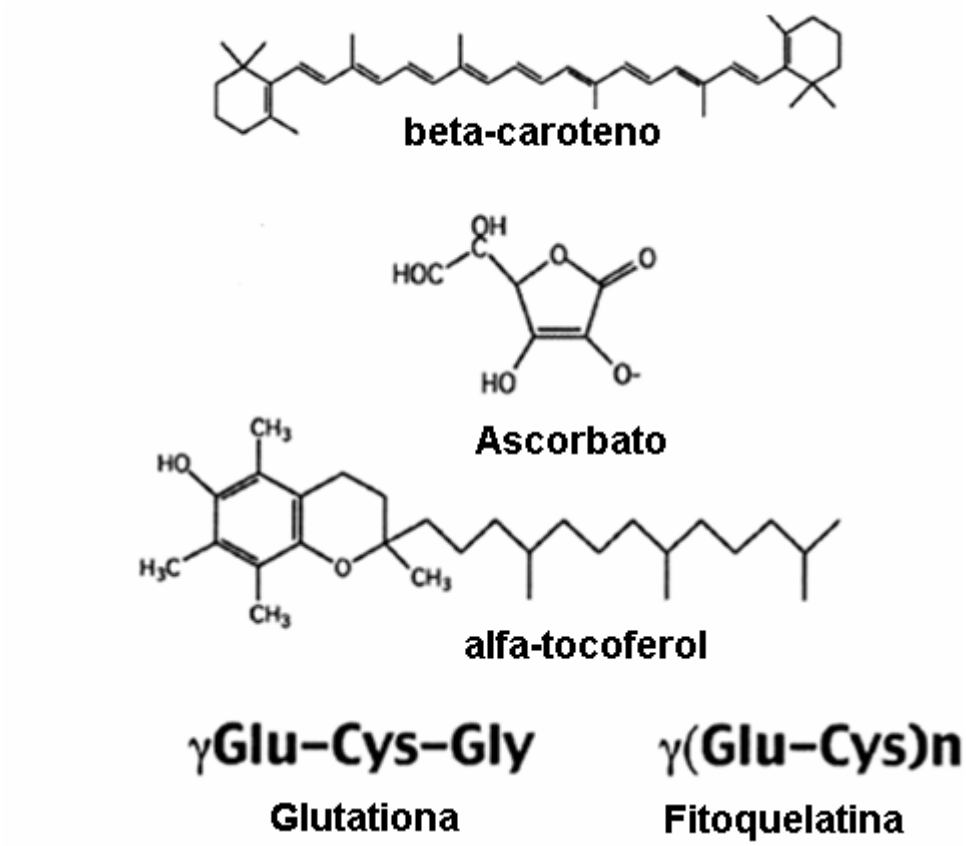

Figura 5. Antioxidantes de baixo peso molecular. (Adaptado de Pinto et al., 2003)

Dessa forma, os metais pesados tornam-se tóxicos para as plantas sempre que seus níveis de acumulação excederem a capacidade de detoxificação. Assim, o fator que determina o estresse oxidativo é a velocidade

com que as plantas ativam suas reservas antioxidantes (RANIERI et al., 1993), aspecto este que confere tolerância ao estresse (SINHA et al., 1996). Sinha et al. (2005) sugerem que a capacidade de tolerância das plantas aos metais depende do balanço entre os fatores que favorecem o estresse oxidativo e os fatores que o reduzem.

2.4. Delta-aminolevulinato desidratase (δ-ALA-D)

A enzima delta-aminolevulinato desidratase (E.C. 4.2.1.24), também conhecida como porfobilinogênio sintase, catalisa a condensação assimétrica de duas moléculas do ácido delta-aminolevulínico (δ-ALA), formando o composto monopirrólico porfobilinogênio (PBG) (Figura 6). O produto final da rota dos tetrapirrólicos, tais como o heme, as clorofilas e as corinas, está envolvido em muitos aspectos do metabolismo, como o transporte de elétrons até a fotossíntese (JAFFE, 2000).

A δ-ALA-D possui grande importância toxicológica, pois alguns metais, tais como o cádmio (NORIEGA et al., 2007), o mercúrio e o chumbo (MORSCH, 2002; PRASAD & PRASAD, 1987), são capazes de inibir esta enzima. A δ-ALA-D é sensível aos agentes oxidantes, tais como metais pesados e ROS, devido a sua natureza sulfidrílica (ROCHA et al., 2001). Além disso, a sua inibição leva à síntese reduzida de clorofila, o que traz prejuízos para o crescimento das plantas. PEREIRA et al. (2006) observaram que o alumínio inibe a atividade da δ-ALA-D de plântulas de *Cucumis sativus*, sendo que esta inibição esteve relacionada com alterações no crescimento das plântulas. Além disso, CHO & PARK (2000) observaram que até mesmo concentrações baixas de mercúrio no substrato reduzem o crescimento de raízes e da parte aérea de plantas de tomate, sendo que essa redução foi concomitante com a indução de radicais livres e a redução nos níveis de clorofilas.

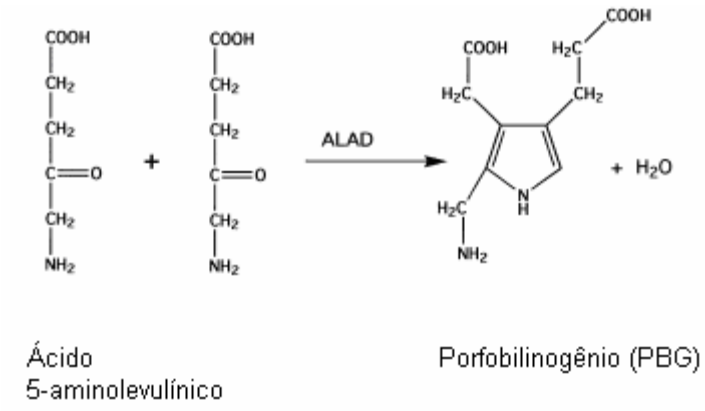

Figura 6 - Formação do porfobilinogênio (PBG). (Adaptado de Senior et al., 1996)

2.5. *Cucumis sativus* L. (Pepino)

O pepino é uma importante espécie cultivada e consumida no Brasil. É uma hortaliça fruto pertence à família Cucurbitaceae. Trabalhos recentes mostraram que o pepino é sensível a uma grande variedade de contaminantes (GORSUCH et al., 1991, PEREIRA et al., 2006) e, em função disso, foi selecionado como uma planta teste para o estudo do metabolismo dos metais em plantas. Além disso, há informação disponível insuficiente sobre a toxicologia de mercúrio nesta espécie e sobre os mecanismos pelo qual esse elemento produz estresse oxidativo em plantas.

2.6. *Zea mays* L. (Milho)

O milho é a mais importante planta comercial com origem nas Américas. Pertencente a família Poaceae, o milho possui sua importância econômica caracterizada pelas diversas formas de sua utilização, que vai desde a alimentação animal até a indústria de alta tecnologia. Na realidade, o uso do milho em grão como alimentação animal representa a maior parte do consumo desse cereal, isto é, cerca de 70% no mundo. Existem várias espécies e variedades de milho, todas pertencentes ao gênero *Zea* (EMBRAPA)

Devido ao fato do milho apresentar um excelente sistema para os modelos de absorção e de translocação de metais do ambiente (CHRYSAFOPOULOU et al., 2005), ele também tem sido usado em muitos estudos de poluição ambiental (WANG et al., 2007). Contudo, no Brasil são

escassos os estudos sobre o impacto de metais pesados no ecossistema. Assim, torna-se importante os estudos que avaliam a toxicologia destes metais em plantas tal como o milho e outras espécies agrícolas.

3. RESULTADOS

3.1. ARTIGO E MANUSCRITOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo e manuscritos, os quais se encontram no item **ARTIGO E MANUSCRITOS**. Esse item, por sua vez, está subdividido em Capitulo I e Capitulo II. Tendo em vista que migrei do Mestrado para o Doutorado na condição de continuar meu estudo, o Capitulo I está constituído pelo artigo **1** e manuscrito **1** que fizeram parte de minha Dissertação de Mestrado, a qual foi apresentada ao Programa de Pós Graduação em Bioquímica Toxicológica da UFSM. No capitulo II estão apresentados os manuscritos **2**, **3** e **4** que representam a continuação de meu projeto inicial. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigo e manuscritos e representam a íntegra deste estudo. Os itens, **DISCUSSÃO E CONCLUSÕES**, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre o artigo e os manuscritos científicos contidos neste trabalho. No item **PERSPECTIVAS** estão expostos as possíveis propostas de trabalho para a continuação do estudo, referentes a esse assunto. As **REFERÊNCIAS BIBLIOGRÁFICAS** contêm somente às citações que aparecem nos itens **INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA** e **DISCUSSÃO**.

3.1.1. ARTIGO E MANUSCRITO CIENTÍFICO: CAPÍTULO I

Efeito do mercúrio no estresse oxidativo, na atividade da δ-ALA-D e no crescimento de plântulas de pepino (*C. sativus* L.).

3.1.1.1. Artigo 1 - Mercury toxicity induces oxidative stress in growing cucumber seedlings.

3.1.1.2. Manuscrito 1 - Effects of mercury on antioxidant system and δ-aminolevulinic acid dehydratase activity of growing cucumber seedlings

3.1.1.1. Efeito do mercúrio no estresse oxidativo e no crescimento de plântulas de pepino (*C. sativus* L.).

Artigo 1

Mercury toxicity induces oxidative stress in growing cucumber seedlings

Denise Cargnelutti, Luciane Almeri Tabaldi, Rosélia Maria Spanevello, Gladis de Oliveira Jucoski, Vanessa Battisti, Marciel Redin, Carlos Eduardo Blanco Linares, Valderi Luiz Dressler, Érico Marlom de Moraes Flores, Fernando Teixeira Nicoloso, Vera Maria Morsch, Maria Rosa Chitolina Schetinger

(Publicado na Revista Chemosphere, v. 65, pp. 999 – 1006, 2006)

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Chemosphere 65 (2006) 999–1006

CHEMOSPHERE

www.elsevier.com/locate/chemosphere

Mercury toxicity induces oxidative stress in growing cucumber seedlings

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Received 20 October 2005; received in revised form 20 February 2006; accepted 18 March 2006
Available online 3 May 2006

Abstract

In this study, the effects of exogenous mercury ($HgCl_2$) on time-dependent changes in the activities of antioxidant enzymes (catalase and ascorbate peroxidase), lipid peroxidation, chlorophyll content and protein oxidation in cucumber seedlings (*Cucumis sativus* L.) were investigated. Cucumber seedlings were exposed to from 0 to 500 μM of $HgCl_2$ during 10 and 15 days. Hg was readily absorbed by growing seedlings, and its content was greater in the roots than the in shoot. Time and concentration-dependent reduction in root and shoot length was observed at all concentrations tested, equally in the roots and shoot, at both 10 and 15 days. At 50 μM $HgCl_2$, root fresh weight of 15-day-old seedlings increased, and at other concentrations, it reduced. For 10-day-old seedlings, reduction in root and shoot fresh biomass was observed. At 15 days, only at 50 μM $HgCl_2$ was there no observed reduction in shoot fresh biomass. Dry weight of roots increased at 500 μM both at 10 and 15 days, though at 250 μM $HgCl_2$ there was only an increase at 15 days. There was a significant effect on shoot dry weight at all concentrations tested. Hg-treated seedlings showed elevated levels of lipid peroxides with a concomitant increase in protein oxidation levels, and decreased chlorophyll content when exposed to between 250 and 500 μM of $HgCl_2$. At 10 days, catalase activity increased in seedlings at a moderately toxic level of Hg, whereas at the higher concentration (500 μM), there was a marked inhibition. Taken together, our results suggest that Hg induces oxidative stress in cucumber, resulting in plant injury.

Keywords: Catalase; Ascorbate peroxidase; Lipid peroxides; Cucumber; Chlorophyll; Protein oxidation

1. Introduction

The effects of certain heavy metals on cellular systems has received a great deal of attention in recent decades due to the increasing exposure of living organisms to these metals in the environment (Cavallini et al., 1999). Amongst heavy metals, mercury is one of the most hazardous pollutants of the environment and originates from various sources, such as gold and silver mining, copper and zinc mining and smelting areas, and in areas close to coal burning and other industrial activities (Du et al., 2005). It is known to accumulate in living organisms (Su et al., 2005), causing serious damage.

Its increasing levels in the soil exert a wide range of adverse effects on the growth and metabolism of plants (Verma and Dubey, 2003; Patra et al., 2004), such as reduced photosynthesis, transpiration, water uptake, chlorophyll synthesis (Godbold and Huttermann, 1986), and increased lipid peroxidation (Cho and Park, 2000).

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0045-6535/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2006.03.037

An important feature of mercury toxicity is the generation of free radicals. The generation of reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) has been proven to be one of the underlying agents in the origin of tissue injury after the exposure of plants to a wide variety of stressful conditions, such as draught, heat, chilling, high light intensity, UV radiation, heavy metals, various organic chemicals and air pollutants (Cho and Park, 2000; Qureshi et al., 2005).

Complex antioxidant systems (Qureshi et al., 2005) such as catalase (E.C.1.11.1.6), ascorbate peroxidase (E.C.1.11.1.11), and superoxide dismutases (SOD, E.C.1.15.1.1) (Nakano and Asada, 1981; Cho and Park, 2000; Verma and Dubey, 2003), which neutralize and scavenge the ROS (Cho and Park, 2000; Mittler, 2002), are very important for plants in order to protect cellular membranes and organelles from the damaging effects of ROS, generated by various environmental stress, as heavy metals.

Cucumis sativus was selected as the test plant species, due to its sensitivity to a wide range of contaminants (Pereira et al., 2006) and also due to the insufficient information available on mercury toxicity in this species. Aiming to contribute to a better understanding of the toxicology of this metal, in this paper we present some data showing changes in antioxidative capacity, plant growth, chlorophyll content, protein oxidation and lipid peroxidation in seedlings of *C. sativus* exposed to mercury chloride.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of cucumber (*C. sativus* L.) obtained from Feltrin Ltd. (Santa Maria, RS) were germinated in glass recipients containing 20 mL of 10% of Murashige and Skoog (1962) medium, supplemented with 0.6% agar and various $HgCl_2$ levels. Seedlings were exposed to 0, 0.5, 50, 250 and 500 μM of $HgCl_2$. The medium pH was adjusted to 5.8. Each experimental unit consisted of six seeds, totalizing 15 replicates per treatment. After the radicle broke through, the seedlings were maintained in a growth chamber with controlled temperature (25 ± 1 °C) and photoperiod (16 h light; light intensity of 35 μmol m^{-2} s^{-1} at plant level) for 10 and 15 days. This time was selected to verify if there would be alterations in the biochemical parameters evaluated at a small interval of time.

2.2. Growth analysis

Cucumber growth was determined by measuring the length of the root system (Tennant, 1975) and of the shoot (measured with a ruler), both expressed in cm $plant^{-1}$. To obtain fresh weight, excess water from root washing was removed with a paper towel. To obtain dry weight, the plants were left at 65 °C to a constant weight. Fresh and dry weight was expressed as g $plant^{-1}$.

2.3. Metal determination

The Hg content was determined in the roots and cotyledons of 10 or 15 day-old cucumber seedlings. Between 20 and 300 mg of cotyledons 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 absorbed was expressed as μg g^{-1} dry weight.

2.4. Protein oxidation

The reaction of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) was used to determine the amount of protein oxidation, as described in Levine et al. (1990). Protein extract was obtained by the homogenization of cucumber seedlings (1 g) with 5 mL, 25 mM K_2HPO_4 (pH 7.0) which contained 10 mL L^{-1} Triton X-100. After the homogenate was centrifuged at 15.000 × *g* for 30 min at 4 °C, the supernatant was used for the immediate determination of protein oxidation. After the DNPH-reaction, the carbonyl content was calculated by absorbance at 370 nm, using the extinction coefficient for aliphatic hydrazones (22 l $mmol^{-1}$ cm^{-1}) and expressed as nmol carbonyl $(mg\ protein)^{-1}$.

2.5. Chlorophyll determination

Cotyledons were weighed and used for chlorophyll determination. Chlorophyll was extracted following the method of Hiscox and Israelstam (1979) and estimated with the help of Arnon's formulae (Arnon, 1949). 0.1 g chopped fresh cotyledons sample was incubated at 65 °C in dimethylsulfoxide (DMSO) until the pigments were completely bleached. Absorbance of the solution was then measured at 663 and 645 nm in a Spectrophotometer (Celm E-205D). Chlorophyll content was expressed as μg g^{-1} fresh weight.

2.6. Estimation of lipid peroxides

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 whole plant samples (0.1 g fresh weight) were ground in 20 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 $10.000 \times g$ 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}.

2.7. Catalase assay

The activity of catalase was assayed according to the method of Aeby (1984) with some modifications. Fresh samples (1 g) 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 $12.000 \times g$ for 20 min at 4 °C and then, the supernatant was used for the enzyme assay. Activity of catalase 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 15 mM H_2O_2 in KPO_4 buffer (pH 7.0) and 30 µl extract. Activity was expressed as ΔE/min/mg protein.

2.8. Ascorbate peroxidase 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 ($E = 2.8$ mM^{-1} cm^{-1}) and activity was expressed as µM ascorbate oxidated min^{-1} mg^{-1} protein.

2.9. 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.

2.10. 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 Duncan's range test. Three pools of five replicates each ($n = 3$) were taken for all analyses from each set of experiments.

3. Results

3.1. Hg content and seedling growth

The content of Hg in tissues of cucumber seedlings was exposure time- and concentration-Hg dependent (Table 1). Hg accumulated at a higher content in the roots than in the cotyledons. Hg content in the roots of 10 and 15-day-old seedlings was, respectively, about 7-fold and 5.6-fold higher than that in cotyledons. The maximum accumulation of Hg was 31 857 µg g^{-1} dry weight in roots treated with 500 µM $HgCl_2$ at 10 days.

Table 1
Mercury content of cucumber seedling growth under increasing concentrations of $HgCl_2$ for 10 or 15 days

Hg treatment (µM $HgCl_2$)	Hg content (µg g^{-1} dry wt.)	
	Cotyledons	Root
Day-10		
0	0.67 ± 0.17	0.60 ± 0.11
0.5	3.40 ± 1.47	6.13 ± 0.74
50	552.33 ± 43.5*	1284.33 ± 61.5*
250	1800.33 ± 50.5*	12498 ± 78*
500	4734.33 ± 63.5*	33377 ± 55*
Day-15		
0	1.4 ± 0.27	0.79 ± 0.05
0.5	3.38 ± 0.13	4.43 ± 0.1
50	759 ± 22*	1474.33 ± 21.5*
250	1816.33 ± 44.5*	12654 ± 45*
500	3698 ± 60*	20545 ± 42*

Data represent mean values ± SD based on independent determination.
* Different from control to $p < 0.05$.

The effect of Hg on the growth of cucumber seedlings, expressed as biomass and length of roots and shoot, are shown in Fig. 1. Hg-exposure induced a significant reduction of root (Fig. 1A) and shoot (Fig. 1B) length, and this effect varied with the time of exposure and the concentration of exogenous Hg. At the higher concentrations of Hg (250 and 500 µM $HgCl_2$), the root length of 10 and 15-day-old seedlings was, respectively, 96% and 98% less than that of the control. However, shoot length was completely impaired.

A low concentration of Hg conversely affected the production of fresh biomass, where, at about 50 µM $HgCl_2$, root fresh weight of 15-day-old seedlings increased (Fig. 1C). Moreover, only a concentration higher than 250 µM $HgCl_2$ reduced root fresh weight. For 10-day-old seedlings, the presence of Hg in substrate caused a continuous reduction in root fresh biomass (Fig. 1C), and shoot fresh biomass (Fig. 1D). At 15 days, only at 50 µM $HgCl_2$ was there no reduction observed in shoot fresh biomass (Fig. 1D). Contrary to the results observed for fresh biomass, the dry weight of roots (Fig. 1E) significantly increased as a function of Hg level in the substrate. In addition, 15-day-old seedlings showed greater dry weight than did 10-day-old seedlings. With relation to shoot dry weight, there was a significant effect at all concentrations of mercury tested (Fig. 1F).

3.2. Chlorophyll levels

The effects of Hg on chlorophyll levels are shown in Fig. 2A. The presence of Hg in the substrate caused a linear decrease of chlorophyll content in the cotyledons, but this

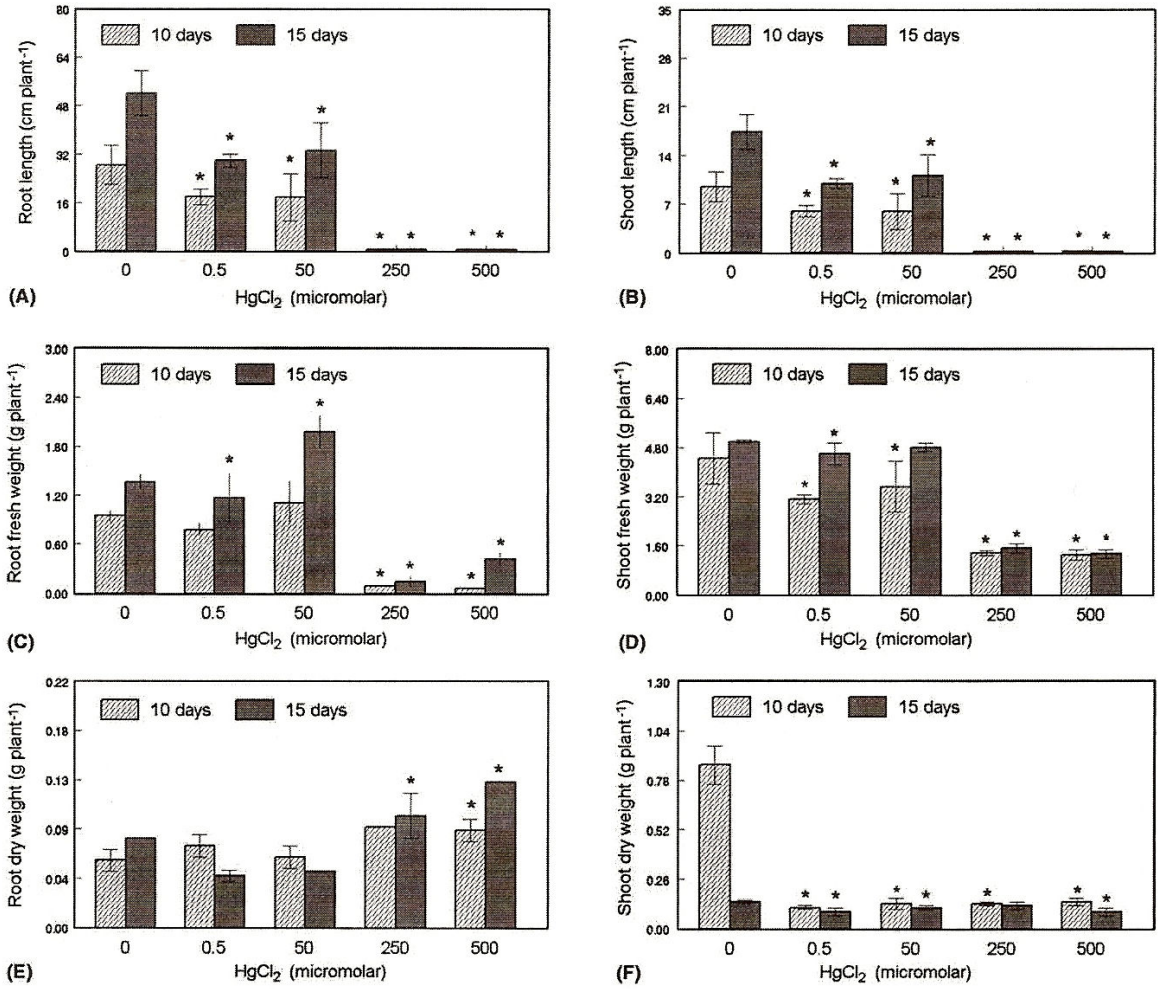

Fig. 1. Effect of increasing concentration of $HgCl_2$ in the growth medium on the length of roots (A), length of shoots (B), root fresh weight (C), shoot fresh weight (D), root dry weight (E) and shoot dry weight (F) of 10- and 15-day old cucumber seedlings. Data represent the mean ± SD of three different experiments. *Different from control to $p < 0.05$.

response varied with the time of exposure and the concentration of exogenous Hg. At the highest levels of Hg (500 μM $HgCl_2$), chlorophyll content was 59% and 94% lower, respectively, than that of the control in 10- and 15-day-old seedlings.

3.3. Lipid peroxidation and protein oxidation

The effects of Hg on lipid peroxidation and protein oxidation are shown in Fig. 2B and C. At the highest level of Hg (500 μM $HgCl_2$), the level of lipid peroxides, measured in terms of TBARS, increased 33% and 250%, respectively, in comparison with the control for both 10- and 15-day-old plants (Fig. 2B). At the concentrations lower than 250 μM $HgCl_2$, the lipid peroxide content was higher in 15-day-old seedlings than in 10-day-old seedlings.

Increasing Hg levels in the substrate caused an enhancement of protein oxidation at 250 and 500 μM $HgCl_2$ (Fig. 2C), where the highest carbonyl levels were found in the 15-day-old seedlings at the concentration of 250 μM $HgCl_2$.

3.4. Soluble protein content

The effects of $HgCl_2$ on soluble protein content are presented in Fig. 3A. The soluble protein content was exposure time- and concentration-Hg dependent. Plants treated with Hg for 10 days showed a higher soluble protein content than those treated for 15 days. In addition, regardless of Hg-exposure time, soluble protein content significantly increased as Hg increased.

3.5. Activities of some antioxidant enzymes

Catalase activity varied as a function of both exposure time and Hg concentration (Fig. 3B). For 10-day-old seed-

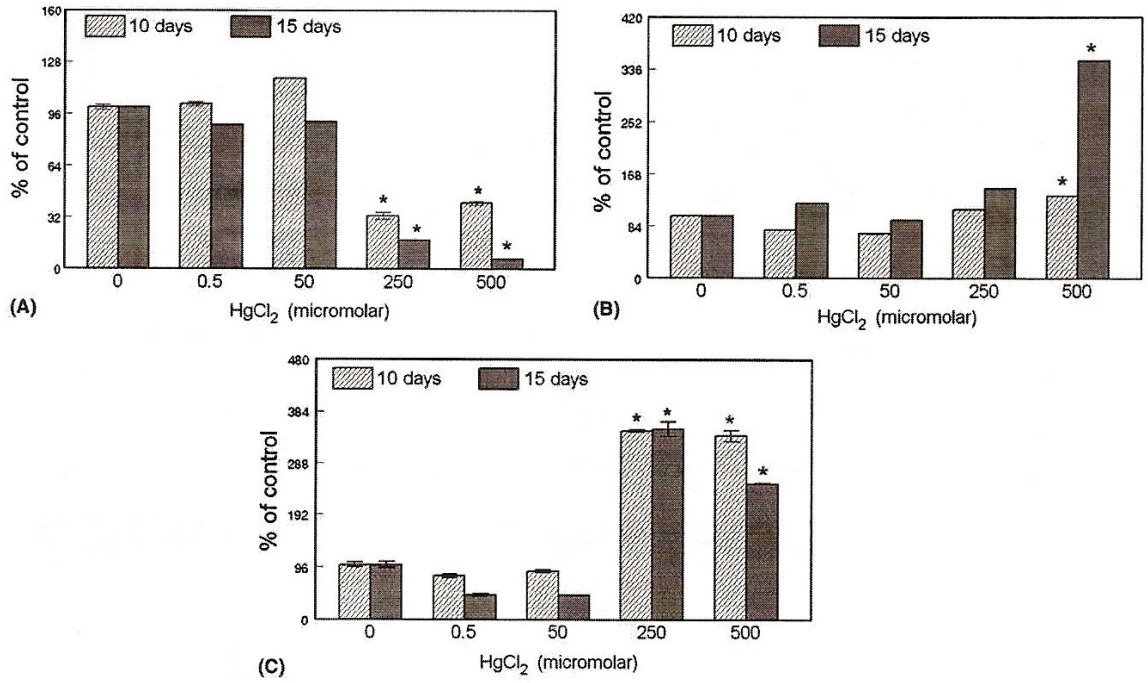

Fig. 2. Effect of increasing concentration of $HgCl_2$ on chlorophyll content (A), lipid peroxides (B) and protein carbonyl (C) of 10- and 15-day old cucumber seedlings. Data represent the mean ± SD of three different experiments. The control specific activity (without mercury) that represents 100% was 11.42 ± 1.71 and 12.72 ± 0.79 mg l^{-1}, 0.18 ± 0.02 and 0.08 ± 0.01 nmol MDA (mg protein)$^{-1}$, and 14.2 ± 4.31 and 20.7 ± 5.50 nmol carbonyl (mg protein)$^{-1}$, for 10 and 15 days, respectively. *Different from control to $p < 0.05$.

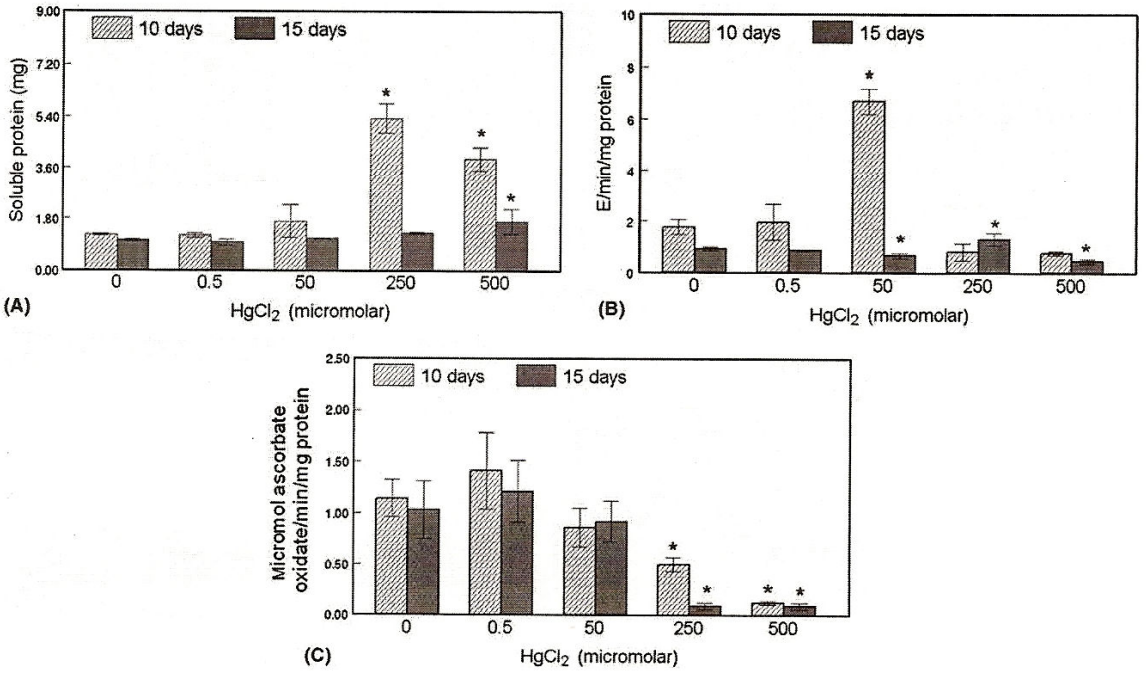

Fig. 3. Effect of increasing concentration of $HgCl_2$ on content soluble protein (A), catalase activity (B) and ascorbate peroxidase activity (C) of 10- and 15-day old cucumber seedlings. Data represent the mean ± SD of three different experiments. *Different from control to $p < 0.05$.

lings, catalase activity peaked at 50 μM $HgCl_2$. On the other hand, 15-day-old seedlings showed the highest level of catalase activity when grown at 250 μM $HgCl_2$ (Fig. 3B). At the concentrations of 50 and 500 μM $HgCl_2$, catalase activity of 15-day-old seedlings was, respectively, 30% and 51% lower than that of the control.

Ascorbate peroxidase activity varied only in accordance with Hg concentration in the substrate (Fig. 3C). A higher inhibition was observed at concentrations of 250 and 500 μM $HgCl_2$, both for 10 or 15 days.

4. Discussion

Mercury is inadvertently added to soils in fertilizer, limestone, natural gypsum, phosphogypsum, manure (especially of marine origin), sewage sludge, etc., and intentionally added in fungicides containing Hg (Andersson, 1979). Mercury concentrations in limestone are generally $<20\ \mu g\ kg^{-1}$, whereas animal manures may have concentrations of the order of $100\ \mu g\ kg^{-1}$. Occasionally, values of up to $100\ mg\ kg^{-1}$ are reported (Steinnes, 1990).

The changes observed in the growth of cucumber seedlings were consistent with the results obtained at low Hg concentrations in tomatoes (Cho and Park, 2000). Suszcynsky and Shann (1995) showed that inhibition of root and shoot growth occurred at $1.0\ \mu g\ mL^{-1}$ Hg and above, with very limited tissue damage at higher levels of treatment. Also, Hg-induced root damage may have serious consequences for nutrient and water supply to above ground plant parts (Godbold and Huttermann, 1986).

Our results indicated that higher concentrations of Hg increased the production of root dry weight (Fig. 2E). This may be explained by mercury-induced formation of gathering in the vegetable tissue. These changes are consistent with the hypothesis that Hg induces an abnormal proliferation of root cells. This also has been observed in studies with cadmium in plants (Arduini et al., 2004).

On the other hand, higher concentrations of Hg dramatically reduced shoot biomass (Fig. 2F). The increase in root fresh weight at lower Hg-concentrations (50 μM $HgCl_2$) might be caused by the hormetic effect. Calabrese (1999) observed a similar effect in *Mentha piperita* to the synthetic plant growth inhibitor phosfon. Growth hormesis represents an overcompensation due to a disruption in homeostasis that has been described in relation to different factors, such as several organic and inorganic chemicals, Al, and the amelioration of a latent deficiency of an essential element or stimulation of defense reactions leading to a general activation of metabolism (Barceló and Poschenrieder, 2002; Calabrese and Blain, 2005).

Results of the present study indicate a continuous increase in the content of Hg in the roots and cotyledons of cucumber seedlings with the increase of the external concentration of Hg. Seedlings of cucumber accumulated a significantly higher Hg content in the roots when compared to the cotyledons, which is in agreement with the findings of other authors (Greger et al., 2005). Hg accumulation in the root system indicates that roots serve as a partial barrier to the transport of Hg to shoots (Cavallini et al., 1999). In this study, a portion of Hg could have been simply sequestered away by epidermal cell walls or cuticles, though in response to the effects of Hg on seedlings, we can suggest that Hg was, in fact, taken by tissue cells.

Zang and Tyerman (1999) reports that Hg is known to inhibit water uptake via aquaporins on plasma membranes in higher plants, which could explain the detrimental effect of higher concentrations of Hg on the fresh weight of seedlings. It is interesting to note that, contrarily, root dry weight significantly increased.

The decreased chlorophyll content observed in our study corroborates with other reports (Cho and Park, 2000). $HgCl_2$ (0.5–500 μM) caused a time-dependent and concentration-dependent decline in chlorophyll content (Fig. 2A) in the cotyledons. In plants, Hg ions may substitute metal ions in photosynthetic pigments, causing a decrease in photosynthesis rates (Xylander et al., 1996). Exposure to Hg was reported to induce a loss of K, Mg, Mn and an accumulation of Fe (Doening, 2000). Several studies have shown that Hg in the substrate decreased the levels of photosynthetic pigment chlorophylls and carotenoids at a prolonged duration of exposure. It also strongly inhibits the photosynthetic electron transport chain, where photosystem II (PS II) is the most sensitive target (Bernier et al., 1993; Bernier and Carpentier, 1995). 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.

Heavy metal toxicity is believed to induce the production of reactive oxygen species (ROS) and may result in significant damage to cellular constituents. Membrane lipids and proteins are especially prone to attack by free radicals, considered to be reliable indicators of oxidative stress in plants (Halliwell and Gutteridge, 1993). It is known that high concentrations of metals in plants can interfere with physiologically important functions, can cause an imbalance of nutrients and have detrimental effects on the synthesis and functioning of biologically important compounds, such as enzymes, vitamins, hormones, etc. (Vangronsveld and Clijsters, 1994).

The peroxidation of lipids probably starts with the hydroxyl radical. Scavengers of $OH^{\cdot}$ do not inhibit the process, and Fe^{2+} bound to the membrane and exposed to the attack of H_2O_2 generates $OH^{\cdot}$ formed will react locally and immediately with the lipids in the membrane (Halliwell and Gutteridge, 1999). Therefore, $O_2^{\cdot}$, H_2O_2 and other ROS such as the hydroxyl radical ($OH^{\cdot}$) could be responsible for Hg-induced membrane damage. Active oxygen species bring about the peroxidation of membrane lipids, which leads to membrane damage (Scandalios, 1993). Since lipid peroxidation is the symptom most easily ascribed to oxidative damage (Zhang and Kirkam, 1996), it is often used as an indicator of increased oxidative damage (Halliwell, 1987).

Malondialdehyde is a common product of lipid peroxidation and a sensitive diagnostic index of oxidative injury (Janero, 1990). In cucumber seedlings, MDA levels were significantly enhanced and were exposure time- and concentration-Hg dependent (Fig. 2B). In tomato plants exposed to 50 μM of $HgCl_2$, MDA content also increased (Cho and Park, 2000). Briefly, increased carbonylation and MDA contents indicate that the cucumber plants experienced substantial oxidative damage when exposed to high concentrations of $HgCl_2$.

Lipids and proteins are common targets for oxidative damage in tissues under environmental stress (Prassad, 1996). Carbonyl content is a sensitive indicator of oxidative damage to proteins (Levine et al., 1994), and levels of carbonylated proteins increase in plants undergoing oxidative stress associated with heavy metals (Boscolo et al., 2003), drought (Boo and Jung, 1999), ozone (Junqua et al., 2000) and low temperatures (Kingston-Smith and Foyer, 2000).

Halliwell and Gutteridge (1999) suggested that the oxidation of proteins from carbonyls occurs via the $OH^{\cdot}$ radical, since neither H_2O_2 nor $O_2^{\cdot-}$ are reactive enough to provoke oxidation, suggesting that really the induce mercury formation of ROS. The formation of carbonyls is a process that involves a site-specific mechanism in proteins (Stadtman and Oliver, 1991). Our data indicates that the differences in protein oxidation at the higher concentrations of Hg in cucumber seedlings are related to the levels of antioxidant defense. The accumulation of carbonyls in the cucumber seedlings, thus, indicate that the quantity of radicals exceeded the capacity of the antioxidant defensive system.

In the present study, a biphasic effect was observed in the catalase activity of 10-day-old seedlings, which also might be attributed to a hormetic dose response. Furthermore, for 10-day-old seedlings, the detrimental effect of Hg on catalase activity coincided with a decrease in soluble protein content. High concentrations of Hg may lead to protein precipitation (Patra and Sharma, 2000), thus reducing the functions of some enzymes, which suggests that plants have lost their system of defense. As at low concentrations with an increased time of Hg exposure, there may occur a similar effect at high concentrations with a short period of time. Moreover, with an increase of exposure time, there may occur an increase in the production of ROS, causing greater damage to tissue cells.

Mercury-stressed (1–10 $mg\,l^{-1}$) plant cells showed increased activities of antioxidants such as catalase in varying degrees and presented positive endogenous protection effects. However, the protection effect disappeared at higher levels (50 $mg\,l^{-1}$) of mercury (Ma, 1998). Higher activity of catalase at a short time of Hg exposure might be related to low levels of MDA, being that plant defense system efficient against the stress generated by metal.

APX could be responsible for the fine modulation of ROS for signaling (Mittler, 2002), and utilizes the reducing power of ascorbic acid to eliminate potentially harmful H_2O_2. Our results showed a steady decrease in the activity of APX in response to increasing levels of Hg in substrate. A decline in both catalase and ascorbate peroxidase activities in Hg-treated plants suggests a possible delay in the removal of H_2O_2 and toxic peroxides mediated by catalase and peroxidase, and hence an enhancement of lipid peroxidation.

In conclusion, the growth reduction of cucumber seedlings might be related to a decreased chlorophyll content with a consequent reduction in the rate of photosynthesis and an increase in membrane damage, which could account for the higher levels of lipid peroxidation and protein oxidation. Therefore, Hg-treatment caused oxidative stress, and the antioxidant system of the seedlings was not sufficient to revert the stress of a prolonged period of Hg exposition.

References

Aeby, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.

Andersson, A., 1979. In: Niriagu, J.O. (Ed.), The Biogeochemistry of Mercury in the Environment. Elsevier, Amsterdam, pp. 79–102.

Arduini, I., Masoni, A., Mariotti, M., Ercoli, L., 2004. Low cadmium application increase iscanthus growth and cadmium translocation. Environ. Exp. Bot. 52, 89–100.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24, 1–15.

Assche, F.V., Clijsters, H., 1990. Effects of metals on enzyme activity in plants. Plant Cell Environ. 13, 195–206.

Barceló, J., Poschenrieder, C., 2002. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. Environ. Exp. Bot. 48, 75–92.

Bernier, M., Carpentier, R., 1995. The action of mercury on the binding of the extrinsic polypeptides associated with the water oxidizing complex of photosystem II. FEBS Lett. 360, 251–254.

Bernier, M., Popovic, R., Carpentier, R., 1993. Mercury inhibition at the donor side of photosystem II is reversed by chloride. FEBS Lett. 321, 19–23.

Boo, Y.C., Jung, J., 1999. Water deficit-induced oxidative stress and antioxidative defenses in rice plants. J. Plant Physiol. 155, 255–261.

Boscolo, P.R.S., Menossi, M., Jorge, R.A., 2003. Aluminum-induced oxidative stress in maize. Phytochemistry 62, 181–189.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Calabrese, E.J., 1999. Evidence that Hormesis Represents an "Overcompensation" Response to a Disruption in Homeostasis. Ecotoxicol. Environ. Safety 42, 135–137.

Calabrese, E.J., Blain, R., 2005. The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. Toxicol. Appl. Pharmacol., Rev. 202, 289–301.

Cavallini, A., Natali, L., Durante, M., Maserti, B., 1999. Mercury uptake, distribution and DNA affinity in durum wheat (*Triticum durum* Desf.) plants. Sci. Total Environ. 243, 119–127.

Cho, U.-H., Park, J.O., 2000. Mercury-induced oxidative stress in tomato seedlings. Plant Sci. 156, 1–9.

Doening, D.W., 2000. Ecological effects, transport, and fate of mercury: a general review. Chemosphere 40, 1335–1351.

Du, X., Zhu, Y.-G., Liu, W.-J., Zhao, X.-S., 2005. Uptake of mercury (Hg) by seedlings of rice (*Oryza sativa* L.) grown in solution culture and interactions with arsenate uptake. Environ. Exp. Bot. 54, 1–7.

El-Moshaty, F.I.B., Pike, S.M., Novacky, A.J., Sehgal, O.P., 1993. Lipid peroxidation and superoxide production in cowpea (*Vigna unguiculata*)

leaves infected with tobacco ringspot virus or southern bean mosaic virus. Physiol. Mol. Plant Pathol. 43, 109–119.
Godbold, D.L., Huttermann, A., 1986. The uptake and toxicity of mercury and lead to spruce seedlings. Water Air Soil Pollut. 31, 509–515.
Greger, M., Wang, Y., Neuschütz, C., 2005. Absence of Hg transpiration by shoot after Hg uptake by roots of six terrestrial plant species. Environ. Pollut. 134, 201–208.
Halliwell, B., 1987. Oxidative damage, lipid peroxidation, and antioxidant protection in chloroplasts. Chem. Phys. Lipids 44, 327–340.
Halliwell, B., Gutteridge, J.M.C., 1993. Free Radicals in Biology and Medicine. Clarendon Press, Oxford.
Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine, thrid ed. Oxford Science Publications, New York.
Hiscox, J.D., Israelstam, G.F., 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. 57, 1132–1334.
Janero, D.R., 1990. Malondialdehyde and thiobabituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic. Biol. Med. 9, 515–540.
Junqua, M., Biolley, J.-F., Pie, S., Kanon, M., Duran, R., Goulas, P., 2000. In vivo occurrence of carbonyl residues in *Phaseolus vulgaris* proteins as a direct consequence of a chronic ozone stress. Plant Physiol. Biochem. 38, 853–861.
Kingston-Smith, A.H., Foyer, C.H., 2000. Bundle sheath proteins are more sensitive to oxidative damage than those of the mesophyll in maize leaves exposed to paraquat or low temperatures. J. Exp. Bot. 51, 123–130.
Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464–478.
Levine, R.L., Williams, J.A., Stadtman, E.R., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. 233, 346–357.
Ma, C., 1998. Hg harm on cell membrane of rape leaf and cell endogenous protection effect. Yingyong Shengtai Xuebao 9, 323–326.
Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Plant Sci. 7 (9), 405–410.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobbacco tissue culture. Plant Physiol. 15, 473–497.
Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22 (5), 867–880.
Patra, M., Sharma, A., 2000. Mercury toxicity in plants. Bot. Rev. 66 (3), 379–422.
Patra, M., Bhowmik, N., Bandopadhyay, B., Sharma, A., 2004. Comparison of mercury systems and the development of genetic tolerance. Environ. Exp. Bot. Rev. 52, 199–223.
Pereira, L.B., Tabaldi, L.A., Gonçalves, J.F., Jucoski, J.O., Pauletto, M.M., Weis, S.N., Nicoloso, F.T., Borher, D., Rocha, J.B.T., Schetinger, M.R.C., 2006. Effect of aluminum on δ-aminolevulinic acid dehydratase (ALA-D) and the development of cucumber (*Cucumis sativus*). Environ. Exp. Bot. 57, 106–115.
Prassad, T.K., 1996. Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: changes in antioxidant system, oxidation of proteins and lipids, and protease activities. Plant J. 10, 1017–1026.
Qureshi, M.I., Israr, M., Abdin, M.Z., Iqbal, M., 2005. Responses of *Artemisia annua* L. to lead and salt-induced oxidative stress. Environ. Exp. Bot. 53, 185–193.
Scandalios, J.G., 1993. Oxygen stress and superoxide dismutase. Plant Physiol. 101, 7–12.
Stadtman, E.R., Oliver, C.N., 1991. Metal-catalyzed oxidation of proteins. J. Biol. Chem. 266, 2005–2008.
Steinnes, E., 1990. In: Alloway, B.J. (Ed.), Heavy Metals in Soils. Wiley, New York, pp. 220–326.
Su, Y.-H., Zhu, Y.-G., Du, X., 2005. Co-uptake of atrazine and mercury by rice seedlings from water. Pestic. Biochem. Physiol. 82, 226–232.
Suszcynsky, E.M., Shann, J.R., 1995. Phytotoxicity and accumulation of mercury in tobacco subjected to different exposure routes. Environ. Toxicol. Chem. 14, 61–67.
Tennant, B.D., 1975. A test of a modified line intersect method of estimating root length. J. Ecol. 63 (3), 995–1001.
Vangronsveld, J., Clijsters, H., 1994. Toxic effect of metals. In: Farago, M.E. (Ed.), Plants and the chemical Elements-Biochemistry, Uptake, Tolerance and Toxicity. VCH, New York, pp. 149–177.
Verma, S., Dubey, R.S., 2003. Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci. 164, 645–655.
Xylander, M., Hagen, C., Braune, W., 1996. Mercury increases light susceptibility in the green alga *Haematococcus lacustris*. Bot. Acta 109, 222–228.
Zhang, J., Kirkam, M.B., 1996. Lipid peroxidation in sorghum and sunflower seedlings as affected by ascorbic acid, benzoic acid, and propyl gallate. J. Plant Physiol. 149, 489–493.
Zang, W.H., Tyerman, S.D., 1999. Inhibition of water channels by $HgCl_2$ in intact wheat root cells. Plant Physiol. 120, 849–857.
Zhu, Z., Wei, G., Li, J., Qian, Q., Yu, J., 2004. Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (*Cucumis sativus* L.). Plant Sci. 167, 527–533.

3.1.1.2. Efeito do mercúrio no sistema antioxidante e na atividade da δ-ALA-D em plântulas de pepino (*C. sativus* L.).

Manuscrito 1

MERCURY TOXICITY ALTERS THE ANTIOXIDANT SYSTEM OF GROWING CUCUMBER SEEDLINGS

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(Em revisão na Revista Biometals)

EFFECTS OF MERCURY ON ANTIOXIDANT SYSTEM AND δ-AMINOLEVULINIC ACID DEHYDRATASE ACTIVITY OF GROWING CUCUMBER SEEDLINGS

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Abstract

The objective of the present study was to verify the response of antioxidant defenses after of exposure to Hg. To investigate the effect of five $HgCl_2$ levels in cucumber (*Cucumis sativus* L.), seedlings were grown for 10 and 15 days and the following parameters were evaluated: the hydrogen peroxide (H_2O_2) concentration, the response of the antioxidant system and delta-aminolevulinic acid dehydratase (δ-ALAD) activity. Hg-treated seedlings have H_2O_2 levels decreased at 10 days at 50 μM $HgCl_2$, whereas at higher concentrations it increased. Superoxide dismutase (SOD) activity was inhibited at the higher Hg levels, but was enhanced at 50 μM $HgCl_2$ at 15 days of exposure. Increased non-protein thiols (NPSH) and ascorbic acid (AsA) levels were observed mainly at 10 days, whereas carotenoid levels were reduced at the higher $HgCl_2$ levels at 10 days. As expected, δ-ALA-D activity was inhibited at the higher $HgCl_2$ levels. Therefore, our results suggest that Hg increased the levels of ROS, provoking an increase in the antioxidant system, which is part of the overall expression of Hg tolerance in the seedlings.

Keywords: Mercury, Cucumber, Superoxide dismutase, Non-protein thiol groups, δ-aminolevulinic acid dehydratase.

Introduction

Heavy metal contamination is one the most serious environmental problems for plant productivity and it is also a threat to human health. Factors such as mining or industrial activities and use of metal-enriched materials such as chemicals fertilizers, farm manures, sewage sludge, and wastewater irrigation can contribute to contamination (Freedman and Hutchinson 1981) of natural ecosystems (Segura-Muñoz et al. 2006).

Mercury (Hg) is regarded as a non-essential element, with no known physiological function in plants. Due to its transition properties, Hg is readily up taken by plants, accumulates at high levels, and consequently results in toxicity or even plant death (Boening 2000). One of the characteristic effects of metal poisoning, observable at an early stage, is a reduction in plant cell proliferation and growth (Schützendübel et al. 2001; Israr and Sahi 2006; Zhou et al. 2008), as well as a reduction in the percentage of seed germination (Street et al. 2007). In addition, Hg alters transpiration (Zhang and Tyerman 1999), mineral nutrition (Boening 2000), chlorophyll metabolism (Morsch et al. 2002; Cargnelutti et al. 2006) and photosynthetic integrity (Israr et al. 2006). Moreover, Ortega-Villasante et al. (2007) reported that by shortening the analysis period a transient oxidative burst was caused by Hg, which preceded cell death. Generally, plants adopt a number of strategies to avoid the build-up of excess metal levels, including the reduction of metal uptake into the cytosol by a number of both ligand and transporters of metals or efflux from the cytosol either into the apoplast or into the vacuole. Plants also make use of strategies to reduce toxicity once a particular metal has been absorbed, for example through phytochelatin (PC) production (Hall 2002). Hg possesses high affinity to

sulfhydryl groups of glutathione (GSH) and this peptide is the main non-protein thiol and an important non-enzymatic antioxidant. In addition, GSH participates in the formation of PC, which might play an important side role in Hg detoxification (Cobbett and Goldsbrough 2002).

Mercury has been demonstrated to stimulate the formation of reactive oxygen species (ROS) (Cho and Park 2000), including superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$), all of which attack proteins (Romero-Puertas et al. 2002; Noriega et al. 2007), lipids (Sandalios et al. 2001) and nucleic acids (Gichner et al. 2006), leading to oxidative stress. Among the proteins that may suffer oxidation and damage is the enzyme δ-aminolevulinic acid dehydratase (δ-ALA-D). δ-ALA-D is sensitive to heavy metals due to its sulfhydrylic nature (Rocha et al. 1995, Morsch et al. 2002). In addition, it catalyzes the asymmetric condensation of 2 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).

In order to combat metal toxicity, plant cells have antioxidants such as carotenoids (Polyakov et al. 2001), GSH (Foyer and Noctor 2005) and ascorbate (Dipierro et al. 2005), and also antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, catalase and glutathione reductase, which participate in scavenging ROS and hence protect cells from injury (Matés 2000).

Cucumis sativus is known to accumulate toxic metals under laboratory conditions and has been selected as one of the test plant species due to its

sensitivity to a wide range of contaminants (Cargnelutti et al. 2006; Pereira et al. 2006; Gonçalves et al. 2007). Utilizing the same experimental conditions described by Cargnelutti et al. (2006), the present study investigated biochemical parameters in cucumber seedlings after both 10 and 15 days of exposure to Hg, in order to better understand the effect of this metal on the antioxidant system and its relation to δ-ALA-D activity.

Material and methods

Plant material and growth conditions

Seeds of cucumber (*Cucumis sativus* L.) obtained from Feltrin Ltd. (Santa Maria, RS) were germinated in glass recipients (100 mL) containing 20 mL of 10% of Murashige and Skoog (1962) medium, supplemented with 0.6% agar and various $HgCl_2$ levels. Seedlings were exposed to 0.5, 50, 250 and 500 µM of $HgCl_2$. These concentrations were chosen considering the range of mercury concentrations found in the soil, from 15 to 300 µg g^{-1} dry weight (55 – 1104.94 µM Hg) (Cavallini et al. 1999). Moreover, in our previous study (Cargnelutti et al. 2006), it was shown that Hg ranging from 50 to 500 µM was toxic to the cucumber seedlings. The medium pH was adjusted to 5.8. Each experimental unit consisted of 6 seeds, totalizing 15 replicates per treatment. After the radicle broke through, the seedlings were maintained in a growth chamber at 25 ± 2ºC on a 16/8-h light/dark cycle with 35 µmol m^{-2} s^{-1} of irradiance.

Determination of hydrogen peroxide

The H_2O_2 contents of both control and treated seedlings were determined according to Loreto and Velikova (2001). Approximately 100 mg of seedlings

were homogenized at 4 °C in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 x g for 15 min and 0.5 mL of 10 mM potassium phosphate buffer, pH 7.0, and 1 mL of 1M KI were added to the supernatant. The H_2O_2 content of the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve. The H_2O_2 content was expressed as μmol g^{-1} fresh weight.

Superoxide Dismutase (SOD; E.C 1.15.1.1)

The activity of SOD was assayed according to Mc Cord and Fridovich (1969). About 200 mg fresh seedlings (whole seedlings) were homogenized in 5 mL of 100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). The extract was filtered through muslin cloth and centrifuged at 22,000 x g for 10 min at 4 °C. The supernatant was used for the assay. The assay mixture consisted of a total volume of 1 mL, containing glycine buffer (pH 10.5), 1 mM epinephrine and the tissue extract. Epinephrine was the last component added. Adrenochrome 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) and non-protein thiol group (NPSH) concentrations

Cucumber seedlings were homogenized in a solution containing 50 mM Tris- HCl and 10 mL L^{-1} Triton X-100 (pH 7.5) and centrifuged at 6,800 x g for 10 min. To the resulting supernatant 10% TCA was added at a proportion of 1:1 (v/v) followed by centrifugation (6,800 x g for 10 min) to remove protein. Determination of AsA was performed as described by Jacques-Silva et al. (2001). 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 DNPH. The DNPH solution contained 2% DNPH, 0.23% thiourea, and 0.27% CuSO4 diluted in 49% H_2SO_4. After 3 h, 500 µL of 65% H_2SO_4 was added and the samples were read at 520 nm. A standard curve was constructed using L(+) ascorbic acid. Non-protein thiol concentration was measured spectrophotometrically with Ellman's reagent (Ellman 1959). An aliquot of the sample (400 µL) was added to a medium containing 550 µL of 1 M Tris-HCl (pH 7.4). The color that developed was read at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 mL). A standard curve using cysteine was used to calculate the concentration of thiol groups in samples.

Carotenoid determination

Carotenoids were extracted following the method of Hiscox and Israeslstam (1979). 0.1 g chopped fresh cotyledon sample was incubated at 65 °C in dimethylsulfoxide (DMSO) until the tissues were completely bleached. Absorbance of the solution was then measured at 470 nm with a

spectrophotometer (Celm E-205D). Carotenoid content was calculated using the formula (Lichtenthaler 1987) given below: carotenoid (mg g^{-1} fresh weight) = ((100 x A_{470}) – (2.27 x chl a) – (81.4 x chl b) / 227) x 50/500.

Estimation of delta-aminolevulinic acid dehydratase (δ-ALA-D; E.C. 4.2.1.24) activity

Cucumber cotyledons 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 x 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 dithiothreitol (DTT). δ-ALA-D activity was assayed as described by Morsch et al. (2002) by measuring the rate of porphobilinogen (PBG) formation. The incubation medium for the assays contained 100 mM Tris-HCl buffer, pH 9.0. For the enzyme assay, the final concentration of ALA was 3.6 mM. Incubation was started by adding 100 μL of the tissue preparation to a final volume of 400 μL. 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 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}.

Protein determination

In all the enzyme preparations, protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by one way analysis to determine the differences between the control group and the $HgCl_2$ exposure group, and by two way analysis to determine the differences between Hg-exposure times (10 and 15 days). The analyses were followed by Tukey's test when *F*-tests were significant ($P<0.05$). The results are the means ± S.D. of at least three independent replicates. For all parameters studied, correlation analyses were performed between Hg-exposure times (10 and 15 days).

Results and Discussion

Mercury is a highly toxic non-essential element and its dispersion in the environment is considered to be a global concern due to its persistent nature (Válega et al. 2008). In Figure 1, it was demonstrated that in 10- and 15-day-old cucumber seedlings, exposed to 0.5, 50, 250 and 500 μM $HgCl_2$, the Hg content in the cotyledons was, on average, 4, 7, 2,000 and 5,000-fold higher than the control, respectively. Moreover, in the Hg-exposed seedlings, Hg accumulated in the root system 8, 2,000, 18,000 and 41,000-fold more than in the control (**redesigned to Cargnelutti et al., 2006**).

As shown in Figure 2, the exposure of cucumber seedlings to 50 μM $HgCl_2$ for 10 days decreased the H_2O_2 content (60%), when compared to the control. The decrease in H_2O_2 content of plants exposed to heavy metals has been related to an increase in enzymatic (CAT and APX) (Cho and Park 2000; Tewari et al. 2008) and non-enzymatic antioxidants (NPSH and AsA) levels (Tiryakioglu et al. 2006). Thus, in the present study, it can be assumed that this decrease in H_2O_2 content may have been probably a result of activation of NPSH and AsA (Figs. 3B and 3C). It is known that the accumulation of ROS such as H_2O_2 is a

result of a complex balance between pro-oxidants and antioxidants. Whenever pro-oxidants are in greater abundance than antioxidants, either by an increase of the oxidative input or by a disruption of the defense systems, the outcome is a condition known as oxidative stress (Scandalios 1993), which leads to accumulation of H_2O_2 in the tissue. In fact, the results of the present investigation showed that only Hg-exposure at levels of 250 and 500 μM $HgCl_2$ resulted in increased H_2O_2 content in 10-day-old seedlings (Fig. 2), which may have been due to a balance between pro-oxidants and antioxidants, where antioxidants were not sufficient to contain the increase of the oxidative input.

Compared with 15-day-old seedlings, 10-day-old seedlings showed higher H_2O_2 levels for control seedlings and for all Hg treatments (Fig. 2). These results may be related to the reduced metabolism with increased plant age. The germination process is characterized by an accelerated metabolism, which culminates with an increasing production of ROS, such as H_2O_2. These changes in ROS levels might induce the oxidation of antioxidant compounds such as AsA. In addition, current evidence suggests that ROS can induce antioxidant enzymes. Therefore, in the present study, increased ROS production may have induced the antioxidant system at 10 days, which efficiently scavenged ROS by day 15, explaining the lower H_2O_2 levels.

The induction of activities of a particular group of enzymes is considered to play an important role in cellular defense strategies against oxidative stress (Van Assche and Clijsters 1990). Among the various enzymes involved in the abolishment of ROS, superoxide dismutase (SOD) can be considered a key enzyme. SOD activity varied as a function of both exposure time and Hg concentration (Fig. 3A). For 10-day-old seedlings, SOD activity decreased at

0.5, 250 and 500 μM $HgCl_2$, whereas it did not change at 50 μM $HgCl_2$. This behaviour may be considered rather erratic. The mechanism of SOD inhibition is not still clear, but an increase in the production of ROS, such as H_2O_2 may inactivate enzymes by oxidizing their thiol groups (Charles and Halliwell 1980; Bowler et al. 1994). However, 15-day-old seedlings showed the highest level of SOD activity at 50 μM $HgCl_2$ (Fig. 3A) and the lowest level at 250 μM $HgCl_2$ (Fig. 3A). Both an increase and decrease in SOD activity have been reported by Qiu et al. (2008). The increase in SOD activity may be linked to an increase in superoxide radical formation as well as to *de novo* synthesis of enzyme protein (Verma and Dubey 2003), which in turn may be associated with an induction of SOD genes caused by superoxide-mediated signal transduction (Fatima and Ahamad 2005). Reduction of SOD activity in 10 and 15-day-old cucumber seedlings at 250 μM $HgCl_2$ indicated that the oxygen scavenging function of SOD was impaired, suggesting accumulation of superoxide anion. Inhibition of SOD activity in response to heavy metals stress has also been found in *Hydrilla verticillata* (Panda and Khan 2004), rice cultivars (Hassan et al. 2005) and in pea plants (Sandalio et al. 2001). The decline in SOD activity might be also attributed to deficiency of metals induced by Hg (Boening 2000) being the cofactors used by respective forms of this enzyme as well as to its inactivation by ROS (Hodgson and Fridovich 1975). Moreover, SOD levels for 10- and 15-day-old seedlings were positively correlated (0.77) (Table 1).

For 10-day-old cucumber seedlings, NPSH content increased for all Hg treatments (Fig. 3A). In contrast, at 15 days NPSH content increased (232%) only at 250 μM $HgCl_2$. Ortega-Villasante et al. (2005) also found increased NPSH content in *Medicago sativa* after a short exposure period. Such results

could be due to the reduction of metal availability by a cytoplasmatic detoxification mechanism. In agreement with Patra et al. (2004), Hg possesses a high affinity for NPSH and the Hg-induced increase in GSH levels might reflect a defensive mechanism against oxidative stress triggered by Hg (Zhou et al. 2007). Moreover, GSH is the substrate for PCs synthesis. PCs are involved in the cellular detoxification mechanism due to their ability to form stable metal-PC complexes (Scarano and Morelli 2002). Rellán-Álvarez et al. (2006) reported that Hg has reduced capacity to induce the synthesis of PCs, in comparison with other toxic elements such as As or Cd. However, to a certain extent PCs are accumulated upon Hg exposure (Iglesia-Turiño et al. 2006). The increase in antioxidant levels decreases the amount of oxidized biomolecules, but these detoxicants might be not be sufficient to protect cells against injury (Ranieri et al. 1993). In fact, in the present study, an abrupt increase in the NPSH concentration at 250 μM $HgCl_2$ both in 10- and 15-day-old seedlings showed an apparently poor response in the H_2O_2 content. Glutathione is the major constituent of NPSH in most plant cells. It is generally present at concentrations of 2 to 3 mM in various plant tissues, primarily in its reduced form (GSH) (Noctor et al. 2002). Because GSH is a major cellular antioxidant, it is regarded as an indicator of a cellular redox state and may indirectly influence many fundamental cellular processes. Glutathione also seems to be important in defending against environmental stress, including that caused by heavy metals (Ball et al. 2004). Therefore, the higher accumulation of NPSH at 250 μM might be attributed to a hormetic dose response (Calabrese and Blain 2005). Moreover, studies have shown that GSH accumulates in response to increased ROS generation (Schmidt and Kunert 1986; Gupta et al. 1991). In the

present study, H_2O_2 was the only ROS measured. Therefore, other ROS such as the superoxide anion and hydroxyl radical may have been present at 250 μM $HgCl_2$, stimulating NPSH accumulation. In addition, decreased SOD activity at 250 μM $HgCl_2$ may be evidence of superoxide anion accumulation both at 10 and 15 days.

In the present investigation, AsA levels increased in all Hg-treatments at 10 and 15 days (Fig. 3C). The maximum accumulation of AsA was 232.6 μg AsA g^{-1} fresh weight in 10-day-old seedlings treated with 500 μM $HgCl_2$ (Fig. 3C). Similarly, Sinha et al. (1996) reported an increase in AsA content in *Baccopa monieri* plants treated with Hg, showing a significant increase in AsA content during the initial period of metal exposure. For Hg-treated cucumber seedlings, as AsA is an important reductant in the removal of H_2O_2 (Noctor and Foyer 1998), therefore protecting the integrity of –SH groups (Rai 1979). Moreover, the positive correlation observed between the exposure times (10 and 15 days) for SOD activity, AsA and NPSH contents (Table 1) indicates that Hg caused a stress pattern that was maintained throughout the period of Hg-exposure. Contrary to AsA levels, carotenoid levels were reduced by about 67% at the highest Hg concentration at 10 days (Fig. 3D). Hg decreased the levels of photosynthetic pigments, chlorophylls and carotenoids at 15 days, and strongly inhibited the photosynthetic electron transport chain in plants (Bernier et al. 1993; Bernier and Carpentier 1995). In addition, carotenoid content for 10- and 15-day-old of cucumber seedlings were positively correlated (r = 0.71) (Table 1).

Hg-exposure induced a significant reduction of δ-ALA-D activity (Fig. 4), which varied with the time of exposure and the concentration of exogenous Hg.

At the highest concentration of Hg (500 μM $HgCl_2$), δ-ALA-D activity was decreased by 99% and 95%, at 10 and 15 days respectively, when compared to the control. Such an inhibitory effect has been attributed to the high bonding affinity between Hg and thiol groups (Clarkson 1997), which are essential for δ-ALA-D activity (Barbosa et al. 1998). However, the cotyledons Hg concentration may be not have been large enough to exert a direct inhibition of δ-ALA-D and the inhibition this enzyme may occur due to other factors such as the ROS presence. Indeed, Cargnelutti et al. (2006) reported that the chlorophyll level was reduced at Hg levels above 50 μM. Due to the sensitivity of δ-ALA-D to Hg treatment, the activity of this enzyme may be a good environmental marker for the presence of mercury in plants.

In conclusion, the decreased SOD and δ-ALA-D activities observed may have been related to the increase in tissue Hg content in cucumber seedlings. Moreover, Hg stress increased the levels of AsA and NPSH in seedlings of *C. sativus*. These antioxidant systems may play an important role in making Hg less harmful, and consequently making the seedlings tolerant at low concentrations. However, the antioxidant systems were not able to protect from the toxicity caused by higher levels of Hg, resulting in the negative effects observed in the growth of cucumber seedlings.

In addition, as reflected by some of the results, a similar pattern was obtained in seedlings grown for 10 and 15 days. This may indicate that the physiological response reaches a plateau at ten days (Ortega-Villasante et al. 2005). Thus, in future studies the exposure time should be shortened in order to examine the effects of Hg in the early stages of exposure. The results may

contribute to a better understanding of the oxidative stress conditions generated by Hg in plants.

Acknowledgements

The authors wish to thank to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação e Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa de Estado do Rio Grande do Sul (FAPERGS).

References

Ball L, Accotto GP, Bechtold U, Creissen G, Funck D, Jimenez A, Kular B, Leyland N, Mejia-Carranza J, Reynolds H, et al (2004) Evidence for a direct link between glutathione biosynthesis and stress defense gene expression in *Arabidopsis*. Plant Cell 16:2448–2462

Barbosa NBV, Rocha JBT, Zeni G, Emanuelli T, Beque MC, Braga AL (1998) Effect of inorganic forms of selenium on delta-aminolevulinate dehydratase from liver, kidney and brain of adult rats. Toxicol Appl Pharmacol 149:143-253

Bernier M, Carpentier R (1995) The action of mercury on the binding of the extrinsic polypeptides associated with the water oxidizing complex of photosystem II. FEBS Lett 360:251–254

Bernier M, Popovic R, Carpentier R (1993) Mercury inhibition at the donor side of photosystem II is reversed by chloride. FEBS Lett 321:19–23

Boening DW (2000) Ecological Effects, Transport, and Fate of Mercury: a general review. Chemosphere 40:1335-1351

Bowler C, Camp VW, Montagu V, Inzé D (1994) Superoxide dismutase in plants. CRC Plant Sc 13:199–218

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Calabrese EJ, Blain R (2005) The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. Toxicol Applied Pharmacol 202:289–301

Cargnelutti D, Tabaldi LA, Spanevello RM, Jucoski GO, Battisti V, Redin M, Linares CEB, Dressler VL, Flores EMM, Nicoloso FT, Morsch VM, Schetinger MRC (2006) Mercury toxicity induces oxidative stress in growing cucumber seedlings. Chemosphere 65:999–1006

Cavallini A, Natali L, Durante M, Maserti B (1999) Mercury uptake, distribution and DNA affinity in durum wheat (*Triticum durum* Desf.) plants. Sci Total Environ 243:119-127

Charles SA, Halliwell B (1980) Effects of hydrogen peroxide on spinach (*Spinacia oleracea*) chloroplast fructose biphosphatase. Biochem J 189:373–376

Cho U-H, Park JO (2000). Mercury-induced oxidative stress in tomato seedlings. Plant Sci 156:1-9

Clarkson TW (1997) The toxicology of mercury. Crit Rev Clin Lab Sci 34:369-403

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53:159–182

Dipierro N, Mondelli D, Paciolla C, Brunetti G, Dipierro S (2005) Changes in the ascorbate system in the response of pumpkin (*Cucurbita pepo* L.) roots to aluminium stress. J Plant Physiol 162:529–536

Ellman GL (1959). Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77

Fatima RA, Ahmad M (2005) Certain antioxidative enzymes of *Allium cepa* as biomarkers for the detection of toxic heavy metals in wastewater. Sci Total Environ 346:256-273

Foyer C, Noctor G (2005) Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiology responses. Current Perspective Assay. Plant Cell 17:1866–1875

Freedman B, Hutchinson TC (1981) Sources of metal and elemental contaminations of terrestrial environments. In: Lepp NW (ed.) Effect of heavy metal pollution on plants: Metals in environment. London and New Jersey, p 35–94.

Gichner T, Patková Z, Száková J, Demnerová J (2006) Toxicity and DNA damage in tobacco and potato plants growing on soil polluted with heavy metals Ecotoxicol Environ Saf 65:420–426

Gibson KD, Neuberger A, Scott JJ (1955) The purification and properties of delta-aminolevulinic acid dehydratase. Biochem J 61:618 – 676

Gonçalves JF, Becker A.G, Cargnelutti D, Tabaldi LA, Pereira LB, Battisti V, Spanevello RM, Morsch VM, Nicoloso FT, Schetinger MRC (2007) Cadmium toxicity causes oxidative stress and induces response of the antioxidant system in cucumber seedlings. Braz J Plant Physiol 19:223-232

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53:1–11

Hassan MJ, Shao G, Zhang G (2005) Influence of cadmium toxicity on growth and antioxidant enzyme activity in rice cultivars with different grain cadmium accumulation. J Plant Nutrit 28:1259 - 1270

Hiscox JD, Israelstam GF (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57:1132-1334

Hodgson MA, Fridovich I (1975) The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: inactivation of the enzyme. Biochemistry 40:5294–5303

Iglesia-Turiño S, Febrero A, Jauregui O, Caldelas C, Araus JL, Bort J (2006) Detection and quantification of unbound phytochelatin 2 in plant extracts of *Brassica napus* grown with different levels of mercury. Plant Physiol 142:742-749

Israr M, Sahi SV (2006) Antioxidative responses to mercury in the cell cultures of *Sesbania drummondii*. Plant Physiol Biochem 44:590–595

Israr M, Sahi S, Datta R, Sarkar D (2006) Bioaccumulation and physiological effects of mercury in *Sesbania drummondii*. Chemosphere 65:591-598

Jacques-Silva MC, Nogueira CW, Broch LC, Flores EMM, Rocha JBT (2001) Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. Pharmacol Toxicol 88:119–125

Jaffe Jaffe EK, Kervinen J, Dunbrack JR, Litwin S, Martins J, Scarrow RC, Volim M, Yeung AT, Yonn E (2000) Pophobilinogen synthase from pea: Expression from an artificial gene, kinetic characterization, and novel implications for subunit interactions. Biochemistry 39:9018-9029

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomemranes. Meth Enzymol 148:350–82

Loreto F, Velikova V (2001) Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quences ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiol 127:1781–7

Ma C (1998) Hg harm on cell membrane of rape leaf and cell endogenous protection effect. Ying Yong Sheng Tai Xue Bao 9:323-326

Matés JM (2000) Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 153:83–104

Mc Cord JM, Fridovich I (1969) Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244:6049–6055

Morsch VM, Schetinger MRC, Martins AF, Rocha JBT (2002) Effects of cadmium, lead, mercury and zinc on δ-aminolevulinic acid dehydratase activity from radish leaves. Biol Plant 45:85–89

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tabbaco tissue culture. Plant Physiol 15:473-497

Noctor G, Foyer CH (1998) Ascorbate and gluthatione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49:249–279

Noctor G, Gornez L, Vanacker H, Foyer CH (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signaling. J Exp Bot 53:1283–1304

Noriega GO, Balestrasse KB, Batlle A, Tomaro ML (2007) Cadmium induced oxidative stress in soybean plants also by the accumulation of δ-aminolevulinic acid. Biometals 20:841-851

Ortega-Villasante C, Rellán-Álvarez R, Del Campo FF, Carpena-Ruiz RO, Hernández LE (2005) Cellular damage induced by cadmium and mercury in *Medicago sativa*. J Exp Boy 56:2239–2251

Ortega-Villasante C, Hernández LE, Rellán-Álvarez R, Del Campo FF, Carpena-Ruiz RO (2007) Rapid alteration of cellular redox homeostasis upon exposure to cadmium and mercury in alfalfa seedlings. New Phytologist 176:96–107

Panda SK, Khan MH (2004) Changes in growth and superoxide dismutase activity in *Hydrilla verticillata* L. under abiotic stress. Braz J Plant Physiol 16:115-118

Patra M, Bhowmik N, Bandopadhyay B, Sharma A (2004) Comparison of mercury systems and the development of genetic tolerance. Environ Exp Bot Review 52:199-223

Pereira LB, Tabaldi LA, Gonçalves JF, Jucoski JO, Pauletto MM, Weis SN, Nicoloso FT, Borher D, Rocha JBT, Schetinger MRC (2006) Effect of aluminum on δ-aminolevulinic acid dehydratase (ALA-D) and the development of cucumber (*Cucumis sativus*). Environ Exp Bot 57:106–115

Polyakov NE, Leshina TV, Konovalova TA, Kispert LV (2001) Carotenoids as scavengers of free radicals in a Fenton reaction: antioxidants or pro-oxidants? Free Radic Biol Med 31:398–404

Qiu R-L, Zhao X, Tang Y-T, Yu F-M, Hu P-J (2008) Antioxidative response to Cd in a newly discovered cadmium hyperaccumulator, *Arabis paniculata* F. Chemosphere 74:6-12

Rai LC (1979) Mercury toxicity to *Chlorella vulgaris*. I. Reduction of toxicity by ascorbic acid and reduced glutathione (GSH). Phykos 18:105–109

Ranieri A, Lencioni L, Schenone G, Soldatini GF (1993) Gluthatione-ascorbic acid cycle in pumpkin plants grown under polluted air in open-top chambers. J Plant Physiol 142:286–290

Rellán-álvarez R, Ortega-Villasante C, Álvarez-Fernández A, Campo FF, Hernández LE (2006) Stress Responses of *Zea mays* to Cadmium and mercury. Plant Soil 279:41–50

Rocha JBT, Pereira ME, Emanuelli T, Christofari RS, Souza D (1995) Effects of methylmercury exposure during the second stage of rapid postnatal brain growth on delta-aminolevulinic acid dehydratase (ALA-D) activity in brain, liver and blood of suckling rats. Toxicology 100:27–37

Romero-Puertas MC, Palma JM, Gómez M, Río LA del, Sandalio LM (2002) Cadmium causes the oxidative modification of proteins in pea plants. Plant Cell Environ 25:677–686

Sassa S (1982) Delta-aminolevulinic acid dehydratase assay. Enzyme 28, 133–145.

Sandalio, L.M., Dalurzo, H.C., Gomez, M., Romero-Puertas, M.C., Río, L.A. del., (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J Exp Bot 52:2115–2126

Scandalios JG (1993) Oxygen stress and superoxide dismutase. Plant Physiol 101:7–12

Scarano G, Morelli E (2002) Characterization of cadmium- and lead-phytochelatin complexes formed in a marine microalga in response to metal exposure. Biometals 15:145–151

Schmidt A, Kunert KJ (1986) Lipid peroxidation in higher plants: the role of glutathione reductase. Plant Physiol 82:700–2

Schützendübel A, Schwanz P, Teichmann T, Gross K, Langenfeld-Heyser R, Goldbold DL, Polle A (2001) Cadmium-induced changes in antioxidative

systems, hydrogen peroxide content, and differentiation in Scots pine roots. Plant Physiol 127:887–898

Segura-Muñoz SI, Oliveira AS, Nikaido M, Trevilato TMB, Bocio A, Takayanagui AMM, Dimingo JL (2006) Metal levels in sugar cane (*Saccharum* spp.) samples from an area under the influence of a municipal landfill and a medical waste treatment system in Brazil. Environ Sci Int 32:52–57

Sen Gupta A, Alscher RG, McCune D (1991) Response of photosynthesis and cellular antioxidants to ozone in *Populus* leaves. Plant Physiol 96:650– 55

Sinha S, Gupta M, Chandra P (1996) Bioccumulation and biochemical effects on mercury in the plant *Bacopa monnieri* (L.). Environ Toxicol Water Qual 11:105–112

Street RA, Kulkarni MG, Stirk WA, Southway C, Van Staden J (2007) Toxicity of Metal Elements on Germination and Seedling Growth of Widely Used Medicinal Plants Belonging to Hyacinthaceae. Bull Environ Contam Toxicol 79:371–376

Tewari A, Singh R, Singh NK, Rai UN (2008) Amelioration of municipal sludge *by Pistia stratiotes* L.: Role of antioxidant enzymes in detoxification of metals. Bioresour Technol 99:8715–8721

Tiryakioglu M, Eker S, Ozkutlu F, Husted S, Cakmak I (2006) Antioxidant defense system and cadmium uptake in barley genotypes differing in cadmium tolerance. J Trace Elem Med Biol 20:181-189

Válega M, Lima AIG, Figueira EMAP, Pereira E, Pardal MA, Duarte AC (2008) Mercury intracellular partitioning and chelation in a salt marsh plant, *Halimione portulacoides* (L.) Aellen: Strategies underlying tolerance in environmental exposure. Chemosphere, *in press*

Van Assche F, Clijsters H (1990) Effects of metals on enzyme activity in plants. Plant Cell Environ 13:195-206

Verma S, dubey RS (2003) Lead toxicity inducer lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci 164:645-655

Zang WH, Tyerman SD (1999) Inhibition of water channels by $HgCl_2$ in intact wheat root cells. Plant Physiol 120:849-857

Zhou ZS, Huang SQ, Guo K, Mehta SK, Zhang PC, Yang ZM (2007) Metabolic adaptations to mercury-induced oxidative stress in roots of *Medicago sativa* L. J Inorg Biochem 101:1–9

Zhou ZS, Wang SJ, Yang ZM (2008) Biological detection and analysis of mercury toxicity to alfalfa (*Medicago sativa*) plants. Chemosphere 70:1500-1509

Table 1

Correlation coefficient between Hg-exposure times (10 and 15 days) for various studied parameters of cucumber seedlings.

Parameters	Pearson's coefficient
ELP content	- 0.15†
SOD activity	+ 0.77**
δ-ALA-D activity	+ 0.24†
carotenoids content	+ 0.71*
H_2O_2 content	- 0.27†
ASA content	+ 0.95**
NPSH content	+ 0.92**

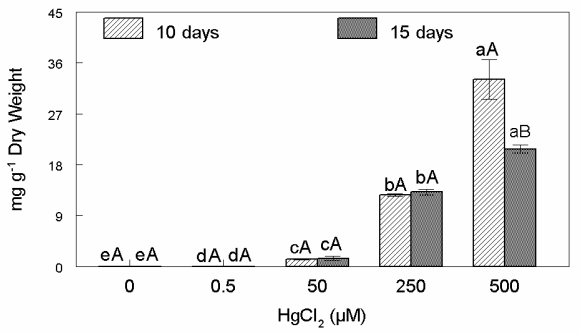
†Statistically non-significant.

*Statistically significant at $p<0.005$.

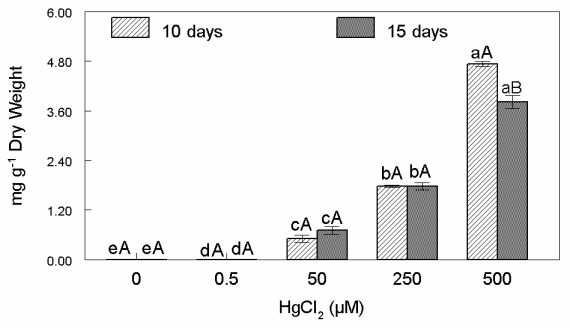
**Statistically significant at $p=0.001$.

Figure 1

(A)


10 days
15 days
mg g-1 Dry Weight
45
36
27
18
9
0
eA eA
dA dA
cA cA
bA bA
aA
aB
0
0.5
50
250
500
HgCl2 (µM)

(B)


10 days
15 days
mg g-1 Dry Weight
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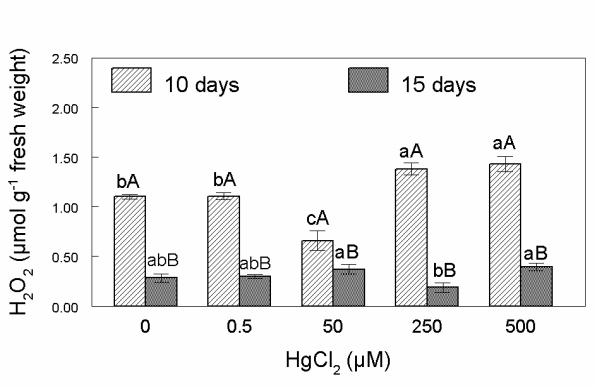
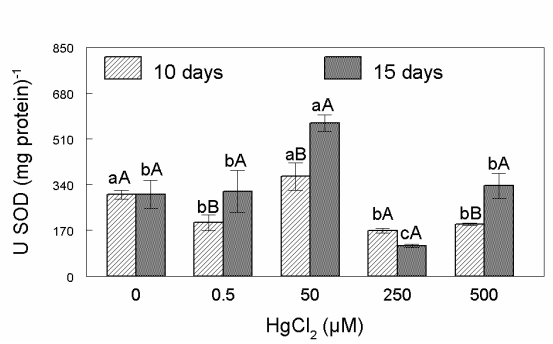
4.80
3.60
2.40
1.20
0.00
eA eA
dA dA
cA cA
bA bA
aA
aB
0
0.5
50
250
500
HgCl2 (µM)

Figure 2

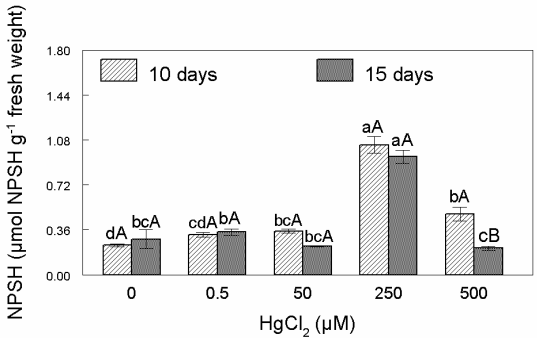

10 days
15 days
H_2O_2 (µmol g^{-1} fresh weight)
2.50
2.00
1.50
1.00
0.50
0.00
bA
abB
bA
abB
cA
aB
aA
bB
aA
aB
0
0.5
50
250
500
$HgCl_2$ (µM)

Figures 3

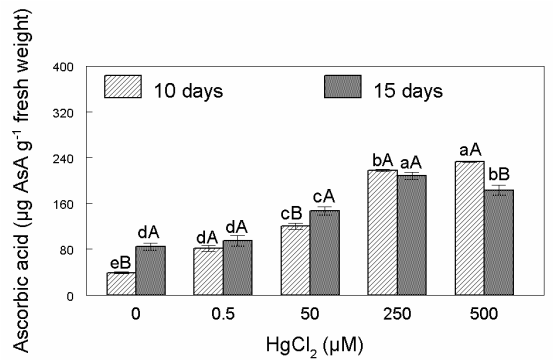
(A)



(B)



(C)



(D)

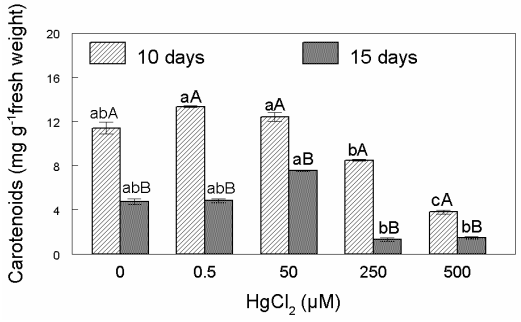
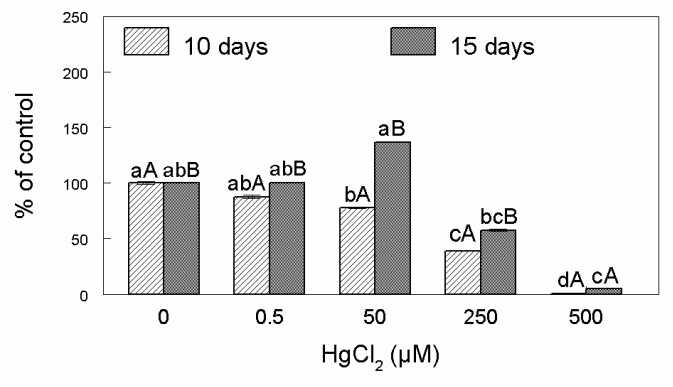


Figure 4


250
200
150
100
50
0
% of control
10 days
15 days
aA abB
abA abB
bA aB
cA bcB
dA cA
0
0.5
50
250
500
HgCl2 (µM)

LEGEND OF THE FIGURES

Figure 1. Effect of increasing concentration of $HgCl_2$ in the root (A) and cotyledons (B) mercury content at 10- and 15-days- old cucumber seedlings. Lower-case letter compare means between Hg levels and upper-case letters compare means between time exposure (10 and 15 days) to $p<0.05$. Data represent the mean ± S.D. of three different experiments.

Figure 2. Effect of increasing concentration of $HgCl_2$ on the hydrogen peroxide content at 10- and 15-days- old cucumber seedlings. Lower-case letter compare means between Hg levels and upper-case letters compare means between time exposure (10 and 15 days) to $p<0.05$. Data represent the mean ± S.D. of three different experiments.

Figure 3. Effect of increasing concentration of $HgCl_2$ on the superoxide dismutase activity (A), and non-protein thiols (B), ascorbic acid (C) carotenoid content (D) of 10- and 15-days- old cucumber seedlings. Lower-case letter compare means between Hg levels and upper-case letters compare means between time exposure (10 and 15 days) to $p<0.05$. Data represent the mean ± S.D. of three different experiments.

Figure 4. Effect of increasing concentration of $HgCl_2$ on delta-aminolevulinic acid dehydratase activity of 10- and 15-days- old cucumber seedlings. Lowercase letter compare means between Hg levels and upper-case letters compare means between time exposure (10 and 15 days) to $p<0.05$. Data represent the mean ±

S.D. of three different experiments. The control specific activity (without mercury) that represents 100% was 11.98± 1.17 and 3.37±0.1 nmol PBG min^{-1} mg^{-1} protein, for 10 and 15 days, respectively.

3.1.2. MANUSCRITOS CIENTÍFICOS: CAPÍTULO II

Interação entre mercúrio e zinco em parâmetros bioquímicos e fisiológicos em híbridos de milho (*Zea mays* L.).

3.1.2.1. Manuscrito 2 - Effect of Hg on δ-Aminolevulinic acid dehydratase activity and growth in three hybrids of maize (*Zea mays* L.)

3.1.2.2. Manuscrito 3 - Zinc alleviates mercury-induced oxidative stress in maize

3.1.2.3. Manuscrito 4 - Zinc protects maize against inhibition on growth and δ-aminolevulic acid dehydratase activity induced by mercury

3.1.2.1. Manuscrito II - Efeito do mercúrio na atividade da δ-Aminolevulinato desidratase e no crescimento de três hibridos de milho (*Zea mayz* L.)

Manuscrito 2

Effect of Hg on δ-Aminolevulinic acid dehydratase activity and growth in three hybrids of maize (*Zea mays* L.)

Denise Cargnelutti, Fernando T. Nicoloso, Liana V. Rossato, Nicéia S. Calgaroto, Luciane B. Pereira, Luciane A. Tabaldi, Gustavo R. Thomé, Vera M. Morsch, Fabiane G. Antes, Valderi L. Dressler, Érico M. M. Flores, Maria R.C. Schetinger

(Em revisão na Revista Plant and Soil)

Effect of Hg on δ-Aminolevulinic acid dehydratase activity and growth in three hybrids of maize (*Zea mays* L.)

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Abstract

In this study, the effects of mercury (Hg) on δ-ALA-D activity, growth and tissue Hg concentration of three maize hybrids (BR205, 30F71 and 32R21) exposed to Hg (0 - 100 μM) in hydroponic solution were investigated, as well the in vitro effect of Hg on δ-ALA-D activity in the BR205. Root Hg concentration in 32R21 and BR205 was greater than that in 30F71 at 100 μM Hg, whereas shoot Hg concentration did not differ between hybrids. The length of shoot and root of the three hybrids decreased in a similar fashion with increasing Hg concentrations. In vivo δ-ALA-D activity decreased in BR205 and 32R21, whereas it increased in 30F71 at 25 and 50 μM Hg. Therefore, δ-ALA-D activity may not be a good biomarker to indicate Hg toxicity in maize. On the other hand, in vitro studies showed that Hg was a potent inhibitor of δ-ALA-D activity in BR205.

Keywords*: Zea mays* L.; Accumulation; Mercury; delta-Aminolevulinic acid dehydratase

Abbreviations

δ-ALA-D – delta-Aminolevulinic acid dehidratase;

ALA – 5-aminolevulinic acid;

DTT – dithiotreitol;

EDTA – ethylenediaminetreaacetic acid;

Hg – mercury;

H_2O_2 – hydrogen peroxide;

Ki – inhibition constant;

K_m – Michaels and Menten constant;

PBG – porphobilinogen;

PVP - polyvinylpyrrolidone;

ROS – reactive oxygen species;

Zn – zinc;

Introduction

Mercury (Hg) is one of the most toxic heavy metals released in the environment (Zilloux et al. 1993; Shaolin and David 1997) through both natural and human processes. Most commonly, the gaseous form is released into the atmosphere, where from it is deposited into land and water, causing pollution. Mercury is also deposited into soils, where concentrations of this chemical element can be high. In soil, Hg can be transformed and evaporate, contributing to its circulation. It can also be absorbed by microorganisms and plants, thus penetrating into the food chain (Kabata-Pendias and Pendias 2001).

Although the availability of soil Hg to plants is low, several studies have shown that plants accumulate Hg when they are exposed to Hg-contaminated soils. Mercury pollution of soils causes toxicity to plants and long-term effects on soil fertility (Wang and Greger 2004). Among other effects, Hg can induce inhibition of plant growth, disturbances in water and nutrient uptake, oxidative stress and significant alteration of enzymatic activity (Cho and Park 2000; Patra and Sharma 2000; Ortega-Villasante et al. 2005, Cargnelutti et al. 2006; López-Berenguer et al. 2006). One important enzyme that may be affected is δ-Aminolevulinic acid dehydratase (δ-ALA-D), which catalyzes the synthesis of porphobilinogen from two molecules of δ-aminolevulinic acid (ALA) (Gibson et

al. 1955) and is a key enzyme of the biosynthesis pathway, leading to the formation of porphyrins, hemes and chlorophylls in higher plants (Jaffe et al. 2000), making it essential for adequate aerobic metabolism and photosynthesis. Furthermore, in several systems, this enzyme has been found to play a major role in the regulation of chlorophyll biosynthesis (Schneider 1976). Rocha et al. (1995) and Morsch et al. (2002) reported that δ-ALA-D is sensitive to heavy metals, such as Hg, due to its sulfhydrylic nature and, therefore, its activity could be used as a biomarker of metal toxicity (Pereira et al. 2006; Vanparys et al. 2008), such as Hg.

Maize is an important agricultural crop worldwide that has been used in many studies of elemental pollution (Sudová and Vosátka 2007; Abbas and Meharg 2008; Wang et al. 2008). Therefore, the purpose of this study was to evaluate the effects of different concentrations of Hg on the in vivo and in vitro leaf δ-ALA-D activity of three maize (*Zea mays* L.) hybrids as a biomarker for Hg toxicity. Moreover, the effect of Hg on growth and tissue Hg concentration were evaluated in order to verify whether there is a correlation between these parameters and Hg-mediated alterations in δ-ALA-D activity.

Material and methods

Plant material and growth conditions

Three hybrids (BR205, 32R21 and 30F75) were evaluated. The BR205 (duple hybrid; obtained from Empresa Brasileira de Pesquisa Agropecuária, EMBRAPA, Minas Gerais, Brazil) is adapted to tropical regions of Brazil,

presents precocity and high productivity, as well as tolerance to aluminum toxicity and water stress. Moreover, it was shown to have high capacity for uptake of mineral elements from soils (Silva et al. 2005). The 32R21 (simple hybrid) and 30F75 (triple hybrid; both obtained from PIONNER company) hybrids were chosen due to their high biomass production and because they have been extensively cultivated in the southern region of Brazil. Seeds were germinated in plastic boxes on filter paper. Seven-day old uniform plantlets were transferred into plastic boxes (10 L) filled with aerated nutrient solution of low ionic strength. The nutrient solution was prepared as described in Tabaldi et al. (2007) (in mg L^{-1}): 8.31 of N; 0.754 of P; 1.154 of S; 9.76 of Ca; 2.37 of Mg; 1.05 of K; 17.68 of Cl; 0.027 of B; 0.005 of Mo; 0.001 of Ni; 0.013 of Zn; 0.003 of Cu; 0.011 of Mn and 0.268 of Fe ($FeSO_4$/Na-EDTA). The plantlets were acclimated for seven days before addition of the treatments. Throughout the acclimation and treatment periods, the plants were grown in a growth chamber at 25 ± 2ºC on a 16/8-h light/dark cycle with 35 μmol m^{-2} s^{-1} of irradiance. The solution pH was adjusted daily to 5.4 ± 0.1 with HCl or NaOH solutions (0.1 M). Treatments consisted of the addition of 0, 25, 50, 75 or 100 μM of Hg^{2+} as $HgCl_2$. Plants remained under treatment for 5 days. At harvest, the plants were divided into shoot and roots. Roots were rinsed twice with distilled water. Subsequently, growth, Hg concentration and δ-ALA-D activity were determined.

Growth analysis

Maize growth was determined by measuring the length of the root system (Tennant 1975) and of the shoot (measured with a ruler), both expressed in cm

plant^{-1}. To obtain dry weight, the plants were left at 65ºC until reaching a constant weight (for approximately two weeks). Dry weight was expressed as g plant^{-1}.

Mercury (Hg) concentration

Between 20 and 300 mg of shoot and root samples were digested with 5 mL HNO_3 and 0.2 mL H_2O in closed Teflon vessels, which were heated at 100ºC for 3h in a digester block (Tecnal TE 007D). The samples were then diluted to 50 mL with high-purity water. Mercury concentrations were determined using a Varian Atomic Absorption Spectrophotometer (Spectr AA 600, Australia) equipped with a vapor generative accessory (Varian VGA-76). The tissue Hg concentration was expressed as μg g^{-1} dry weight. Peach leaves NIST 1547 and apple leaves NIST 1515 (National Institute of Standards and Technology, Gaithersburg, USA), used as certified reference material, were analyzed to evaluate the accuracy of the sample preparation and Hg determination methods.

Estimation of δ-Aminolevulinic acid dehydratase (ALA-D; E.C. 4.2.1.24) activity

Maize leaves 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 x 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 dithiothreitol (DTT). ALA-D activity was assayed as described by Morsch et al. (2002) by

measuring the rate of porphobilinogen (PBG) formation. The incubation medium for the assays contained 100 mM Tris-HCl buffer, pH 9.0. For the enzyme assay, the final concentration of ALA was 3.6 mM. Incubation was started by adding 100 μL of the tissue preparation in a final volume of 400 μL. The reaction product was determined with the Ehrlich reagent at 555 nm using a molar absorption coefficient of 6.1×10^4 L mol^{-1} cm^{-1} (Sassa 1982) for the Ehrlich-porphobilinogen salt. ALA-D activity was expressed as nmol PBG mg^{-1} protein h^{-1}.

Kinetic determinations

The kinetics of the interaction between Hg and δ-ALA-D were investigated using the Lineweaver and Burk (1934), double reciprocal plot. The x-axis represents the inverse of the aminolevulic acid concentration (1/S) and the y-axis represents the inverse of enzyme activity (1/V). Substrate concentrations ranged from 0.36 mM to 36 mM in the absence and in the presence of Hg (40–60 μM). K_m values were obtained by two different estimations, 1/V vs. 1/S (Lineweaver and Burk 1934) and V vs. V/S (Hofstee 1952; Dowd and Riggs 1965). The Ki average values were obtained using Cornish–Bowden plots of S/V vs. [I] and the Dixon and Webb (1964) plot using 1/V vs. [I]. IC_{50} was calculated according to the Dixon and Webb (1964) plot using 1/V vs. [I].

Protein determination

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 mL^{-1}.

Statistical analysis

Data were submitted to variance analyses (two-way ANOVA) and treatment means were compared by Duncan range at 5% of error probability using the SOC statistic package (Software Científico: NTIA/EMBRAPA). Treatments were presented as mean ± S.D. of three replicates.

Results

Effects of Hg on seedling growth

The effects of Hg on root and shoot length varied among the different hybrids and Hg treatments (Figs. 1A and B). Mercury had an inhibitory effect on root elongation of BR205, 30F71 and 32R21 at all Hg concentrations after 5 days of exposure. Shoot length of the three maize hybrids was similarly reduced by Hg (Fig. 1B). At the highest Hg concentration tested (100 μM), shoot length of BR205, 30F71 and 32R21 were 36%, 24% and 27% lower, respectively, than that of the respective controls.

Root dry weight (RDW) was also reduced by Hg (Fig. 1C), where at 75 and 100 μM, 30F71 and 32R21 hybrids were more negatively affected than

BR205. Conversely, shoot dry weight (SDW) of the three hybrids responded differently to Hg (Fig. 1D). The 32R21 hybrid showed the least reduction of SDW, where no significant difference was found at levels of Hg ranging from 25 to 100 μM. The response of SDW in the 30F71 hybrid was linear and negative, whereas in the BR205 hybrid it decreased only at 50 and 100 μM Hg, when compared to the control.

Tissue Hg concentration

The results obtained for Hg concentration in the certified reference materials analyzed were 0.032 ± 0.003 $\mu g\ g^{-1}$ and 0.043 ± 0.002 $\mu g\ g^{-1}$ for NIST 1547 and NIST 1515, respectively. These values are in good agreement with the certified Hg concentrations which are, respectively, 0.031 ± 0.007 $\mu g\ g^{-1}$ and 0.044 ± 0.004 $\mu g\ g^{-1}$ for NIST 1547 and NIST 1515. Mercury concentration in roots and shoots of BR205, 30F71 and 32R21 hybrids was concentration-dependent (Table 1). The Hg concentration in roots and shoots of the three maize hybrids increased significantly with increasing Hg concentrations in the nutrient solution. Mercury accumulated mainly in the roots, and small amounts were transferred to shoots. At the highest Hg concentration tested (100 μM), root Hg concentration of the 32R21 hybrid was greater than that of BR205 and 30F71. On the other hand, no significant difference in the shoot Hg concentration was found between the three genotypes in all Hg treatments.

In vivo δ-Aminolevulinic acid dehydratase (ALA-D; E.C. 4.2.1.24) activity

Leave δ-ALA-D activities measured in the three maize hybrids were quite different (Fig. 2). In the presence of Hg, the 30F71 hybrid showed higher δ-ALA-D activity than that of the other two hybrids. Moreover, at 25 and 50 μM of Hg, δ-ALA-D activity increased in 30F71 when compared to the control, whereas it decreased in BR205 and was not altered in 32R21. δ-ALA-D activity decreased linearly in the BR205 hybrid, whereas it was only reduced in 32R21 upon addition of Hg at levels 75 and 100 μM. On the basis of these results, we studied the mechanism by which Hg inhibited δ-ALA-D activity in the BR205 hybrid, where the highest reduction was observed.

Kinetic determinations

Mercury inhibited the in vitro δ-ALA-D activity in BR205 (Fig. 3A). Statistical analysis revealed a concentration dependent inhibition. Analysis of kinetic data indicated that the inhibition of δ-ALA-D activity was mixed. The Km and V_{max} values, measured by 1/V versus 1/S, were increased and decreased, respectively, confirming the mixed-type inhibition (Fig. 3B). The IC_{50} obtained by the method of the Dixon plot (1/V versus [I]) with saturable substrate concentration was 47.22 μM. The average value of inhibition constant (Ki) obtained using Cornish-Bowden plots of S/V versus [I] and the Dixon and Webb (1964) plot using 1/V vs. [I] were 140.14 (Table 2).

Discussion

In the present study there was a high uptake of Hg by all maize hybrids used. It is generally accepted that plant uptake of toxic ions like Hg^{2+} takes

place via the same uptake process used for essential micronutrient ions (Patra and Sharma 2000). Hg^{2+} preferentially binds with amino acids that contain sulfur- and nitrogen- rich ligants (Nieboer and Richardson 1980). In the present study, the Hg concentration in maize hybrids increased with increasing amounts of Hg over 5 days of exposure. At 100 µM Hg, the Hg concentration in roots (6,359 and 4,936 µg g^{-1} DW for 32R21 and BR205, respectively) was higher than in shoot. Similar results were reported for other maize hybrids (*Zea mays* cv. Dekalb DK 604) (Rellán-Álvarez et al. 2006) treated with Hg. Moreover, the results of the present study showed that there were high Hg concentrations in both shoots and roots in the controls (Table 1) which may be related at leaf absorption of gaseous Hg in the climate chamber, because Hg may be volatilized into air (Ericksen et al. 2003; Schwesig and Krebs 2003; Caille et al. 2005) and the plants can absorb elemental Hg through stomatal pores in leaves (Hanson et al., 1995).

In addition, the reported differences in both root and shoot Hg concentration might be explained by the fact that one of the normal functions of roots is to selectively acquire ions from the soil solution (Salt et al. 1997). For Hg tolerance, seedlings must be able to prevent the adsorption of excess Hg or detoxify the Hg after it has been absorbed. Esteban et al. (2008) reported high Hg uptake for lupin, and suggested that metal was uptaken in part, by calcium channels or even aquaporins. Mercury blocks the water channels by binding to a cysteine residue in the pore (Vanderleur et al. 2005). In agreement, $HgCl_2$ was found to reduce the hydraulic conductivity of wheat root cells and rapidly depolarized the membrane potential of the root cells (Zhang and Tyerman 1999).

In this study, there was an inhibition of root and shoot length in plants exposed to Hg, where hybrids responded in a similar manner to all Hg treatments (Fig. 1A and 1B). The Hg-mediated inhibition of plant growth has been studied in several plants species, such as *Medicago sativa* (Zhou et al. 2007), tomato (Cho and Park 2000) and cucumber (Cargnelutti et al. 2006). Odjegba and Fasidi (2004) reported that growth inhibition could be result of one, or a combination, of the following reasons: (1) high solute potential of the external medium that inhibit of nutrient uptake; (2) direct inhibition of enzymes that are of physiological importance; (3) inhibition of mitotic division of the meristematic cells. In fact, the direct inhibition of enzymes of physiological importance was confirmed in part in the present work by the results observed for δ-ALA-D activity. Plant dehydratases are localized in plastids and are needed for chlorophyll synthesis in addition to other cellular tetrapyrroles (Smith 1988). They share 35 to 50% identity with non-plant enzymes, but activity requires Mg rather than Zn. The peptide region in the plant enzyme corresponding to the Zn domain in animals lacks cysteine and histidine residues and contains aspartate, alanine, or threonine instead (Boese et al. 1991).

One mechanism involved in heavy metal toxicity entails the ability to form strong bonds with reactive groups of proteins, modifying both their structure and functions (Wang 1999). Heavy metals may compete with other divalent cations such as Zn^{2+} and Mg^{2+} replacing them in their physiological roles. In the present study, BR205 and 32R21 hybrids under Hg stressed conditions showed lower ALA-D activity than that of 30F71, although there was no significant difference in the shoot Hg concentration among these hybrids. In fact, Hg accumulated massively in roots and in most cases the shoot Hg concentration was about

4.77% of the amount measured in roots. Possibly, the shoot Hg concentration was not great enough to exert a direct inhibition of δ-ALA-D. Therefore, δ-ALA-D activity may not be a good biomarker for the presence of Hg in maize. Moreover, δ-ALA-D activity was inhibited in the BR205 and 32R21 hybrids by about 52% and 20%, respectively at the highest dose of Hg (Fig. 2), while it was not altered in the 30F71 hybrid. These results also suggest that the 30F71 hybrid possess some mechanism to reduce Hg bioavailability (Table 1) and toxicity on δ-ALA-D activity (Fig. 2). However, the lack of Hg inhibitory effect in the δ-ALA-D activity of the 30F71 hybrid was not effective in prevent the dry weigth decreasing (Fig 1C). As shown in Fig 1C, root dry weight of both hybrids was reduced by Hg exposure.

To understand the mechanism by which Hg could affect BR205 δ-ALA-D activity, the hybrid more affected by Hg exposure, we performed experiments with three Hg (40, 50 and 60 μM) concentrations and variable substrate concentrations (0.36 – 36 mM). The analysis of the kinetic data indicated that Hg inhibited δ-ALA-D activity of the BR205 hybrid in a concentration-dependent manner (Fig. 3A). It was clearly observed that K_m values increased and V_{max} decreased with increasing Hg concentrations (Fig. 3B). In addition, analysis of the IC_{50} (47.22 μM) and Ki (140.143 μM) data obtained in the present study revealed that Hg was a potent inhibitor of δ-ALA-D activity. The nature of the inhibition caused by Hg seemed to be mixed. Nevertheless, in the present study, the enzymatic assay was performed with crude extract, which might contain interfering substances, and hence could be insufficient to sustain the exact type of inhibition. In fact, Pereira et al. (2006) reported that $Al_2(SO_4)_3$ did not inhibit δ-ALA-D activity from cucumber cotyledons when aluminum salt was

mixed directly with the crude extract and inhibition of δ-ALA-D activity was only observed after gel filtration. The Hg-mediated decrease in V_{max} of δ-ALA-D activity from crude extract could have produced a conformational change in the enzyme. Mercury may interact with δ-ALA-D at either the δ-ALA-D-2(ALA) complex stage or at the regulatory site of free δ-ALA-D. In either case, Hg may form a complex with δ-ALA-D or δ-ALA-D-2(ALA), due to its high affinity to thiol groups, which are essential for catalytic activity (Tsukamoto et al. 1979), such as Hg-(thiol)δ-ALA-D or Hg-(thiol)ALA-D-2(ALA), thereby decreasing δ-ALA-D activity. In line with this, the formation of porphobilinogen (PBG), the first condensation product of two ALA molecules, was decreased with increasing concentrations of Hg. Mercury could also act by displacing Mg in the allosteric site or by forming a complex with the free enzyme. Due to the communication between the allosteric Mg site and the active site of the enzyme (Coates et al. 2004), this displacement could lead to δ-ALA-D inhibition. However, in the present study, either the interference of Hg with an allosteric Mg site of the enzyme or the existence of different conformations is highly speculative. However, it was clearly observed, from kinetic parameters, that Hg had a negative effect on δ-ALA-D activity in the BR205 hybrid both in vitro and in vivo. In addition, the extract preparation used excludes most interfering substances and favors the presence of δ-ALA-D enzyme. The hypothesis that Hg brought about a mixed-type inhibition of δ-ALA-D activity could only be proved by using purified δ-ALA-D, instead of a crude enzymatic extract. Thus, in future studies we intend to study the effect of Hg on the activity of purified δ-ALA-D in order to confirm this hypothesis.

In conclusion, the present study demonstrated that there was not a direct correlation between the effect of Hg treatments on biomass and δ-ALA-D activity. Therefore, this enzyme may not be a good biomarker to indicate Hg intoxication in plants. Further research should focus on δ-ALA-D activity using the purified enzyme in order to clarify the interaction between Hg and the enzyme.

Acknowledgements

The authors wish to thank the Coordenação e Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa de Estado do Rio Grande do Sul (FAPERGS).

References

Abbas MHH, Meharg AA (2008) Arsenate, arsenite and dimethyl arsinic acid (DMA) uptake and tolerance in maize (*Zea mays* L.). Plant Soil 304:277–289

Boese QF, Spano AJ, Li J, Timko MP (1991) Aminolevulinic acid dehydratase in pea (*Pisum sativum* L.). Identification of an unusual metal-binding domain in the plant enzyme. J Biol Chem 266:17060–17066

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Caille N, Vauleon C, Leyval C, Morel J (2005) Metal transfer to plants grown on a dredged sediment: use of radioactive isotope ^{203}Hg and titanium. Sci Total Environ 341:227-239

Cargnelutti D, Tabaldi LA, Spanevello RM, Jucoski GO, Battisti V, Redin M, Linares CEB, Dressler Flores EMM, Nicoloso FT, Morsch VM, Schetinger MRC (2006) Mercury toxicity induces oxidative stress in growing cucumber seedlings. Chemosphere 65:999–1006

Cho UH, Park JO (2000) Mercury-induced oxidative stress in tomato seedlings. Plant Sci 156:1–9

Coates L, Beave G, Erskine PT, Beale SI, Avissar YJ, Gill R, Mohammed F, Wood SP, Shoolingin-Jordan P, Cooper JB (2004) The X-ray Structure of the Plant like 5-Aminolaevulinic Acid Dehydratase from Chlorobium vibrioforme Complexed with the Inhibitor Laevulinic Acid at 2.6 A°Resolution. J Mol Biol 342:563–570

Dixon M, Webb EC (1964) Enzyme kinetics, 2nd edn. Longman, London, UK, p 54

Dowd JE, Riggs DS (1965) A comparison of estimates of Michaelis menten kinetic constants from various linear transformations. J Biol Chem 240:863–869

EPA. Clean Air Mercury Rule (2005) U.S. Environmental Protection Agency: Washington, DC

Ericksen JA, Gustin MS, Schorran DE, Johnson DW, Lindberg SE, Coleman JS (2003) Accumulation of atmospheric mercury in forest foliage. Atmos Environ 37:1613-1622

Esteban E, Moreno E, Peñalosa J, Cabrero JI, Millán R, Zornoza P (2008) Short and long-term uptake of Hg in white lupin plants: Kinetics and stress indicators Environ Exp Bot 62:316–322

Grazia CA, Pestana MHD (2005) Mercury contaminated soils in gold mining areas of Lavras do Sul, RS, Brazil. In: INTERNATIONAL CONFERENCE ON HEAVY METALS IN THE ENVIRONMENT, 13, Rio de Janeiro. 4p

Gibson KD, Neuberger A, Scott JJ (1955) The purification and properties of delta-aminolevulinic acid dehydratase. Biochem J 61:618-676

Hanson PJ, Lindberg SE, Tabberer TA, Owens JG, Kim KH (1995) Water Soil Pollut 80:373-382

Hofstee BH (1952) On the evaluation of the constant V_m and constant K_m enzyme reactions. Science 116:329–331

Jaffe EK, Kervinen J, Dunbrack JR, Litwin S, Martins J, Scarrow RC, Volim M, Yeung AT, Yonn E (2000) Porphobilinogen synthase from pea: Expression from an artificial gene, kinetic characterization, and novel implications for subunit interactions. Biochemistry 39:9018-9029

Kabata-Pendias A, Pendias H (2001) Trace elements in soils and plants. CRC, Boca Ranton, FL

Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56:658–666

López-Berenguer C, García-Viguera C, Carvajal M (2006) Are root hydraulic conductivity responses to salinity controlled by aquaporins in broccoli plants? Plant Soil 279:13–23

Meagher R B, Rugh C L, Kandasamy M K, Gragson G, Wang NJ (2000) Engineered phytoremediation of mercury pollution in soil and water using

bacterial genes. In Phytoremediation of Contaminated Soil and Water; Terry N, Bañuelos G, (eds) Ann Arbor Press Inc.: Berkeley CA, pp 201–219

Morsch VM, Schetinger MRC, Martins AF, Rocha, JBT (2002) Effects of cadmium, lead, mercury and zinc on δ-aminolevulinic acid dehydratase activity from radish leaves. Biol Plant 45:85–89

Nieboer E, Richardson DHS (1980) The replacement of the nondescript term heavy metals by a biologically and chemically significant classification of metal ions. Environ Pollut 1:3–26

Odjegba VJ, Fasidi IO (2004) Accumulation of Trace Elements by Pistia stratiotes: Implications for phytoremediation. Ecotoxicology 13:637–646

Ortega-Villasante C, Rellán-Álvarez R, Del Campo FF, Carpena-Ruiz RO, Hernández LE (2005) Cellular damage induced by cadmium and mercury in *Medicago sativa*. J Exp Bot 56:2239–2251

Patra M, Sharma A (2000) Mercury toxicity in plants. Bot Rev 66:379– 422

Pereira LA, Tabaldi LA, Gonçalves JF, Jucoski GO, Pauletto MM, Weis SN, Nicoloso FT, Borher D, Rocha JBT, Schetinger MRC (2006) Effect of aluminum on δ-aminolevulinic acid dehydratase (ALA-D) and the development of cucumber (*Cucumis sativus*). Environ Exp Bot 57:106-115

Pilon-Smits E (2005) Phytoremediation. Annu Rev Plant Biol 56:15–39

Rellán-álvarez R, Ortega-Villasante C, Álvarez-Fernández A, Campo FF, Hernández LE (2006) Stress responses of *Zea mays* to cadmium and mercury. Plant Soil 279:41–50

Rocha JBT, Pereira ME, Emanuelli T, Christofari RS, Souza D (1995) Effects of methylmercury exposure during the second stage of rapid postnatal brain

growth on delta-aminolevulinic acid dehydratase (ALA-D) activity in brain, liver and blood of suckling rats. Toxicology 100:27–37

Salt DE, Pickering IJ, Prince RC, Gleba D, Dushenkov S, Smith RD, Raskin I (1997) Metal accumulation by aquacultured seedlings of Indian mustard. Environ Sci Technol 31:1636-1644

Sassa S (1982). Delta-aminolevulinic acid dehydratase assay. Enzyme 28:133–145

Schneider HA (1976) Enzymic capacities for chlorophyll biosynthesis. Activation and *de novo* synthesis of enzymes. Z Naturforsch C: Biosci 31:55-63

Schwesig D, Krebs O (2003) The role of ground vegetation in the uptake of mercury and methylmercury in a forest ecosystem Plant Soil 253: 445–455

Shaolin C, David BW (1997) Construction and characterization of genetically engineered for bioremediation of Hg^{2+} contaminated environments. Appl Environ Microbiol 63:2442–2445

Silva CJC, Lima MGS, Carvalho CM, Eloi WM, Pedroza MM, Silva CJC (2005) Efeito do lodo de estação de tratamento de despejos de curtume na fase inicial do crescimento do milho. Rev Biol Ciênc Terra 5

Smith AG (1988) Subcellular localization of two porphyrin-synthesis enzymes in *Pisum sntivum* (pea) and *Arum* (cuckoo-pint) species. Biochem J 249:423-428

Steinnes E (1997) Trace element profiles in ombrogenous peat cores from Norway: evidence of long-range atmospheric transport. Water Air Soil Pollut 100:405–413

Sudová R, Vosátka M (2007) Differences in the effects of three arbuscular mycorrhizal fungal strains on P and Pb accumulation by maize plants. Plant Soil 296:77–83

Tabaldi LA, Nicoloso FT, Castro GY, Cargnelutti, D, Gonçalves JF, Rauber R, Skrebsky EC, Schetinger MRC, Morsch VM, Bisognin DA (2007) Physiological and oxidative stress responses of four potato clones to aluminum in nutrient solution. Braz J Plant Physiol 19:211-222

Tennant BD (1975) A test of a modified line intersect method of estimating root length. J Ecol 63:995-1001

Tsukamoto I, Youshinag T, Sano S (1979) The role of zinc with special reference to the essential thiol groups in delta-aminolevulinic acid dehydratase of bovine liver. Biochim Biophys Acta 570:167– 178

Vanderleur et al. 2005 Vandeleur R, Niemietz C, Tilbrook J, Tyerman SD (2005) Roles of aquaporins in root responses to irrigation. Plant Soil 274:141–161

Vanparys C, Dauwe C, Campenhout KV, Bervoets L, Coen WD, Blust R, Eens M (2008) Metallothioneins (MTs) and δ-aminolevulinic acid dehydratase (ALAd) as biomarkers of metal pollution in great tits (*Parus major*) along a pollution gradient. Science of the Total Environment 401:184–193

Zhang WH, Tyerman SD (1999) Inhibition of water channels by $HgCl_2$ in intact wheat root cells. Plant Physiol 120:849– 858

Zhou ZS, Huang SQ, Guo K, Methta K, Zhnag PC, Yang ZM (2007) Metabolic adaptations to mercury-induced oxidative stress in roots of *Medicago sativa* L. J Inorg Biochem 101:1–9

Zilloux EJ, Porcella DB, Benott JM (1993) Mercury cycling and effects in freshwater wetland ecosystems. Environ Toxicol Chem 12:2245–2264

Wang LY (1999) Conditional stability of the HemA protein (glutamyl-tRNA reductase) regulates heme biosynthesis in Salmonella typhimurium. J Bacteriol 181:1211–1219

Wang Y, Greger M (2004) Clonal differences in mercury tolerance, accumulation, and distribution in willow. J Environ Qual 33:1779–1785

Wang Y, Stauffer C, Keller C, Greger M (2005) Changes in Hg fractionation in soil induced by willow. Plant Soil 275:67–75

Wang ZH, Zhang JL, Christie P, Li X-L (2008) Influence of inoculation with *Glomus mosseae* or *Acaulospora morrowiae* on arsenic uptake and translocation by maize. Plant Soil 311:235–244

Wenzel WW (2008) Rhizosphere processes and management in planttyu-assisted bioremediation (phytoremediation) of soils. Plant Soil DOI 10.1007/s11104-008-9686-1

Legends

Figure 1. Effect of increasing concentration of Hg on the length of roots (A), length of shoots (B), root dry weight (C) and shoot dry weight (D) of maize hybrids. Data represent the mean ± S.D. of three replicates. Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids at $p < 0.05$.

Figure 2. Effect of increasing concentration of Hg on δ-Aminolevulinic acid dehydratase activity of maize hybrids. Data represent the mean ± S.D. of three replicates. Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids at $p < 0.05$.

Figure 3. Maize BR205 hybrid δ-Aminolevulinic acid dehydratase activity in the presence and absence of different concentrations of Hg, in vitro (A) and kinetic analysis of the inhibition of δ-Aminolevulinic acid dehydratase by Hg in maize BR205 hybrid (B). * Different from control at $p < 0.05$.

Table 1

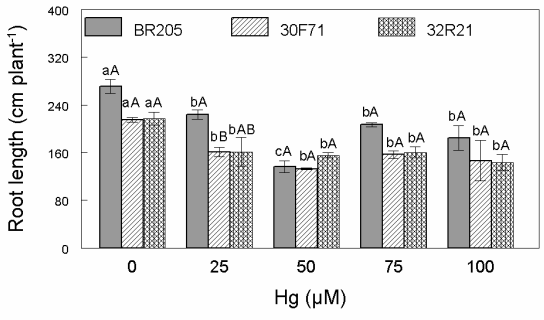
Mercury concentration of maize seedlings grown under increasing concentrations of Hg.

Hybrids	Treatment (μM)	Root (μg g DW^{-1})	Shoot (μg g DW^{-1})
BR 205	Control	10±1cA	29±3cA
	25	3455±499bA	125±25bcA
	50	3014±504bA	150±47bA
	75	4771±916aA	218±98abA
	100	4937±460aB	297±69aA
30F71	Control	39±53cA	25±3cA
	25	2859±448bA	129±28bA
	50	3526±782abA	170±60bA
	75	4232±842aA	187±70bA
	100	3783±481abC	339±70aA
32R21	Control	62±81dA	34±6bA
	25	2686±513cA	102±42abA
	50	3138±438cA	126±18aA
	75	4555±740bA	177±72aA
	100	6360±671aA	158±19aA

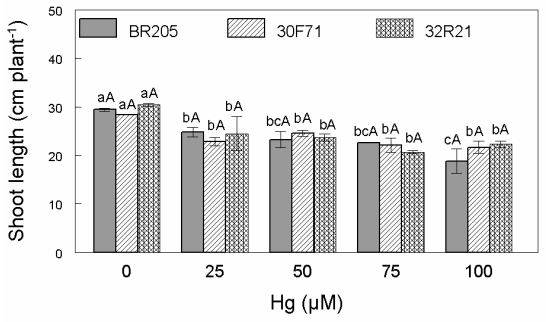
Data represent mean values ± SD based on three independent determinations. Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids at $p < 0.05$

Figure 1

(A)



(B)



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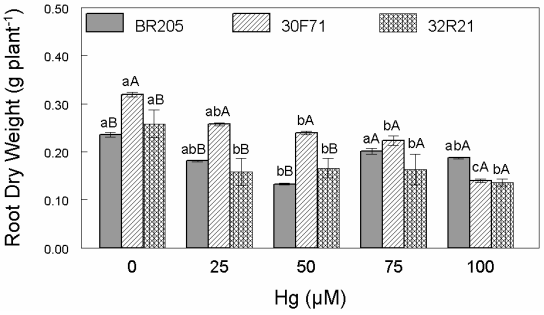


(D)

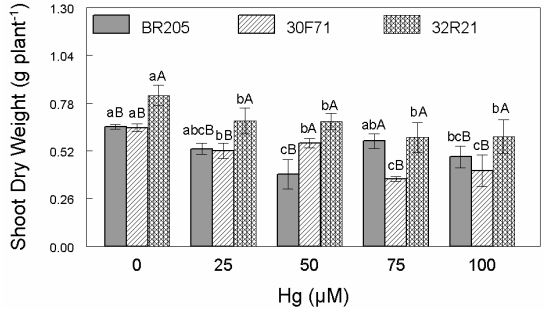


Figure 2

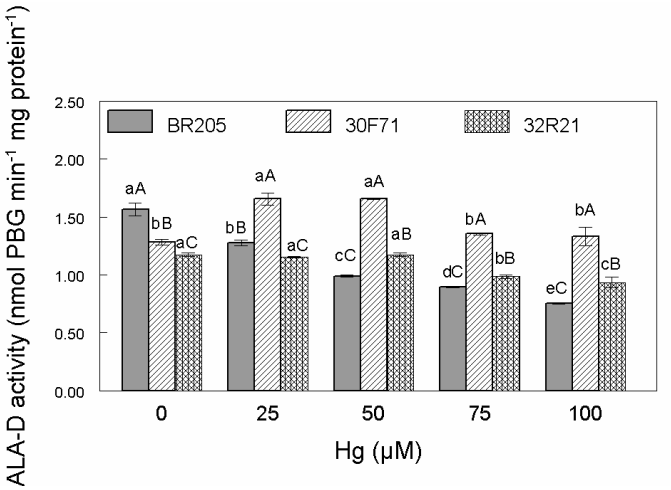
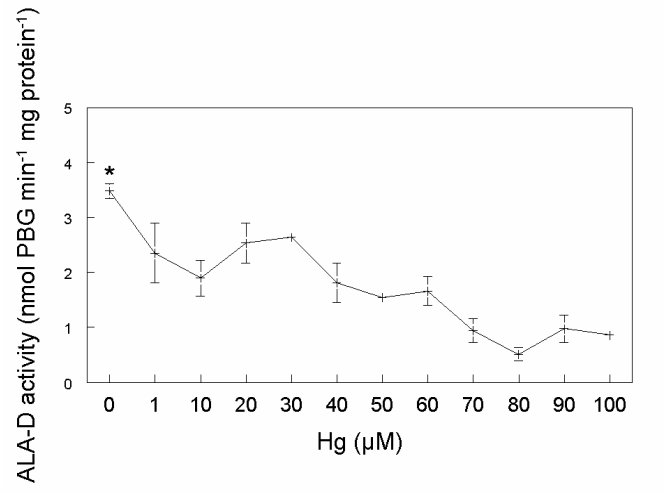
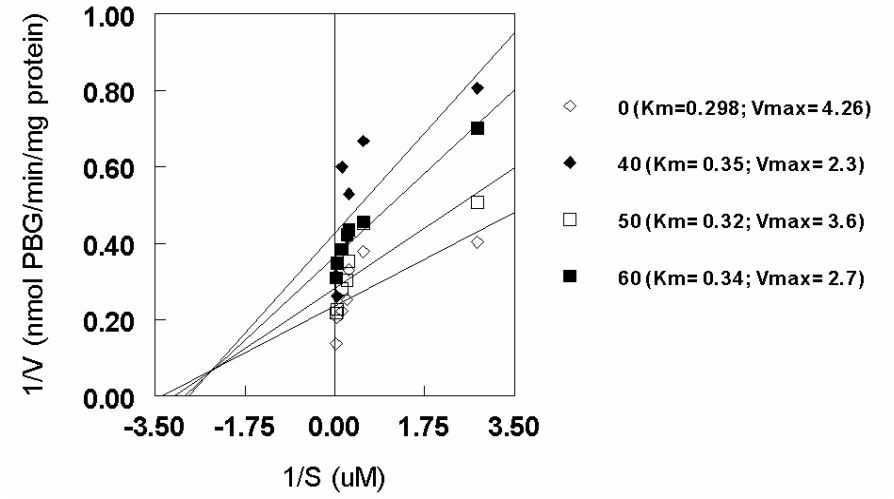

ALA-D activity (nmol PBG min-1 mg protein-1)
2.50
2.00
1.50
1.00
0.50
0.00
BR205
30F71
32R21
aA
bB
aC
bB
aA
aC
cC
aA
aB
dC
bA
bB
eC
bA
cB
0
25
50
75
100
Hg (µM)

Figure 3

(A)


ALA-D activity (nmol PBG min-1 mg protein-1)
*
0 1 2 3 4 5
0 1 10 20 30 40 50 60 70 80 90 100
Hg (µM)

(B)


1/V (nmol PBG/min/mg protein)
1.00
0.80
0.60
0.40
0.20
0.00
-3.50 -1.75 0.00 1.75 3.50
1/S (uM)
0 (Km=0.298; Vmax= 4.26)
40 (Km= 0.35; Vmax= 2.3)
50 (Km= 0.32; Vmax= 3.6)
60 (Km= 0.34; Vmax= 2.7)

3.1.2.2. Efeito do zinco no estresse oxidativo induzido pelo mercúrio em dois híbridos de milho (*Zea mays* L.)

Manuscrito 3

Zinc alleviates mercury-induced oxidative stress in maize

Denise Cargnelutti, Fernando T. Nicoloso, Vera M. Morsch, Liana V. Rossato, Nicéia C. Spanholi, Luciane B. Pereira, Luciane A. Tabaldi, Gustavo R. Thomé, Valderi L. Dressler, Érico M.M. Flores, Maria R.C. Schetinger

(Resubmetido à Revista Plant Physiology and Biochemistry)

Zinc alleviates mercury-induced oxidative stress in maize

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Abstract

The potential mechanism by which zinc (Zn) antagonizes mercury (Hg) toxicity was investigated in two maize (*Zea mays* L.) hybrids (BR205 and 32R21). Maize seedlings were grown for 5 days in nutrient solution using one concentration of Hg (25 μM) and three of Zn (50, 100 and 200 μM) either singly or in combination. Hg-treated seedlings accumulated more in roots than in shoot, whereas in the treatments supplemented with 50 μM Zn for both hybrids the Hg concentration was decreased in roots, when compared to treatment with Hg alone. Mercury at level of 25 μM reduced Chl b content in hybrids, while in treatments with Hg applied together with Zn (100 μM) it was increased to control levels. The carbonyl and hydrogen peroxide (H_2O_2) concentrations were increased on Hg-treatment, while in Hg-treated seedlings with supplemented Zn they were reduced. Mercury-treated hybrids showed reduced NPSH levels, but when supplemented with Zn levels were increased. In general, superoxide dismutase (SOC, E.C. 1.15.1.1), ascorbate peroxidase (APX, EC. 1.11.1.11) and catalase (CAT, EC. 1.11.1.6) activities varied with Hg-25 μM alone and in Hg-treated seedlings with supplemented Zn their activities were increased, in most treatments. In vitro studies suggested that Hg induced ROS production due the SOD activation. Moreover, in general APX was reduced by Hg in vitro while in vivo it was increased. Therefore, Zn seems to play a role in the induction of NPSH groups and antioxidant enzymes, reducing oxidized biomolecules, preventing destruction of photosynthetic pigments, and reestablishing the seedling growth reduced by Hg exposure.

Keywords: Antioxidant enzymes; Chlorophyll, Mercury; Non-protein thiols; Zinc, *Zea mays* L.

Abbreviations: Chl, chlorophyll; DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylenediaminetreaacetic acid; Hg, mercury; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; TCA, trichloroacetic acid; Zn, zinc;

1. Introduction

Soil contamination with heavy metals has become a worldwide problem leading to losses in agricultural yield and hazardous health effects as they enter into the food chain [1]. Mercury (Hg) is non-essential and toxic, whereas zinc (Zn), an essential nutrient, is an important component of many vital enzymes, a structural stabilizer for proteins, membranes and DNA-binding proteins (Zn-fingers) [2]. Relationships between Hg toxicity and oxidative stress have been studied in many systems and heavy metal contamination has often been implicated as the root cause of oxidative injury to plants. The key step in oxidative stress is the production of reactive oxygen species (ROS) which initiate a variety of autooxidative chain reactions in membrane unsaturated fatty acids, producing lipid hydroperoxides and a subsequent cascade of reactions ultimately leading to the destruction of organelles and macromolecules [3].

The generation of reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$) has been proven to be one of the underlying agents in the origin of tissue injury after the exposure of plants to a wide variety of stressful conditions, such as drought, heat, chilling, high light intensity, UV radiation, heavy metals, various organic chemicals and air pollutants [3, 4]. Complex antioxidant systems such as catalase (E.C.1.11.1.6), ascorbate peroxidase (E.C.1.11.1.11), and superoxide dismutases (E.C.1.15.1.1) [4], which neutralize and scavenge ROS [4], are very important for plants in order to protect cellular membranes and organelles from the damaging effects of ROS.

Zinc is an essential mineral nutrient and a co-factor of over 300 enzymes and proteins involved in cell division, nucleic acid metabolism, and protein synthesis [5]. There are several well-known Zn requiring enzymes that have been studied in plants, such as copper/zinc superoxide dismutase (Cu/Zn SOD). This enzyme plays an important role in protecting plants against oxidative damage catalyzed by reactive oxygen. Zinc is known to have a stabilizing and protective effect on biomembranes against oxidative and peroxidative damage, loss of plasma membrane integrity and also alteration of membrane permeability [6]. Zinc ions bind to ligands containing sulfur, nitrogen and, to a lesser extent, oxygen and preferentially bind to membrane proteins [6].

The balance between free radical generation and free radical defense determines the survival of the system. Therefore, Zn may have a role in modulating free radicals and their related processes through its antioxidant properties [7]. Thus, studies on interactions of metals would elucidate stress and resistance mechanisms exhibited by plants exposed to heavy metal. However, no information is available on the role of nutrients against toxic metals in this system. Hence, the purpose of this study was to investigate Zn-Hg interactions with respect to oxidative stress in maize (*Zea mays* L.) hybrids. In addition, in the present investigation, the effect of Hg alone was compared to the effect of Hg with Zn supplementation on metal uptake in seedlings using inductively coupled plasma atomic emission spectrometry (ICP-AES).

2. Results

Maize shoot and root growth were sensitive to Hg exposure. In previous experiments, treatments with 25, 50, 75 and 100 µM Hg gradually inhibited the shoot and root growth, as expressed by length (data not shown). The length of roots treated with 25 µM Hg decreased by about 25% as compared to the control (Hg-free) (data not shown). Therefore, 25 µM Hg was used for the evaluate biochemical responses in the presence of Zn.

2.1.1. Mercury (Hg) and zinc (Zn) concentration

The results obtained in the certified reference materials analyzed were 0.032 ± 0.003 $\mu g\ g^{-1}$ and 17.4 ± 1.1 $\mu g\ g^{-1}$ for Hg and Zn in NIST 1547, respectively, and 0.043 ± 0.002 $\mu g\ g^{-1}$ and 12.7 ± 0.83 $\mu g\ g^{-1}$ for Hg and Zn in NIST 1515, respectively. These values are in good agreement with the certified Hg and Zn concentrations which are, respectively, 0.031 ± 0.007 $\mu g\ g^{-1}$ and 17.9 ± 0.4 $\mu g\ g^{-1}$ for NIST 1547 and 0,044 ± 0.004 $\mu g\ g^{-1}$ and 12.5 ± 0.3 $\mu g\ g^{-1}$ for NIST 1515. Mercury-treated seedlings accumulated more Hg in roots than in shoot (on average 23.8-fold and 45.2-fold greater in root than in shoot, respectively for BR205 and 32R21) (*Figures 1 and 2*), whereas in treatments supplemented with 50 µM Zn the Hg concentration was decreased up to 3,212.5 and 3,643.0 µg Hg g^{-1} DW in roots BR205 and 32R21, respectively, when compared to treatment with Hg alone (*Figures 1A and 2A*). Zn-treated seedlings without any Hg treatment showed higher uptake of Zn (Table I), indicating a competition between Hg and Zn in seedlings treated with both Hg and Zn.

2.1.2. Chlorophyll concentration

Mercury at level of 25 µM induced little change in chlorophyll (Chl). Chl *b* content in shoot of BR205 and 32R21 reduced with Hg-25 µM alone (1.3-fold smaller than control). However, in Hg+Zn-supplemented (100 µM) seedlings it was increased to control levels (*Figure 3B*). In addition, treatments with Hg

applied together with Zn at level of 100 µM increased the BR205 chlorophyll *a* and *b* (*Figures 3A* and *3B*), when compared to treatment with Hg alone. Seedlings with Zn treatments alone showed little alteration in the chlorophyll content of BR205 and 32R21 hybrids (Table II).

2.1.3. Hydrogen peroxide and carbonyl content

The root carbonyl concentrations were increased in the Hg-treated 32R21. However, when supplemented with Zn (50, 100 and 200 µM) they were 22.5-fold, 7.8-fold and 2.9-fold lower than in Hg-25 µM alone (*Figure 4A*). However, in root of BR205, Hg-treated seedlings with supplemented 50 µM Zn showed a reduction in carbonyl concentrations, while those supplemented with 200 µM Zn showed an increase of about 62%, when compared to Hg-25 µM alone (*Figure 4A*). In shoot of BR205 hybrid, carbonyl concentrations were increased by about 172% in Hg-25 µM alone, while in the Hg-treated seedlings with supplemented Zn (100 and 200 µM) they were reduced to control levels (*Figure 5A*). Seedlings with Zn treatments alone showed little alteration in carbonyl concentrations (Table III).

The BR205 hybrid H_2O_2 concentration was increased (18% and 131%, for shoot and root, respectively) (*Figures 4B and 5B*) with Hg-25 µM alone, whereas in Hg-treated seedlings with supplemented Zn (50, 100 and 200 µM) it was reduced when compared to Hg-25 µM alone (*Figure 5B*), except in the shoot of Hg-treated seedlings supplemented with 200 µM Zn, where the H_2O_2 levels increased. In root of 32R21 hybrid, an increase in the H_2O_2 concentration was only observed for Hg-treated seedlings supplemented with Zn (50 µM) (*Figure 4B*). However, the shoot H_2O_2 concentration of 32R21 hybrid was 1.6-fold times lower in the Hg-alone treatment when compared to the control, while supplementation with 100 and 200 µM Zn increased H_2O_2 by about 25% and 47% higher than Hg-25 µM alone (*Figure 5B*). Seedlings with Zn treatments alone showed little alteration in H_2O_2 concentrations, except for BR205 where the H_2O_2 concentration was increased in both shoot and root (Table III).

2.1.4. Non-protein thiol group (NPSH) concentration

In the roots, Hg-treated BR205 and 32R21 hybrids showed NPSH levels reduced by about 39% and 14%, but when supplemented with Zn (in all concentrations) NPSH levels were increased to control levels. The maximum NPSH content (0.187 and 0.171 n mol SH min^{-1} mg $protein^{-1}$) in roots of BR205 and 32R21 was observed for Hg+Zn (50 µM), which was about 1.4-fold higher than 25 µM Hg alone (*Figure 6A*). Shoot NPSH content of BR205 was not changed in the presence of 25 µM Hg alone, while supplementation with Zn (50 and 100 µM) increased NPSH levels. However, shoot NPSH content of BR205 was reduced by Hg+Zn (200 µM)-treatment, when compared to 25 µM Hg-alone (*Figure 6B*). Shoot NPSH content for 32R21 Hg-alone treated was about 1.2-fold lower than the control (free-Hg), while supplementation with Zn restored NPSH to control levels (*Figure 6B*). In general, Zn treatments alone showed increased NPSH levels (Table IV).

2.1.5. Antioxidant enzyme activity

Treatment with Hg applied alone reduced by about 46% the root SOD activity of BR205, whereas an increase of 8% and a reduction of 42% were observed for Hg-treated seedlings supplemented with Zn at levels of 50 and 200 µM, respectively (*Figure 7A*). In the 32R21 hybrid an increase in root SOD activity occurred in Hg-25 µM alone. However, in Hg-treated seedlings supplemented with Zn (50 and 100 µM) SOD was decreased by about 16% and 14% than compared to treatment with Hg-25 µM alone (*Figure 7A*). The BR205 shoot SOD activity decreased in Hg-25 µM alone, whereas in Hg-treated seedlings supplemented with Zn (50, 100 and 200 µM) it was increased by about of 45% when compared to treatment with Hg-25 µM alone (*Figure 8A*). Maize seedlings with Zn treatments alone showed an increase in shoot SOD activity in both BR205 and 32R21 hybrids (Table V).

Root CAT activity was only altered in BR205, where it decreased by about 25% in the treatment with 25 µM Hg (*Figure 7B*). However, Hg-treatments supplemented with Zn (50 and 100 µM) showed increases of 48% and 34%, respectively. Shoot CAT activity showed no alteration in the Hg-treated seedlings, while in the Hg-treated 32R21 hybrid supplemented with Zn (100 and

200 μM) there was an increase in CAT activity, when compared to treatment with Hg alone (*Figure 8B*).

APX activity in shoot and roots of BR205 hybrid increased with Hg-25 μM alone. However, in Hg+Zn-supplemented seedlings it was either reduced at Hg+50 μM Zn or increased at Hg+100 μM Zn (*Figures 7C* and *8C*). Contrarily, in 32R21, APX activity decreased in Hg-25 μM alone, both in shoot and root. In the root, APX activity was only increased (114%) in Hg+200 μM Zn-supplemented 32R21 hybrid (*Figure 7C*). However, in the shoot of Hg+Zn-supplemented seedlings (50 and 100 μM), APX activity was increased by about 54% and 155%, respectively, when compared to Hg-25 μM alone (*Figure 8C*). Zn-treated BR205 and 32R21, however, showed a general increase in SOD, CAT and APX activities (Table V).

2.2. Metals effect in the extract

2.2.1. Antioxidant enzyme activity

Two-way ANOVA revealed significant effects of Hg^{2+} (25 μM) treatment on antioxidants enzymes activities (*Figures 9* and *10*). Post-hoc comparisons by Duncan's multiple range test showed that Hg^{2+} (25 μM) treated BR205 and 32R21 hybrids presented an increase in root SOD activity of about 40% and 31% (*Figure 9A*), and an increase in shoot SOD of about 20% and 14% (*Figure 10A*). In both BR205 and 32R21, Zn^{2+} significantly increased basal SOD activity in most of the concentrations tested (Table VI), but Zn^{2+} had little effect on the Hg-induced increase of SOD activity in both root and shoot of BR205 (*Figures 9A and 10A*).

Hg^{2+} (25 μM) significantly inhibited CAT activity in shoot of BR205 and 32R21 (*Figure 10B*). However, Zn^{2+} (50 and 100 μM) significantly increased CAT activity in shoot BR205 and 32R21 which was reduced by treatment with Hg alone (*Figure 10B*). Treatments with Zn at levels of 50, 100 and 200 μM, significantly increased the Hg-induced reduction of CAT activity in shoot of 32R21 (*Figure 10B*). In general, treatments with Zn alone increased CAT activity (Table VI)

Mercury at level of 25 μM significantly inhibited root and shoot BR205, and shoot 32R21 APX activity by about 74%, 37% and 85%, respectively (*Figures 9C* and *10C*). Zinc, at levels of 100 and 200 μM, increased the APX activity of both root BR205 and shoot 32R21 which was reduced by treatment with Hg alone (*Figures 9C* and 10*C*). Treatments Zn significantly inhibited shoot 32R21 APX activity, but showed a small effect on root BR205 APX activity (Table VI).

3. Discussion

This study was undertaken to establish Zn antagonism Hg induces in maize and to identify the mechanisms through which this metal is brought about. The BR205 hybrid was selected because of its capacity to extract elements from the soil [8] and the 32R21 hybrid was chosen because it is cultivated in the southern region of Brazil. Studying interactions between two metals leads to a better understanding of the metabolic pathways in order to improve defense strategies using various parameters. In *Ceratophyllum demersum*, Zn alleviates Cd-induced oxidative stress by inducing and activating antioxidant enzymes such as SOD, CAT and POD [9,10], which prevent oxidative injury to membranes, proteins [11], chloroplasts and their associated photosynthetic pigments [10]. However, cellular and metabolic aspects involved in the interaction between Hg and Zn have not been studied.

Hg is known to accumulate primarily in roots because the majority of the metal that enters the roots is compartmentalized into the vacuole as the free cation or complexed with thiol-rich peptides known as phytochelatins (PCs) [12]. In fact, in the present study, only limited amounts of Hg was translocated to the shoot (*Figures 1* and *2*). Differently than studies using Cd and Zn in *C. demersum*, the present investigation showed no changes in the Hg uptake with increasing Zn concentrations, although a decrease in Zn accumulation was observed in the plant tissue, when compared to treatments with Zn alone (Table I). The absence of suppression in Hg uptake and the increase in Zn accumulation suggest a low competition between Zn and Hg in plant absorption. However, the reduced Zn uptake in Hg-treated seedlings supplemented with Zn, suggest that the Zn transporters are damaged by Hg exposure and hence Zn accumulation diminishes. This response may be due to fact that the transport of non-essential elements such as Hg is most likely to occur via transporters of essential cations [13].

It has been reported that heavy metals [14] such as Hg reduce levels of chlorophylls [15]. Mercury also strongly inhibits the photosynthetic electron transport chain, where photosystem II (PS II) is the most sensitive target [16, 17]. In accordance with Patra et al. [18] Hg ions may substitute metal ions in photosynthetic pigments, causing a decrease in photosynthesis rates. We

observed reduced chlorophyll *b* levels for BR205 and 32R21 hybrids upon Hg exposure, suggesting destruction of photosynthetic pigments. Similarly, Prasad and Prasad [19] reported decreased chlorophyll a and b in *Pennisetum typhoideum* seedlings upon Hg exposure and suggested the impairment of chlorophyll biosynthesis by Hg in seedlings. The enzyme δ-aminolevulinic acid dehydratase (δ-ALA-D) are involved in chlorophyll metabolism. It is known that δ-ALA-D is sensitive to heavy metals due the sulfidrilic nature [20]. In fact, in our previous studies was shown that Hg is a potent inhibitor from maize ALA-D activity (Cargnelutti et al., unpublished data). Nonetheless, in the present study, Zn at level of 100 μM counteracted the chlorophyll *b* reduction in maize hybrids caused by 25 μM Hg. Chavapil [21] reported that Zn prefers binding to –SH groups of the membrane protein moiety and protects phospholipids and proteins from thiol oxidation and disulphide formation. This result demonstrates an apparent stability of the enzymes, membrane proteins and lipid structure [22, 23], which hence affords protection from Hg-induced sulfhydryl oxidation and structural damage. Therefore, if Zn protects against the oxidation of proteins and membranes in the photosynthetic apparatus, we may suggest that Zn (especially 100 μM) interacted to reduce the oxidized biomolecules of the chloroplast, reducing the chlorophyll destruction induced by Hg.

Carbonyl content is a sensitive indicator of oxidative damage to proteins [24], and levels of carbonylated proteins increased in plants undergoing oxidative stress associated with heavy metal [25], such as Hg [15]. Our data indicate that higher carbonyl protein levels (especially in the shoot of BR205) are related to the H_2O_2 and antioxidant defense system levels (especially SOD activity). On the other hand, Hg-treated maize hybrids supplemented with Zn, showed reduced protein oxidation levels, which is related to the low H_2O_2 levels and activation of antioxidant enzymes. Cakmak [26] reported that Zn is required for detoxification of ROS, including $O_2^{\bullet-}$ and H_2O_2. Because of its fundamental role in the activation and expression of genes [2], Zn might be involved in oxidative stress-induced expression of gene-encoding-antioxidative-defense enzymes such as H_2O_2-scavenging ascorbate peroxidase and glutathione reductase [27]. Therefore, our results suggested that Zn may depress the

generation of ROS due the induction of antioxidant enzyme expression, reducing the oxidation of cellular components, such as proteins.

The results of the present study indicate that the tolerance of maize hybrids to Hg was associated with a greater accumulation of non-protein thiol compounds (NPSH) promoted by Zn. It is known that Hg has poor capability as a phytochelatin (PC) synthesis inductor when compared with other heavy metals such as Cd and Cu [12], while Zn is a stronger inductor [28] than Hg. In fact, we observed a general increase in NPSH accumulation for Hg-treated hybrids supplemented with Zn, suggesting that due the amount of NPSH, the synthesis of PCs might be constantly promoted by Zn exposure. This hypothesis confirms the indirect role of Zn in Hg detoxification in maize hybrids.

In the present study, there was a compensatory mechanism for antioxidant enzymes. The reduction in SOD and CAT activities were compensated by an increase in APX activity in BR205 exposed to 25 μM Hg alone. However, the reduction in APX activity was compensated by an increase in SOD activity in 32R21 exposed to 25 μM Hg alone. The same trend was also seen in Hg-treated seedlings supplemented with Zn, indicating the ROS scavenging activity in the system. In order to characterize the potential mechanism by which Zn antagonizes Cd toxicity in *C. demersum*, a free floating freshwater macrophyte, Aravind and Prasad [9] found greater increases in SOD and CAT activities in Cd+Zn treatments, due to extreme oxidative stress induced by Cd and the subsequent Zn-mediated protection induced by high levels of antioxidant enzymes. The same authors suggested that Zn is able to increase the biosynthesis of antioxidant enzymes [26]. A similar hypothesis could be reached in our study on Hg toxicity. In seedlings treated with Hg alone, the Hg-mediated production of ROS may have inactivated the antioxidant enzymes. It has been suggested that very high levels of ROS inhibited Cu/Zn SOD [29] through Cu^{2+} to Cu^{+} reduction and that the formation of excess hydroxyl radicals also inhibits Cu/Zn SOD. This pattern was observed in BR205 which high H_2O_2 levels may have inactivated SOD and CAT activities. However, a reduction in H_2O_2 levels and activation in SOD and CAT activities was observed from BR205 hybrid Hg-treated supplemented with Zn (*Figures 6A* and *6B*, *7A* and *7B*). Therefore, theses results indicated that BR205 was more sensitive to Hg exposure than 32R21. Similarly, the CAT enzyme is also

sensitive to $O_2^{\bullet-}$ and can be inactivated by its increasing levels [26]. However, in vitro studies showed enhanced SOD activity when maize hybrids were exposed to Hg-alone (*Figures 9A* and *10A*), suggesting that Hg induced the in vitro production of ROS such as $O_2^{\bullet-}$. Nonetheless, Hg-treated maize and supplemented with Zn at level of 50 µM had restored 32R21 SOD activity to control levels, whereas in BR205 its activity was not altered. Unlike SOD activity, shoot CAT activity was decreased by Hg exposure, but it was increased in maize seedlings exposed to Hg and supplemented with Zn. Theses results suggested that Zn may play a role in the modulation of ROS in plant cells [26].

Hg-25 µM treatments did not enhance APX activity to a great extent in roots (1.4-fold) (*Figure 7C*) and shoot (1.6-fold) (*Figure 8C*) of BR205. However, it was reduced in roots (2.9-fold) (*Figure 7C*) and shoot (1.3-fold) (*Figure 8C*) of 32R21. Probably, the higher levels of H_2O_2 formed in Hg-treatments became inhibitory to APX. In Zn supplemented (especially 100 µM) Hg-treatments, a much higher induction of root BR205 APX (1.6-fold) was observed when compared to Hg treatments alone, indicating an efficient control of H_2O_2 levels [30]. Our in vitro studies showed that Hg had an inhibitory effect on APX activity, while Zn had an antagonistic effect, increasing APX activity (*Figures 9C* and *10C*). Taken together, our results suggested the *de novo* synthesis of APX in maize seedlings exposed to Hg and indicated the protective effect of Zn in the modulation of ROS.

Therefore, Zn protects maize hybrids from Hg-induced oxidative stress by inhibiting the formation of carbonyl groups and H_2O_2 production, as well increasing ROS scavenging antioxidant enzyme activity, which indicate the possible role of Zn as an antioxidant and its action against oxidative stress Moreover, Zn seems to play an important role in the induction of NPSH groups, protecting them from oxidation as well as protecting biomolecules, thus preventing destruction of photosynthetic pigments and promoting normal growth of seedlings.

However, further studies are needed to investigate the Hg-Zn interaction and its relation with the antioxidant system as well as to better understand Hg and Zn uptake by plants.

4. Materials and Methods

4.1. Plant material and growth conditions

Two hybrids (BR205 and 32R21) were evaluated. The BR205 (duple hybrid; obtained from Empresa Brasileira de Pesquisa Agropecuária, EMBRAPA, Minas Gerais, Brazil) is adapted to tropical regions of Brazil, presents precocity and high productivity, as well as tolerance to aluminum toxicity and water stress. Moreover, it was shown to have high capacity for uptake of mineral elements from soils [8]. The 32R21 (simple hybrid, obtained from PIONNER company) hybrid was chosen due to its high biomass production and because it has been extensively cultivated in the southern region of Brazil. Seeds were germinated in plastic boxes on filter paper. Seven-day old uniform plantlets were transferred into plastic boxes (10 L) filled with aerated nutrient solution of low ionic strength. The nutrient solution was prepared as described in Tabaldi et al. [31] (in mg L^{-1}): 8.31 of N; 0.754 of P; 1.154 of S; 9.76 of Ca; 2.37 of Mg; 1.05 of K; 17.68 of Cl; 0.027 of B; 0.005 of Mo; 0.001 of Ni; 0.013 of Zn; 0.003 of Cu; 0.011 of Mn and 0.268 of Fe ($FeSO_4$/Na-EDTA). The plantlets were acclimated for seven days before addition of the treatments. Throughout the acclimation and treatment periods, the plants were grown in a growth chamber at 25 ± 2ºC on a 16/8-h light/dark cycle with 35 μmol m^{-2} s^{-1} of irradiance. The solution pH was adjusted daily to 5.4 ± 0.1 by titration with HCl or NaOH solutions (0.1 M). Treatments consisted of the addition of Hg (0 and 25 μM) and/or Zn (0, 50, 100, 200 μM) for 5 days. At harvest, the plants were divided into shoot and roots. Roots were rinsed twice with distilled water. Subsequently, Hg uptake, index of oxidative stress and antioxidant enzymes were determined. Hg at a concentration of 25 μM was found to decrease the root length by 25% (data not shown), which was the concentration used for the estimation of physiological and biochemistry parameters. The concentrations of Zn were based on studies using *Ceratophyllum demersum* L. [9 - 11].

4.2- Mercury (Hg) and zinc (Zn) determination in tissues

Dried (65^{o}C) plant tissues (root and shoot) were ground and digested (using 10 to 200 mg) initially with 5 ml of concentrated HNO_3 at 90 ^{o}C during 2 h. Sample decomposition was carried out in an open digestion system, using a heating block from Velp Scientifica (Milano, Italy) equipped with glass vessels. Furthermore, 1 ml H_2O_2 was added and heated to 90 °C for 1 h. The relatively low temperature was used to avoid Hg losses. Moreover, plastic caps were fitted to the vessels to prevent analyte losses by volatilization and contamination. The decomposed sample solution was diluted to 30 mL with purified water. Analyte determinations were performed directly in these solutions. The certified reference materials peach leaves NIST 1547 and apple leaves, NIST 1515 (National Institute of Standards and Technology, Gaithersburg, USA) were analyzed to evaluate the accuracy of the sample preparation and Hg and Zn determination methods.

Zinc concentrations were 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 line used was 213.617nm. 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.

Mercury determination was performed by flow injection cold vapor generation hyphenated to inductively coupled plasma optical emission spectrometry (FI-CV-ICP OES), using the Hg emission line on 253.650 nm. The FI-CV system was adapted from Kaercher et al. [32]. The FI system consists of a peristaltic pump (Gilson, minipuls 3, France), a manual injector, and a U type gas/liquid separator. Tygon pump tubing of different internal diameters (i.d.) was used for carrying the solutions. All other tubing was of PTFE with 0.8 mm i.d.

The tissue Hg and Zn concentration were expressed as μg g^{-1} dry weight.

4.3. Chlorophyll concentration determination

Chlorophyll (chl) was extracted following the method of Hiscox and Israeslstam [33]. Fresh leaf (0.1 g) samples were incubated at 65ºC in

dimethylsulfoxide (DMSO) until the pigments were completely bleached. Absorbance of the solution was then measured at 663 and 645 nm for chl a and b with a spectrophotometer (Celm E-205D). Chlorophyll content was expressed as mg g^{-1} fresh weight. Chlorophyll content was calculated using the formulae [34] given below:

chlorophyll a (mg=g FW)= (11.75 x A663 – 2.35 x A645) x 50/500;
chlorophyll b (mg=g FW)= (18.61 x A645 – 3.96 x A663) x 50/500.

4.4- Protein oxidation

The reaction of carbonyls with 2,4- dinitrophenylhydrazine (DNPH) was used to determine the amount of protein oxidation, as described by Levine et al. [35]. Shoot and root of maize hybrids were homogenized in a 25 mM K-phosphate buffer containing 10 mL L^{-1} Triton X-100, pH 7.0, at a proportion of 1:5 (w/v). The homogenate was centrifuged at 13,000 *g* for 30 min at 4°C. After the DNPH-reaction, the carbonyl concentration was calculated by absorbance at 370 nm, using the molar extinction coefficient 21 x 103 mM cm^{-1}.

4.5- Hydrogen peroxide content

The H_2O_2 concentration was determined according to Loreto and Velikova [36]. Approximately 0.1 g of both roots and shoots was homogenized at 4ºC in 2 mL of 0.1% trichloroacetic acid (TCA) (w/v). The homogenate was centrifuged at 12,000 *g* for 15 min at 4ºC. Then, 0.5 mL of the supernatant was added to 0.5 mL of 10 mM K 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 standard calibration curve. Hydrogen peroxide concentration was expressed as µmol g^{-1} FW.

4.6. Non-protein thiol group (NPSH) concentration

Shoot and roots of maize hybrids were homogenized in a solution containing 50 mM Tris- HCl and 10 mL L^{-1} Triton X-100 (pH 7.5) and centrifuged at 6,800 *g* for

10 min. To the resulting 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. Non-protein thiol concentration was measured spectrophotometrically with Ellman's reagent [37]. An aliquot of the sample (400 µL) was added to a medium containing 550 µL of 1 M Tris-HCl (pH 7.4). The developed color was read at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 mL). A standard curve using cysteine was used to calculate the concentration of thiol groups in samples.

4.7- Antioxidant enzyme activity

Fresh leaf samples of both cultivars at 5 and 10 days after salt treatment were used for enzyme analysis. One gram of leaves was homogenized in 3ml of 0.05M sodium phosphate buffer (pH 7.8) including 1 mM EDTA and 2% (w/v) PVP. The homogenate was centrifuged at 13,000 *g* for 20 min at 4°C. The supernatant was used for enzyme activity and protein content assays. All steps in the preparation of the enzyme extract were carried out at 4°C.

The activity of SOD was assayed according to McCord and Fridovich [38]. The assay mixture consisted of a total volume of 1 mL, containing glycine buffer (pH 10.5), 1 mM epinephrine and enzyme material. Epinephrine was the last component added. Adrenochrome 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.

CAT activity was carried out according to the method of Aebi [39] with some modifications. The reaction mixture in a total volume of 2 ml contained 25 mM sodium phosphate buffer (pH 7.0), 15 mM H_2O_2. The reaction was initiated by the addition of 30 µL of enzyme extract and activity was determined by

measuring the initial rate of disappearance of H_2O_2 at 240 nm (E = 39.4 mM^{-1} cm^{-1}) for 30 s.

Ascorbate peroxidase activity was determined according to the modified method of Zhu et al. [40]. The reaction mixture in a total volume of 2 mL consisted of 25 mM sodium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H_2O_2 and 100 µL extract. The H_2O_2-dependent oxidation of ascorbate was followed by a decrease in absorbance at 290 nm using the molar extinction coefficient 2.8 mM cm^{-1}.

In vitro antioxidant enzymes (SOD, CAT and APX) activity was carried out as described above, except that untreated seedlings were used, and tissue supernatant were pre-incubated at 37°C for 60 min in the medium containing Hg (0 and 25 µM) and/or Zn (0, 50, 100 and 200 µM) before the SOD, CAT and APX assay were carried out.

4.8- Protein determination

In all the enzyme preparations, protein was measured by the Coomassie Blue method according to Bradford [41] using bovine serum albumin as standard.

4.9- Statistical analysis

Data were submitted to variance analyses (two-way ANOVA) and treatment means were compared by Duncan range at 5% of error probability using the SOC statistic package (Software Científico: NTIA/EMBRAPA). Treatments were presented as mean ± S.D. of three replicates.

Acknowledgements

The authors wish to thank the Coordenação e Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa de Estado do Rio Grande do Sul (FAPERGS).

References

[1] E.M. Suszcynsky, J.R. Shann, Phytotoxicity and accumulation of mercury in tabacco subjected to different exposure routes, Environ. Toxicol. Chem. 14 (1995) 61-67.

[2] B.L. Vallee, K.H. Falchuk, The biochemical basis of zinc physiology, Physiol. Rev. 73 (1993) 79-118.

[3] B.P. Shaw, S.K. Sahu, R.K. Mishra, Heavy metal induced oxidative damage in terrestrial plants. In: Prasad, M.N.V., (ed), Heavy metal stress in plants-from biomolecules to ecosystems, 2nd ed, (2004) pp.84-126. Springer-Verlag, Narosa, New Delhi.

[4] U.-H. Cho, J.O. Park, Mercury-induced oxidative stress in tomato seedlings, Plant Sci. 156 (2000) 1-9.

[5] H. Marschner, Mineral nutrition of higher plants, 2nd ed. (1995) London, UK: Academic Press.

[6] W.J. Bettger, B.L. O'Dell, A critical physiological role of zinc in the structure and function of biomembranes, Life Sci. 28 (1981) 1425–1438.

[7] M.P. Zago, P.I. Oteiza, The antioxidant properties of zinc: interactions with iron and antioxidants, Free Rad. Biol. Med. 31 (2001) 266-274.

[8] C.J.C. Silva, M.G.S. Lima, C.M. Carvalho, W.M. Eloi, M.M. Pedroza, C.J.C. Silva, Efeito do lodo de estação de tratamento de despejos de curtume na fase inicial do crescimento do milho, Ciência de Biologia e Ciências da Terra, v. 5, (2005) n. 2.

[9] P. Aravind, M.N.V. Prasad, Zinc alleviates cadmium-induced oxidative stress in *Ceratophyllum demersum* L.: a free floating freshwater macrophyte, Plant Physiol. Biochem. 41 (2003) 391-397.

[10] P. Aravind, M.N.V. Prasad, Zinc protects chloroplasts and associated photochemical functions in cadmium exposed *Ceratophyllum demersum* L., a freshwater macrophyte, Plant Sci. 166 (2004) 1321-1327.

[11] P. Aravind, M.N.V. Prasad, Cadmium-Zinc interactions in a hydroponic system using *Ceratophyllum demersum* L.: adaptative ecophysiology, biochemiostry and molecular toxicology, Braz. J. Plant Physiol. 17 (2005c) 3-20.

[12] R. Rellán-álvarez, C. Ortega-Villasante, A. Álvarez-Fernández, F.F. Campo, L.E. Hernández, Stress Responses of *Zea mays* to Cadmium and mercury, Plant Soil 279 (2006) 41–50.
[13] T.C. Fox, M.L. Guerinot, Molecular biology of cation transport in plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 669-696.
[14] J.F. Gonçalves, A.G Becker, D. Cargnelutti, L.A. Tabaldi, L.B. Pereira, V. Battisti, R.M. Spanevello, V.M. Morsch, F.T. Nicoloso, M.R.C Schetinger, Cadmium toxicity causes oxidative stress and induces response of the antioxidant system in cucumber seedlings, Braz. J. Plant Physiol. 19 (2007) 223-232.
[15] D. Cargnelutti, L.A. Tabaldi, R.M. Spanevello, G.O. Jucoski, V. Battisti, M. Redin, C.E.B. Linares, V.L. Dressler, E.M.M. Flores, F.T. Nicoloso, V.M. Morsch, M.R.C. Schetinger, Mercury toxicity induces oxidative stress in growing cucumber seedlings, Chemosphere 65 (2006) 999–1006.
[16] M. Bernier, R. Carpentier, The action of mercury on the binding of the extrinsic polypeptides associated with the water oxidizing complex of photosystem II, FEBS Lett. 360 (1995) 251–254.
[17] M. Bernier, R. Popovic, R. Carpentier, Mercury inhibition at the donor side of photosystem II is reversed by chloride, FEBS Lett. 321 (1993) 19–23.
[18] M. Patra, N. Bhowmik, B. Bandopadhyay, A. Sharma, Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance, Environ. Exp. Bot. 52 (2004) 199–223.
[19] D.D.K. Prasad, A.R.K. Prasad, Porphyrin metabolism in lead and mercury treated bajra (*Pennisetum typhoideum*) seedlings, J. Biosci. 15 (1990) 271-279.
[20] J.B.T. Rocha, M.E. Pereira, T. Emanuelli, R.S. Christofari, D. Souza, Effects of methylmercury exposure during the second stage of rapid postnatal brain growth on delta-aminolevulinic acid dehydratase (ALA-D) activity in brain, liver and blood of suckling rats, Toxicology 100 (1995) 27–37.
[21] M. Chavapil, New aspects in the biological role of zinc: a stabilizer of macromolecules and biological membranes, Life Sci. 13 (1973) 1041-1049.
[22] T.M. Bray, W.J. Bettger, The physiological role of zinc as an antioxidant, Free Radical Biol. Med. 8 (1990) 281-291.

[23] S.R. Powell, The antioxidant properties of zinc, J. Nutr. 130 (2000) 1447-1454.

[24] R.L. Levine, J.A. Williams, E.R. Stadtman, E. Shacter, Carbonyl assays for determination of oxidatively modified proteins, Methods Enzymol. 233 (1994) 346-357.

[25] P.R.S. Boscolo, M. Menossi, R.A. Jorge, Aluminum-induced oxidative stress in maize, Phytochemistry 62 (2003) 181-189.

[26] I. Cakmak, Possible roles of zinc in protecting plant cells from damage by reactive oxygen species, New Phytol. 146 (2000) 185-205.

[27] J. Gressel, E. Galun, Genetic controls of photooxidant tolerance, In: Foyer CH, Mullineaux P, eds. Causes of photooxidative stress and amelioration of defense systems in plants. Boca Raton, FL, USA: CRC Press, (1994) 237-273.

[28] A. Tukendorf, Phytochelatin synthesis in maize seedlings in response to excess zinc, Biol. Plant 38 (1996) 137–140.

[29] L.C. Casano, L.D. Gomez, H.R. Lascano, A.C. Gonzalez, V.S. Trippi, Inactivation and degradation of CuZn/SOD by active oxygen species in wheat chloroplasts exposed to photooxidative stress, Plant Cell Physiol. 38 (1997) 433-440.

[30] P. Aravind, M.N.V. Prasad, Modulation of cadmium-induced oxidative stress in *Ceratophyllum demersum* by zinc involves ascorbate-glutathione cycle and glutathione metabolism, Plant Physiol. Biochem. 43 (2005a) 107-116.

[31] L.A. Tabaldi, F.T. Nicoloso, G.Y. Castro, D. Cargnelutti, J.F. Gonçalves, R. Rauber, E.C. Skrebsky, M.R.C. Schetinger, V.M. Morsch, D.A. Bisognin, Physiological and oxidative stress responses of four potato clones to aluminum in nutrient solution, Braz J Plant Physiol 19 (2007) 211-222.

[32] L.E. Kaercher, F. Goldschmidt, J.N.G. Paniz, E.M.M. Flores, V.L. Dressler, Determination of inorganic and total mercury by vapor generation atomic absorption spectrometry using different temperatures of the measurement cell, Spectrochim. Acta B 60 (2005) 705-710.

[33] J.D. Hiscox, G.F. Israelstam, A method for the extraction of chlorophyll from leaf tissue without maceration, Can. J. Bot. 57 (1979) 1132-1334.

[34] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Meth. Enzymol. 148 (1987) 350–82.

[35] R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, S. Shaltiel, E.R. Stadtman, Determination of carbonyl content in oxidatively modified proteins, Metthods Enzymol. 186 (1990) 464-478.

[36] F. Loreto, V. Velikova, Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quences ozone products, and reduces lipid peroxidation of cellular membranes, Plant. Physiol. 127 (2001) 1781–7.

[37] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.

[38] J.M. Mc Cord, I. Fridovich, Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein), J. Biol. Chem. 244 (1969) 6049–6055.

[39] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121-126.

[40] Z. Zhu, G. Wei, J. Li, Q. Qian, J. Yu, Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (*Cucumis sativus* L.), Plant Sci. 167 (2004) 527-533.

[41] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

Table I

Zn accumulation in two maize hybrids, BR205 and 32R21.

Zn^{2+} (μM)	Zn content (μg g^{-1} dry weight)	
Root	BR205	32R21
0	124.5±6.5 dB	187.5±30.5 dA
50	1,143.5±44.5 cB	1,535.5±217 cA
100	2,497.5±288 bA	2,391.5±22.5 bB
200	2,908.5±496 aB	4,965.5±938 aA
Shoot		
0	137.5±10.5 cA	138±17 dA
50	760.5±10.5 bB	860±125 cA
100	1,798.5±123 aA	1,142±47 bB
200	1,849.5±3.5 aA	1,467±75 aB

Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Table II

Influence of Zn on chlorophyll a and b content in two maize hybrids, BR205 and 32R21.

Zn^{2+} (µM)	Chl a (mg g^{-1} FW)		Chl b (mg g^{-1} FW)	
Shoot	BR205	32R21	BR205	32R21
0	1.23±0.09 abA	1.70±0.06 aA	0.85±0.07 aA	1.07±0.03 aA
50	0.99±0.2 bA	1.54±0.3 aA	0.68±0.1 bA	1.09±0.25 aA
100	1.43±0.09 aA	0.90±0.4 bA	0.94±0.07 aA	0.6±0.27 bA
200	1.41±0.2 aA	1.46±0.3 aA	0.91±0.11 aA	0.99±0.24 aA

Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Table III

Influence of Zn on carbonyl and hydrogen peroxide content in root and shoot of two maize hybrids, BR205 and 32R21.

Zn^{2+} (μM)	Carbonyl content (nmol carbonyl/mg protein)		H_2O_2 content (μmol g^{-1} FW)	
Root	BR205	32R21	BR205	32R21
0	12.6±8.8 aA	8.79±0.3 aA	0.212±0.01 bB	0.317±0.03 cA
50	19.35±0.6 aA	5.71±2.5 bB	0.266±0.04 aB	0.749±0.02 aA
100	18.25±2.9 aA	10.93±1.2 aA	0.295±0.03 aB	0.745±0.03 aA
200	17.08±6.3 aA	3.75±2.1 bB	0.155±0.02 cB	0.554±0.03 bA
Shoot				
0	4.79±3.1 bcA	1.16±0.1 bA	0.272±0.02 cB	0.605±0.04 aA
50	2.74±0.4 cA	3.03±0.8 aA	0.347±0.04 bA	0.310±0.05 bA
100	7.33±2.1 abA	3.29±0.4 aA	0.299±0.01 cA	0.313±0.08 bA
200	9.23±1.0 aA	2.3±1.4 abB	0.439±0.02 aA	0.320±0.09 bA

Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Table IV

Influence of Zn on non-protein thiol content in root and shoot of two maize hybrids, BR205 and 32R21.

Zn^{2+} (μM)	NPSH content (nmol SH mg $protein^{-1}$)	
Root	BR205	32R21
0	0.136±0.005 bA	0.124±0.003 cA
50	0.14±0.003 bA	0.153±0.002 bA
100	0.153±0.004 aA	0.173±0.006 aA
200	0.163±0.001 aA	0.119±0.01 cA
Shoot		
0	0.156±0.004 bA	0.186±0.005 aA
50	0.174±0.013 aA	0.157±0.002 bA
100	0.17±0.003 aA	0.155±0.002 bA
200	0.131±0.002 cA	0.187±0.006 aA

Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Table V

Influence of Zn on superoxide dismutase, catalase and ascorbate peroxidase activities in root and shoot of two maize hybrids, BR205 and 32R21.

Zn^{2+} (μM)	SOD activity (U SOD mg^{-1} protein)		CAT activity (ΔE min^{-1} mg^{-1} protein)		APX activity (μmol AsA oxidate min^{-1} mg^{-1} protein)	
Root	BR205	32R21	BR205	32R21	BR205	32R21
0	2,038.9±274 aA	1,501.7±91.7 bB	12.99±0.87 aA	4.76±1.3 aB	8.91±0.7 aA	4.18±0.1 aB
50	2,805.7±93.4 bA	1,118.4±86 cB	7.74±1.84 bA	4.74±1.8 aA	5.53±0.6 bA	1.45±0.1 bB
100	3,058.2±35.9 bA	2,041.1±169 aB	6.77±0.51 bA	5.74±2.7 aA	4.54±0.4 bA	3.89±0.7 aA
200	2,246.4±129 cA	1,373.6±57.4 bB	7.05±0.59 bA	4.84±0.2 aA	4.03±1.3 bA	2.46±0.1 bA
Shoot						
0	1,377.9±41.9 dA	944.9±16.2 cB	2.3±0.17 bA	2.51±0.83 aA	3.45±0.1 bA	1.28±0.2 cB
50	2,783.7±41.8 aA	1,107.9±74 aB	6.35±1.8 aA	2.61±1.0 aA	4.72±0.25 aA	1.90±0.6 bB
100	2,102.7±2.5 bA	1,022.8±66 bcB	5.10±3.1 abA	2.38±0.81 aA	4.99±0.25 aA	2.1±0.03 abB
200	1,711.9±61.3 cA	1,053.0±23 abB	4.03±1.6 abA	2.46±1.09 aA	3.28±0.14 bA	2.56±0.3 aA

Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Table VI

Influence of Zn on superoxide dismutase, catalase and ascorbate peroxidase activities in root and shoot of two maize hybrids, BR205 and 32R21, in vitro.

Zn^{2+} (μM)	SOD activity (U SOD mg^{-1} protein)		CAT activity ($\Delta E\ min^{-1}\ mg^{-1}$ protein)		APX activity (μmol AsA oxidate $min^{-1}\ mg^{-1}$ protein)	
Root	BR205	32R21	BR205	32R21	BR205	32R21
0	614.476±1.1 cB	995.61±4.0 bA	0.34±0.04 bB	1.024±0.04 aA	1.98±0.4 aA	0.227±0.02 bB
50	636.135±16.3 cB	910.25±133 bA	0.243±0.05 bB	0.931±0.09 aA	1.58±0.04 bA	0.83±0.01 aB
100	709.605±36.4 bB	1001.89±14.4 bA	0.73±0.05 aA	1.025±0.03 aA	2±0.36 aA	0.2±0.04 bB
200	785.74±22.5 aB	1177.51±67.4 aA	1.024±0.05 aA	1.257±0.03 aA	1.55±0.08 bA	0.19±0.01 bB
Shoot						
0	359.402±5.6 cB	567.558±0.5 bA	0.77±0.01 bA	0.515±0.02 cA	2.38±0.38 aA	1.595±0.09 aB
50	408.393±12.1 bB	641.661±2.3 aA	0.925±0.04 aA	1.082±0.04 abA	2.05±0.15 aA	1.285±0.04 bA
100	412.013±7.0 bB	566.262±12.6 bA	1.109±0.09 aA	1.245±0.03 abA	2.15±0.03 aA	1.02±0.02 cB
200	485.02±64.4 aB	635.183±27.8 aA	0.693±0.05 bA	0.902±0.01 bcA	2.55±0.21 aA	0.51±0.02 dB

Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

LEGEND OF THE FIGURES

Figure 1. Metal accumulation in root (A) and shoot (B) of BR205 hybrid treated with Hg-25 µM and Zn (50, 100 and 200 µM). Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations (two-way ANOVA/Duncan; $p<0.05$).

Figure 2. Metal accumulation in root (A) and shoot (B) of 32R21 hybrid treated with Hg-25 µM and Zn (50, 100 and 200 µM). Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations (two-way ANOVA/Duncan; $p<0.05$).

Figure 3. Chlorophyll a (A) and chlorophyll b (B) content in two maize hybrids, BR205 and 32R21, treated with Hg-25 µM and Zn (50, 100 and 200 µM). Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Figure 4. Root carbonyl protein (A) and hydrogen peroxide (B) content in two maize hybrids, BR205 and 32R21, treated with Hg-25 µM and Zn (50, 100 and 200 µM) concentrations. Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Figure 5. Shoot carbonyl protein (A) and hydrogen peroxide (B) content in two maize hybrids, BR205 and 32R21, treated with Hg-25 µM and Zn (50, 100 and 200 µM) concentrations. Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Figure 6. Root (A) and shoot (B) non-protein thiols concentration in two maize hybrids, BR205 and 32R21, treated with Hg-25 µM and Zn (50, 100 and 200 µM). Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters

represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Figure 7. Root superoxide dismutase (A), catalase (B) and ascorbate peroxidase (C) activities in two maize hybrids, BR205 and 32R21, treated with Hg-25 µM and Zn (50, 100 and 200 µM) concentrations. Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

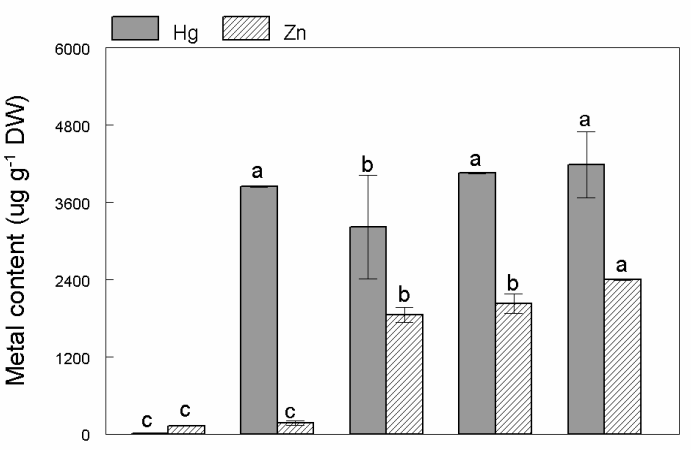
Figure 8. Shoot superoxide dismutase (A), catalase (B) and ascorbate peroxidase (C) activities in two maize hybrids, BR205 and 32R21, treated with Hg-25 µM and Zn (50, 100 and 200 µM) concentrations. Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Figure 9. Root superoxide dismutase (A), catalase (B) and ascorbate peroxidase (C) activities in two maize hybrids, BR205 and 32R21, treated with Hg-25 µM and Zn (50, 100 and 200 µM) concentrations, in vitro. Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Figure 10. Shoot superoxide dismutase (A), catalase (B) and ascorbate peroxidase (C) activities in two maize hybrids, BR205 and 32R21, treated with Hg-25 µM and Zn (50, 100 and 200 µM) concentrations, in vitro. Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Figure 1

(A)


Hg
Zn
Metal content (ug g-1 DW)
6000
4800
3600
2400
1200
0
a
b
a
a
c
c
c
b
b
a

(B)

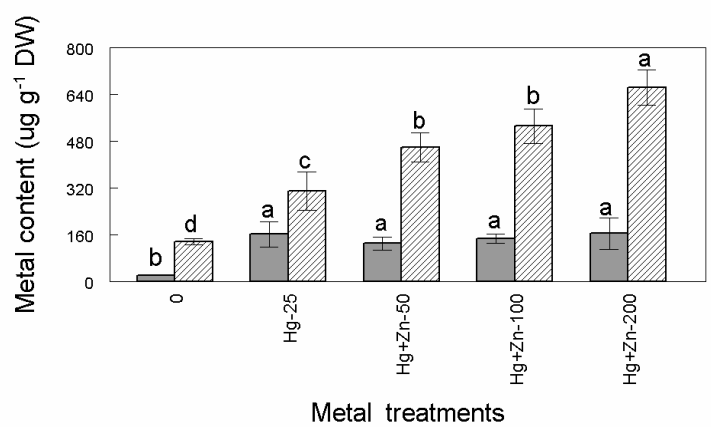
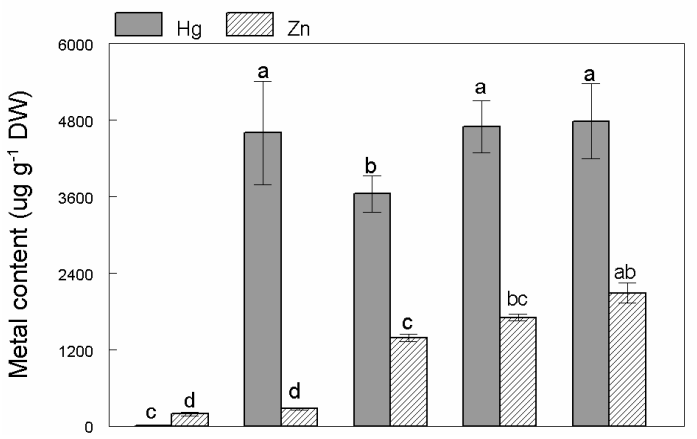

Metal content (ug g-1 DW)
800
640
480
320
160
0
b
d
a
c
a
b
a
b
a
a
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal treatments

Figure 2

(A)


Hg
Zn
Metal content (ug g-1 DW)
6000
4800
3600
2400
1200
0
a
d
b
c
bc
ab

(B)

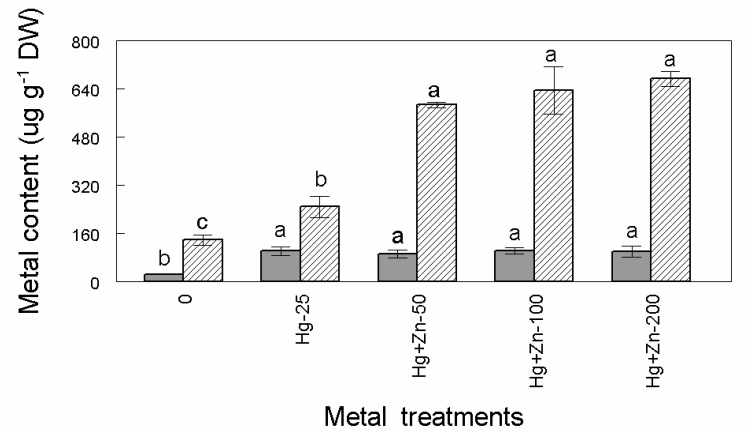

Metal content (ug g-1 DW)
800
640
480
320
160
0
b
c
a
b
a
a
a
a
a
a
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal treatments

Figure 3

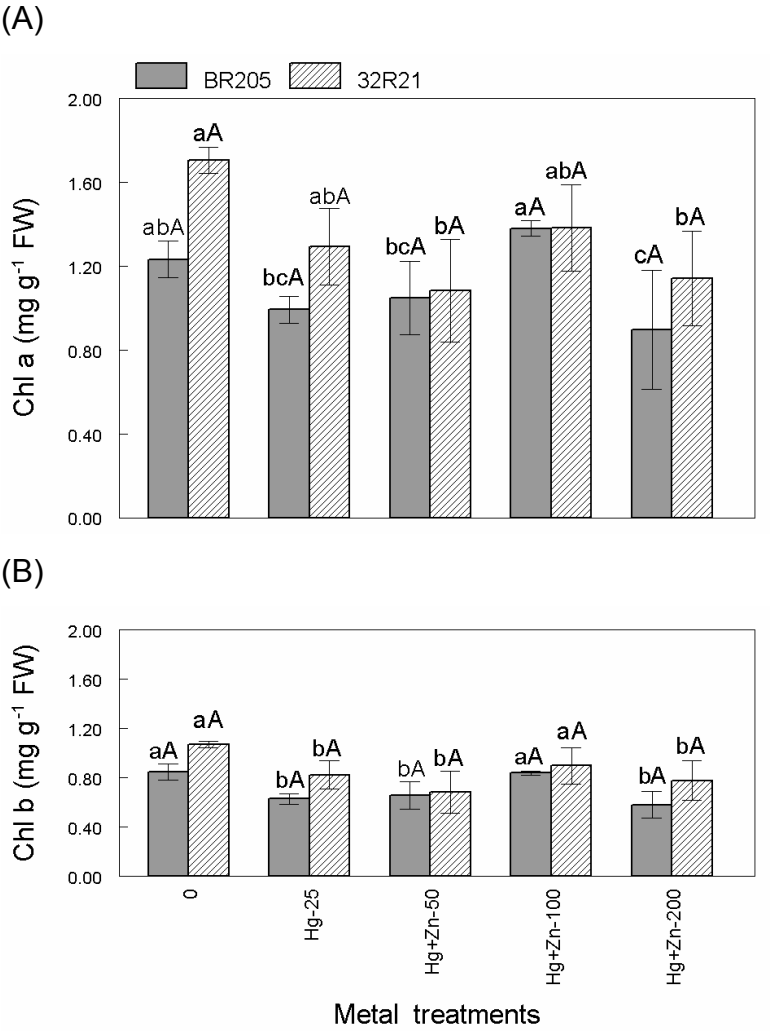
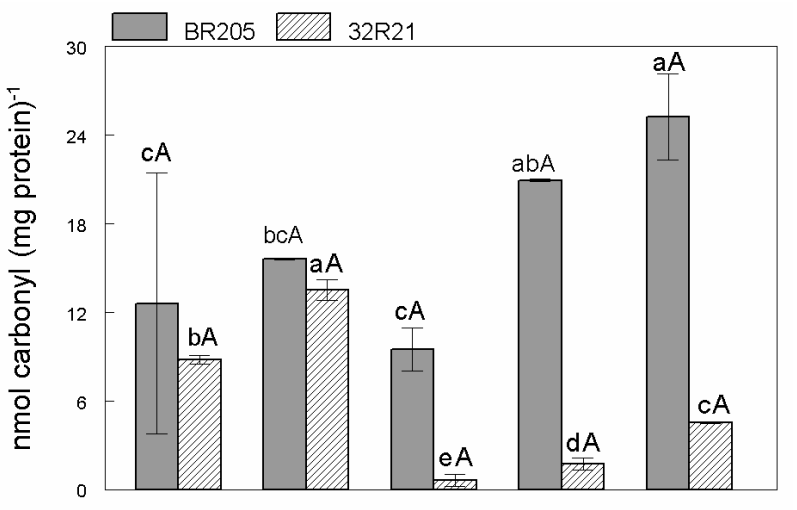

(A)
BR205
32R21
Chl a (mg g-1 FW)
2.00
1.60
1.20
0.80
0.40
0.00
aA
abA
abA
bcA
bcA
bA
aA
abA
cA
bA
(B)
Chl b (mg g-1 FW)
2.00
1.60
1.20
0.80
0.40
0.00
aA
aA
bA
bA
bA
bA
aA
aA
bA
bA
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal treatments

Figure 4

(A)


BR205
32R21
nmol carbonyl (mg protein)-1
30
24
18
12
6
0
cA
bA
bcA
aA
cA
eA
abA
dA
aA
cA

(B)

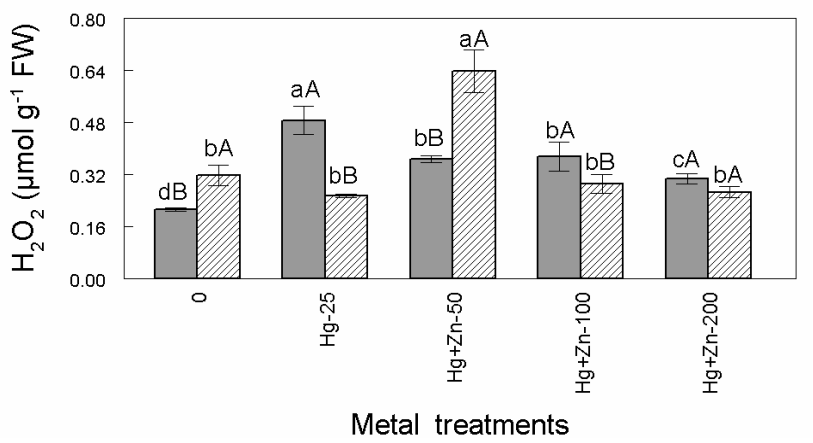
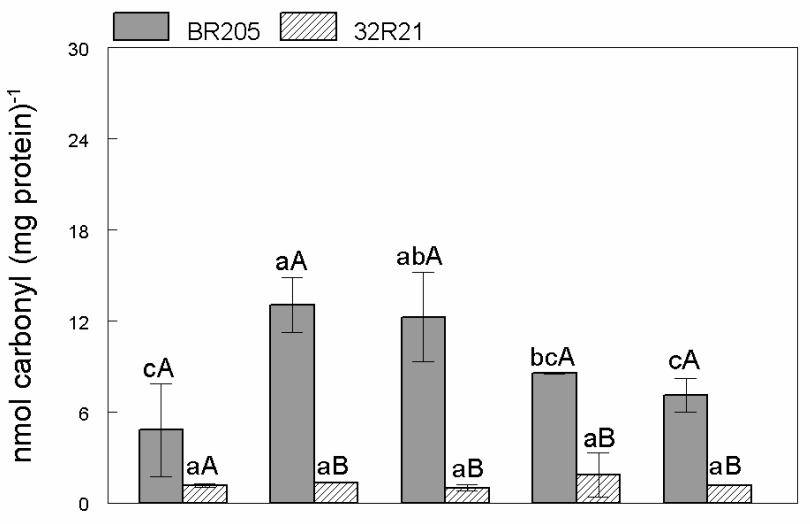

H2O2 (µmol g-1 FW)
0.80
0.64
0.48
0.32
0.16
0.00
dB
bA
aA
bB
bB
aA
bA
bB
cA
bA
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal treatments

Figure 5

(A)


BR205
32R21
nmol carbonyl (mg protein)-1
30
24
18
12
6
0
cA
aA
aA
aB
abA
aB
bcA
aB
cA
aB

(B)

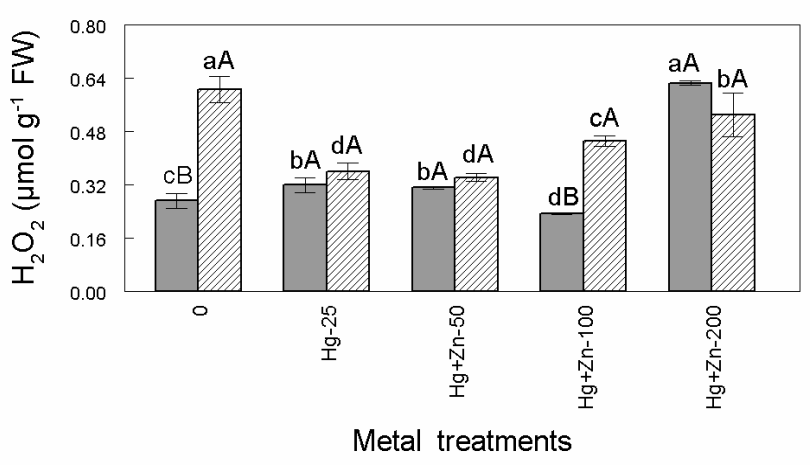
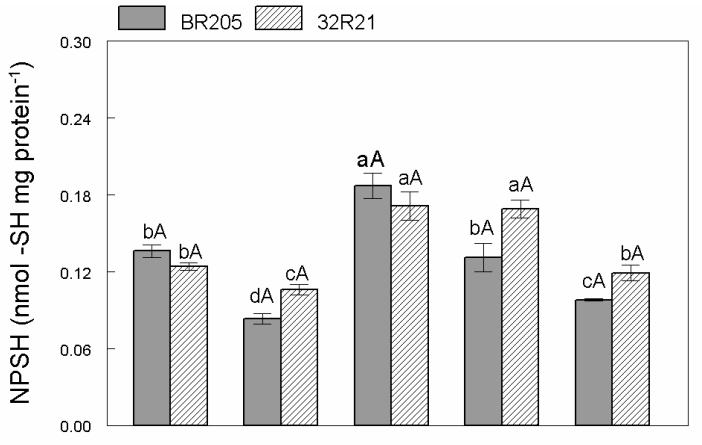
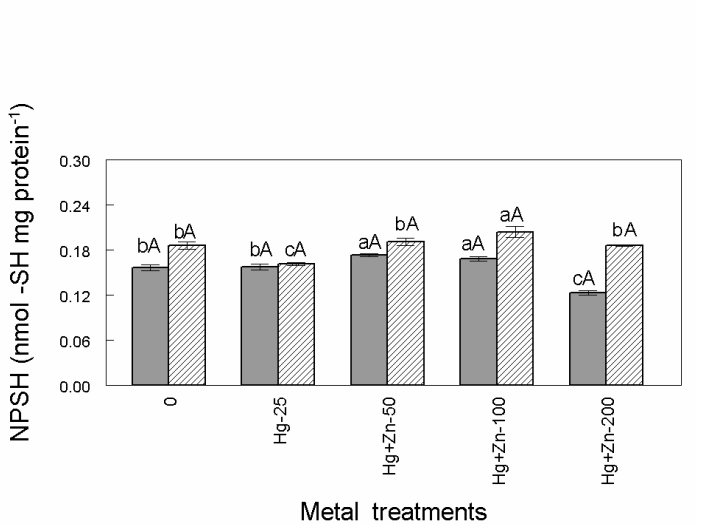

H2O2 (µmol g-1 FW)
0.80
0.64
0.48
0.32
0.16
0.00
cB
aA
bA
dA
bA
dA
dB
cA
aA
bA
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal treatments

Figure 6

(A)


BR205
32R21
NPSH (nmol -SH mg protein-1)
0.30
0.24
0.18
0.12
0.06
0.00
bA
bA
dA
cA
aA
aA
bA
aA
cA
bA

(B)

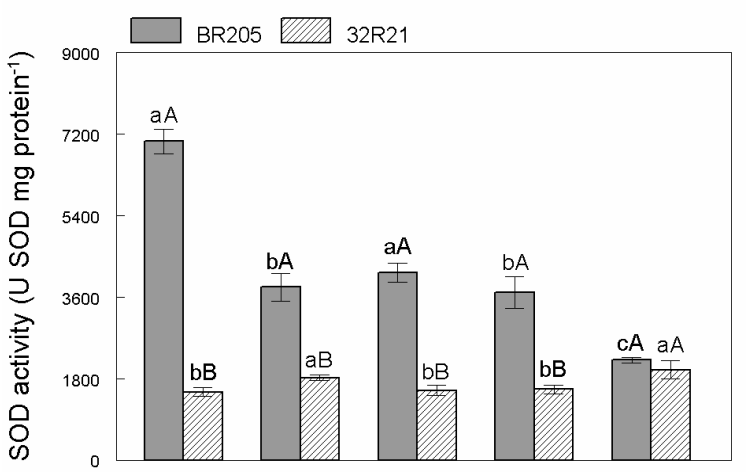

NPSH (nmol -SH mg protein-1)
0.30
0.24
0.18
0.12
0.06
0.00
bA
bA
bA
cA
aA
bA
aA
aA
cA
bA
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal treatments

176

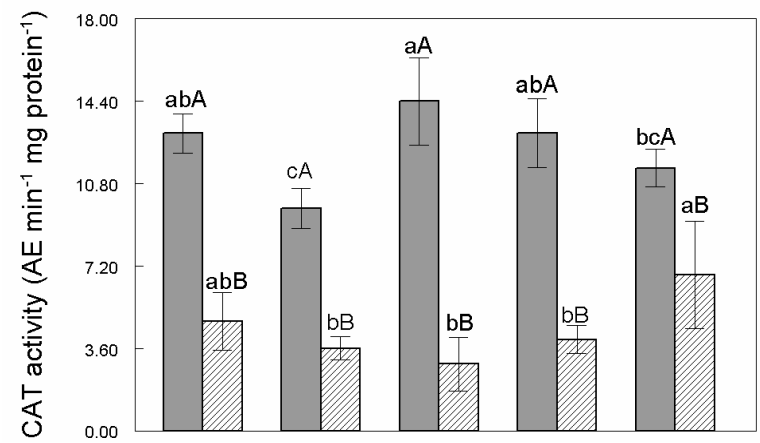
177 **Figure 7**

178

(A)



(B)



(C)

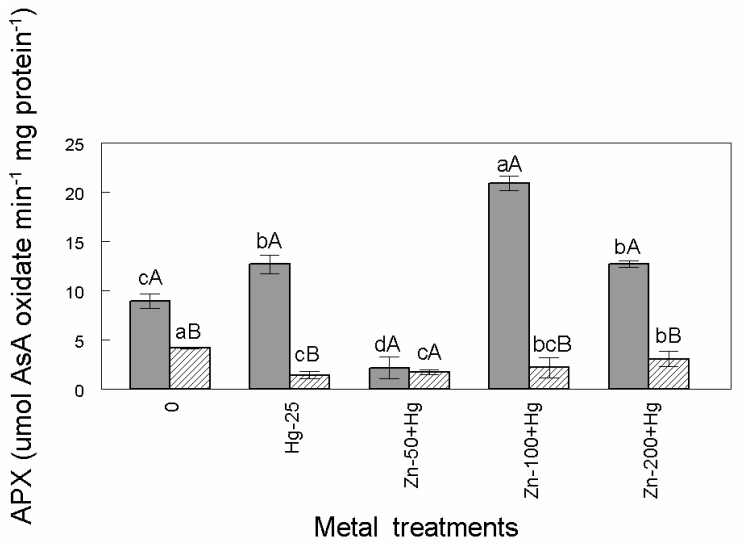
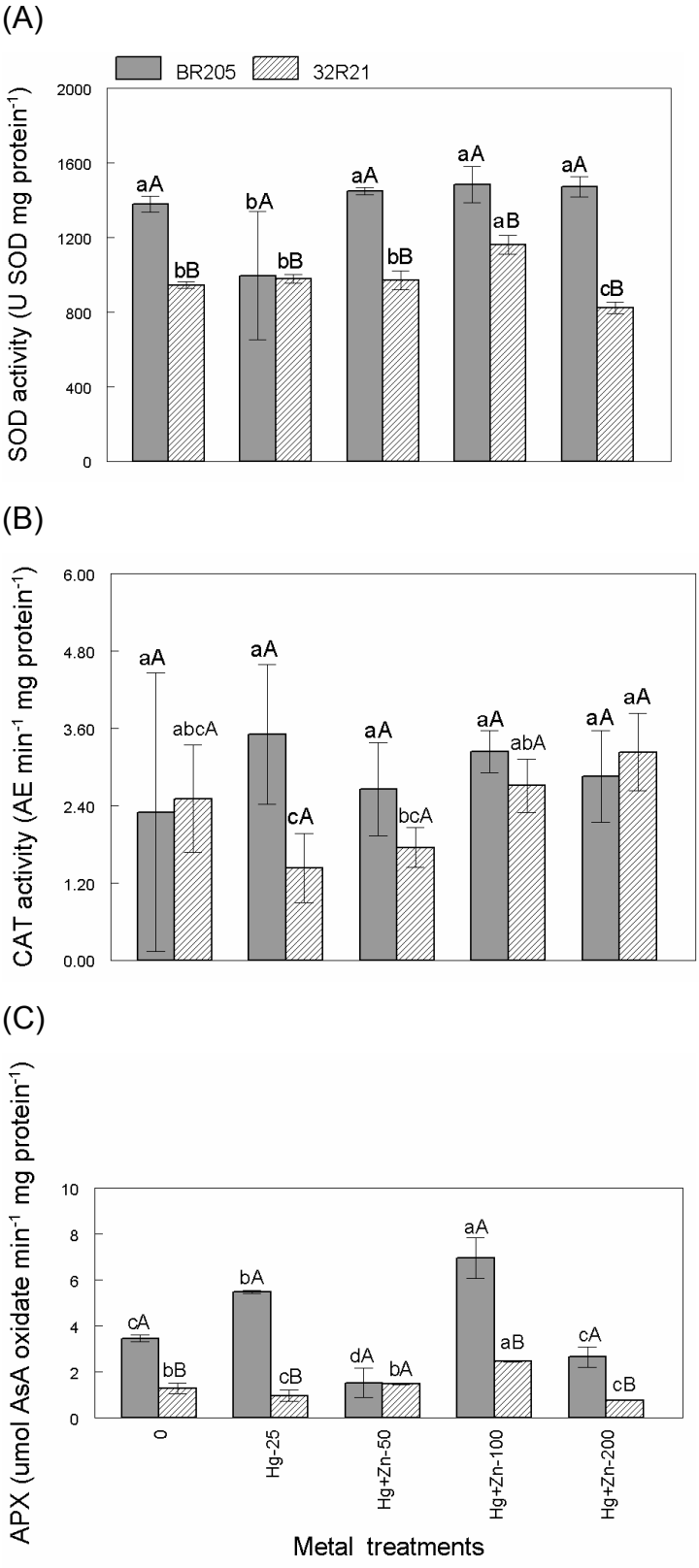
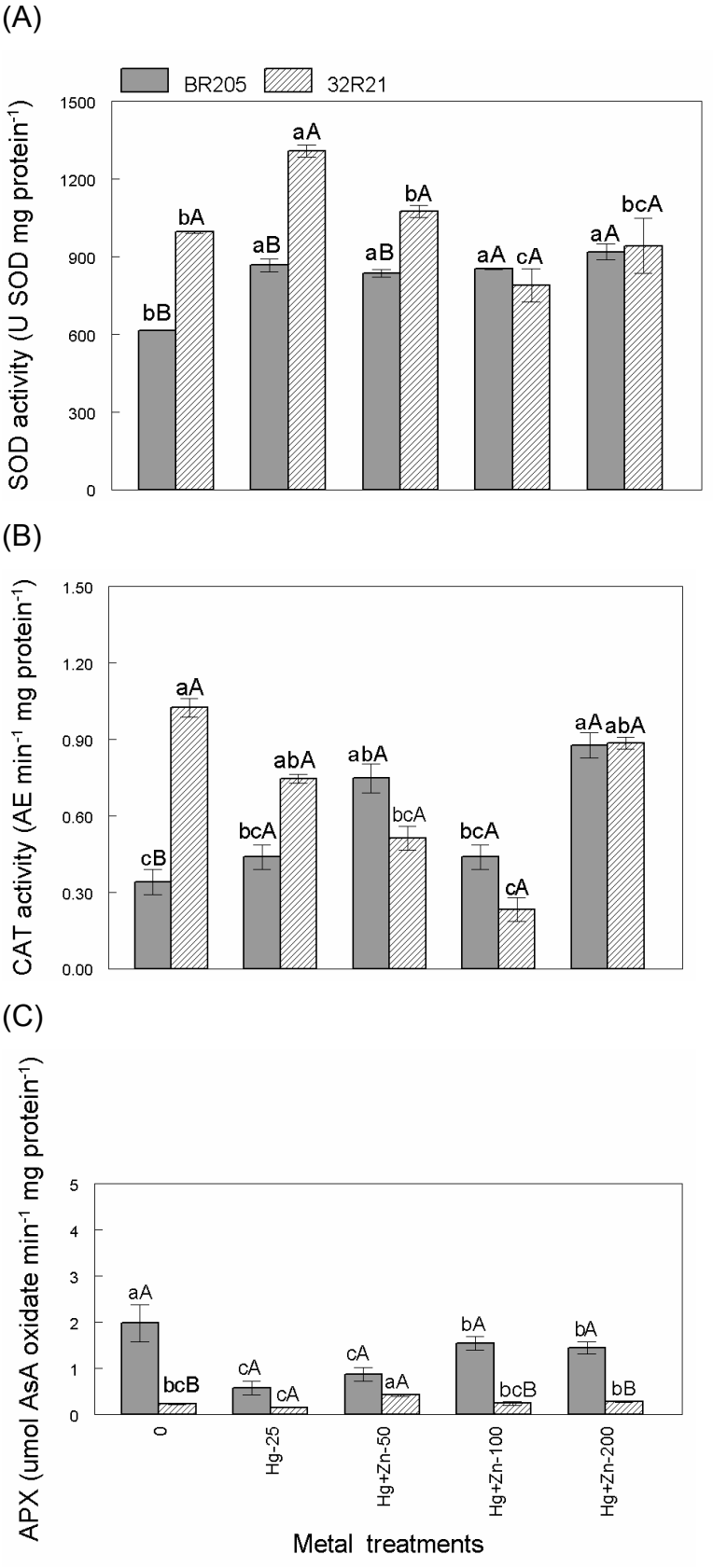


Figure 8


(A)
BR205
32R21
SOD activity (U SOD mg protein-1)
2000
1600
1200
800
400
0
aA
bB
bA
bB
aA
bB
aA
aB
aA
cB
(B)
CAT activity (AE min-1 mg protein-1)
6.00
4.80
3.60
2.40
1.20
0.00
aA
abcA
aA
cA
aA
bcA
aA
abA
aA
aA
(C)
APX (umol AsA oxidate min-1 mg protein-1)
10
8
6
4
2
0
cA
bB
bA
cB
dA
bA
aA
aB
cA
cB
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal treatments

182 **Figure 9**

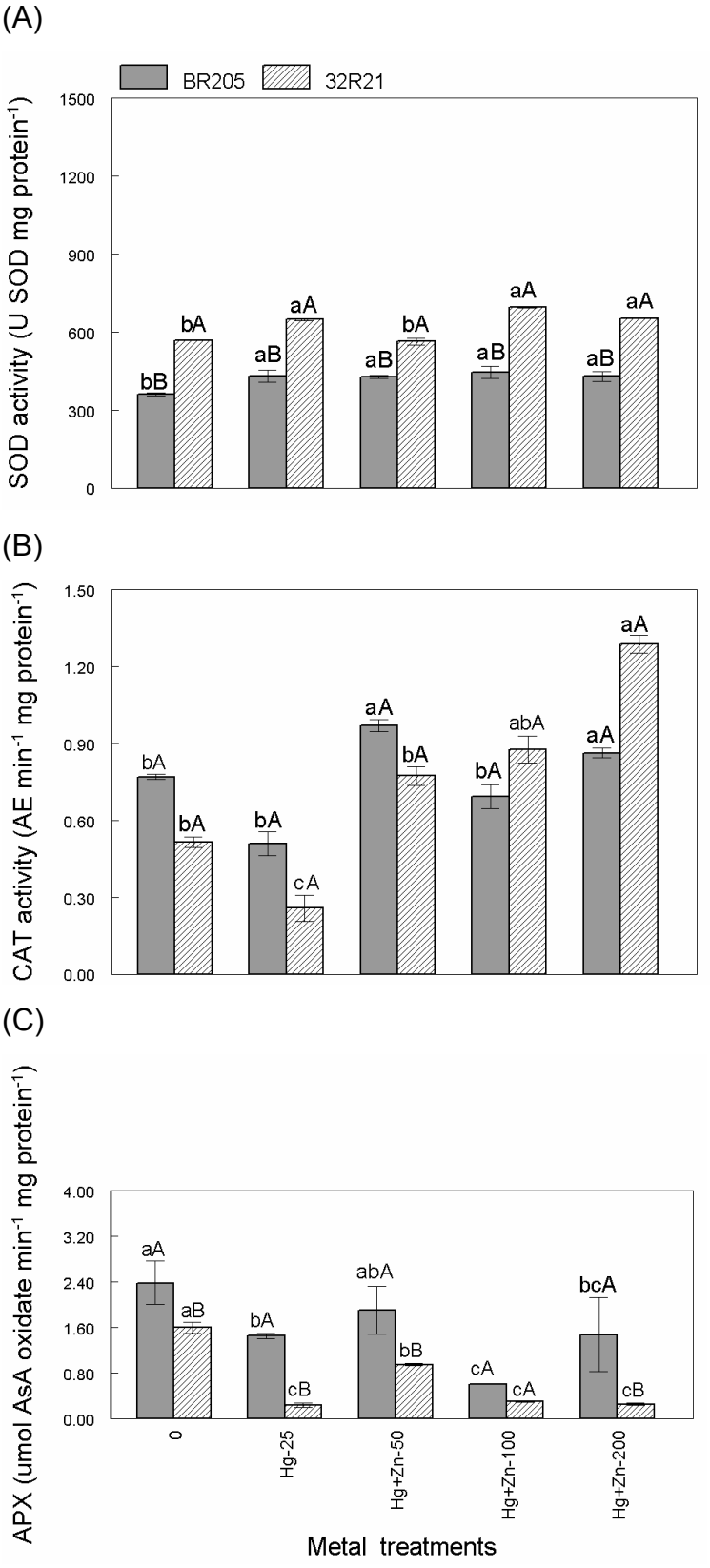
183



184

185 **Figure 10**

186



187

188

3.1.2.3. Zinco protege híbridos de milho contra a inibição induzida pelo mercúrio no crescimento e na atividade δ-Aminolevulinato desidratase

Manuscrito 4

Zinc protects maize against inhibition on growth and δ-aminolevulic acid dehydratase activity induced by mercury

Denise Cargnelutti, Fernando T. Nicoloso, Luciane B. Pereira, Liana V. Rossato, Nicéia C. Spanholi, Luciane A. Tabaldi, Jamile F. Gonçalves, Gabriel Y. Castro, Vera M. Morsch, Maria R.C. Schetinger

(Submetido à Plant cell reports)

Zinc protects maize against inhibition on growth and δ-Aminolevulic acid dehydratase activity induced by mercury

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Abstract

The interactions of Zn and Hg on growth, ascorbic acid (AsA) concentration and in the δ-Aminolevulinic acid dehydratase (δ-ALAD; E.C. 4.2.1.24) activity in two hybrids maize (*Zea mayz* L.) (BR205 and 32R21) were investigated. Hybrids were exposed for 5 days in nutrient solution using 25 μM Hg and/or Zn (50 - 200 μM). The length, fresh weight (FW), dry weight (DW) and AsA concentration were reduced by Hg in both hybrids, whereas in treatments supplemented with Zn there was an increase on length and FW. The BR205 δ-ALA-D activity was inhibited by Hg, whereas Zn at all levels hampered the negative effect of Hg on this enzyme. Moreover, treatments with Hg either alone applied or in combination with Zn reduced BR205 and 32R21 δ-ALA-D activity in vitro, suggesting that Zn plays a role in δ-ALA-D induction mainly in the BR205 and reestablishing the growth reduced by Hg.

Keywords*:* δ-Aminolevulinic acid dehydratase, ascorbic acid, growth, mercury; zinc.

253 **Introduction**

254

255 Mercury (Hg) is a non-essential nutrient in higher plants, and exposure to relatively low concentrations
256 results in serious toxicity (Salt et al. 1995). However, zinc (Zn) is an important component of many vital
257 enzymes having a catalytic, co-catalytic or structural role as a stabilizer of proteins, membranes and DNA-
258 binding proteins (Zn-fingers) (Vallee and Auld 1990), but is toxic in high concentrations (Sterckeman et al.
259 2000). Since both Hg and Zn are transition group elements with similar electronic configuration and valence,
260 they have similar geochemical and environmental properties (Patra and Sharma 2000; Du et al. 2005).

261 Mercury is considered one of the most readily accumulated toxic metal elements. It accumulates in
262 living organisms causing harmful damage (Ortega-Villasante et al. 2005; Cargnelutti et al. 2006; Rellán-Álvarez
263 et al. 2006; Zhou et al. 2007; 2008). Among the different forms of mercury, Hg^{2+} is highly water-soluble and
264 reactive, and strongly interacts with sulfhydryl groups of vital enzymes and proteins in cell apoplasts (Assche
265 and Clijsters 1990). For example, Hg^{2+} is able to bind with water channel proteins (aquaporins) in the membrane
266 (Ionenko et al. 2003) of root cells causing a physical obstruction to water flow (Maggio and Joly 1995) and
267 consequently affecting transpiration (Zhang and Tyerman 1999). Another toxic symptom of mercury
268 accumulation in plants is the decrease in plant growth (Du et al. 2005; Cargnelutti et al. 2006) and decreases
269 (Cargnelutti et al. 2006) or increase (Esteban et al. 2008) in chlorophyll levels. Mercury is known to induce the
270 formation of reactive oxygen species (ROS) that initiate oxidative stress in plants, an important mechanism of
271 cell injury (Ortega-Villasante et al. 2005; Cargnelutti et al. 2006; Moreno et al. 2008). There are also reports
272 indicating that Hg accumulation in roots blocks the uptake and transport of nutrients (Boening 2000). However,
273 biochemical and molecular mechanisms of Hg phytotoxicity remain to be elucidated (Zhou et al. 2007).

274 Amongst proteins effected by Hg exposure is the δ-Aminolevulinic acid dehydratase (δ-ALA-D), which
275 catalyzes the synthesis of porphobilinogen from two molecules of δ-aminolevulinic acid (δ-ALA) (Gibson et al.
276 1955), is a key enzyme of the biosynthesis pathway, leading to the formation of porphyrins, hemes and
277 chlorophylls in higher plants (Jaffe et al. 2000) and is therefore essential for adequate aerobic metabolism and
278 photosynthesis. Rocha et al. (1995) and Morsch et al. (2002) reported that δ-ALA-D is sensitive to heavy metals,
279 such as Hg, due to its sulfhydrylic nature. Therefore, Hg cause toxic effect in the δ-ALA-D activity due at
280 interaction with sulfhydryl groups of proteins or displacement of essential elements such as Mg^{2+}. However, the
281 effect of interactions between Zn, a micronutrient essential with antioxidant potential, and the Hg in the δ-ALA-
282 D activity has not yet been studied.

Zinc ions bind to ligands containing sulfur, nitrogen and, to a lesser extent, oxygen and preferentially bind to membrane proteins (Bettger and O'Dell 1981). The balance between free radical generation and free radical defense determines the survival of the system. Therefore, Zn may have a role in modulating free radicals and related processes through its antioxidant properties (Zago and Oteiza 2001). Hence, the purpose of this study was to investigate Zn-Hg interactions with respect to growth and δ-ALA-D activity in maize (*Zea mays* L.) hybrids. In addition, the ascorbic acid (AsA) and carotenoids concentrations were assessed with the aim of verifying the protection capacity of these antioxidants on Hg treatment alone and with Zn supplementation.

Materials and Methods

Plant material and growth conditions

Two hybrids (BR205 and 32R21) were evaluated. The BR205 (duple hybrid; obtained from Empresa Brasileira de Pesquisa Agropecuária, EMBRAPA, Minas Gerais, Brazil) is adapted to tropical regions of Brazil, presents precocity and high productivity, as well as tolerance to aluminum toxicity and water stress. Moreover, it was shown to have high capacity for uptake of mineral elements from soils (Silva et al. 2005). The 32R21 (simple hybrid, obtained from PIONNER company) hybrid was chosen due to it high biomass production and because it has been extensively cultivated in the southern region of Brazil. Seeds were germinated in plastic boxes on filter paper. Seven-day old uniform plantlets were transferred into plastic boxes (10 L) filled with aerated nutrient solution of low ionic strength. The nutrient solution was prepared as described in Tabaldi et al. (2007) (in mg L^{-1}): 8.31 of N; 0.754 of P; 1.154 of S; 9.76 of Ca; 2.37 of Mg; 1.05 of K; 17.68 of Cl; 0.027 of B; 0.005 of Mo; 0.001 of Ni; 0.013 of Zn; 0.003 of Cu; 0.011 of Mn and 0.268 of Fe ($FeSO_4$/Na-EDTA). The plantlets were acclimated for seven days before addition of the treatments. Throughout the acclimation and treatment periods, the plants were grown in a growth chamber at 25 ± 2ºC on a 16/8-h light/dark cycle with 35 μmol m^{-2} s^{-1} of irradiance. The solution pH was adjusted daily to 5.4 ± 0.1 by titration with HCl or NaOH solutions (0.1 M). Treatments consisted of the addition of Hg (0 and 25 μM) and/or Zn (0, 50, 100, 200 μM) for 5 days. At harvest, the plants were divided into shoot and roots. Roots were rinsed twice with distilled water. Subsequently, Hg and Zn uptake, growth, non-enzymatic antioxidant concentration and δ-ALA-D activity were determined. Mercury at level of 25 μM was found to decrease the root length by 25% (data not shown), which

312 was the concentration used for the estimation of physiological and biochemical parameters. The concentrations
313 of Zn were based on studies using *Ceratophyllum demersum* L. (Aravind and Prasad 2003, 2004, 2005).
314
315 Growth analysis
316
317 Maize growth was determined by measuring the length of the root system (Tennant 1975) and of the shoot
318 (measured with a ruler), both expressed in cm plant^{-1}. To obtain fresh weight, excess water from root washing
319 was removed with a paper towel. To obtain dry weight, the plants were left at 65°C to a constant weight
320 (approximately two weeks). Fresh and dry weight were expressed as g plant^{-1}.
321
322 Mercury (Hg) and zinc (Zn) determination in tissues
323
324 Dried (65^{o}C) plant tissues (root and shoot) were ground and digested (using 10 to 200 mg) initially with
325 5 ml of concentrated HNO_3 at 90^{o}C during 2 h. Sample decomposition was carried out in an open digestion
326 system, using a heating block from Velp Scientifica (Milano, Italy) equipped with glass vessels. Furthermore, 1
327 ml H_2O_2 was added and heated to 90°C for 1 h. The relatively low temperature was used to avoid Hg losses.
328 Moreover, plastic caps were fitted to the vessels to prevent analyte losses by volatilization and contamination.
329 The decomposed sample solution was diluted to 30 mL with purified water. Analyte determinations were
330 performed directly in these solutions. The certified reference materials peach leaves NIST 1547 and apple leaves,
331 NIST 1515 (National Institute of Standards and Technology, Gaithersburg, USA) were analyzed to evaluate the
332 accuracy of the sample preparation and Hg and Zn determination methods.
333 Zinc concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP
334 OES) using a PerkinElmer Optima 4300DV (Shelton, USA) equipped with a cyclonic spray chamber and a
335 concentric nebulizer. The emission line used was 213.617nm. Instrumental parameters were adjusted according
336 manufacturer recommendations. Nebulizer, intermediate and principal gas flow rates were set to 0.65, 0.20 and
337 14 L min^{-1}, respectively.
338 Mercury determination was performed by flow injection cold vapor generation hyphenated to
339 inductively coupled plasma optical emission spectrometry (FI-CV-ICP OES), using the Hg emission line on
340 253.650 nm. The FI-CV system was adapted from Kaercher et al. (2005). The FI system consists of a peristaltic
341 pump (Gilson, minipuls 3, France), a manual injector, and a U type gas/liquid separator. Tygon pump tubing of

different internal diameters (i.d.) was used for carrying the solutions. All other tubing was of PTFE with 0.8 mm i.d. The tissue Hg and Zn concentration were expressed as μg g^{-1} dry weight.

Ascorbic acid (AsA) concentration

Shoot and roots of maize hybrids were homogenized in a solution containing 50 mM Tris- HCl and 10 mL L^{-1} Triton X-100 (pH 7.5) and centrifuged at 6,800 *g* for 10 min. To the resulting 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. Determination of AsA was performed as described by Jacques-Silva et al. (2001). 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 DNPH. The DNPH solution contained 2% DNPH, 0.23% thiourea, and 0.27% CuSO4 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.

Estimation of δ-Aminolevulinic acid dehydratase (ALA-D; E.C. 4.2.1.24) activity

Maize leaves 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 dithiothreitol (DTT). ALA-D activity was assayed as described by Morsch et al. (2002) by measuring the rate of porphobilinogen (PBG) formation. The incubation medium for the assays contained 100 mM Tris-HCl buffer, pH 9.0. For the enzyme assay, the final concentration of ALA was 3.6 mM. Incubation was started by adding 100 μL of the tissue preparation in a final volume of 400 μL. The reaction product was determined with the Ehrlich reagent at 555 nm using a molar absorption coefficient of 6.1×10^4 L mol^{-1} cm^{-1} (Sassa 1982) for the Ehrlich-porphobilinogen salt. ALA-D activity was expressed as nmol PBG mg^{-1} protein h^{-1}. In vitro δ-ALA-D activity was carried out as described above, except that untreated seedlings were used, and tissue supernatant were pre-incubated at 37°C for 60 min in the medium containing Hg (0 and 25 μM) and/or Zn (0, 50, 100 and 200 μM) before the δ-ALA-D activity assay were carried out.

370

371 Protein determination

372

373 In all the enzyme preparations, protein was measured by the Coomassie Blue method according to
374 Bradford (1976) using bovine serum albumin as standard.

375

376 Statistical analysis

377

378 Data were submitted to variance analyses (two-way ANOVA) and treatment means were compared by
379 Duncan range at 5% of error probability using the SOC statistic package (Software Científico:
380 NTIA/EMBRAPA). Treatments were presented as mean S.D. of three replicates.

381

382 **Results**

383

384 Maize shoot and root growth was sensitive to Hg exposure. In previous experiments, treatments with
385 25, 50, 75 and 100 μM Hg gradually inhibited shoot and root growth, as expressed by length (data not shown).
386 The roots length treated with 25 μM Hg decreased by about 25% when compared to the control (Hg-free) (data
387 not shown). Therefore, Hg at level of 25 μM was used for estimation of physiological and biochemical responses
388 in the Zn presence.

389

390 Growth analysis

391

392 Treatments with 25 μM Hg applied alone showed reduced root (by about 50%) and shoot (by about
393 30%) length for both BR205 and 32R21 (Figs. 1A and 2A), when compared to the control. However, treatments
394 with 25 μM Hg either in the presence of 50 and 200 μM Zn increased significantly (about of 64% and 102%,
395 respectively) the BR205 root length (Fig. 1A). The same effect was observed in 32R21 root treated with 25 μM
396 Hg in the presence of 100 μM Zn. In addition, Hg-treated BR205 and 32R21 and supplemented with increasing
397 levels of Zn increased significantly shoot length (varying from 12% to 29%), when compared to Hg-alone
398 treatment (Fig. 2A). Zinc-alone treated seedlings showed no alteration in the length of both hybrids (Table 1).

399 The root fresh weight (FW) decreased significantly by about 44% and 27% with 25 μM Hg,
400 respectively for, BR205 and 32R21. However, treatments with 25 μM Hg in the presence of 50 and 100 μM Zn
401 increased significantly (about of 46% and 40%, respectively) the 32R21 root FW (Fig. 1B). The similar effect
402 was observed in BR205 root FW treated with 25 μM Hg in the presence of 100 μM Zn (35% of increase), when
403 compared to treatment with Hg alone. Moreover, treatments with 25 μM Hg decreased shoot FW in the both
404 hybrids of maize, whereas the supplementation with Zn at levels of 50, 100 and 200 μM at Hg-treatments
405 increased shoot FW (varying from 23% to 30%) (Fig. 2B). Treatments either with 25 μM Hg applied alone or
406 together with increasing Zn levels showed smaller root and shoot dry weight (DW) than to control for both
407 hybrids (Figs. 1C and 2C). Zinc-alone treated seedlings showed little alteration in the FW of BR205 and 32R21
408 hybrids. However, treatments with Zn-alone reduced the DW of BR205 and 32R21 hybrids (Table 1).
409
410 Mercury and zinc concentration
411
412 Hg-treated seedlings accumulated more in roots than in shoot (on average of 23.8-fold and 45.2-fold
413 greater in root than in shoot, respectively for BR205 and 32R21) (Fig. 3A and 4A), whereas, the Hg
414 concentration in roots of BR205 and 32R21 was decreased respectively, by up to 3,212.5 and 3,643.0 μg Hg g^{-1}
415 DW in the treatments with supplemented 50 μM Zn. However, root Zn accumulation was decreased to 124.5 and
416 187.5 μg g^{-1} DW in Hg-treated BR205 and 32R21 supplemented with 200 μM Zn, respectively (Fig. 3A and
417 4A), when compared to treatment with 200 μM Zn alone. Zn-treated seedlings without any Hg treatment showed
418 higher uptake of Zn (Table 1), indicating a competition between Hg and Zn in seedlings treated with both Hg
419 and Zn.
420
421 Ascorbic acid concentration
422 Treatments containing 25 μM Hg applied together with Zn at levels of 50, 100 and 200 μM on BR205
423 root and shoot, and those treatments containing 25 μM Hg together with Zn at levels of 100 and 200 μM on
424 32R21 root had tissue ascorbic acid (AsA) concentration reduced, when compared to the control (Fig. 5A). In
425 addition, treatments containing Hg applied together with Zn at levels of 50 and 100 μM on 32R21 shoot had
426 tissue AsA concentration reduced, when compared to the control (Fig 5B). In general, Zn-alone treated seedlings
427 showed AsA levels reduced (Table 2).
428

Interactions between Zn and Hg in the δ-ALA-D activity

Treatments with 25 μM Hg applied together with Zn at levels of 50, 100, and 200 μM showed greater BR205 δ-ALA-D activity than treatments with 25 μM Hg, in which δ-ALA-D activity was 19% smaller than to control. Treatment with 25 μM Hg added together with 100 μM Zn increased 32R21 δ-ALA-D levels by about 12%, when compared to the control (Fig. 6A). However, in vitro treatments either with 25 μM Hg applied alone or together with increasing Zn levels showed smaller BR205 and 32R21 δ-ALA-D activity than to control (Fig. 6B). In vivo and in vitro treatments with Zn applied alone at level of 50 and 100 μM showed greater tissue δ-ALA-D activity than control, both for BR205 (in the range between 10% – 30% when compared to control) and 32R21 (in the range between 10% – 68% when compared to control) (Table 3).

Discussion

The present study analyzed the responses of growth, ascorbic acid concentration, and δ-ALA-D activity in two maize hybrids, BR205 and 32R21, grown in hydroponic medium with Hg and Zn, either singly or in combination. In a previous study, we showed that Hg induced oxidative stress in maize (Cargnelutti et al. unpublished data) and cucumber (Cargnelutti et al. 2006) seedlings, and supplementation with Zn was able to regulate Hg-induced oxidative stress in the maize hybrids (Cargnelutti et al. unpublished data). In the present study, Hg reduced root and shoot growth in both maize hybrids (Fig. 1A and 2A). Growth inhibition caused by Hg exposure has been reported for *Cucumis sativus* (Cargnelutti et al. 2006), *Medicago sativa* (Ortega-Villasante et al. 2005; Zhou et al. 2007, 2008) and Hyacinthaceae species (Street et al. 2007). Suszcynsky and Shann (1995) showed that inhibition of root and shoot growth occurred at 1.0 μg mL^{-1} Hg and above, with very limited tissue damage at higher levels of treatment. Zhou et al. (2007) reported that the elevated peroxidase activity might contribute to the stiffening of the cell wall, and consequently the blockage of root growth by Hg. In the present study, treatments with Zn at levels of 50, 100 and 200 μM almost completely counteracted the growth inhibition of maize hybrids caused by 25 μM Hg. Natale et al. (2002) and Grunes et al. (1961) reported positive effects of Zn in moderate concentrations in shoot and roots of plants. The same authors reported that Zn plays an important role in auxin synthesis, which stimulates the development and elongation of young parts of the plants (Malavolta et al. 1997). In the present study, due to its high mobility, Zn was more uptaken by roots and transported to the

458 shoot than Hg (Figs. 3 and 4). The limited translocation of Hg to shoot tissues has been well documented in the
459 literature (Cargnelutti et al. 2006; Rellán-Álvarez et al. 2006; Moreno et al. 2008).

460 In our previous study (Cargnelutti et al. unpublished data) it was observed that maize seedlings treated
461 with Hg showed reduced fresh weight (FW). Interestingly, in the present study the addition of Zn at all levels
462 together with Hg was effective to increase shoot FW of both hybrids, suggesting that Zn could either directly act
463 in the displacement of Hg ions in aquaporins or indirectly via enhanced action of some antioxidant processes. It
464 is known that Hg reduce the FW of plants due it effect inhibitory on aquaporins, likely due to oxidation
465 mechanisms (Maurel et al. 2008). Mercury blocks water channels by binding to a cysteine residue in the pore
466 (Vanderleur et al. 2005). Since Hg and Zn are both considered divalent cations of group II transition metals with
467 eight electrons in their outer orbital, Hg can readily inhibit most Zn-dependent processes (Siedlecka 1995) and
468 hence increased Zn concentrations are able to replace a non-physiological metal such as Hg and may bind to the
469 crucial and functional membrane and enzyme active side and inactivate its functions (Shaw et al. 2004; Assche
470 and Clijsters 1990). This could explain our findings about the increased growth, FW and δ-ALA-D activity in
471 treatments with Zn added together with Hg.

472 In the present study, Hg reduced dry weight (DW) of both maize hybrids (Figs. 1C and 2C), which it is
473 well reported in the literature (Cargnelutti et al. 2006). However, when Zn was added together to treatments with
474 Hg, either the DW was not changed (root and shoot of the BR205 and root of the 32R21) (Fig. 1C) or it was
475 reduced (32R21 shoot) (Fig. 2C). These results suggest that 32R21 shoot may be more sensitive to Hg exposure.
476 This sensitivity may have induced high ROS levels damaging biomolecules, and when Zn is applied together
477 with Hg treatments, it may not prevent Hg toxicity. However, treatments with Hg applied together with Zn at all
478 levels did not change BR205 DW, when compared to treatment with 25 μM Hg.

479 A wide range of the non-enzymatic antioxidants such as GSH and AsA are involved in the oxidative
480 defense of plants. Ascorbate is known to operate as an antioxidant either in direct chemical interaction with
481 reactive oxygen species, or during the reaction catalyzed by APX in chloroplasts and other cell compartments
482 (Shigeoka et al. 2002). Under normal conditions, 90% ascorbate pool is in the reduced form (Foyer 1993). In the
483 present study, assays showed that Hg significantly reduced ascorbic acid (AsA) only in shoot BR205 (Fig. 5B).
484 Interestingly, Hg added together with Zn reduced AsA concentration below of control levels in both hybrids
485 (Fig. 5). This results suggest that addition of Zn at Hg-treated hybrids induced the reactive oxygen species
486 (ROS) production which may have been involved in the oxidation of AsA to dehydroascorbic acid, leading to

reduction in the AsA content of plants (Pignocchi and Foyer 2003). Moreover, in this status of oxidative stress, may be that the AsA levels are no longer necessary in the prevention of oxidative stress.

As mentioned above, there was an inhibition of root and shoot growth in plants exposed to Hg, amongst other factors, which may be due direct inhibition of enzymes that are of physiological importance. In fact, the direct inhibition of enzymes of physiological importance was confirmed in part in the present work by the results observed for δ-Aminolevulic acid dehydratase (δ-ALA-D) activity. Plant dehydratases are localized in plastids and are needed for chlorophyll synthesis in addition to other cellular tetrapyrroles (Smith 1988). They share 35 to 50% identity with non-plant enzymes, but activity requires Mg rather than Zn. The peptide region in the plant enzyme corresponding to the Zn domain in animals lacks cysteine and histidine residues and contains aspartate, alanine, or threonine instead (Boese et al. 1991).

One mechanism involved in heavy metal toxicity entails the ability to form strong bonds with reactive groups of proteins, modifying both their structure and functions (Wang 1999). Heavy metals may compete with other divalent cations such as Zn^{2+} and Mg^{2+} replacing them in their physiological roles. In the present study, BR205 under Hg stressed conditions in vivo showed lower δ-ALA-D activity than 32R21. However, Zn at all levels applied together with 25 μM Hg was effective in increase the BR205 δ-ALA-D activity to control levels (Fig. 6A). Possibly, in the BR205, Zn was more effective in inducing antioxidant defense mechanisms which prevent the ALA-D inhibition induced by Hg. Moreover, high Zn concentration may have displaced Hg of the δ-ALA-D active site. Chavapil (1973) reported that zinc prefers binding to –SH groups of the membrane protein moiety and protects phospholipids and proteins from thiol oxidation and disulphide formation either directly or through a side close to the sulfhydryl groups or through a conformational change. This result demonstrates an apparent stability of the enzymes, membrane proteins and lipid structure (Bray and Bettger 1990; Powell 2000), which hence affords protection from Hg-induced sulfhydryl oxidation and structural damage. This suggested that Zn protects against the oxidation of proteins such as δ-ALA-D. Nonetheless, our in vitro studies showed that Zn do not prevent δ-ALA-D activity inhibition when added together at 25 μM Hg (Fig. 6B). Theses results suggested that Zn did not act on Hg displacement of δ-ALA-D active site, but possibly in the antioxidant defense mechanisms activation.

In conclusion, the results of the present study showed that despite the fact that supplementation with Zn has not been effective in restoring the dry matter production of both hybrids reduced by Hg, Zn seems to play an important role in δ-ALA-D activation, mainly in the BR205, thus promoting normal growth of hybrids.

Acknowledgements

The authors wish to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação e Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa de Estado do Rio Grande do Sul (FAPERGS).

References

Aravind P, Prasad MNV (2003) Zinc alleviates cadmium-induced oxidative stress in *Ceratophyllum demersum* L.: a free floating freshwater macrophyte. Plant Physiol Biochem 41:391-397

Aravind P, Prasad MNV (2004) Zinc protects chloroplasts and associated photochemical functions in cadmium exposed *Ceratophyllum demersum* L., a freshwater macrophyte. Plant Sci 166:1321-1327

Aravind P, Prasad MNV (2005) Cadmium-Zinc interactions in a hydroponic system using *Ceratophyllum demersum* L.: adaptative ecophysiology, biochemistry and molecular toxicology. Braz J Plant Physiol 17:3-20

Assche FV, Clijsters H (1990) Effects of metals on enzyme activity in plants. Plant Cell Environ. 13, 195–206.

Bettger WJ, O'Dell BL (1981) A critical physiological role of zinc in the structure and function of biomembranes. Life Sci 28:1425–1438

Boening DW (2000) Ecological effects, transport, and fate of mercury: a general review. Chemosphere 40:1335-1351

Boese QF, Spano AJ, Li J, Timko MP (1991) Aminolevulinic acid dehydratase in pea (*Pisum sativum* L.). Identification of an unusual metal-binding domain in the plant enzyme. J Biol Chem 266:17060–17066

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Bray TM, Bettger WJ (1990) The physiological role of zinc as an antioxidant. Free Radical Biol Med 8:281-291

Cargnelutti D, Tabaldi LA, Spanevello RM, Jucoski GO, Battisti V, Redin M, Linares CEB, Dressler VL, Flores EMM, Nicoloso FT, Morsch VM, Schetinger MRC (2006) Mercury toxicity induces oxidative stress in growing cucumber seedlings. Chemosphere 65:999–1006

Chavapil M (1973) New aspects in the biological role of zinc: a stabilizer of macromolecules and biological membranes. Life Sci 13:1041-1049

546 Du X, Zhu YG, Liu WJ, Zhao XS (2005) Uptake of mercury (Hg) by seedlings of rice (*Oryza sativa* L.) grown
547 in solution culture and interactions with arsenate uptake. Environ Exp Bot 54:1–7
548 Esteban E, Moreno E, Peñalosa J, Cabrero JI, Millán R, Zornoza P (2008) Short and long-term uptake of Hg in
549 white lupin plants: Kinetics and stress indicators. Environ Exp Bot 62:316–322
550 Foyer C (1993) Ascorbic acid, in: R.G. Alscher JL Hess (Eds.), Antioxidants in Higher Plants, CRC Press, Boca
551 Raton, FL, pp. 31–58
552 Gibson KD, Neuberger A, Scott JJ (1955) The purification and properties of delta-aminolevulinic acid
553 dehydratase. Biochem J 61:618-676
554 Grunes DL, Boawn LC, Carlson CW, Viets JFG (1961) Zinc deficiency of corn and potatoes, as related to soil
555 and plant analysis. Agronomy J 53:68-71
556 Ionenko IF, Anisimov AV, Romanov AV (2003) Effect of Water Stress and Mercuric Chloride on the
557 Translational Diffusion of Water in Maize Seedling Roots. Russ J Plant Physiol 50:79–83
558 Jacques-Silva, M.C., Nogueira, C.W., Broch, L.C., Flores, E.M.M., Rocha, J.B.T., 2001. Diphenyl diselenide
559 and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. Pharmacol
560 Toxicol 88:119–125
561 Jaffe EK, Kervinen J, Dunbrack JR, Litwin S, Martins J, Scarrow RC, Volim M, Yeung AT, Yonn E (2000)
562 Pophobilinogen synthase from pea: Expression from an artificial gene, kinetic characterization, and novel
563 implications for subunit interactions. Biochemistry 39:9018-9029
564 Kaercher LE, Goldschmidt F, Paniz JNG, Flores EMM, Dressler VL (2005) Determination of inorganic and total
565 mercury by vapor generation atomic absorption spectrometry using different temperatures of the
566 measurement cell. Spectrochim Acta B 60:705-710
567 Maggio A, Joly RJ (1995) Effects of Mercuric Chloride on the Hydraulic Conductivity of Tomato Root Systems
568 (Evidence for a Channel-Mediated Water Pathway). Plant Physiol 109:331–335
569 Malavolta E, Vitti GC, Oliveira AS (1997) Avaliação do estado nutricional das plantas: princípios e aplicações.
570 Piracicaba: POTAFÓS, 319p
571 Maurel C, Verdoucq L, Luu D-T, Santoni V (2008) Plant Aquaporins: Membrane Channels with Multiple
572 Integrated Functions. Ann Rev Plant Biol 59:595-624
573 Moreno FN, Anderson CWN, Stewart RB, Robinson BH (2008) Phytofiltration of mercury-contaminated water:
574 volatilisation and plant-accumulation aspects. Environ Exp Bot 62:78–85

575 Morsch VM, Schetinger MRC, Martins AF, Rocha JBT (2002) Effects of cadmium, lead, mercury and zinc on δ-
576 aminolevulinic acid dehydratase activity from radish leaves. Biol Plant 45:85–89
577 Natale W, Prado RM, Corrêa MCM, Silva MAC, Pereira L (2002) Response of guava to zinc application. Rev
578 Bras Frutic 24:770-773
579 Ortega-Villasante C, Rellán-Álvarez R, Del Campo FF, Carpena-Ruiz RO, Hernández LE (2005) Cellular
580 damage induced by cadmium and mercury in *Medicago sativa*. J Exp Bot 56:2239–2251
581 Patra M, Sharma A (2000) Mercury toxicity in plants. Bot Rev 66:379–422
582 Pignocchi C, Foyer CH (2003) Apoplastic ascorbate metabolism and its role in the regulation of cell signalling.
583 Curr. Opin. Plant Biol 6:379-389
584 Powell SR (2000) The antioxidant properties of zinc. J Nutr 130:1447-1454
585 Rellán-álvarez R, Ortega-Villasante C, Álvarez-Fernández A, Campo FF, Hernández LE (2006) Stress responses
586 of *Zea mays* to cadmium and mercury. Plant Soil 279:41–50
587 Rocha JBT, Pereira ME, Emanuelli T, Christofari RS, Souza D (1995) Effects of methylmercury exposure
588 during the second stage of rapid postnatal brain growth on delta-aminolevulinic acid dehydratase (ALA-D)
589 activity in brain, liver and blood of suckling rats. Toxicology 100:27–37
590 Salt DE, Blaylock M, Kumar NPBA, Dushenkov V, Ensley BD, Chet I, Raskin I (1995) Phytorremediation: a
591 novel strategy for the removal of toxic metals from the environment using plants. Biotechnol 13:468-474
592 Sassa S (1982) Delta-aminolevulinic acid dehydratase assay. Enzyme 28:133–145
593 Shaw BP, Sahu SK, Mishra RK (2004) Heavy metal induced oxidative damage in terrestrial plants. In: Prasad
594 MNV (ed), Heavy metal stress in plants-from biomolecules to ecosystems, 2nd ed., pp.84-126. Springer-
595 Verlag, Narosa, New Delhi
596 Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, Yoshimura K (2002) Regulation and
597 function of ascorbate peroxidase isoenzymes. J Exp Bot 53:1305–1319
598 Siedlecka A (1995) Some aspects of interactions between heavy metals and plant mineral nutrients. Acta Soc Bot
599 Pol 3:265-272
600 Silva CJC, Lima MGS, Carvalho CM, Eloi WM, Pedroza MM, Silva CJC (2005) Efeito do lodo de estação de
601 tratamento de despejos de curtume na fase inicial do crescimento do milho. Rev Biol Ciênc Terra 5
602 Smith AG (1988) Subcellular localization of two porphyrin-synthesis enzymes in *Pisum sntivum* (pea) and *Arum*
603 (cuckoo-pint) species. Biochem J 249:423-428

Sterckeman T, Douay F, Proix N, Fourrier H (2000) Vertical distribution of Cd, Pb and Zn in soils nears smelters in the north of France. Environ Pollut 107:377-389

Street RA, Kulkarni MG, Stirk WA, Southway C, Van Staden J (2007) Toxicity of Metal Elements on Germination and Seedling Growth of Widely Used Medicinal Plants Belonging to Hyacinthaceae. Bull Environ Contam Toxicol 79:371–376

Suszcynsky EM, Shann JR (1995) Phytotoxicity and accumulation of mercury in tabacco subjected to different exposure routes. Environ Toxicol Chem 14:61-67

Tabaldi LA, Nicoloso FT, Castro GY, Cargnelutti D, Gonçalves JF, Rauber R, Skrebsky EC, Schetinger MRC, Morsch VM, Bisognin DA (2007) Physiological and oxidative stress responses of four potato clones to aluminum in nutrient solution. Braz J Plant Physiol 19:211-222

Tennant BD (1975) A test of a modified line intersect method of estimating root length. J Ecol 63:995-1001

Vallee BL, Auld DS (1990) Zinc coordination, function, and structure of zinc enzymes and other proteins. Biochemistry 29:5647-5659

Vandeleur R, Niemietz C, Tilbrook J, Tyerman SD (2005). Roles of aquaporins in root responses to irrigation. Plant Soil 274:141–161

Zago MP, Oteiza PI (2001) The antioxidant properties of zinc: interactions with iron and antioxidants. Free Rad Biol Med 31:266-274

Zhang WH, Tyerman SD (1999) Inhibition of Water Channels by $HgCl_2$ in Intact Wheat Root Cells. Plant Physiol 120:849–857

Zhou ZS, Huang SQ, Guo K, Mehta SK, Zhang PC, Yang ZM (2007). Metabolic adaptations to mercury-induced oxidative stress in roots of *Medicago sativa* L. J Inorg Biochem 101:1–9

Zhou ZS, Wang SJ, Yang ZM (2008) Biological detection and analysis of mercury toxicity to alfalfa (*Medicago sativa*) plants. Chemosphere 70:1500–1509

Wang LY (1999) Conditional stability of the HemA protein (glutamyl-tRNA reductase) regulates heme biosynthesis in Salmonella typhimurium. J Bacteriol 181:1211–1219

630 **Table 1**

631

632 Influence of Zn on length, fresh weight, dry weight and Zn content in root and shoot of two maize hybrids, BR205 and 32R21.

Zn^{2+} (μM)	Length (cm)		FW (g $plant^{-1}$)		DW (g $plant^{-1}$)		Zn content (μg g^{-1} dry weight)	
Root	BR205	32R21	BR205	32R21	BR205	32R21	BR205	32R21
0	234.9±8.9 aA	282.9±7.8 abA	0.59±0.07 aA	0.56±0.08 bA	0.65±0.07 aA	0.65±0.08 aA	124.5±6.5 dB	187.5±30.5 dA
50	214.4±11 aA	336.9±8.1 aA	0.54±0.01 aB	0.70±0.03 aA	0.66±0.01 aA	0.51±0.03 bA	1,143.5±44.5 cB	1,535.5±217 cA
100	263.0±4.6 aA	268.4±8.2 abA	0.56±0.04 aA	0.59±0.1 bA	0.48±0.04 bA	0.53±0.1 bA	2,497.5±288 bA	2,391.5±22.5 bB
200	263.3±6.8 aA	239.9±12 bA	0.54±0.07 aA	0.56±0.02 bA	0.40±0.07 bA	0.37±0.02 cA	2,908.5±496 aB	4,965.5±938 aA
Shoot								
0	35.2±3.9 abA	40.2±1.0 aA	1.35±0.16 aB	1.81±0.03bA	0.86±0.03 aA	0.5±0.001 aB	137.5±10.5 cA	138±17 dA
50	38.2±1.7 aA	40.5±0.5 aA	1.38±0.1 aB	2.03±0.02aA	0.55±0.02 bA	0.2±0.013 bB	760.5±10.5 bB	860±125 cA
100	32.6±0.8 bA	40±0.3 aA	1.23±0.04 aB	1.8±0.1bA	0.55±0.04 bA	0.49±0.02 aA	1,798.5±123 aA	1,142±47 bB
200	32.3±0.7 bA	35.3±3.2 bA	1.31±0.03 aB	1.75±0.08bA	0.31±0.01 cA	0.21±0.01 bA	1,849.5±3.5 aA	1,467±75 aB

633 Data represent mean values ± S.D. based on independent determination. *Different from control at $p < 0.05$.

Table 2

Influence of Zn on ascorbic acid (AsA) concentration in root and shoot of two maize hybrids, BR205 and 32R21.

Zn^{2+} (μM)	AsA content (μg AsA g^{-1} FW)	
Root	BR205	32R21
0	148.12±4.53 aA	152.75±12.1 aA
50	136.39±9.15 bcA	137.84±1.81 aA
100	124.66±4.51 cB	158.69±5.43 aA
200	146.09±1.33 abA	98.75±18.25 bB
Shoot		
0	392.38±7.4 aA	438.13±37.5 aA
50	286.68±1.0 bA	326.79±116 bA
100	398.31±22.4 aA	299.13±77.1 bA
200	233.11±10.0 cB	409.9±6.4 aA

Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Table 3

Influence of Zn on ALA-D activity in two maize hybrids, BR205 and 32R21, *in vivo*.

Zn^{2+} (μM)	ALA-D activity (nmol PBG min^{-1} mg $protein^{-1}$) in vivo		ALA-D activity (nmol PBG min^{-1} mg $protein^{-1}$) in vitro	
	BR205	32R21	BR205	32R21
0	1.57±0.04 cA	1.62±0.02 cA	2.397±0.021 cA	2.13±0.026 cA
50	1.72±0.08 bB	2.72±0.08 aA	2.733±0.051 aA	2.42±0.03 aA
100	2.04±0.06 aA	1.83±0.07 bA	2.56±0.026 bA	2.35±0.02 abA
200	1.57±0.05 cA	1.56±0.02 cA	2.4±0.02 cA	2.25±0.021 bA

Data represent mean values ± S.D. based on independent determination. Different from control at $p < 0.05$.

LEGEND OF THE FIGURES

Figure 1. Root length (A), weight fresh (B) and weight dry (C) in two maize hybrids, BR205 and 32R21, treated with Hg-25 μM and Zn (50, 100 and 200 μM). Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Figure 2. Shoot length (A), weight fresh (B) and weight dry (C) in two maize hybrids, BR205 and 32R21, treated with Hg-25 μM and Zn (50, 100 and 200 μM). Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

Figure 3. Metal accumulation in root (A) and shoot (B) of BR205 hybrid treated with Hg-25 μM and Zn (50, 100 and 200 μM). Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations (two-way ANOVA/Duncan; $p<0.05$).

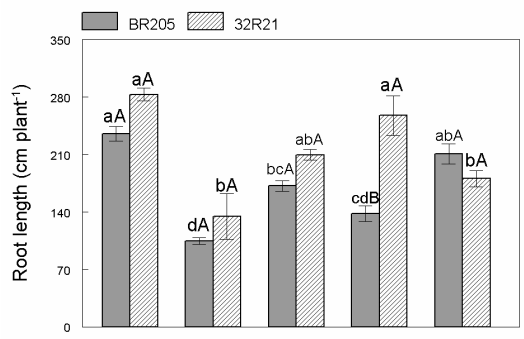
Figure 4. Metal accumulation in root (A) and shoot (B) of 32R21 hybrid treated with Hg-25 μM and Zn (50, 100 and 200 μM). Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations (two-way ANOVA/Duncan; $p<0.05$).

Figure 5. Root (A) and shoot (B) ascorbic acid in two maize hybrids, BR205 and 32R21, treated with Hg-25 μM and Zn (50, 100 and 200 μM). Data are mean ± S.D. of three pools of 5 replicates each (n=3). Lowercase letters represent differences among concentrations and capital letters represent differences among hybrids (two-way ANOVA/Duncan; $p<0.05$).

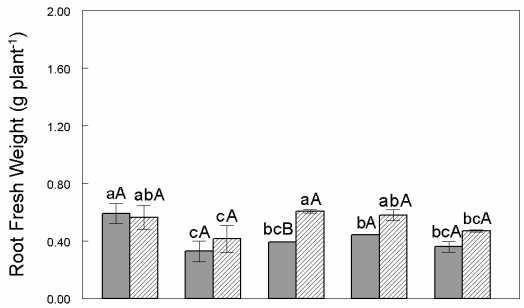
Figure 6. ALA-D activity of two maize hybrids, BR205 and 32R21, treated with Hg-25 μM and Zn (50, 100 and 200 μM) concentrations in vivo (A) and ALA-D activity of BR205 hybrid treated with Hg-25 μM and Zn (50, 100 and 200 μM) concentrations in vitro (B). Data represent mean values ± S.D. based on independent determination. Activity is reported as nmol porphobilinogen (PBG) h^{-1} mg^{-1} protein. * Different from control at $p < 0.05$.

Figure 1

(A)


BR205
32R21
Root length (cm plant-1)
350
280
210
140
70
0
aA
aA
dA
bA
bcA
abA
cdB
aA
abA
bA

(B)


Root Fresh Weight (g plant-1)
2.00
1.60
1.20
0.80
0.40
0.00
aA
abA
cA
cA
bcB
aA
bA
abA
bcA
bcA

(C)

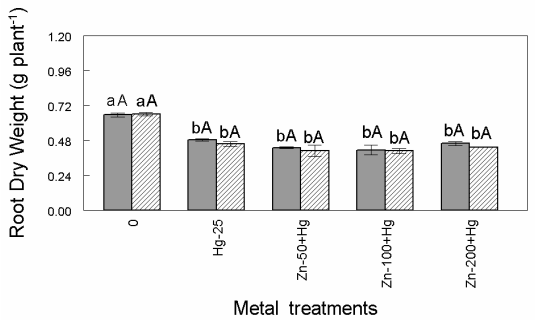
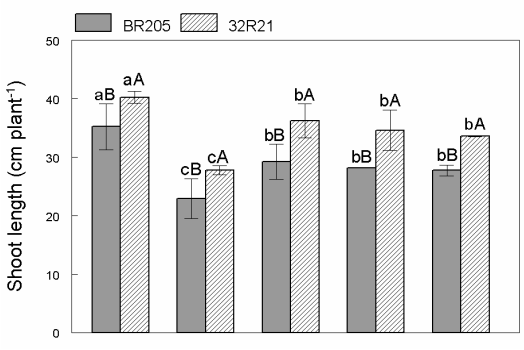
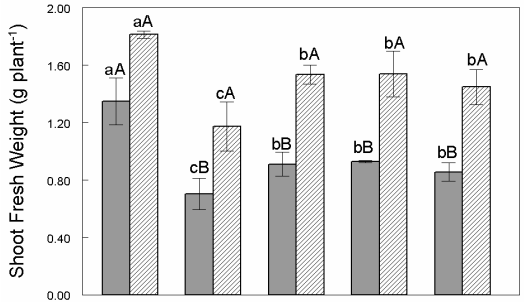

Root Dry Weight (g plant-1)
1.20
0.96
0.72
0.48
0.24
0.00
aA aA
bA bA
bA bA
bA bA
bA bA
0
Hg-25
Zn-50+Hg
Zn-100+Hg
Zn-200+Hg
Metal treatments

Figure 2

(A)


BR205
32R21
Shoot length (cm plant-1)
50
40
30
20
10
0
aB
aA
cB
cA
bB
bA
bB
bA
bB
bA

(B)


Shoot Fresh Weight (g plant-1)
2.00
1.60
1.20
0.80
0.40
0.00
aA
aA
cB
cA
bB
bA
bB
bA
bB
bA

(C)

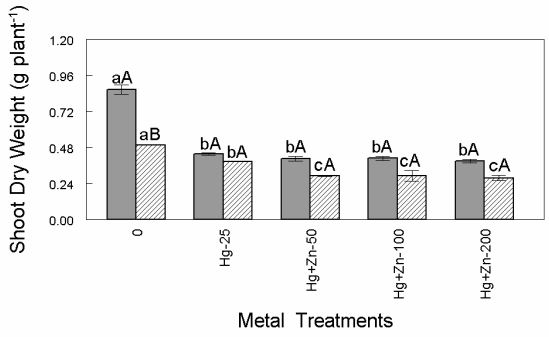
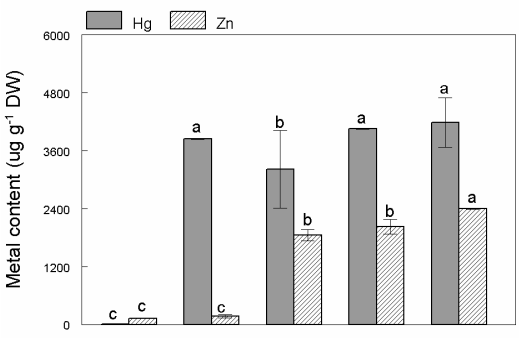

Shoot Dry Weight (g plant-1)
1.20
0.96
0.72
0.48
0.24
0.00
aA
aB
bA
bA
bA
cA
bA
cA
bA
cA
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal Treatments

Figure 3

(A)


Hg
Zn
Metal content (ug g-1 DW)
6000
4800
3600
2400
1200
0
a
b
a
a
c
c
b
b
c
c
a
a

(B)

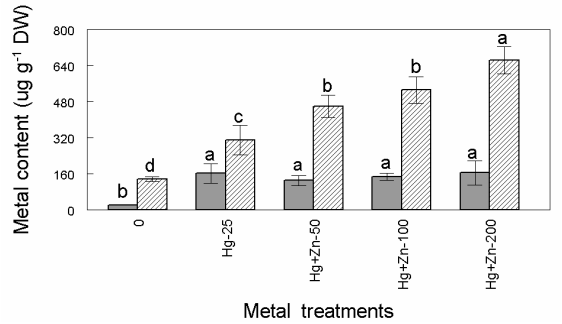
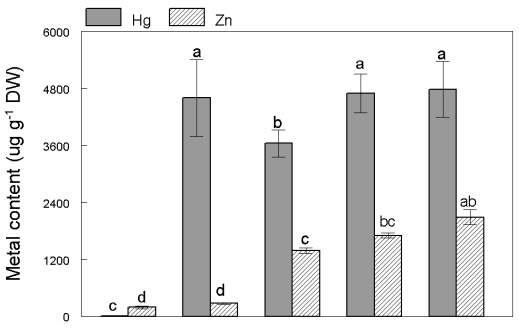

Metal content (ug g-1 DW)
800
640
480
320
160
0
b
d
a
c
a
b
a
b
a
a
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal treatments

Figure 4

(A)


Hg
Zn
Metal content (ug g-1 DW)
6000
4800
3600
2400
1200
0
a
d
b
c
a
bc
a
ab

(B)

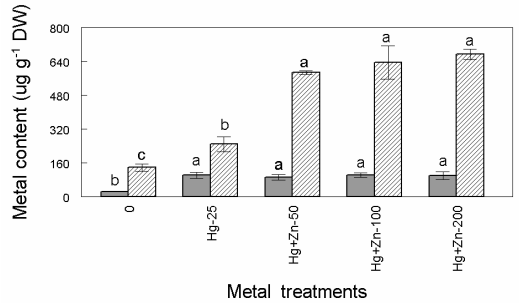
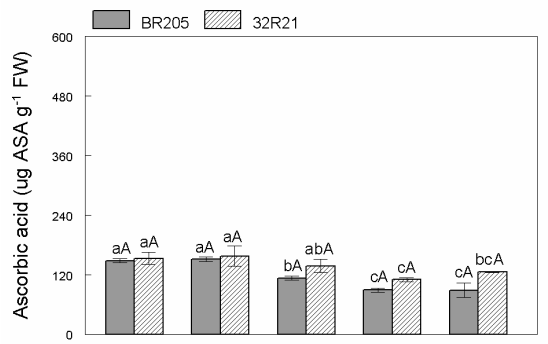

Metal content (ug g-1 DW)
800
640
480
320
160
0
b
c
a
b
a
a
a
a
a
a
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal treatments

Figure 5

(A)


BR205
32R21
Ascorbic acid (ug ASA g-1 FW)
600
480
360
240
120
0
aA aA
aA aA
bA abA
cA cA
cA bcA

(B)

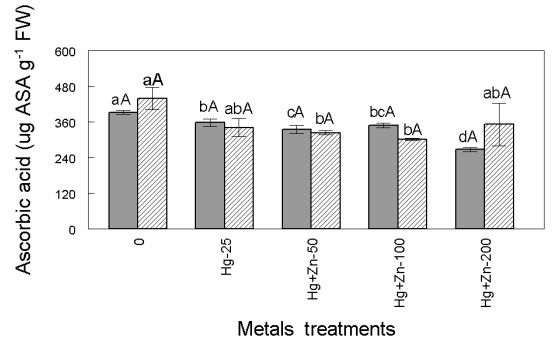
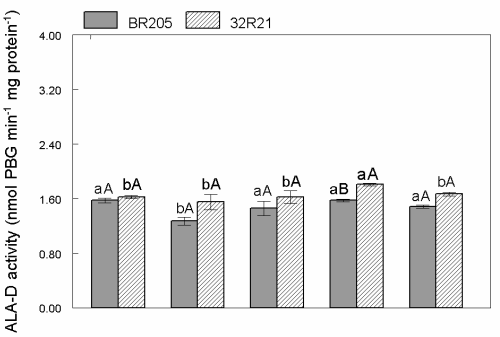
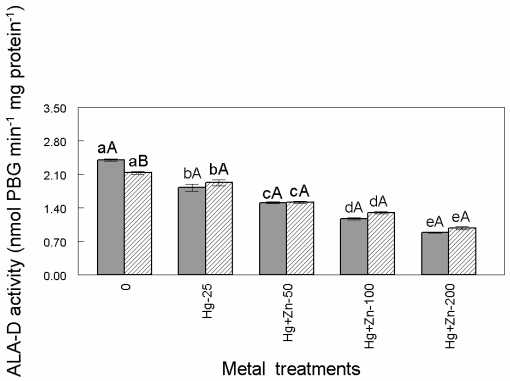

Ascorbic acid (ug ASA g-1 FW)
600
480
360
240
120
0
aA aA
bA abA
cA bA
bcA bA
dA abA
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metals treatments

Figure 6

(A)


BR205
32R21
ALA-D activity (nmol PBG min-1 mg protein-1)
4.00
3.20
2.40
1.60
0.80
0.00
aA bA
bA bA
aA bA
aB aA
aA bA

(B)


ALA-D activity (nmol PBG min-1 mg protein-1)
3.50
2.80
2.10
1.40
0.70
0.00
aA aB
bA bA
cA cA
dA dA
eA eA
0
Hg-25
Hg+Zn-50
Hg+Zn-100
Hg+Zn-200
Metal treatments

5. DISCUSSÃO

Ferri (1985) relatou que o estudo do metabolismo dos metais é melhor observado em plântulas devido a alguns fatores; entre eles o fato de que nesse período de plântula, é observado um metabolismo acelerado, com divisão e expansão celular, e formação dos tecidos, dessa forma vários processos relacionados ao metabolismo do mercúrio seriam detectáveis. Ainda, o período que vai da germinação até a época em que a plântulas se tornam estabelecidas como um organismo independente é o período de maior susceptibilidade à injúria por diversos fatores como a presença do mercúrio. Assim, neste trabalho foram estudados os efeitos do mercúrio em plântulas de pepino e milho.

Os resultados apresentados no **artigo 1** sugerem que o mercúrio induz estresse oxidativo em plântulas de pepino (*Cucumis sativus* L.). Essa conclusão é baseada nos resultados mostrados nas figuras 1, 2 e 3 do referido artigo. Os resultados indicaram que os íons de mercúrio foram absorvidos pelas plântulas de pepino, e seu conteúdo foi maior no sistema radicular (SR) do que na parte aérea (PA) (Tabela 1, artigo 1). Como esperado, foi observada uma redução no comprimento do SR e da PA que foi dependente do tempo de exposição e da concentração testada (Figuras 1A e 1B, artigo1). Aos 15 dias de exposição de *C. sativus* ao mercúrio em meio Murashige & Skoog, a massa fresca (MF) do SR foi aumentada pela exposição a 50 μM $HgCl_2$, mas foi reduzida nas demais concentrações testadas (Figura 1C, artigo 1). Esta resposta é bem documentada na literatura, a qual é referrida como hormesis ou efeito bifásico (CALABRESE & BALDWIN, 2000). Este efeito representa uma super-estimulação do parâmetro observado, neste caso a MF do SR, em concentrações baixas do metal. Contudo, em concentrações altas esta resposta foi negativa. Em plântulas com 10 dias de exposição, foi observada uma redução na MF do SR e PA (Figuras 1C e 1D, artigo 1). Aos 15 dias, o mercúrio induziu uma redução na MF da PA em todas as concentrações testadas, exceto na concentração de 50 μM (Figura 1D, artigo 1). No entanto, a exposição às concentrações altas de $HgCl_2$ (250 e 500 μM) induziu um aumento na massa seca (MS) do SR de *C. sativus*, tanto aos 10 quanto aos 15 dias (Figura 1E, artigo 1). Da mesma forma, Arduini et al. (2004) verificaram efeito similar na MS de plantas expostas ao cádmio, relatando a formação de agregados nos

tecidos vegetais devido à exposição ao metal. Este efeito pode ter ocorrido em plântulas de pepino expostas ao $HgCl_2$ devido a uma proliferação anormal das células da raiz e subseqüente acréscimo no MS se comparado com as plântulas controle. Contudo, a MS da PA foi reduzida em todas as concentrações testadas (Figura 1F, artigo 1).

Nossos resultados confirmaram a geração de estresse oxidativo em plântulas de pepino pelo $HgCl_2$, uma vez que este metal aumenta a peroxidação lipídica e a oxidação de proteínas em concentrações altas do metal (Figuras 2B e 2C, artigo 1) com concomitante redução nos níveis de clorofila (Figura 2A, Artigo) e aumento nos níveis de proteínas (Figura 3A, artigo 1). Além disso, os resultados apresentados no **Manuscrito 1** mostraram que os níveis de tióis não-protéicos (NPSH), ácido ascórbico (AsA) e carotenóides (Figuras 3B, 3C e 3D, manuscrito 1) foram aumentados ou permaneceram não alterados em *C. sativus* exposto ao $HgCl_2$. Nossos resultados confirmaram a inibição na síntese da clorofila, observados através da atividade da δ-ALA-D (Figura 4, manuscrito 1). Por ser uma enzima sensível a metais pesados devido a sua natureza sulfidrílica (Morsch et al., 2002), a atividade desta enzima pode ser considerada um biomarcador para a presença de mercúrio em *C. sativus*. Concomitante aos danos a lipídios e proteínas (artigo 1), as concentrações altas de $HgCl_2$ induziram a geração de EROs em plântulas de pepino aos 10 dias (manuscrito 1). Essas mudanças nos níveis de EROs podem induzir a oxidação de compostos antioxidantes, tais como o AsA (manuscrito 1). Além disso, em plântulas com 10 e 15 dias de exposição ao $HgCl_2$, foi observado um aumento na atividade da catalase (CAT) (Figura 3B, artigo 1), respectivamente, nas concentrações de 50 e 250 μM a qual foi relacionada com aumentos nos níveis de peróxido de hidrogênio (H_2O_2) (Figura 2, manuscrito 1). Contudo, aos 10 dias, a atividade desta enzima foi reduzida pela exposição a 50 e 500 μM (Figura 3B, artigo 1). Uma resposta hormética, similar a observada para a MF da PA em concentrações baixas de $HgCl_2$, também foi observada para a atividade da superóxido dismutase (SOD). A ativação desta enzima em concentrações baixas do metal poderia indicar síntese *de novo*, fenômeno este que pode estar relacionado à Hormesis (Figura 3A, manuscrito 1). Contudo, a inibição da SOD em altas concentrações do metal, tanto aos 10 quanto aos 15 dias, sugere a interferência direta do metal na estrutura da enzima, ou a oxidação dos seus grupamentos –SH pelo ânion superóxido. Já, a ascorbato peroxidase (APX) de plântulas de pepino foi

sensível às concentrações altas de $HgCl_2$, a qual teve sua atividade próxima à zero (Figura 3C, artigo 1). Portanto, os resultados obtidos em plântulas de pepino expostas ao $HgCl_2$ aos 10 e 15 dias, mostraram que esta planta pode ser utilizada como um bioindicador para a presença de mercúrio. Além disso, foi observado que em concentrações baixas de $HgCl_2$, em torno de 30% a 40% do metal foi transportado para a PA e o restante ficou retido no SR. Contudo, nas concentrações altas de $HgCl_2$, 80% a 90% do mercúrio absorvido pelas plantas permaneceu retido no sistema radicular onde interage principalmente com os componentes da parede celular das células da raiz (WANG, 2004). Com base no exposto, pode-se concluir que *C. sativus* é sensível ao mercúrio.

Neste trabalho, também foram estudados os mecanismos de toxicidade do mercúrio em três híbridos de milho, BR205, 30F71 e BR205, em solução nutritiva (hidroponia). Os resultados apresentados no **manuscrito 2** indicaram uma alta captação do mercúrio pelos híbridos de milho, BR205, 30F71 e 32R21, em solução nutritiva. Contudo, o mercúrio foi mais acumulado no SR, se comparado com a PA (Tabela 1, manuscrito 2). Devido a esta acumulação, o crescimento do SR e da PA foi reduzido em todas as concentrações de mercúrio testadas (Figuras 1A e 1B, Manuscrito 2). Resposta similar também foi observada para a MS (Figuras 1C e 1D, manuscrito 4) e MF (dados não mostrados) dos SR e PA, sobretudo, a MF de ambos os híbridos foi bastante sensível à presença do mercúrio. PATRA et al. (2004) relataram que o mercúrio inibe as aquaporinas de plantas. Assim, a redução na MF observada neste estudo, pode ser devido, em parte, a inibição das aquaporinas pelo mercúrio. O híbrido 30F71 apresentou sintomas de clorose, murchamento das folhas, escurecimento do SR e morte das plântulas. Por este motivo, este híbrido foi excluído das demais análises fisiológicas e bioquímicas.

Embora não tenham sido observadas diferenças na acumulação de mercúrio entre os híbridos, BR205 e 32R21 apresentaram atividade da δ-ALA-D menor quando comparados com 30F71, onde a atividade da enzima não foi alterada (Figure 2, manuscrito 2). Estes resultados sugerem que o mercúrio pode não ter sido acumulado o suficiente na PA para exercer inibição direta na atividade da δ-ALA-D, ou a δ-ALA-D do híbrido 30F71 não é sensível ao mercúrio, possivelmente devido à presença de mecanismos que previnem a inibição da δ-ALA-D pelo mercúrio. Portanto, a atividade desta enzima pode não ser um bom biomarcador para a

presença de mercúrio em milho. Contudo, para conhecer o mecanismo pelo qual o mercúrio poderia afetar a atividade da δ-ALA-D, foram realizados estudos *in vitro*. Nestes estudos, o híbrido BR205 foi selecionado devido à alta sensibilidade da δ-ALA-D ao mercúrio *in vivo*, se comparado com os demais híbridos estudados. Os estudos *in vitro* mostraram que o mercúrio inibe a atividade da δ-ALA-D (Figura 3A, manuscrito 2) de maneira dependente da concentração indicando interação direta do metal com os grupamentos sulfidrílicos da enzima ou atuando no deslocamento do Mg^{2+} no sítio ativo da enzima (MORSCH, et al., 2002; PEREIRA et al., 2007). Aliado a isso, os estudos cinéticos mostraram uma inibição do tipo mista na atividade da δ-ALA-D frente ao mercúrio, onde este potente inibidor (Tabela 2, manuscrito 2) pode atuar competindo pelo substrato no sítio ativo da enzima ou pode estar se ligando em um sítio alostérico da enzima, ocasionando alterações estruturais da mesma, refletindo na atividade diminuída da mesma.

O zinco é um micronutriente essencial, com papel importante na indução de antioxidantes enzimáticos e não-enzimáticos responsáveis pela destoxificação das EROs (GRESSEL & GALUN, 1994; ALLEN, 1995; ALSCHER et al., 1997). Neste sentido, o zinco pode ser utilizado em associação com o mercúrio com o objetivo de aliviar os seus efeitos tóxicos (TSUJI et al., 2002). Para estes estudos, a concentração de 25 μM Hg foi escolhida, pois inibiu por cerca de 25% o crescimento dos híbridos de milho (Figuras 1A e 1B, manuscrito 2). Sendo assim, os resultados apresentados no **manuscrito 3** indicaram uma competição pelo transporte dos metais no tratamento utilizando 25 μM Hg + 50 μM Zn, onde os níveis de mercúrio foram reduzidos pela presença do zinco para ambos os híbridos (Figuras 1A e 2A, manuscrito 3). Contudo, estas mudanças não refletiram em alterações na PA, onde o conteúdo de mercúrio não foi aterado pela presença do zinco (Figuras 1B e 2B, manuscrito 3). Apesar dos efeitos benéficos do zinco descritos na literatura, apenas a concentração de 100 μM Zn preveniu a redução nos níveis de clorofila b os quais foram reduzidos pelo mercúrio (Figura 3B, manuscrito 3). Estes resultados demonstram uma aparente estabilidade das enzimas, proteínas de membrana e estrutura de lipídios (BRAY & BETTEGER, 1990; POWELL, 2000) pelo qual o zinco fornece proteção da oxidação de biomoléculas e do dano estrutural induzido pelo mercúrio. O zinco (especialmente na concentração de 100 μM) interage reduzindo a

oxidação das biomoléculas nos cloroplastos, reduzindo a destruição da clorofila induzida pelo mercúrio.

Para avaliar o estresse oxidativo causado pela exposição ao mercúrio e/ou zinco, os níveis de proteína carbonil e a concentração de H_2O_2 foram determinados nos híbridos de milho. No híbrido 32R21, os tratamentos com zinco aplicado sózinho mostraram poucas alterações (Figura 5A, manuscrito 3). Contudo, a suplementação com zinco aos tratamentos utilizando 25 μM Hg foi efetiva em reduzir ambos os níveis de proteína carbonil do SR em 32R21 e os de H_2O_2 em BR205, aumentados pelos tratamentos com mercúrio (Figuras 4A e 5A, manuscrito 3). Esta resposta pode ser devido à indução pelo zinco da expressão de genes que codificam para enzimas antioxidantes tais como APX e GPX as quais removem o H_2O_2 (GRESSEL & GALUN, 1994). Estes resultados sugerem que os híbridos apresentaram respostas diferenciadas aos tratamentos. Contudo, a PA do híbrido BR205 mostrou um efeito compensatório em relação às enzimas antioxidantes. Os resultados mostraram uma inibição pelo mercúrio das enzimas SOD e CAT, enquanto a APX foi ativada (Figuras 7A, 7B e 7C, manuscrito 3). No entanto, a suplementação com zinco aumentou as atividades da CAT e APX. Para 32R21, o tratamento somente com mercúrio reduziu a atividade da APX da PA, e somente a concentração maior de zinco foi capaz de aumentar a atividade da enzima. Portanto, estes resultados sugerem que o zinco protege as enzimas antioxidantes da oxidação pelas EROs e pelo metal, e a enzima SOD parece ser mais sensível do que a APX e a CAT à presença do mercúrio. Além disso, os estudos *in vitro* sugerem que o mercúrio induz a produção de EROs devido à ativação das enzimas antioxiantes principalmente na PA. O zinco parece ter papel importante na ativação da APX do SR e da PA, mas em concentrações superiores a 50 μM (Figuras 9C e 10C, manuscrito 3) e na ativação da CAT da PA (Figura 10B, manuscrito 3). Contudo, o zinco apresentou pouco efeito para a SOD do SR e da PA e a CAT do SR (Figuras 9A, 10A e 9B, manuscrito 3). Além disso, em geral, a APX foi inibida por Hg *in vitro*, e nos estudos *in vivo* ela foi ativada.

Nossos resultados indicaram que a tolerância dos híbridos de milho ao mercúrio foi associada com uma acumulação de grupos tióis não-protéicos (NPSH) promovida pelo zinco (Figuras 6A and 6B, manuscrito 3). Os grupos NPSH são peptídeos que agem quelando os íons mercuriais reduzindo a sua biodisponibilidade e seus efeitos tóxicos. Além disso, dentre os NPSH, a glutationa (GSH) representa

quase 80% destes tióis. A partir da GSH são sintetizadas as fitoquelatinas (PCs). Tsuji et al. (2002) relataram o potencial maior de quelação de mercúrio e remoção de EROs pelas PCs se comparado com antioxidantes tais como a GSH e o ácido ascórbico. Além disso, diferentemente do mercúrio, o zinco é um bom indutor na síntese de PCs. Contudo, o zinco se liga com menor estabilidade às PCs que o mercúrio. Portanto, estes resultados sugerem que o zinco poderia estar induzindo a síntese de PCs e protegendo os híbridos de milho contra os danos causados pelo mercúrio.

Os resultados apresentados no **manuscrito 4** mostraram uma inibição do crescimento de BR205 e 32R21 expostos a 25 µM Hg (Figuras 1A e 1B, manuscrito 4) também observadas para *C. sativus* (Figuras 1A e 1B, artigo 1). Provavelmente a redução no crescimento pode estar relacionada com o enrijecimento da parede celular induzida pelo mercúrio (ZHOU et al., 2007). Contudo, devido ao seu papel na modulação dos radicais livres e proteção de membranas e enzimas, a suplementação com zinco preveniu em parte a inibição do crescimento dos híbridos de milho o qual foi reduzido por 25 µM Hg (Figuras 1A e 2A, manuscrito 4). Da mesma forma, a MF foi reduzida em plântulas expostas ao mercúrio, mas a suplementação com zinco restabeleceu a MF dos híbridos aos níveis do controle (Figuras 1B e 2B, manuscrito 4). Sabe-se que o mercúrio reduz o peso fresco das plantas devido ao seu efeito inibitório nas aquaporinas e esta inibição provavelmente ocorre através de mecanismos de oxidação (MAUREL et al., 2008). Portanto, nossos resultados sugerem que o zinco poderia agir ou diretamente no deslocamento dos íons Hg nas aquaporinas, ou indiretamente via aumento da ação de alguns processos antioxidantes tal como os NPSH (Figura 6, manuscrito 3). Ao contrário do crescimento e MF, a suplementação com zinco aos tratamentos com mercúrio não foi efetiva em prevenir a inibição da MS induzida pelo mercúrio para ambos os híbridos (Figuras 1C e 2C, manuscrito 4).

Compostos com atividade antioxidante tal como o ácido ascórbico (AsA) são capazes de remover EROs, as quais reduzem os níveis de AsA nas plantas (PIGNOCCHI & FOYER, 2003). Nossos resultados mostraram que o mercúrio reduziu os níveis de AsA somente na PA de BR205 (Figura 5, manuscrito 4). No entanto, a suplementação com zinco aos tratamentos com mercúrio não foi efetiva em aumentar os níveis de AsA . Contrário ao efeito nos níveis de AsA, o zinco previniu a inibição da atividade da δ-ALA-D reduzida pelo mercúrio. Contudo, este

efeito somente foi observado para o híbrido BR205 (Figura 6A, manuscrito 4). Estes resultados sugerem que em BR205, o zinco foi efetivo na indução do sistema de defesa antioxidante, o qual previne a inibição da atividade da δ-ALA-D induzida pelo mercúrio possivelmente através da proteção contra a oxidação da δ-ALA-D (CHAVAPIL, 1973). Além disso, concentrações altas de zinco poderiam ter deslocado o mercúrio do sítio de ligação na enzima. Contudo, nos estudos *in vitro* mostraram que o zinco não previne a inibição da δ-ALA-D induzida pelo mercúrio, para ambos os híbridos (Figura 6B, manuscrito 4), sugerindo que o zinco pode não estar agindo no deslocamento dos íons Hg os quais se ligam junto aos grupos –SH da enzima, mas possivelmente atuando na ativação dos mecanismos de defesa antioxidante.

Com base no exposto, nossos resultados sugerem que o mercúrio induz estresse oxidativo em plântulas de pepino e milho. Associado a isso, o Zn foi utilizado como um nutriente com potencial protetor, desempenhando um papel importante no combate à toxicidade induzida pelo mercúrio, atuando na modulação de ROS e indução de NPSH que reduzem a biodisponibilidade do metal e restabelecem o crescimento dos híbridos de milho.

6. CONCLUSÕES

- O mercúrio induziu estresse oxidativo em plântulas de pepino, tanto aos 10 quanto aos 15 dias de exposição ao metal, resultando em injúria nas plântulas. O mercúrio foi mais acumulado no sistema radicular do que nos cotilédones das plântulas. Esta acumulação induziu peroxidação lipídica, oxidação de proteínas e redução nos níveis de clorofila, com conseqüente redução no crescimento das plântulas. Além de reduzir a atividade da catalase, o mercúrio inibiu a ascorbato peroxidase nas concentrações maiores.

- O mercúrio aumentou os níveis de peróxido de hidrogênio e reduziu os níveis de carotenóides. Além disso, este metal inibiu as atividades das enzimas superóxido dismutase e δ-aminolevulinato desidratase as quais estiveram relacionadas com a acumulação de mercúrio nos tecidos. Além disso, um papel importante contra o estresse gerado pelo mercúrio foi observado para os níveis de ácido ascórbico e grupos tióis não-protéicos. Contudo, esse sistema antioxidante não foi efetivo na proteção contra os danos causados pelo metal, resultando em efeitos negativos no crescimento das plântulas de pepino.

- O crescimento dos híbridos de milho BR205, 30F71 e 32R21, foi reduzido pela exposição ao mercúrio em solução nutritiva, sendo que o híbrido 30F71 foi extremamente sensível à presença do mercúrio. Nestes híbridos a atividade da ALA-D foi reduzida pela presença do metal, exceto para 30F71, onde a atividade da enzima não foi afetada. Além disso, os estudos *in vitro* mostraram que o mercúrio é um potente inibidor da atividade da ALA-D em BR205, produzindo uma inibição do tipo mista.

- Em geral, o mercúrio aumentou os níveis de peróxido de hidrogênio e proteína carbonil, e reduziu a atividade das enzimas antioxidantes, bem como os níveis de clorofila b e grupos tiólicos não-protéicos. Contudo, os tratamentos com mercúrio associado ao zinco reduziram os índices de estresse oxidativo e aumentam as defesas antioxidantes enzimáticas e não-enzimáticas, prevenindo a destruição dos pigmentos fotossintéticos.

- O crescimento e a massa fresca dos híbridos de milho BR205 e 32R21, reduzido por 25 μM Hg, foram restabelecidos pelo tratamento com mercúrio associado ao zinco. Um efeito similar foi observado para a atividade da δ-ALA-D de BR205, onde o tratamento com mercúrio inibiu a atividade desta enzima, ao passo que a suplementação com zinco previniu desta inibição. Contudo, a suplementação com zinco não foi efetiva em reestabelecer o peso seco e os níveis de ácido ascórbico em ambos os híbridos.

7. PERSPECTIVAS

- Avaliar em solução nutritiva e em solo o efeito do pré-tratamento com zinco e/ou enxofre e após, o tratamento com mercúrio nos híbridos de milho a fim de induzir a síntese prévia de fitoquelatinas nestes híbridos. Estas plantas pré-expostas ao zinco poderão apresentar tolerância na presença do mercúrio.

- Avaliar a expressão de enzimas antioxidantes induzidas pela pré-exposição ao zinco e/ou enxofre em híbridos de milho expostos ao mercúrio.

- Avaliar o potencial de produção de fitoquelatinas induzidas pelo zinco em híbridos de milho, bem como a sua capacidade de quelação do mercúrio.

8. REFERÊNCIAS BIBLIOGRÁFICAS

AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA – ANVISA. Resolução RE nº 528, de 17 de abril de 2001. **DOU** de 8/6/01.

ALLEN, R.D. Dissection of oxidative stress tolerance using transgenic plants. **Plant Physiology**, v. 107, p. 1049 – 1054, 1995.

ALSCHER, R.G., DONAHUE, J.L., CRAMER, C.L. Reactive oxygen species and antioxidants: relationships in green cells. **Physiologia Plantarum**, v. 100, p. 224 - 233, 1997.

ARAVIND,P., PRASAD, M.N.V. Zinc alleviates cadmium-induced oxidative stress in *Ceratophyllum demersum* L.: a free floating freshwater macrophyte. **Plant Physiology and Biochemistry**, v. 41, p. 391 – 397, 2003.

ARAVIND,P., PRASAD, M.N.V. Zinc protects chloroplasts and associated photochemical functions in cadmium exposed *Ceratophyllum demersum* L., a freshwater macrophyte. **Plant Science**, v. 166, p. 1321 – 1327, 2004.

ARAVIND,P., PRASAD, M.N.V. Cadmium-Zinc interactions in a hydroponic system using *Ceratophyllum demersum* L.: adaptative ecophysiology, biochemistry and molecular toxicology. **Brazilian Journal of Plant Physiology**, v. 17, p. 3-20, 2005.

ARDUINI, I., MASONI, A., MARIOTTI, M., ERCOLI, L. Low cadmium application increase iscanthus growth and cadmium translocation. **Environmental and Experimental Botany**, v. 52, p. 89–100, 2004.

ASSCHE, F.V., CLIJSTERS, H. Effects of metals on enzyme activity in plants. **Plant Cell and Environment**, v. 13, p. 195-206, 1990.

BAHIA, M.O. **Le potentiel génotoxique du mercure**: mutations HPRTet effects cytogénétiques. 1997. 119p. Dissertação (Mestrado) - Universidade de Quebéc, Montreal, 1997.

BARREIROS, A. L. B. S.; DAVID, J. M.; DAVID, J. P. Estresse oxidativo: relação entre geração de espécies reativas e defesa do organismo. Química Nova v. 29, p. 113, 2006.

BARTOSZ, G. Glutathione as antioxidant and electrophile sacavenger. **Pollution Journal Environmental Study**, v. 5, p. 87 – 88, 1996.

BERG, J.M., SHI, Y. The galvanization of biology: a growing appreciation for the roles of zinc. **Science**, v. 271, p. 1081 – 1085, 1996.

BISINOTI, M. C., JARDIM, W. F. Behavior of methylmercury in the environment. **Química Nova**, v. 27(4), p. 593-600, 2004.

BOENING, D.W. Ecological Effects, Transport, and Fate of Mercury: a general review. **Chemosphere**, v. 40, p. 1335 - 1351, 2000.

BOUDOU, A., DELNOMDEDIEU, M., GEORGESCAULD, D., RIBEYRE, F. AND SAOUTER, E. Fundamental roles of biological barriers in mercury accumulation and transfer in freshwater ecosystems (analysis at organism, organ, cell and molecular levels). **Water, Air and Soil Pollution**, v. 56, p. 807 – 822, 1991.

BRAY, T.M., BETTGER, W.J. The physiological role of zinc as an antioxidant. **Free Radical Biology and Medicine**, v. 8, p. 281-291, 1990.

BRUINS, M.R., KAPIL, S., OEHME, F.W. Microbial resistence to metals in the environment. **Ecotoxicology and Environmental Safety**, v. 45, p. 198 – 207, 2000.

BUCKNER, B., JOHAL, G.S., JANICK-BUCKNER, D. Cell death in maize. **Physiology Plant**, v. 108, p. 231 – 239, 2000.

BUETTNER, G. R. The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. **Archives of biochemistry and biophysics**, v. 300, p. 535-543, 1993.

CAKMAK, I. Possible roles of zinc in protecting plant cells from damage by reactive oxygen species, **New Phytologist**, v. 146, p. 185-205, 2000.

CAKMAK, I., ATLI, M., KAYA, R., EVLIYA, H., MARSCHNER, H. Association of high light and zinc deficiency in cold induced leaf chlorosis in grapefruit and mandarin trees. **Journal of Plant Physiology**, v. 146, p. 355 – 360, 1995.

CAKMAK, I., BRAUN, H.J. **Genotypic variation for zinc efficiency**. In: Reynolds, M.P., Ortiz-Monasterio, J.I., McNab, A. (eds). Application of physiology in wheat breeding. pp. 183 – 199, Mexico, D.F. CIMMYT.

CAKMAK, I., ENGELS, C. **Role of mineral nutrients in photosynthesis and yield formation**. In: RENGEL, Z., (ed) Mineral nutrition of crops. New York, USA: Haworth Press, p. 141 – 168, 1999.

CAKMAK, I., MARSCHNER, H. Enhanced superoxide radical production in roots of zinc deficient plants. **Journal of Experimental Botany**, v. 39, p. 1449 – 1460, 1988.

CALABRESE ,E.J., BLAIN, R. The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. **Toxicology and Applied Pharmacology**, v. 202, p. 289 – 301, 2005.

CARVALHO, W.A. **Efeito do Metilmercúrio Sobre a Secreção de Prolactina Induzida por Estresse de Imobilização em Ratos Wistar**. 2001. 54p. Dissertação (Mestrado em Ciências Biológicas) - Universidade Federal do Pará, Pará, 2001.

CETESB, Companhia de Tecnologia de Saneamento Ambiental. Relatório de estabelecimento de valores orientadores para solos e águas subterrâneas. São Paulo, 2001. 245 p.

CHANEY, R.L., 1993. **Zinc phytotoxicity**. In: Robson, A.D. (Ed.), Zinc in Soil and Plants. Kluwer Academic Publishers, Dordrecht, pp. 135–150.

CHANG, A. C. Developing Human Health-related Chemical Guidelines for Reclaimed Water and Sewage Sludge Applications in Agriculture. **World Health Organization**. May, 2002.

CHANG, T.C., YEN, J.H. On-site mercury-contaminated soils remediation by using thermal desorption technology. **Journal of Hazardous Materials**, v. 128, p. 208 – 217, 2006.

CHAVAPIL, M. New aspects in the biological role of zinc: a stabilizer of macromolecules and biological membranes. **Life Science**, v. 13, p. 1041-1049, 1973.

CHEN, J., GOLDSBROUGH, P.B. Increased activity of γ-glutamyl cysteine synthetase in tomato cells selected for cadmium tolerance. **Plant Physiology**, v. 106, p. 233 – 239, 1994.

CHO, U., PARK, J. Mercury-induced oxidative stress in tomato seedlings. **Plant Science**, v. 156, p. 1- 9 , 2000.

CHOW, P.V.T., CHUA, T.H., TANG, K.F. Dilute acid digestion procedure for the determination of lead, copper, mercury in traditional Chinese medicines by atomic absorption spectrometry. **Analyst**, v. 120, p. 1221 – 1223, 1995.

CHRYSAFOPOULOU, E., KADUKOVA, J., KALOGERAKIS, N. A whole-plant mathematical model for the phytoextraction of lead (Pb) by maize. **Environmental International**, v. 31, p. 255 – 262, 2005.

CLEMENS, S. Molecular mechanisms of plant metal homeostasis and tolerance. **Planta**, v. 212, p. 475 – 486, 2001.

CLEMENS, S. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. **Biochimie**, v. 88, p. 1707 – 1719, 2006.

COCKING, D., ROHRER, M., THOMAS, R., WALKER, J., WARD, D. Effects of root morphology and Hg concentration in the soil on uptake by terrestrial vascular plants. **Water, Air and Soil Pollution**, v. 80, p. 1113 – 1116, 1995.

DAUGHNEY, C.J., SICILIANO, S.D., RENCZ, A.N., LEAN, D., FORTIN, D. Hg (II) absorption by bacteria: a surface complexation model and its application to shallow acidic lakes and wetlands in kejimbujic National Park, Nova Escócia, Canadá. **Environmental Science and Technology**, v. 36, p. 1546 - 1553, 2002.

DEPLEDGE, M.H., WEEKS, J.M., BJERREGAARD., P. **Metals**. In: Callow, P. (ed.) Handbook of Ecotoxicology. Blackwell Scientific Publications, v. 2, p 79 – 105, 1994.

DIETZ, K.J., BAIER, M., KRAMER, U. **Free radical and reactive oxygen species an mediators of heavy metal toxicity in plants**. In: Prasad, M.N.V., Hagemeyer, J. (Eds.), Heavy Metal stress in Plants: From Molecules to Ecosystems. Springer-Verlag, Berlin, Germany, p. 73 – 97, 1999.

DU, S. H., FANG, S. C. Uptake of elemental mercury vapor by C_3 and C_4 species. **Environmental and Experimental Botany**, v. 22, p. 437 – 443, 1982.

DU, X., ZHU, Y.G., LIU, W.J., ZHAO, X.S. Uptake of mercury (Hg) by seedlings of rice (*Oryza sativa* L.) grown in solution culture and interactions with arsenate uptake. **Environmental and Experimental Botany**, v. 54, p. 1–7, 2005.

EBBS, S.D., KOCHIAN, L.V. Toxicity of zinc and copper to *Brassica* species: implication for phytoremediation. **Journal of Environment Quality**, v. 26, p. 776–781, 1997.

EIDE, D.J. The molecular biology of metal ion transport in *Saccharomyces cerevisiae*. **Annual Review of Nutrition**, 18, 441 – 469, 1998.

ELSTNER, E.F. Mechanisms of oxygen activation in different compartments of plant cells. In: Pell E.J., Steffen K.L., eds. Active oxygen/oxidative stress and plant metabolism. Rockville, M.D: **American Society of Plant Physiologists**, p. 13-25, 1991.

ESTEBAN, E., MORENO, E., PEÑALOSA, J., CABRERO, J.I., MILLÁN, R., ZORNOZA, P. Short and long-term uptake of Hg in white lupin plants: Kinetics and stress indicators. **Environmental and Experimental Botany**, v. 62, p. 316–322, 2008.

FARRELL, R.E., GERMIDA, J.J, HUANG, P.M. Biotoxicity of mercry as influenced by mercury (II) speciation. **Applied and Environmental Microbiology**, v. 56, p. 3006 - 3016, 1990.

FENGXIANG, X. HAN, YI SU, DAVID L. MONTS, CHARLES A. WAGGONER, PLODINEC, M.J. Binding, distribution, and plant uptake of mercury in a soil from Oak Ridge, Tennessee, USA. **Science of the Total Environment**, v. 368, p. 753 – 768, 2006.

FERRI, M.G. Fisiologia Vegetal. 2 ed., v. 1, São Paulo: EPU, 361 p.,1985.

FOYER, C.H., LELANDAIS, M., KUNERT, K.J. Photooxidative stress in plants. **Physiology Plant**, v. 92, p. 696 – 717, 1994.

FREAR, D.E.H., DILLS, L.E.Mechanism of the insecticidal action of mercury and mercury salts. **Journal of Economic Entomology**, v. 60, p. 970 – 974, 1967.

FRIDOVICH, I., HANDLER, P. Detection of free radicals generated during enzymic oxidation by the initiation of sulphite oxidation. **The Journal of Biological Chemistry**, v. 236, p. 1836 – 1840, 1961.

GAITHER, L.A., EIDE, D.J. Eukaryotic zinc transporters and their regulation. **BioMetals**, v. 14, p. 251 – 270, 2001.

GARCIA-GUINEA, J., HARFFY, M. Mercury mining: profit or loss? **Nature**, v. 390, p. 112, 1997.

GIROTTI, A.W., THOMAS, J.P., JORDAN, J.E. Inihibitory effect of zinc(II) on free radical lipid peroxidation in erythrocyte membranes. **Free Radical Biology and Medicine**, v. 1, p. 395 – 401, 1985.

GNAMUS, A., BYRNE, A.R., HORVAT, M. Mercury in the soil-plant-deer-predator food chain of a temperate forest in Slovenia. **Environmental Science and Technology**, v. 34, p. 3337 – 3345, 2000.

GODBOLD, D. L. AND HÜTTERMANN, A. Inhibition of photosynthesis and transpiration in relation to mercury-induced root damage in spruce seedlings. **Physiology Plant**, v. 74, p. 270 – 275, 1988.

GODBOLD, D.L. Mercury-induced root damage in spruce seedlings. **Water, Air and Soil Pollution**, v. 56, p. 823 – 831, 1991.

GOREN, R., SIEGEL, S.M. Mercury-induced ethylene formation and abscission in *Citrus* and *Coleus* explants. **Plant Physiology**, v. 57, p. 628 – 631, 1976.

GORSUCH, J.W., LOWER, W.R., LEVIS, M.A., WANG, W. **Plant for Toxicity Assessment**, v. 2, p. 1115, 1991.

GRANT, GRANT, C.M.; MACIVER, F.H., DAWES, I.W. Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide gammaglutamylcysteine. **Molecular Biology of the Cell**, v. 8, p. 1699 - 1707, 1997.

GRAZIA, C.A., PESTANA, M.H.D. **Mercury contaminated soils in gold mining areas of Lavras do Sul, RS, Brazil.** In: INTERNATIONAL CONFERENCE ON HEAVY METALS IN THE ENVIRONMENT, 13, Rio de Janeiro. 4p, 2005

GRESSEL, J., GALUN, E. **Genetic controls of photooxidant tolerance**. In: Foyer, C.H., Mullineaux, P., (eds). Causes of photooxidative stress and amelioration of defense systems in plants. Boca Raton, FL, USA: CRC Press, pp. 237 – 273, 1994

GUERINOT, M.L. The ZIP family of metal transporters. **Biochimica et Biophysica Acta**, v. 1465, p. 190 – 198, 2000.

HALLLIWELL, B., GUTTERIDGE, J.M.C. Role of free radicals and catalytic metal ions in human disease: an overview. **Methods in Enzymology**, v. 186, p. 1 - 5, 1990.

HALLIWELL, B., GUTTERIDGE, J.M. **Free Radicals in biology and medicine**. 3ª Ed. Oxford, New York, 543 p., 2000.

HALLIWELL, B. Oxidative damage, lipid peroxidation, and antioxidant protection in chloroplasts. **Chemistry and Physics of Lipids**, v. 44, p. 327 – 340, 1987.

HAZARDOUS SUBSTANCES DATA BANK (HSDB) –. **Mercury**. In: TOMES CPS SYSTEM. Toxicology, ccupational medicine and environmental series. Englewwod: Micromedex, 2000. CD-ROM.

HEATON, A.C.P., RUGH, C.L., WANG, N.J., MEAGHER, R.B. Physiological responses of transgenic merA-tabacco (*Nicotina tabacum*) to foliar and root mercury exposure. **Water, Air and Soil Pollution**, v. 161, p. 137 – 155, 2005.

HEGEDÜS, A., ERDEI, S., HORVÁTH, G. Comparative studies of H_2O_2 detoxifying enzymes in green and greening barley seedlings under cadmium stress. **Plant Science**, v. 160, p. 1085 - 1093, 2001.

ISRAR, M., SAHI, S., DATTA, R., SARKAR, D. Bioaccumulation and physiological effects of mercury in *Sesbania drummondii*. **Chemosphere**, v. 65, p. 591 - 598, 2006.

JAFFE, E.K., KERVINEN, J., DUNBRACK, J.R., LITWIN, S., MARTINS, J., SCARROW, R.C., VOLIM, M., YEUNG, A.T., YONN, E. Pophobilinogen synthase from pea: Expression from an artificial gene, kinetic characterization, and novel implications for subunit interactions. **Biochemistry**, v. 39, p. 9018 - 9029, 2000.

KLUG, A., RHODES, D. 'Zinc fingers': a novel protein motif for nucleic acid recognition. Trends in Biochemical Science, v. 12, p. 464 – 469, 1987.

KOVIDEVA, J., ROY, S., VRANJIC, J.A., HAUKIOJA, E., HUGHES, P.R., HANMINEN, O. Antioxidants responses to stimulated acid rain and heavy metal deposition in birch seedlings. **Environmental Pollution**, v. 95, p. 249 – 258, 1997.

KNOX, J.P., DODGE, A.D. Singlet oxygen and plants. **Phytochemistry**, v. 24, p. 889–896, 1985.

LENTI, K., FODOR, F., BODDI, B. Mercury inhibits the activity of the NAPH: protochorophyllide oxidoreductase. **Photosynthetica**, v. 40, p. 145 – 151, 2002.

LIN C. J., PEHKONEN S. O. The chemistry of atmospheric mercury: a review. **Atmospheric Environment**, v. 33, p. 2067 – 2079, 1999.

MAGGIO, A., JOLY, R.J. Effects of mercury chloride on the hydraulic conductivity of tomato root systems (evidence for a channel – mediated water pathaway). **Plant Physiology**, v. 109, p. 331 – 335, 1995.

MARSCHNER, H. **Mineral nutrition of higher plants**, 2nd ed. (1995) London, UK: Academic Press.

MARSCHNER H, CAKMAK I. High light intensity enhances chlorosis and necrosis in leaves of zinc-, potassium- and magnesium-deffcient bean (*Phaseolus vulgaris*) plants. **Journal of Plant Physiology**, v. 134, p. 308 - 315, 1989.

MASON, R.P., FITZGERALD, W.F., MOREL, F.M.M. The biogeochemical cycling of elemental mercury: anthropogenic influences. **Geochimica et Cosmochimica Acta**, v. 58, p. 3191 – 8, 1994.

MAUREL, C. Aquaporins and water permeability of plant membranes. **Annual Review of Plant Physiology (Plant and Molecular Biology)**, v. 48, p. 399 – 429, 1997.

MAUREL, C., VERDOUCQ, L., LUU, D.-T., SANTONI, V. Plant Aquaporins: Membrane Channels with Multiple Integrated Functions. **Annual Review of Plant Biology**, v. 59, p. 595 – 624, 2008.

MEAGHER, R.B., RUGH, C.L. **Phytoremediation of heavy metal pollution: Ionic and methyl mercury.** In: OECD Biotechnology for Water Use and Conservation Workshop, eds (Cocoyoc, Mexico: Organization for Economic Co-Operation and Development), 305-321, 1996.

MORENO, FN., ANDERSON, C.W.N., STEWART, R.B., ROBINSON, B.H., GHOMSHEI, M., MEECH, J.A. Induced plant uptake and transport of mercury in the presence of sulphur-containing ligands and humic acid. **New Phytologist**, v. 166, p. 445 – 454, 2005.

MORSCH, V.M., SCHETINGER, M.R.C., MARTINS, A.F., ROCHA, J.B.T. Effects of cadmium, lead, mercury and zinc on δ-aminolevulinic acid dehydratase activity from radish leaves. **Biologia Plantarum**, v. 45, p. 85 – 89, 2002.

NASCIMENTO, E.S., CHASIN, A.A.M. **Ecotoxicologia do mercúrio e seus compostos**. Série cadernos de referência ambiental, v. 1, 176 p., 2001. Salvador.

NELSON, N. Metal ion transporters and homeostasis. **European Molecular Biology Organization Journal**, v. 18, p. 4361 – 4371, 1999.

MIQUEL, J. **Historical introduction to free radical and antioxidant biomedical research**. In: Miquel, J., Quintanilha, A.T., Weber, H. (Eds.), CRC Hand Book of Free

Radicals and Oxidants in Biomedicine. CRC Press, Boca Raton, FL, pp. 3 – 11, 1989.

MITTLER, R. Oxidative stress, antioxidants and stress tolerance. **Plant Science**, v. 7, p. 405 - 410, 2002.

NOCTOR, G., FOYER, C.H. Ascorbate and gluthatione: keeping active oxygen under control. **Annual Reviewer of Plant Physiology and Plant Molecular Biology**, v. 49, p. 249 – 279, 1998.

NORIEGA, G.O., BALESTRASSE, K.B., BATLLE, A., TOMARO, M.L. Cadmium induced oxidative stress in soybean plants also by the accumulation of δ-aminolevulinic acid. **Biometals**, v. 20, p. 841 - 851, 2007.

OLIVEIRA, C., AMARAL SOBRINHO, N. M. B., MAZUR, N. Evaluación del potencial de contaminación de barro cloacal enriquecido con cadmio y cinc en dos suelos agrícolas. **Revista Terra**, México, v.21, p.351-363, 2003.

OLMOS, K., HERNÁNDEZ, J.A., SEVILLA, F., HELLÍN, E. Induction of several antioxidant enzymes in the selection of a SALT-tolerance cell line of *Pisum sativum*. **Journal of Plant Physiology**, v. 144, p. 594 – 598, 1994.

ORTEGA-VILLASANTE, C., RELLÁN-ÁLVAREZ, R., DEL CAMPO, F.F., CARPENA-RUIZ, R.O., HERNÁNDEZ, L.E. Cellular damage induced by cadmium and mercury in *Medicago sativa*. **Journal of Experimental Botany**, v. 56, p. 2239 – 2251, 2005.

PAULSEN, I.T., SAIER JR, M.H. A novel family of ubiquitous heavy metal ion transport proteins. **Journal of Membrane Biology**, v. 156, p. 99 – 103, 1997.

PATRA, M., SHARMA, A. Mercury toxicity in plants. **Botany Reviewer**, v. 66, p. 379 – 422, 2000.

PATRA, M., BHOWMIK, N., BANDOPADHYAY, B., SHARMA, A. Comparison of mercury systems and the development of genetic tolerance. **Environmental and Experimental Botany**, Review, v. 52, p. 199 - 223, 2004.

PEREIRA, L.B., TABALDI, L.A., GONÇALVES, J.F., JUCOSKI, J.O., PAULETTO, M.M., WEIS, S.N., NICOLOSO, F.T., BORHER, D., ROCHA, J.B.T., SCHETINGER, M.R.C. Effect of aluminum on δ-aminolevulinic acid dehydratase (ALA-D) and the development of cucumber (*Cucumis sativus*). **Environmental and Experimental Botany**, v. 57, p. 106 – 115, 2006

PIGNOCCHI, C., FOYER, C.H. Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. **Current Opinion in Plant Biology**, v. 6, p. 379 - 389, 2003.

PINTO, E., SIGAUD-KUTNER, T.C.S., LEITÃO, M.A.S., OKAMOTO, O.K., MORSE, D., COLEPICOLO, P. Heavy metal–induced oxidative stress in algae. **Journal Phycology**, v. 39, p. 1008–1018, 2003.

PINTON, R., CAKMAK, I., MARSCHNER, H. Zinc deficiency enhanced NAD(P)H-dependent superoxide radical production in plasma membrane vesicles isolated from roots of bean plants. **Journal of Experimental Botany**, v. 45, p. 45 – 50, 1994.

POWELL, S.R., DONNA, H., AIUTO, L., WAPNIR, R.A., TEICHBERG, S., TORTOLANI, A.J. Zinc improves postischemic recovery of isolated rat hearts through inhibition of oxidative stress. **American Journal of Physiology**, v. 266, p. 2497 – 2507, 1994.

POWELL, S.R. The antioxidant properties of zinc. **Journal Nutrition**, v. 130, p. 1447-1454, 2000.

PRASAD, A.S. Zinc: an overview. **Nutrition**, v. 11, p. 93 – 99, 1995.

PRASAD, D.D.K., PRASAD, A.R.K. Effect of lead and mercury on chlorophyll synthesis in mung bean seedlings. **Phytochemistry**, v. 26, p. 881 – 883, 1987.

PRASAD, R., KAUR, D., KUMAR, V. Kinetics characterization of zinc binding to brush border membranes from rat kidney cortex: interaction with cadmium. **Biochimica et Biophysica Acta**, v. 1284, p. 69 – 78, 1996.

QUEIROZ, I.R. **Determinação de metilmercúrio em peixes de região de garimpo**. 1995. 109f. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo.

RANIERI, A., LENCIONI, L., SCHENONE, G., SOLDATINI, G.F. Gluthatione-ascorbic acid cycle in pumpkin plants grown under polluted air in open-top chambers. Journal of Plant Physiology, v. 142, p. 286–290, 1993.

RAUSER, W.E. Structure and function of metal chelators produced by plants. **Cell Biochemistry and Biophysics**, v. 31, p. 19 – 33, 1999.

RAVICHANDRAN, M. Interactions between mercury and dissolved organic mater — a review. **Chemosphere**, v. 55, p. 319 – 31, 2004.

RELLÁN-ÁLVAREZ, R., ORTEGA-VILLASANTE, C., ÁLVAREZ-FERNÁNDEZ, A., CAMPO, F.F., HERNÁNDEZ, L.E. Stress responses of *Zea mays* to cadmium and mercury. **Plant and Soil**, v. 279, p. 41 – 50, 2006.

REVIS, N.W., OSBORNE, T.R., HOLDSWORTH, G., HADDEN, C., Distribution of mercury species in soil from a mercury-contaminated site. **Water, Air and Soil Pollution**, v. 45, p. 105 – 13, 1989.

RHODES, D., KLUG, A. Zinc fingers. **Scientific Americam**, v. 268, p. 32 – 39, 1993.

ROCHA, J.B.T., ROCHA, L.K., EMANUELLI, T., PEREIRA, M.E. Effect of mercuric chloride and lead acetate treatment during the second stage of rapid post-natal brain growth on the behavioral response to chlorpromazine and on δ-ALA-D activity in weaning rats. **Toxicology Letters**, v. 125, p. 143–150, 2001.

RICHTER, C., SCHEWEITZER, M. **Oxidative stress in mitochondria**. In: Scandalios, J.G. (Ed.), Oxidative stress and the Molecular Biology of Antioxidant Defenses, Monograph Series, v. 34. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, p. 169 – 200, 1997.

ROSEN, B.P. Biochemistry of arsenic detoxification. FEBS; **Federation of European Biochemical Societies, Letters**, v. 529, p. 86 – 92, 2002.

SALT, D.E., BLAYLOCK, M., KUMAR, N.P.B.A., DUSHENKOV, V., ENSLEY, B.D., CHET, I., RASKIN, I. Phytorremediation: a novel strategy for the removal of toxic metals from the environment using plants. **Biotechnology**, v. 13, p. 468 - 474, 1995.

SANTA MARIA, G.E., COGLIATTI, D.H. Bidirectional Zn-fluxes and compartmentation in wheat seedling roots. **Journal of Plant Physiology**, v. 132, p. 312 – 315, 1988.

SCHNEIDER, S., BERGMANN, L. Regulation of glutathione synthesis in suspention cultures parsley and tobacco. **Botanica Acta**, v. 108, p. 34 – 40, 1995.

SCOTT, N., HATLELID, K.M., MAC KENZIE, N.E., CARTER, D.E. Reactions of arsenic (III) and arsenic (V) species with glutathione. **Chemical Research in Toxicology**, v. 6, p. 102 – 106, 1993.

SEIGNEUR, C., VIJAYARAGHAVAN, K., LOHMAN, K., KARAMCHANDANI, P., SCOTT, C. Global source attribution for mercury deposition in the United States. **Environmental Science and Technology**, v. 38, p. 555 – 69, 2004.

SHALATA, A., TAL, M. The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerance relative *Lycopersicon pennellii*. **Physiology Plant**, v. 104, p. 169 – 174, 1998.

SIERRA, M.J., MILLÁN, R., ESTEBAN, E. Potential use of Solanum melongena in agricultural areas with high mercury background concentrations. **Food and Chemical Toxicology**, v. 46, p. 2143–2149, 2008.

SINHA, S., GUPTA, M., CHANDRA, P. Bioccumulation and biochemical effects on mercury in the plant *Bacopa monnieri* (L.). **Environmental Toxicology and Water Quality**, v. 11, p. 105 – 112, 1996.

SINHA, S., SAXENA, R., SINGH, S. Chromium induced lipid peroxidation the plants of Pistia stratiotes L.: role of antioxidants and antioxidant enzymes. **Chemosphere**, v. 58, p. 595 – 604, 2005.

SMIRNOFF, N. Ascorbic acid: Metabolism and functions of a multifaceted molecule. **Current Opinion in Plant Biology**, v. 3, p. 229 – 235, 2000

STEFANIDOU, M., MARAVELIAS, C., DONA, A., SPILIOPOULOU, C. Zinc: a multipurpose trace element. **Archives of Toxicology**, v. 80, p. 1–9, 2006.

TABALDI, L.A., RUPPENTHAL, R., CARGNELUTTI, D., MORSCH, V.M., PEREIRA, L.B., SCHETINGER, M.R.C. Effects of metal elements on acid phosphatase activity in cucumber (*Cucumis sativus* L.) seedlings. **Environmental and Experimental Botany**, v. 59, p. 43 – 48, 2007.

TORRES, B.B. **Nutrição e esporte uma abordagem bioquímica**. Departamento de Bioquímica, Instituto de Química, USP, 2003.

TSUJI, N., HIRAYANAGI, N., OKADA, M., MIYASAKA, H., HIRATA, K., ZENK, M.H., MIYAMOTO, K. Enhancement of tolerance to heavy metals and oxidative stress in Dunaliella tertiolecta by Zn-induced phytochelatin synthesis. **Biochemical and Biophysical Research Communications**, v. 293, p. 653–659, 2002.

USEPA (UNITED STATES ENVIRONMENTAL PROTECTION AGENCY). **Mercury Study**, Report to Congress. EPA-452/R-97-005, 1997b.

USEPA (UNITED STATES ENVIRONMENTAL PROTECTION AGENCY). **National Estuary Program**. Coastlines November 2003-Issue 13.5.

VALLEE, B.L., AULD, D.S. Zinc coordination, function, and structure of zinc enzymes and other proteins. **Biochemistry**, v. 29, p. 5647 - 5659, 1990.

VALLEE, B.L., FALCHUK, K.H. The biochemical basis of zinc physiology, **Physiology Reviewer**, v. 73, p. 79 – 118, 1993.

VAN DER ZAAL, B.J., NEUTEBOOM, L.W., PINAS, J.E., CHARDONNENS, A.N., SCHAT, H., VERKLEIJ, J.A.C., HOOYKAAS, P.J.J. Over-expression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. **Plant Physiology**, v. 119, p. 1047 – 1055, 1999.

WANG, Y., GREGER, M. Clonal differences in mercury tolerance, accumulation and distribution in willow. **Journal of Environmental Quality**, v. 33, p. 1779 – 1785, 2004.

WILLIAMS, R.J.P. **An introduction to the biochemistry of zinc**. In: Zinc in human biology. London, UK: Springer-Verlag, 15 – 31, 1988.

WILLIAMS, L.E., PITTMAN, J.K., HALL, J.L. 2000. Emerging mechanisms for heavy metal transport in plants. **Biochimica et Biophysica Acta**, v. 1465, p. 104 – 126, 2000.

WANG, M., ZOU, J., DUAN, X., JIANG, W., LIU, D. Cadmium accumulation and its effects on metal uptake in maize (*Zea mays* L.). **Bioresource Technology**, v. 98, p. 82 – 88, 2007.

WORLD HEALTH ORGANIZATION – WHO **Conference on intoxicaton due to alkylmercury-treated seed**. Geneva, 1978. Environmental Health Criteria, v.1, p. 148, 1978.

WILLSON RL**. Zinc and iron in free radical pathology and cellular control**. In: Mills CF, ed. Zinc in human biology. London, UK: Springer-Verlag, p. 147 - 172. 1988.

WOJCIECHOWSKA-MAZUREK, M., ZAWADZKA, T., KARLOWSKI, K., CWIEK-LUDWICKA, K., BRULINSKA-OSTROWSKA, E. Content of lead, cadmium, mercury, zinc and copper in fruit from various regions of Poland. **Rocz Panstw Zakl Hig**, v. 46, p. 223 – 238, 1995.

WOOLHOUSE, H.W. **Toxicity and tolerance in the responses of plants to metals**. In: Physiological Plant Ecology 3. Encyclopaedia of Plant Physiology. Lange, O.L., Nobel, P.S., Osmond, C.B., Ziegler, H.. Springer-Verlag, (Eds.), v.. 12, p. 246 – 300, 1983.

YIN, Y., ALLEN, H.E., LI, Y., HUANG CP, SANDERS, P.F. Adsorption of mercury (II) by soil: effects of pH, chloride and organic matter. **Journal of Environmental Quality**, v. 25, p. 837 – 44, 1996.

ZENK, M.H. Heavy metal detoxication in higher plants, a review. **Gene**, v. 179, p. 21 – 30, 1996.

ZHANG, L., WONG, M.H. Environmental mercury contamination in China: Sources and impacts. **Environmental International**, v. 33, p. 108 – 121, 2007.

ZANG, W.H., TYERMAN, S.D. Inhibition of water channels by $HgCl_2$ in intact wheat root cells**. Plant Physiology**, v. 120, p. 849-857, 1999.

ZHOU, Z.S., HUANG, S.Q., GUO, K., MEHTA, S.K., ZHANG, P.C., YANG, Z.M. Metabolic adaptations to mercury-induced oxidative stress in roots of *Medicago sativa* L. **Journal of Inorganic Biochemistry**, v. 101, p. 1 – 9, 2007.

ZHOU, Z.S., WANG, S.J., YANG, Z.M. Biological detection and analysis of mercury toxicity to alfalfa (*Medicago sativa*) plants. **Chemosphere**, v. 70, p. 1500 – 1509, 2008.

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