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

EFEITO DO MERCÚRIO NO ESTRESSE OXIDATIVO, NA ATIVIDADE DA DELTA-ALA-D E NO CRESCIMENTO DE PLÂNTULAS DE PEPINO (*Cucumis sativus* L.)

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
2007

EFEITO DO MERCÚRIO NO ESTRESSE OXIDATIVO, NA ATIVIDADE DA DELTA-ALA-D E NO CRESCIMENTO DE PLÂNTULAS DE PEPINO (*Cucumis sativus* L.)

Por

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

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Santa Maria, RS, Brasil

2007

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 Dissertação de Mestrado

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elaborada por

Denise Cargnelutti

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

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Santa Maria, 01 de fevereiro de 2007.

DEDICATÓRIA

Aos meus pais: Celita e Selito

Pela vida, amor, incentivo e compreensão.

Aos meus irmãos: Jocelito, Ademir, Joceli e Jocelaine

Pela amizade, amor, carinho e incentivo.

Ao meu namorado: Marciel

Pelo amor, amizade e compreensão.

AGRADECIMENTOS

À orientadora, Prof.ª Maria Rosa Schetinger, pelas orientações, incentivos e auxílios em todos os momentos; pela dedicação e pelo modo dinâmico com os quais conduziste a tua orientada, muito obrigada.

À co-orientadora, Prof.ª Vera Maria Morsch, pelo conhecimento, atenção, amizade, confiança e dedicação, muito obrigada.

Ao professor Fernando Nicoloso, pelo auxílio, conhecimento e atenção, obrigada.

Às amigas, Luciane Belmonte, Rosélia e Luciane Tabaldi, pelo auxílio, atenção e incentivo, obrigada.

Aos demais amigos, pela convivência, pelas contribuições, especialmente os amigos Carlos Eduardo e Mushtaq Ahmed, e as amigas Vanessa, Jamile, Joseila, Renata, Liana e Margarete.

À UFSM, ao Curso, aos professores, à CAPES, e a todos que de alguma forma contribuíram para a realização deste trabalho, obrigada.

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LISTA DE ABREVIATURAS

ALA – ácido 5-aminolevulínico

ANOVA – análise de variância

ASA – ácido ascórbico

$CuSO_4$ – sulfato de cobre

DMSO – dimetilsulfóxido

DNPH – dinitrofenilidrazina

DTNB – ácido 5-5' –ditio-bis-(nitobenzóico)

DTT – ditiotreitol

EDTA - ácido etilenodiaminotetracético

ELP – porcentagem de vazamento de eletrólitos

GSH – glutationa reduzida

HCl – ácido clorídrico

Hg – mercúrio

Hg^{2+} - íon mercúrio

$HgCl_2$ – cloreto 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

PBG - porfobilinogênio

PVP- polivinilpirrolidona

ROS – espécies reativas de oxigênio

Rpm – rotações por minuto

TBA – ácido tiobarbitúrico

TCA - ácido tricloroácetico

-SH – grupos tiólicos não-protéicos

δ-ALA-D – delta-aminolevulinato desidratase

RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

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EFEITO DO MERCÚRIO NO ESTRESSE OXIDATIVO, NA ATIVIDADE DA DELTA-ALA-D E NO CRESCIMENTO DE PLÂNTULAS DE PEPINO (*Cucumis sativus* L.)

Autora: Denise Cargnelutti

Orientadora: Maria Rosa Chitolina Schetinger

Co-Orientadora: Vera Maria Morsch

Data e local de defesa: Santa Maria, 01 de fevereiro de 2007.

Neste estudo, foram investigados os efeitos do mercúrio ($HgCl_2$) em plântulas de pepino (*Cucumis sativus* L.) através da análise de parâmetros bioquímicos e fisiológicos. Os parâmetros bioquímicos analisados foram: as atividades de enzimas antioxidantes [catalase (CAT), ascorbato peroxidase (APX) e superóxido dismutase (SOD)] e os níveis de antioxidantes não-enzimáticos (ácido ascórbico (ASA), carotenóides e tióis não-protéicos (-SH)). O dano aos lipídios de membrana [a peroxidação lipídica e a porcentagem de vazamento de eletrólitos (ELP)], o conteúdo de clorofila e a oxidação de proteínas foram determinadas. Foram também determinados os níveis de peróxido de hidrogênio (H_2O_2) e a atividade da delta-aminolevulinato desidratase (δ-ALAD). O crescimento das plântulas de pepino foi avaliado baseado na matéria seca e fresca e no comprimento de raízes e parte aérea. As plântulas de pepino foram expostas a 0; 0,5; 50; 250 e 500 μM de $HgCl_2$ durante 10 e 15 dias. Os resultados demonstraram que o mercúrio foi absorvido pelas plântulas, e seu conteúdo foi maior nas raízes que na parte aérea. Além disso, uma redução no comprimento das raízes e da parte aérea, ambos aos 10 e 15 dias, que foi dependente do tempo e da concentração, foi observada em todas as concentrações testadas. Na concentração de 50 μM de $HgCl_2$ o peso fresco das raízes das plântulas aos 15 dias aumentou, no entanto, ele reduziu nas outras concentrações. Para as plântulas com 10 dias, foi observada uma redução na massa fresca de raízes e parte aérea. Nenhuma redução na massa fresca da parte aérea foi observada na concentração de 50 μM de $HgCl_2$, aos 15 dias. Em relação ao peso seco, houve um aumento a 500 μM, ambos a 10 e 15 dias, entretanto, na concentração de 250 μM de $HgCl_2$ houve um aumento aos 15 dias. Além disso, foi observada uma redução significativa no peso seco da parte aérea em todas as concentrações testadas. Os resultados mostraram níveis elevados de peróxidos lipídicos, assim como aumento na oxidação de proteínas, e redução no conteúdo de clorofila quando as plântulas foram expostas a 250 e 500 μM de $HgCl_2$. Em relação às enzimas antioxidantes, houve um aumento na atividade da CAT aos 10 dias de exposição ao $HgCl_2$, a 50 μM. No entanto, na concentração mais alta (500 μM) de $HgCl_2$, houve uma

marcada inibição. Também, tanto aos 10 quanto aos 15 dias, foi observada uma inibição na atividade da enzima APX nas concentrações de $HgCl_2$ mais elevadas (250 e 500 μM). A SOD, outra enzima do sistema de defesa antioxidante, mostrou atividade aumentada na concentração abaixo de 50 μM $HgCl_2$, e atividade reduzida nas concentrações mais altas. Em relação à ELP, foram observadas alterações somente na concentração mais elevada (500 μM de $HgCl_2$) aos 15 dias de exposição ao metal. Além disso, as plântulas com 10 dias de exposição ao metal, tiveram seus níveis de H_2O_2 reduzidos na concentração de 50 μM de $HgCl_2$, mas o H_2O_2 aumentou na concentração mais alta. Em relação aos antioxidantes não-enzimáticos, foram observados níveis de SH aumentados em todas as concentrações aos 10 dias de exposição. Os níveis de ASA também aumentaram em todas as concentrações testadas aos 10 e 15 dias de exposição ao metal. Ainda, os níveis dos carotenóides aumentaram em baixas concentrações e foram reduzidos em altas concentrações, ambos aos 10 e 15 dias de exposição ao mercúrio. A atividade da ALA-D aumentou a 50 μM de $HgCl_2$ aos 15 dias, e diminuiu em concentrações mais altas. Portanto, os resultados obtidos das análises bioquímicas e fisiológicas sugerem que a exposição ao mercúrio induz estresse oxidativo em plântulas de pepino, resultando em injúria nos tecidos o que leva a redução no crescimento e perda de matéria seca das plântulas.

Palavras-chave: *Cucumis sativus*; antioxidantes; espécies reativas de oxigênio.

ABSTRACT

Master Dissertation

Biological Sciences: Toxicological Biochemistry Post-Graduation

Universidade Federal de Santa Maria

MERCURY EFFECT IN THE OXIDATIVE STRESS, IN THE DELTA-ALA-D ACTIVITY AND ON GROWNHT OF CUCUMBER SEEDLINGS (*Cucumis sativus* L.)

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Oriented by: Maria Rosa Chitolina Schetinger

Co-oriented by: Vera Maria Morsch

Place and date: Santa Maria, February 01, 2007.

In this study, the effects of mercury ($HgCl_2$) in cucumber seedlings (*Cucumis sativus* L.) were investigated through the analysis of the physiological and biochemical parameters. The biochemical parameters analyzed were: the 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 (SH)). The damage at the membrane lipids (lipid peroxidation, electrolytic leakage percentage (ELP)), the chlorophyll content, and protein oxidation were determined. The hydrogen peroxide levels (H_2O_2) and the δ-aminolevulinic acid dehydratase (δ-ALAD) activity were also determined. The growth of cucumber seedlings was evaluated based on the dry and fresh matter, and on the root and shoot length. Cucumber seedlings were exposed to 0 to 500 μM of $HgCl_2$ during 10 and 15 days. The results showed that Hg was absorbed by the growing seedlings, and its content was greater in the roots than in the shoot. Moreover, a reduction in the root and shoot length, at both 10 and 15 days, which was dependent on time and concentration, was observed at all concentrations tested. At the concentration of 50 μM $HgCl_2$ the root fresh weight of 15-day-old seedlings increased, however, it reduced at the other concentrations. For 10-day-old seedlings, a reduction in root and shoot fresh biomass was observed. No reduction in shoot fresh biomass was observed at the concentration of 50 μM $HgCl_2$, at 15 days. Regarding dry weight, there was an increase at 500 μM, both at 10 and 15 days, however, at the concentration of 250 μM $HgCl_2$, there was an increase at 15 days. Moreover, a significant reduction in the dry weight of shoot in all tested concentrations was observed. The results showed higher levels of lipid peroxides, as well as a protein oxidation increase, and chlorophyll content reduction when seedlings were exposed to 250 and 500 μM $HgCl_2$. In relation to the antioxidant enzymes, there was an increase in the CAT activity at 10 days of exposure to $HgCl_2$, at 50 μM. However, in the higher concentration (500 μM) of mercury, there was a marked inhibition. Besides, at both 10 and 15 days, an inhibition of APX enzyme in the mercury higher concentrations (250 and 500 μM) was observed. The SOD, another enzyme of the antioxidant

system, showed an increased activity in the concentration below 50 μM $HgCl_2$, and a reduced activity in the higher concentrations. Regarding ELP, alterations only in the higher concentrations (500 μM $HgCl_2$) and at 15 days of exposure to metal were observed. Furthermore, seedlings with 10 days of exposure to $HgCl_2$ had their reduced H_2O_2 levels at 50 μM $HgCl_2$, but the H_2O_2 increased at the higher concentration. In relation to non-enzymatic antioxidants, increasing SH levels at all the concentrations at 10-days of exposure were observed. ASA levels also increased at all tested concentrations at 10 and 15 days of exposure at metal. Yet, the carotenoids levels increased at low concentrations and decreased at high concentrations, both at 10 and 15 days of exposure to Hg. δ-ALA-D activity increased at 50 μM $HgCl_2$ at 15 days, and was inhibited at higher concentrations. Therefore, the results obtained from the biochemical and physiological analyses suggest that mercury induces oxidative stress in cucumber seedlings, resulting in injuries in the tissues, which leads to a reduction in the growth, and loss of dry matter of the seedlings.

Keywords: *Cucumis sativus*; antioxidants; reactive oxygen species.

1. INTRODUÇÃO

Os metais são componentes essenciais em diferentes processos nos organismos vivos. 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 para as plantas. No entanto, outros elementos metálicos, como por exemplo, o cádmio, o chumbo e o mercúrio, não têm um papel biológico conhecido (BRUINS et al., 2000). A similaridade química aos 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 tanto em animais aquáticos (PASSOS et al., 2006) e terrestres (PEROTONI et al., 2004) quanto em plantas aquáticas e terrestres (ISRAR et al., 2006; RELLÁN-ÁLVAREZ et al., 2006; CHO & PARCK, 2000).

Apesar da sua toxicidade, o mercúrio é extensivamente utilizado no processo de mineração do ouro (VEIGA & HINTON, 2002). Isso leva à contaminação do ambiente devido a sua liberação na atmosfera. Uma fração significativa este elemento também contamina a água e os solos depois da descarga dos resíduos do processo de amalgamação (VEIGA & HINTON, 2002). Além disso, este metal pesado é muito utilizado na indústria e, por conseqüência, é inadequadamente disposto na natureza. O mercúrio no ambiente pode originar-se de várias fontes, como áreas de mineração, áreas poluídas e com intensa atividade industrial (CHO & PARK, 2000; CATHUM et al., 2005). Ainda, a utilização do mercúrio em indústria de papel, tintas,

baterias, pesticidas e fertilizantes contribuem significativamente para a sua presença no ambiente (SINHA et al., 1996).

O mercúrio que é libertado na superfície dos solos geralmente é retido na fase sólida por adsorção a sulfitos, partículas de argila, e pela matéria orgânica (EVANS, 1989). Estas formas de mercúrio são insolúveis e, relativamente, imóveis. Porém, as reações de troca podem acontecer na solução do solo, levando ao aumento da solubilidade e da mobilidade do mercúrio no solo. Os íons cloreto (Cl^-) e hidróxido (OH^-) ocorrem naturalmente nos solos. O Hg^{2+} quando complexado com o Cl^-, forma o $HgCl_2$ que é bastante solúvel em água. Os complexos de Hg(OH)Cl e de $Hg(OH)_2$ são as espécies de mercúrio predominantes em ambientes bem-oxigenados (SCHUSTER, 1991). O mercúrio tem uma forte afinidade por grupos tiólicos, e na sua especiação sob condições de anóxia prepondera os complexos sulfatos e bissulfatos (MOREL et al., 1998).

Em solos, o mercúrio leva a redução no crescimento, no metabolismo (ISRAR et al., 2006), na fotossíntese (GODBOLD & HUTTERMANN, 1986), na transpiração e na absorção de água das plantas, e induz o aumento da peroxidação lipídica (CHO & PARCK, 2000). Além disso, o mercúrio causa a inibição do crescimento da raiz e da parte aérea (SUSZCYNSKY & SHANN, 1995), alterando assim o desenvolvimento normal da planta.

DIETZ et al. (1999) relataram que o excesso de metais pesados, entre eles o mercúrio, induz à formação de radicais livres e de espécies reativas de oxigênio (ROS), resultando em estresse oxidativo em plantas. As ROS tais como, o anion 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 ROS causam dano às membranas, 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 balanço de ROS 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). Entre os antioxidantes não-enzimáticos estão o ácido ascórbico, a glutationa reduzida (GSH), os carotenóides e outros grupos tiólicos não protéicos que removem diferentes tipos de ROS e protegem a célula contra a injúria e a disfunção dos tecidos (HALLIWELL, 1987; FOYER et al., 1994).

A síntese de clorofila pode ser afetada por a uma diminuição na atividade da enzima delta–aminolevulinato desidratase (δ-ALA-D) a qual é sensível a metais pesados, entre eles, o mercúrio, devido a sua natureza sulfidrílica (MORSCH et al., 2002). Esta enzima catalisa a condensação assimétrica de duas moléculas de ácido δ-aminolevulínico (ALA) originando o porfobilinogênio (GIBSON et al., 1955). A síntese do porfobilinogênio promove a formação de porfirinas, hemes, e clorofila, que são essenciais para o metabolismo da clorofila e da fotossíntese (JAFFE et al., 2000). A síntese diminuída de clorofila provoca a diminuição no crescimento devido a menor taxa fotossintética da planta.

O *Cucumis sativus* (pepino), uma importante espécie cultivada e consumida no Brasil, foi selecionado como uma planta teste, devido a sua sensibilidade para uma grande variedade de contaminantes (GORSUCH et al.,

1991, PEREIRA et al., 2006). 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.

Tendo em vista que é de grande importância o estudo da toxicologia do mercúrio no metabolismo das plantas devido ao aumento crescente da contaminação dos solos devido ao uso de pesticidas agrícolas em solos, despejo do lixo industrial em locais inadequados, utilização do lodo de esgotos e as atividades de mineração, os objetivos deste trabalho foram:

1.1. Objetivos

1.1.2. Objetivo Geral

Avaliar o efeito de diferentes concentrações de mercúrio em parâmetros oxidativos e de crescimento de plântulas de pepino durante os primeiros 10 e 15 dias de germinação.

1.1.3. Objetivos Específicos

- Avaliar a atividade de enzimas antioxidantes (catalase, ascorbato peroxidase e superóxido dismutase) e os níveis de antioxidantes não-enzimáticos (carotenóides, ácido ascórbico e tióis não-protéicos) em plântulas de pepino após exposição ao mercúrio;

- Determinar os níveis de peroxidação lipídica, o conteúdo de peróxido de hidrogênio, as proteínas oxidadas e a porcentagem de vazamento de eletrólitos após exposição ao mercúrio;

- Avaliar a atividade da enzima delta-ALA-D e o conteúdo de clorofila em plântulas de pepino após exposição ao mercúrio;

- Avaliar as alterações no crescimento, e determinar o conteúdo de mercúrio absorvido pelas plântulas de *C. sativus* após exposição ao mercúrio.

2. REVISÃO DA LITERATURA

2.1. Mercúrio

O mercúrio é um dos metais pesados mais tóxico encontrado no ambiente que inclui a litosfera, a hidrosfera, a atmosfera e a biosfera (ZHANG & WONG, 2006). 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 meio 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 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 o íon mercuroso (Hg_2^{++}) e o íon mercúrico (Hg^{++}) (NASCIMENTO & CHASIN, 2001). Os sais de mercúrio mais importantes são o $HgCl_2$ (cloreto de mercúrio), 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 prevalentes existindo no ambiente, e CH_3HgCl e CH_3HgOH 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 os mais considerados por sua toxicidade, mas 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 (orgânicas e inorgânicas) geralmente encontradas no ambiente, e algumas formas de mercúrio geradas através da atividade antropogênica.

Tabela 1- Formas orgânicas e inorgânicas do mercúrio. Adaptado de QUEIROZ (1995).

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

2.1.2. Fontes

O mercúrio na sua forma natural 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). 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 com o uso de fertilizantes nos solos. A grande poluição com mercúrio no ambiente 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 ($\mu g.g^{-1}$).

2.1.3. Ciclo biogeoquímico 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 do mercúrio 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 (ANDERSON, 1979).

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 Hgº (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).

2.1.4. Mercúrio nos solos

Patra et al. (2004) relataram que as concentrações de mercúrio encontradas normalmente em solos são baixas e não são tóxicas. O limite máximo estabelecido para o mercúrio em solos fica na faixa de 0,6 mg Kg^{-1}, que representa um solo não contaminado. Para solos contaminados, os níveis de mercúrio podem alcançar valores acima de 120 mg Kg^{-1} (EPA, 1997). Cavallini et al. (1999) mostraram que em solos contaminados com mercúrio em concentrações que variavam de 15 a 200 $\mu g\ g^{-1}$, as plantas absorveram concentrações de mercúrio altas nas folhas (2,6 $\mu g\ g^{-1}$ de peso seco) e nas raízes (4,5 $\mu g\ g^{-1}$ de peso seco). Além disso, 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}.

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., 1989b). 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 e como Hg^{2+} ou 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). MUNZUROGLU & GECKIL (2002) relataram que em solos, o efeito de um metal é determinado sinergisticamente ou antagonisticamente por outros cátions metálicos e seus ânions associados. Assim, alguns elementos naturais ou artificiais dos solos como o húmus e as ciclodextrinas, podem formar complexos estáveis com o mercúrio (MIERLE & INGRAM, 1991; WANG et al., 1995; CATHUM et al., 2005) reduzindo tanto a quantidade de mercúrio absorvida pelas plantas quanto a sua disponibilidade na solução do solo, além de reduzir a toxicidade dos solos contaminados. Além disso, há uma forte afinidade do Hg^{2+} e seus compostos inorgânicos às substâncias que contêm enxofre (grupos –SH e cisteína). O mercúrio se liga a esses compostos formando um complexo que limita grandemente a mobilidade do mercúrio em solos (USEPA, 1997a). 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.

2.1.5. Toxicidade

Embora alguns metais tais como o Mn, o Cu, o Zn, o Mo e o Ni sejam micronutrientes essenciais ou benéficos para microorganismos, plantas e animais, em altas concentrações, têm fortes efeitos tóxicos e são uma ameaça ambiental (NEDELKOSKA & DORAN 2000). Esta ameaça pode ser experimentada primeiro pelas plantas, os produtores primários, principalmente

pela contaminação dos solos, que tem aumentado paralelamente à industrialização (KLAASSEN et al., 1986). Stefanov et al. (1995) relataram que as espécies de plantas diferem na sua sensibilidade aos metais. As plantas que crescem em habitats com altas concentrações de metais provavelmente têm a habilidade para inativar estes elementos. Este processo acontece devido a formação de complexos entre o íon metálico e os grupos –SH produzidos pelas plantas. Também, as plantas que crescem em habitats metalíferos, mudam a composição química e a organização física das suas membranas celulares, impedindo que os íons sejam absorvidos pelas células.

Em relação ao mercúrio, estudos envolvendo o efeito da especiação sob diferentes condições, como por exemplo, o pH, as espécies ligantes e a concentração das espécies de mercúrio, foi observado que a sua toxicidade é influenciada grandemente pela natureza dos íons de mercúrio, por exemplo, a toxicidade do $Hg(CH_3COO)_2$ é similar a do $Hg(NO_3)_2$ (FARRELL et al., 1990).

Salt el al. (1995) observaram que mesmo a exposição à concentrações relativamente baixas de mercúrio pode resultar em toxicidade para as plantas. Além de outros fatores, a toxicidade do mercúrio em baixas concentrações é devido a alta solubilidade das diversas formas do mercúrio em água. Dentre as diferentes formas do mercúrio, o Hg^{2+} é altamente solúvel em água e é reativo (HEATON et al., 2005). Assim como outros metais pesados, tais como o cádmio, o cobre, o chumbo e o zinco, 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 de raiz (ASSCHE & CLIJSTERS, 1990). Assim, o alvo primário de toxicidade do mercúrio em plantas seriam os resíduos sulfidrílicos das

proteínas (WOOLHOUSE, 1983). Por exemplo, o Hg^{2+} pode ligar-se à 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), níveis reduzidos de clorofila e proteínas (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). Porém, mecanismos bioquímicos e moleculares da fitotoxicidade do mercúrio ainda são desconhecidos (CHO & PARK, 2000).

Portanto, o maior risco para a saúde humana e para as cadeias alimentares é quando as plantas desenvolvem mecanismos de tolerância a metais e quando essas plantas são incorporadas as cadeias alimentares (MUNZUROGLU & GECKIL, 2002). Em peixes, o nível máximo aceitável de mercúrio é de 1 mg kg^{-1} (GUPTA & GUPTA, 1998; NATIONAL RESEARCH COUNCIL, 1996; WILLIAMS, 1975) e, em drogas e plantas, o limite aceitável de mercúrio em é de 0,5 μg de Hg g^{-1} (CHOW et al., 1995). Contudo, essas concentrações podem estar bem acima dos valores aceitáveis.

Sendo assim, para resolver o problema da contaminação dos solos com mercúrio, estudos tem focalizado na utilização de plantas biorremediadoras Esta tecnologia faz o uso de plantas tolerantes ao mercúrio, que absorvem o metal e descontaminam os solos (CHANG & YEN).

2.2. Espécies reativas de oxigênio

No ambiente contaminado com metais pesados, as raízes das plantas são a zona de contato primário com os poluentes do solo. A fim de sobreviver, as plantas desenvolvem mecanismos pelos quais quantidades excessivas de metais pesados são absorvidos e transformados em formas fisiologicamente toleráveis (COBBETT, 2000; HALL, 2002).

O excesso de metais pesados tóxicos, entre eles o mercúrio, induz à formação de radicais livres e de espécies reativas de oxigênio (CHO & PARCK, 2000), resultando em estresse oxidativo (DIETZ et al., 1999). Além disso, o mercúrio pode participar nas reações de Haber-Weiss e de Fenton e assim propiciar a formação de radicais hidroxil (HALLIWELL & GUTTERIDGE, 1990), que iniciam o processo de peroxidação lipídica e de oxidação protéica.

Sob condições fisiológicas normais, as células produzem espécies reativas de oxigênio (ROS) por meio da redução do oxigênio molecular. A produção dos derivados tóxicos de oxigênio é aumentada como resultado de vários tipos de estresse biótico ou abiótico (FOYER et al., 1994). A geração de ROS, tais como o anion 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 a uma variedade de condições de estresse. São considerados fatores de estresses em plantas: a seca, o frio, a alta intensidade luminosa, a radiação UV, os metais pesados e alguns compostos químicos orgânicos (HEGEDÜS et al., 2001).

As ROS 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 (LIN & KAO, 2000; SHALATA & TAL, 1998; OLMOS et al., 1994; FOYER et al., 1994). Além disso, a alta afinidade de ligação do mercúrio aos compostos contendo enxofre, nitrogênio e grupos funcionais contendo oxigênio, nas moléculas biológicas, pode induzir a inativação e ao dano dessas moléculas (NELSON, 1999; CLEMENS, 2001).

2.3. Sistema de defesa antioxidante

Para o combate da toxicidade do metal e proteção das membranas celulares e organelas dos efeitos danosos das ROS, as células das plantas possuem um sistema de defesa antioxidante, formado por componentes enzimáticos e não enzimáticos que normalmente mantêm um balanço de ROS 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 ROS (FOYER et al., 1994) e protegem a célula contra a injúria e a disfunção dos tecidos (MIQUEL, 1989). Além disso, em plantas, os carotenóides também possuem efeito antioxidante importante no sistema fotossintético (HALLIWELL, 1987).

A SOD é um componente essencial do sistema de defesa antioxidante em plantas, dismutando dois radicais superóxido ($O_2^{\bullet-}$) até água e oxigênio molecular (Figura 1a-d) (VERMA & DUBEY, 2003; MITTLER, 2002). Contudo, o H_2O_2 é também tóxico para a célula e deve ser detoxificado pela catalase e/ou peroxidases. 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 altas concentrações, pois apresenta baixa afinidade pelo H_2O_2 (MITTLER, 2002). A CAT, pertence a 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.

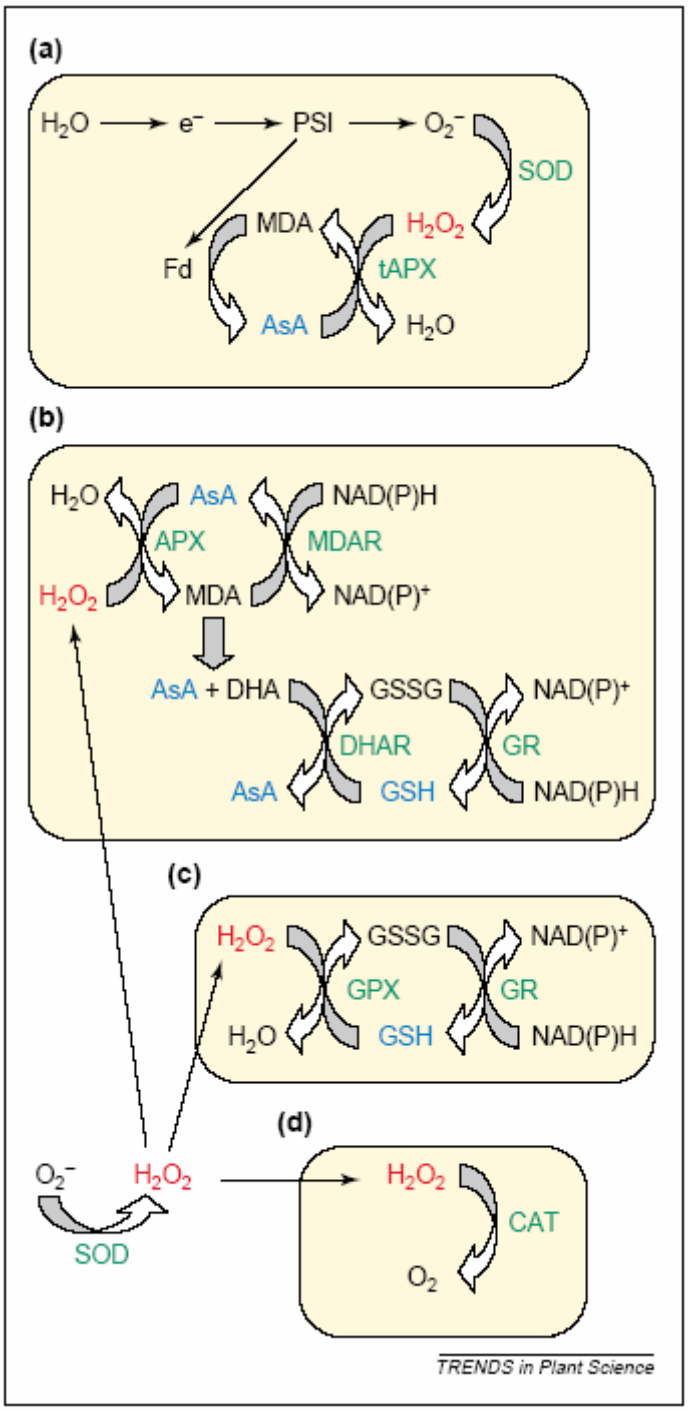

Figura 1 - Caminho das espécies reativas de oxigênio e sua remoção nas plantas (a) Ciclo água-água. (b). Ciclo ascorbato glutationa (c). Ciclo glutationa peroxidase (d). ROS estão indicadas em vermelho, antioxidantes em azul e enzimas removedoras de ROS em verde (Adaptado de Mittler, 2002).

Essas enzimas reduzem de forma eficiente as ROS sob circunstâncias normais, mas se a redução completa não ocorrer, como em condições de produção aumentada, 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).

Além do sistema de defesa antioxidante enzimático, as defesas antioxidantes não-enzimáticas são de fundamental importância para as células. O ácido L-ascórbico é encontrado em concentrações baixas e 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, na elongação das raízes e no transporte dos 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 dehidroascórbico, o qual é regenerado posteriormente até ácido ascórbico (Figura 2) (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).

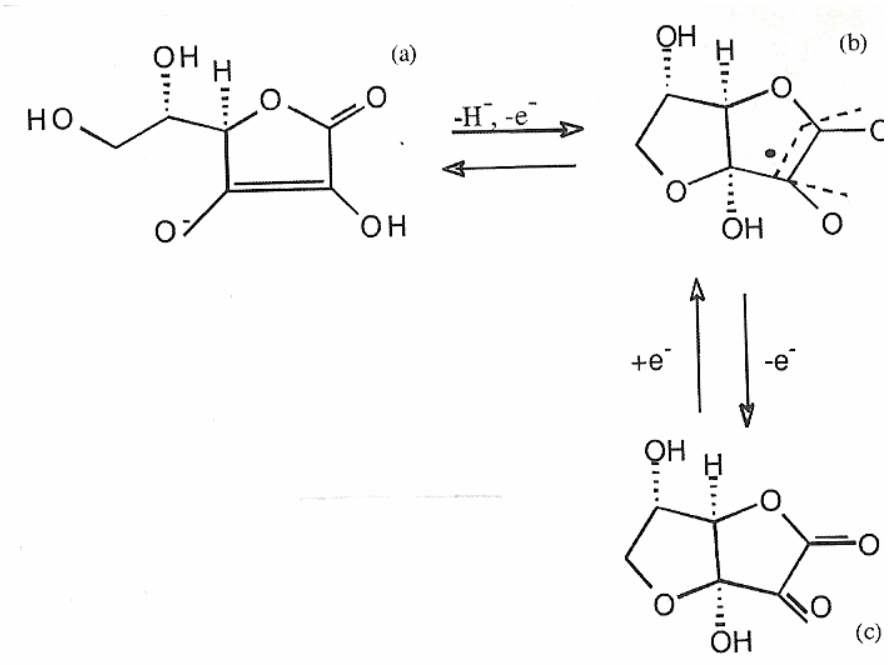

Figura 2 – Estrutura do ácido ascórbico atuando na estabilização dos radicais livres. a) ascorbato, b) radical ascorbil, c) ácido ascórbico. Adaptado de Machlin (1991).

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 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). Estudos mostram que níveis elevados de GSH celular estão associados à tolerância à 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). Além disso, a GSH pode reagir quimicamente com o oxigênio singlete, com o radical superóxido e hidroxila, funcionando como removedor de radicais livres. É também o precursor das fitoquelatinas que agem como peptídeos 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).

Dessa forma, os metais pesados tornam-se tóxicos para as plantas sempre que seus níveis de acumulação exceder a capacidade de destoxificação. 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 citoplasmática 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 (ácido delta-aminolevulínico, δ-ALA), formando o composto monopirrólico porfobilinogênio (PBG) (Figura 3). O produto final do caminho 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), mercúrio e chumbo (MORSCH, 2002; PRASAD & PRASAD, 1987), são capazes de inibir esta enzima. A δ-ALA-D é sensível à agentes oxidantes, tais como metais pesados e ROS, devido a sua natureza sulfidrílica (ROCHA et al., 2001). Além disso, a sua inibição leva a 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 baixas concentrações 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.

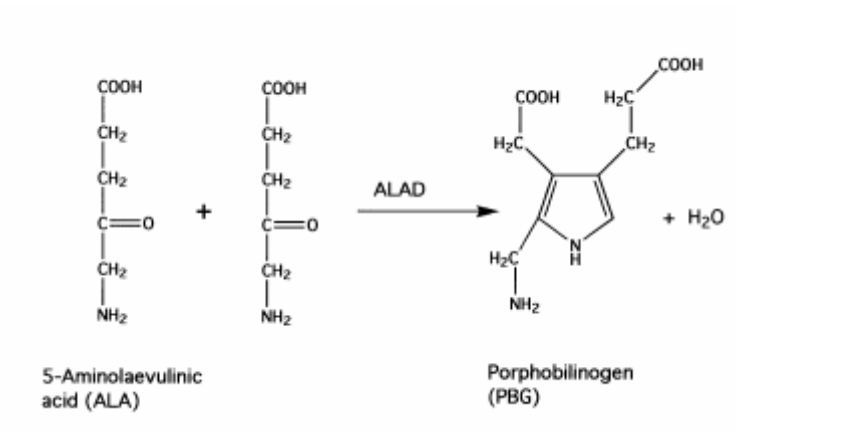

Figura 3 - Formação do porfobilinogênio (PBG) (Adaptado de Senior et al., 1996).

2.5. *Cucumis sativus* L. (Pepino)

O pepino é uma importante espécie cultivada e consumida no Brasil. Trabalhos recentes mostraram que o pepino é sensível para 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.

Ferri (1985) relatou que o estudo do metabolismo dos metais é melhor observado em plântulas devido a alguns fatores; no 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. Além disso, em uma plântula em emergência as substâncias nutrientes são tomadas do material de estoque das sementes e apenas água e oxigênio que a plântula absorve do meio. Ainda, o período que vai da germinação até a época em que a plântulas se torna

estabelecida como um organismo independente constitui a fase mais crucial da história de vida da planta. Durante esse período a planta é mais susceptível a injúria por diversos fatores como a presença do mercúrio.

3. RESULTADOS

3.1. Artigo

Mercury toxicity induces oxidative stress in growing cucumber seedlings

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

ELSEVIER

Chemosphere 65 (2006) 999–1006

CHEMOSPHERE

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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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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

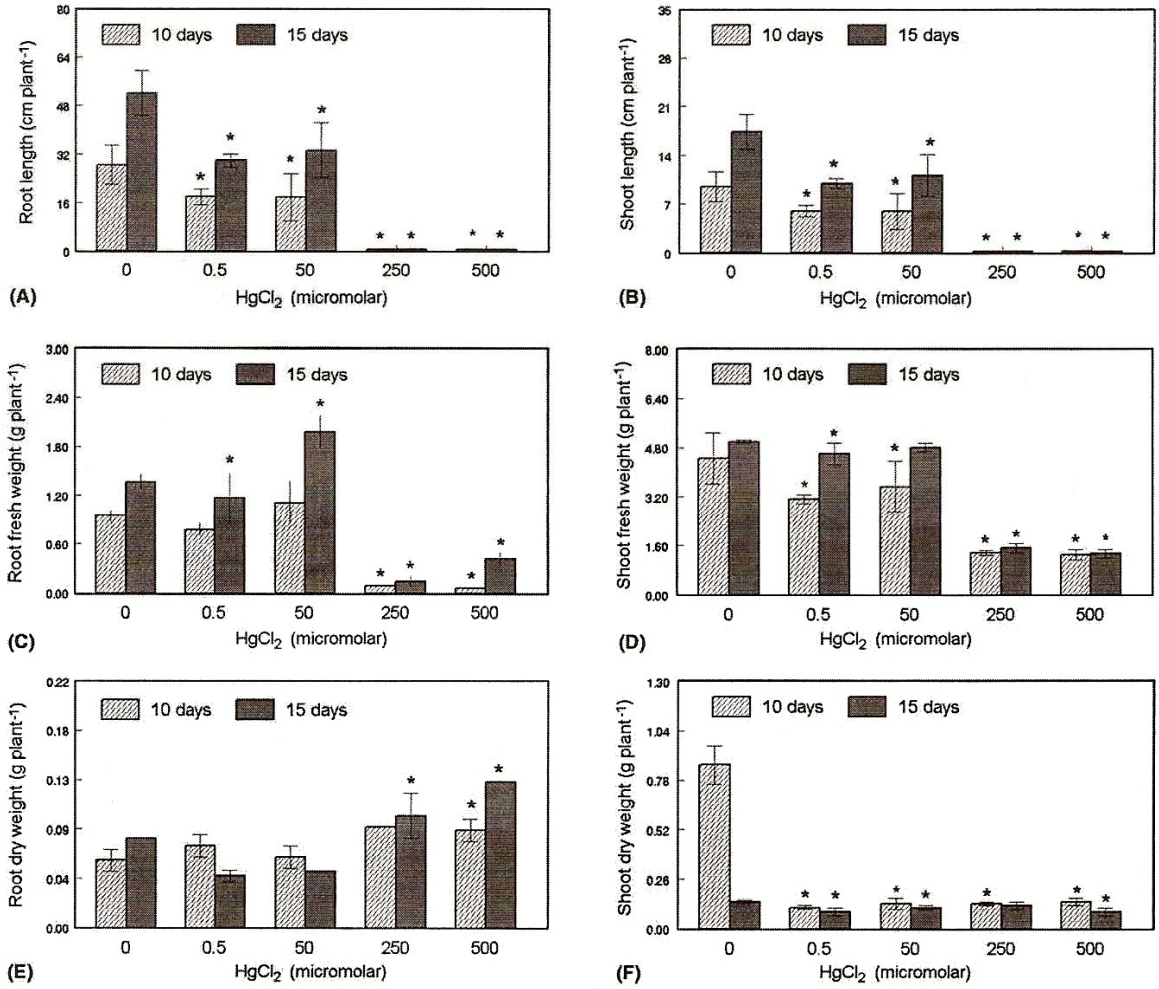

Fig. 1. Effect of increasing concentration of $HgCl_2$ in the growth medium on the length of roots (A), length of shoots (B), root fresh weight (C), shoot fresh weight (D), root dry weight (E) and shoot dry weight (F) of 10- and 15-day old cucumber seedlings. Data represent the mean ± SD of three different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

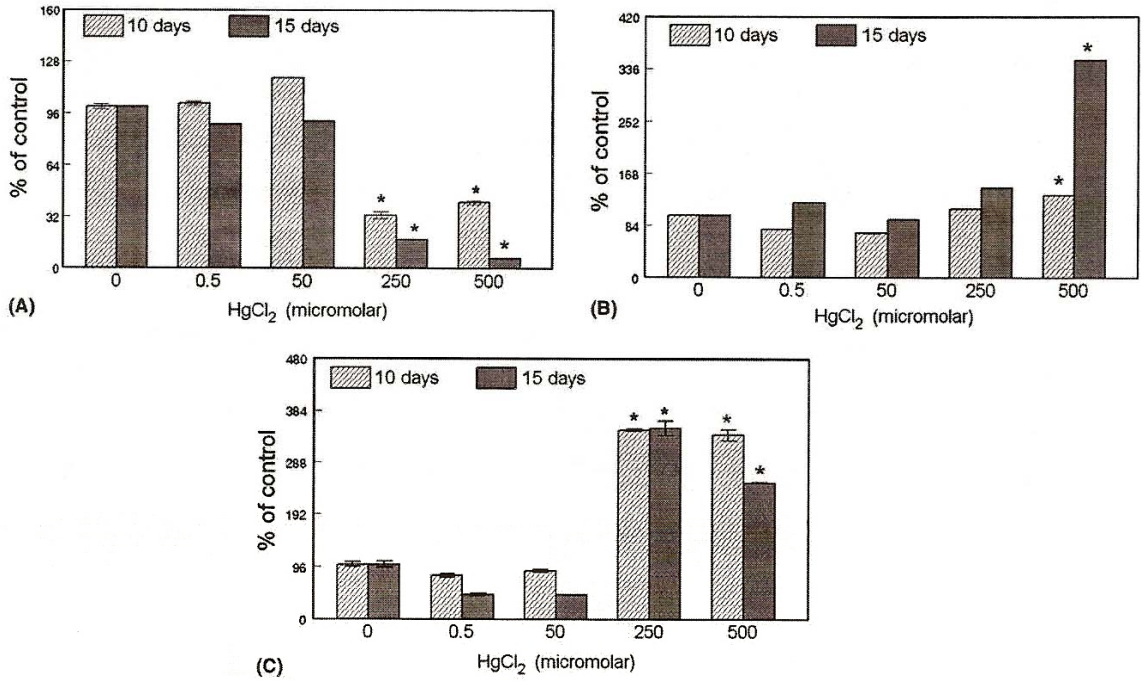

Fig. 2. Effect of increasing concentration of $HgCl_2$ on chlorophyll content (A), lipid peroxides (B) and protein carbonyl (C) of 10- and 15-day old cucumber seedlings. Data represent the mean ± SD of three different experiments. The control specific activity (without mercury) that represents 100% was 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 $p < 0.05$.

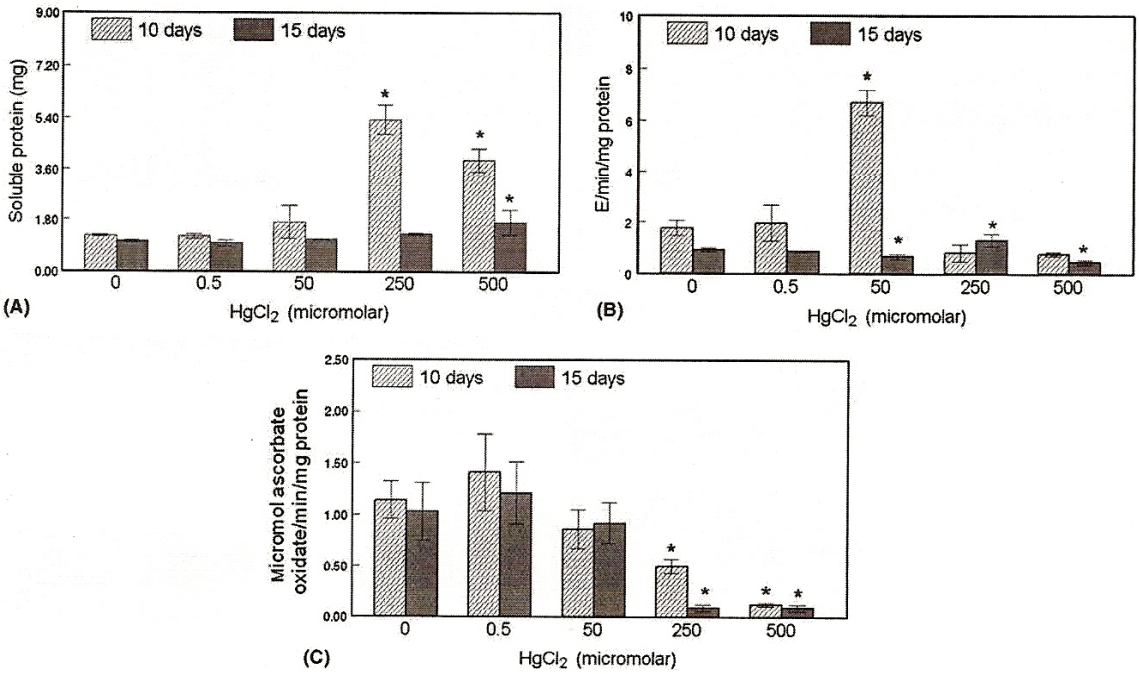

Fig. 3. Effect of increasing concentration of $HgCl_2$ on content soluble protein (A), catalase activity (B) and ascorbate peroxidase activity (C) of 10- and 15-day old cucumber seedlings. Data represent the mean ± SD of three different experiments. *Different from control to $p < 0.05$.

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 μg kg^{-1}, whereas animal manures may have concentrations of the order of 100 μg 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 μg 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.

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3.2. Manuscrito

MERCURY TOXICITY ALTERS THE ANTIOXIDANT SYSTEM OF GROWING CUCUMBER SEEDLINGS

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(Submetido à Revista Chemosphere)

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Abstract

In this study, the effects of mercury on the electrolytic leakage percentage (ELP), hydrogen peroxide (H_2O_2) levels, superoxide dismutase (SOD) activity, non-enzymatic antioxidants (ascorbic acid (ASA), carotenoids, non-protein thiol content (SH)), and δ-aminolevulinic acid dehydratase activity (ALAD) were investigated in Cucumis sativus L. seedlings. Cucumber seedlings were exposed to 0 to 500 μM of $HgCl_2$ during 10 and 15 days. Hg-treated seedlings showed elevated ELP only at 500 μM $HgCl_2$ at 15 days. H_2O_2 levels decreased at 10 days at a moderately toxic level of Hg, but H_2O_2 increased at the highest concentration. Increased SOD activity occurred at concentrations lower than 50 μM $HgCl_2$, and decreased at higher concentrations. Increased SH levels at all concentrations were observed at 10 days. ASA content increased at all concentrations with a concomitant decrease at higher concentrations. Carotenoids levels increased at the lowest concentrations, both at 10 and 15 days. ALA-D activity increased at 50 μM $HgCl_2$ at 15 days, and it was inhibited at higher concentrations. Therefore, our results suggest that Hg increased the levels of ROS, provoking an increase in the antioxidant system, which makes up part of the overall expression of Hg tolerance in the seedlings. In addition, the decrease in carotenoids levels and ALA-D activity is a consequence of Hg toxicity.

Keywords: superoxide dismutase, carotenoids, hydrogen peroxide, non-protein thiol groups, cucumber, δ-aminolevulinic acid dehydratase.

1. INTRODUCTION

Heavy metal contamination is one the most serious environmental problems for plant productivity and it is a threat to human health. Due to diverse human activities, such as mining and smelting, metal pollution is becoming a major risk to many ecosystems. Among the pollution-production metals, mercury (Hg) is regarded as a non-essential element, with no known physiological function for plants.

However, anthropogenic inputs associated with agricultural practices, mineral extraction, industrial processes and solid waste management are important contributors to heavy metal contamination of natural ecosystems (Alumaa et al., 2002; Segura-Muñoz et al., 2006). The exposure of several plants species to heavy metals could also arise from the use of some pesticides and fertilizers (Falahi-Ardakani, 1984). 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).

Mercury has been demonstrated to stimulate the formation of reactive oxygen species (ROS) (Cho and Park, 2000), which include superoxide radicals ($O_2^{\bullet -}$) hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$), either by direct electron transfer involving metal cations, or as a consequence of metal mediated inhibition of metabolic reactions (Stohs and Bagchi, 1995). Under normal conditions, the ROS are necessary for the correct functioning of plants and can play a role in inter- and intracellular signaling (Foyer and Noctor, 1999).

Hydrogen peroxide is produced during different metabolic processes such as photorespiration in chloroplasts or during the formation of lignin in cell walls (Asada, 1992; Schopfer, 1996). Hydrogen peroxide affects the integrity of cells

because it is a precursor of highly reactive oxygen species such as the hydroxyl radical, which attacks proteins, lipids and nucleic acids (Foyer et al., 1994).

Plants are endowed with a complex antioxidant system to cope with ROS (Smirnoff, 1993). However, when the accumulation of ROS under heavy metal stress conditions exceeds the removing capacity of the antioxidant system, the effects of oxidative damage appears, including oxidation of cellular lipids and proteins, destruction of photosynthetic pigments and inactivation of photosynthetic enzymes (Smirnoff, 1993).

The enzyme δ-aminolevulinic acid dehydratase (ALA-D) is sensitive to heavy metals due to its sulfhydrylic nature (Rocha et al., 1995, Morsch et al., 2002). ALA-D 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, glutathione (GSH) and ascorbate, and also antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR), which participate in scavenging ROS. Glutathione, a sulfur containing tripeptide, plays a prominent role in the defense against free radicals in plants under oxidative stress conditions (De Vos et al., 1992; Ranieri et al., 1993).

Major ROS scavenging mechanisms of plants include SOD and CAT. The balance between SOD and CAT activities in cells is crucial in determining the steady state level of superoxide radicals and hydrogen peroxide (Matés, 2000;

Camp et al., 1997). SOD destroys the free radical superoxide by converting it to peroxide, which can in turn be destroyed by CAT (Matés, 2000). Reactive O_2 species (ROS) are produced in both unstressed and stressed cells. Plants have well-developed defense systems against ROS, which involve both limiting the formation of ROS, as well as improving their scavenger capacity (Foyer et al., 1994, Miquel, 1989).

Heavy metals are toxic to plants if their accumulation levels exceed the detoxification capacity of the plant tissue. Thus, a potentially decisive factor in determining the outcome of oxidative stress is the speed with which plants can activate their antioxidant reserves (Ranieri et al., 1993). Correlation studies have indicated that this response is an important aspect of stress tolerance (Sinha et al., 1996).

In animal tissues, it has been demonstrated that mercury induces changes of the antioxidant status either by increasing lipid peroxidation and metallothionein (Aschner, et al., 1997), or by decreasing the enzymatic and non-enzymatic antioxidants (Perottoni et al., 2004). However, less information is available for plants. In a previous study with cucumber, we showed the enhancement of lipid peroxidation and alterations in growth and in the activity of some antioxidative enzyme such as catalase and ascorbate peroxidase (Cargnelutti et al., 2006).

Cucumis sativus is known to accumulate toxic metals under laboratory conditions and have 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).

Thus, the objective of the present study was to contribute to the understanding of the toxicology of mercury. In order to obtain these results, cucumber seedlings were used to evaluate the effect of this metal on the antioxidant system and its relation to ALA-D activity (an enzyme involved in the metabolism of chlorophyll).

2. MATERIAL AND METHODS

2.1. Plant material and growth conditions

Seeds of cucumber (*Cucumis 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 0.5, 50, 250 and 500 µM of $HgCl_2$. Seedlings without $HgCl_2$ treatment served as a control group. These concentrations were chosen due the highest mercury concentrations found on contaminated soil ranging from 15 to 300 µg/g dry weight (Cavallini et al., 1999). Moreover, higher mercury content was recorded in plants growing close to highly industrialized areas (Wojciechowska-Mazurek et al., 1995). 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 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. Determination of electrolyte leakage percentage

ELP was used to assess membrane permeability and it was measured using an electrical conductivity meter. The procedure used was based on the method of Zhu et al. (2004), with some modifications. Plant samples were separated into 5 g segments and placed in individual stoppered vials containing 50 mL of distilled water after washes with distilled water to remove surface contamination. These samples were incubated at room temperature (25ºC) on a shaker (100 rpm) for 24 h. Electrical conductivity of the bathing solution (EC1) was read after incubation. Samples were then placed in a thermostatic water bath at 95ºC for 15 min and the second reading (EC2) was determined after cooling the bathing solutions to room temperature. ELP was calculated as EC1/EC2 and expressed in percentage (%).

2.3. 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 4ºC in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000g for 15 min. 0.5 mL of supernatant was added at 0.5 mL of 10 mM potassium phosphate buffer pH 7.0 and 1 mL of 1M KI. 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 fresh weight.

2.4. Estimation of antioxidants

2.4.1. Superoxide Dismutase (E.C 1.15.1.1)

The activity of superoxide dismutase was assayed according to Mc Cord and Fridovich (1969). About 200 mg fresh tissues were homogenized in 5 ml of 100 mM potassium 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 and centrifuged at 22,000 x g for 10 min at 4ºC, and the supernatant was utilized for assays. The assay mixture consisted of a total volume of 1 mL, containing 50 mM glycine buffer (pH 10.5), 60 mM epinephrine and enzyme. Epinephrine was the last component to be added. The adrenochrome formation in the next 4 min was recorded at 480 nm with a UV- Vis spectrophotometer. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions.

2.4.2. Non-protein thiol content

Non-protein thiol content in seedlings (mg) was measured spectrophotometrically with Ellman's reagent (Ellman, 1959). Reaction was read at 412 nm after the addition of 50 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 ml). Treated seedlings were homogenized in 10 mM Tris/HCl, pH 7.5, centrifuged at 3,000 x g for 10 min, and supernatants were used for total thiol group determination. Non-protein thiol groups were determined in the fraction obtained after mixing 1 volume of supernatant with 1 volume of 10% trichloroacetic acid followed by centrifugation and neutralization (to pH 7.5) as described by Jacques-Silva et al. (2001). A standard curve using cysteine was used to calculate the content of thiol groups in samples, and was expressed as µmol SH g^{-1} fresh weight.

2.4.3. Ascorbic acid content

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Briefly, seedlings were homogenized in 50 mM Tris/HCl, pH 7.5, centrifuged at 3,000 x g for 10 min and protein was removed by dilution with 1 volume of 10% trichloroacetic acid followed by centrifugation. An aliquot of the sample was incubated at 37ºC in a medium containing 4.5 mg/ml dinitrophenylhydrazine, 0.6 mg/ml thiourea, 0.075 mg ml^{-1} $CuSO_4$, and 0.675 mol/l H_2SO_4 (final volume 1 ml). After 3 h, 1 ml of 65% H_2SO_4 was added and samples were read at 520 nm and were expressed as µg ASA g^{-1} fresh weight. A standard curve was constructed using ascorbic acid.

2.4.4. Chlorophyll and carotenoids determination

Cotyledons were weighed and used carotenoid determination. Carotenoids were extracted following the method of Hiscox and Israeslstam (1979) and estimated with the help of Arnon's formulae (Arnon, 1949). 0.1 g chopped fresh cotyledon sample was incubated at 65ºC in dimethylsulfoxide (DMSO) until the pigments were completely bleached. Absorbance of the solution was then measured at 470 nm with a spectrophotometer (Celm E-205D). Carotenoid content were expressed as mg g^{-1} fresh weight.

2.5. Estimation of delta-aminolevulinic acid dehydratase (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. At supernatant were added 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 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} $M^{-1}cm^{-1}$ (Sassa, 1982) for the Ehrlich-porphobilinogen salt. ALA-D activity was expressed as nmol PBG /mg protein/h.

2.6. Protein determination

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

2.7. Statistical analysis

The analyses of variance were computed on statistically significant differences determined based on the variance analiys (one-way ANOVA). The results are the means ± S.D. of at least three independent experiments. The mean differences were compared utilizing Duncan's range test.

3. RESULTS

Electrolyte leakage percentage (ELP) represents cell membrane injury. Fig. 1A shows that increased ELP only occurred at a prolonged period of Hg

exposition (15 days), where at 500 μM $HgCl_2$ there was a significant increase of 172.7%, compared to the control.

The effect of $HgCl_2$ on H_2O_2 content, shown in Fig. 1B. The exposure of cucumber seedlings to 50 μM $HgCl_2$ for 10 days decreased the levels of endogenous H_2O_2 by about 60 % in comparison with the control. At the higher concentrations (250 and 500μM $HgCl_2$), a significant increase of H_2O_2 content was observed. On the other hand, 15-day-old seedlings showed increasing H_2O_2 content at the concentrations of 50 and 500 μM $HgCl_2$, while at 250 μM $HgCl_2$ the content of H_2O_2 was decreased by 39%.

Among the various enzymes involved in the abolishment of reactive oxygen species (ROS), superoxide dismutase (SOD) can be considered a key enzyme. SOD activity varied as a function of both exposure time and Hg concentration (Fig. 2A). For 10-day-old seedlings, SOD activity decrease at 0.5 μM $HgCl_2$, increased at 50 μM $HgCl_2$ and decreased again at 250 and 500 μM $HgCl_2$, by about 45% and 37%, respectively. Similarly, 15-day-old seedlings showed the highest level of SOD activity at 50 μM $HgCl_2$ (Fig. 2A) and the lowest level at 250 μM $HgCl_2$ (Fig. 2A).

SH content also varied as a function of both exposure time and Hg concentration (Fig. 2B). For 10- day- old seedlings, SH content increased with all Hg concentrations tested. On the other hand, at the highest exposure time (15 days), SH content increased by about 20% and 232%, at 0.5 and 250 μM $HgCl_2$ respectively, and decreased by about 80% and 75%, at 50 and 500 μM $HgCl_2$ respectively.

The effects of Hg on ascorbic acid content are shown in Fig. 2C. Regardless of the time of Hg exposure, the ASA content increased as a function of Hg

concentration. The maximum accumulation of ASA was 232.6 µg ASA g^{-1} fresh weight in seedlings treated with 500 µM $HgCl_2$ at 10 days of exposure.

The effects of Hg on carotenoid levels of cucumber seedlings are shown in Fig. 2D. Hg-exposure induced a significant increase in carotenoid content up to 50 µM Hg at both 10 and 15 days followed by decrease at higher metal concentrations. At 500 µM HgCl2, the carotenoid content decreased by 67% and 30% at 10- and 15- days, respectively, in comparison with the control.

Hg-exposure induced a significant reduction of ALA-D activity (Fig. 3), and these effects varied with the time of exposure and the concentration of exogenous Hg. At the highest concentration of Hg (500 µM HgCl2), ALA-D activity at 10 and 15- days, was decreased by 99% and 95%, respectively when compared to the control. However, for 50 µM HgCl2 at 15 days, ALA-D activity was increased.

4- DISCUSSION

Heavy metal contamination of soils has markedly increased in the past few decades. Factors such as mining or industrial activities, automotive emission, and use of metal-enriched materials as chemicals fertilizers, farm manures, sewage sludge, and wastewater irrigation can contribute to this contamination (Webber, 1981; Freedman and Hutchinson, 1981).

Our results indicated that in cucumber seedlings, electrolyte leakage percentage (ELP) levels were significantly enhanced, and exposure time and concentration dependent (Fig. 1A). Another study showed that cucumber plants exposed to cadmium the ELP content also increased (Mishra et al., 2006). In a previous study realized by our laboratory, when cucumber seedlings were

exposure by 10 and 15 at mercury, at same concentrations utilized in this experiment, was observed an increase in the mercury levels in both roots and shoot. At 10-and-15-days-old cucumber seedlings, in the concentrations of 0.5, 50, 250 and 500 μM $HgCl_2$, Hg content in the cotyledons was, respectively, 5, 824, 2,686 and 7,066-fold, and 2.4, 542, 1,297 and 2,641-fold higher than the control. Moreover, Hg was more accumulated in root system, which was 10, 2,140, 20,830 and 55,628, and 6, 1,865, 16,018 and 26,006-fold higher than the control, at 10 and 15 days, respectively (Cargnelutti et al., 2006), confirming the Hg intoxication. These observations and others such as an increase in MDA (Cargnelutti et al., 2006) indicate that cucumber plants experienced substantial oxidative damage when exposed to high concentrations of $HgCl_2$ for a prolonged time.

When plants are exposed to environmental stressors such as heavy metals, oxidative damage can be caused either directly or indirectly by triggering an increased level of production of reactive oxygen species (ROS) (Shah et al., 2001; Patra et al., 2004). These ROS include superoxide radicals ($O2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), which are produced during membrane linked electron transport activities as well as by a number of metabolic pathways (Shah et al., 2001) and in turn cause damage to biomolecules such as membranes, proteins and nucleic acids (Sharma and Talukder, 1989). Although the mechanism of Hg-induced H_2O_2 formation is not yet understood known, heavy metals are known to be involved in many ways in the production of ROS (Luna et al., 1994). H_2O_2 is moderately reactive and is a relatively long-lived molecule (half-life of 1 ms), which can be diffused away from its production side. H_2O_2 may inactivate enzymes by oxidizing their thiol

groups. For example, enzymes of the Calvin cycle and superoxide dismutase are inactivated by H_2O_2 (Charles and Halliwell, 1980; Bowler et al., 1994).

Our results clearly indicate that Hg-exposure resulted in increased H_2O_2 content in seedlings (Fig. 1B), which may be due the a decrease in catalase. However, the decreased H_2O_2 accumulation observed at the concentrations of 50 and 250 μM $HgCl_2$, at 10 and 15 days, respectively, could be related to the increased catalase activity. CAT is one of the key enzymes involved in the removal of toxic peroxide, and it decomposes H_2O_2 to water and molecular oxygen (Lin and Kao, 2000). Our results showed that CAT activity may be critical in the scavenging of H_2O_2.

Superoxide dismutase (SOD) scavenges $O_2^{\bullet-}$radicals to protect from cellular oxidative damage. The control of the steady-state $O_2^{\bullet-}$ levels by SOD is an important protective mechanism against oxidative damage, since $O_2^{\bullet-}$ acts as a precursor of more cytotoxic or highly reactive oxygen derivatives, such as peroxynitrite and $HO^{\bullet}$ (Halliwell and Gutteridge, 1999). Therefore, SOD is usually considered the first line of defense against oxidative stress.

In the present study a biphasic effect was observed in the SOD activity at 10 and 15-days. This result also might be attributed to the hormetic dose response. Low concentrations and short exposure times, may produce an effect similar to high concentrations at any period of time. This trend was compatible to SOD activity reported in grape leaves, seedlings of tomato, and in seedlings of Sesbania drummondii cultivated under Hg treatments (Ma, 1998; Cho and Park, 2000; Israr et al., in press). Moreover, with an increase of both exposure time and Hg concentration, there may be an increase in the production of ROS, causing greater damage to tissue cells. Low levels of SOD may be related with

the increase of H_2O_2 levels, because H_2O_2 may inactivate enzymes by oxidizing their thiol groups, as for instance SOD (Charles and Halliwell, 1980; Bowler et al., 1994).

Inorganic mercury in the Hg^{2+} form has a great affinity for SH groups of endogenous biomolecules (Clarkson, 1997). Thus, it is invariably found in cells and tissues attached to thiol-containing proteins and small-molecular-weight thiols such as cysteine and glutathione (GSH). At relatively low toxic or nontoxic doses, mercury increases the renal content of GSH, probably due to the induction of GSH synthesis (Zalups, 2000). However, due to its ability to form stable complexes with Cl^-, OH^-, S^{2-} and S-containing functional groups of organic ligands (Cotton and Wilkinson, 1972), the free Hg^{2+} ion is rarely found under natural field conditions.

Varying responses of Hg induced oxidative stress might be related to the concentration of thiolic groups, since they are consequently able to counteract oxidative stress. Furthermore, the antioxidant property of thiols depends on the oxidation of –SH groups of the tripeptide form transforming it to the disulphide form (Toppi and Gabbrielli, 1999). An increase in the thiol content in Sesbania drummondii was found by Israr et al. (2006) for a short exposure period, which could be due to the inactivation of the reactivity of the metals by a cytoplasmatic detoxification mechanism. Our results showed a higher concentration of the –SH group in Hg-treated seedlings of cucumber at a short exposure time (Fig. 2B). In agreement with Patra et al. (2004), Hg possesses a high affinity for the SH groups, making it a defense mechanism against damage caused by metals. The fast mercury-induced accumulation of GSH and the high stability of Hg $(GSH)_2$ mercaptide complexes suggest that GSH functions as an effective

scavenger of Hg^{2+} ions (Sinha et al., 1996). Our results suggest that thiols also play an important role in Hg detoxification. Moreover, with an increase of exposure time, -SH levels increased at 0.5 and 250 μM $HgCl_2$, and decreased at 50 and 500 μM $HgCl_2$ (Fig. 2B), demonstrating disturbances in the oxidant system.

L-ascorbic acid (ASA) is found in milimolar concentrations in leaves and plays an important role in plant tolerance to stress. ASA is involved in the regulation of photosynthesis, cell expansion, root elongation, and trans-membrane electron transport (Smirnoff, 2000). ASA is an important component of the plant antioxidant defense system and serves as a reductant for the removal of H_2O_2 among other peroxides (Noctor and Foyer, 1998). Vitamin C is the first line defense against oxygen radicals in the water-soluble compartment (Nordberg and Arner, 2001). This vitamin reacts directly with the superoxide, hydroxyl radical and oxygen singlet. ASA was also demonstrated to detoxify mercury in Chlorella vulgaris by donating electrons to free radicals, thus protecting the integrity of –SH groups (Rai, 1979). The increase in antioxidant levels reduces oxidized biomolecules, but these detoxificants are not completely sufficient to protect against visible injury (Ranieri et al., 1993).

In the present investigation, ASA levels increased at all the concentrations of Hg at 10 and 15 days. Similarly, Sinha et al. (1996) reported an increase in ascorbic acid content in Baccopa monieri plants treated with Hg, showing a significant increase in ASA content during the initial period of metal exposure.

Carotenoids are a part of the photosynthetic pigment, playing an important role in the protection of chlorophyll under stress conditions. Moreover, they are known to quench the photodynamic reactions leading to loss of chlorophylls,

replace peroxidation and collapse of membrane in chloroplasts (Knox and Dodge, 1985). In the present study, an increase in the carotenoid content was found at low concentrations (up to 50 μM $HgCl_2$), corroborating with a study in chromium treated Pistia stratiotes (Sinha et al., 2005). These findings demonstrate that the antioxidant power of carotenoids protects chlorophyll against the attack of free radicals. Contrarily, above 250 μM Hg, a significant decline in carotenoid content was observed at both 10- and 15- days (Fig. 2D). Several studies have shown that Hg in the substrate decreased the levels of photosynthetic pigments, chlorophylls and carotenoids, at a prolonged duration of exposure. Hg also strongly inhibits the photosynthetic electron transport chain, being that photosystem II (PS II) is the most sensitive target (Bernier et al., 1993; Bernier and Carpentier, 1995).

The reduced synthesis of porphobilinogen, the committed precursor of chlorophyll, in chill- and heat-stressed wheat seedlings, demonstrated that the inhibition of chlorophyll biosynthesis is partly due to the impairment of ALA biosynthesis (Tewari and Tripathy, 1998). In this study a decrease in ALA-D activity was observed at concentrations higher than 50 and 250 μM $HgCl_2$, at 10 and 15 days of exposure, respectively. Similarly, Morsch et al. (2002) showed that Hg was a potent inhibitor of the ALA-D activity in radishes. ALA-D reduced activity, that is sensitive to the mercury due to his nature sulfidrílica (Rocha et al., 1995), may lead the inhibition of chlorophyll biosynthesis (Sinha et al, 1996). Therefore, the ALA-D activity could be used as a sensitive marker for the presence of heavy metals in soils.

At a low concentration (50 μM $HgCl_2$) and at a longer exposure time (15 days), an increase in ALA-D activity was observed (Fig. 3). This effect may be

due to an increase of enzyme synthesis by the plant homeostasis system. At low metal concentrations, the pool of enzymatic activity was not inhibited.

In conclusion, the increase of ELP and H_2O_2 production in cucumber seedlings might be related to decreased superoxide dismutase content with a consequent increase in membrane and protein damage (such as ALA-D). Mercury stress increased the levels of ascorbic acid, total SH, and carotenoids in seedlings of C. sativus at the initial period of exposure. These antioxidant systems in seedlings seem to bind the metal in a form that renders its harmless, making the seedlings tolerant at low concentrations and short exposure times. However, the antioxidant systems are not able to protect from the toxicity caused by higher levels of Hg and increased exposure times, resulting in the negative effects observed in the growth of cucumber seedlings. Moreover, at 50 μM $HgCl_2$ a compensatory effect was observed in relation to the antioxidant defense system, causing the possible induction of enzyme synthesis, as for instance, SOD. Considering the results as a whole, we hope to contribute to a better understanding of the oxidative stress conditions generated by mercury in plants.

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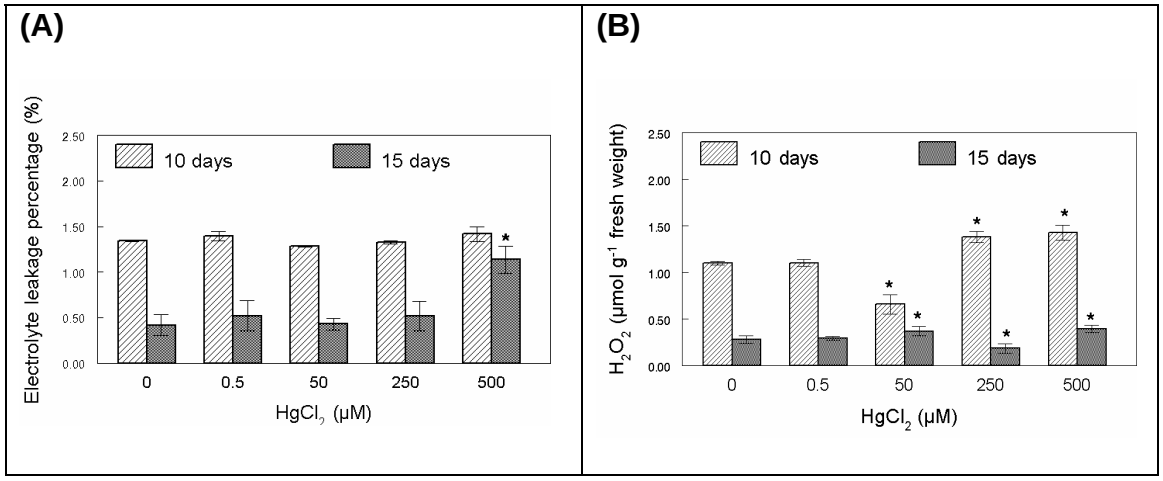
LEGEND OF THE FIGURES

Figure 1. Effect of increasing concentration of $HgCl_2$ on the electrolyte leakage percentage (A) and hydrogen peroxide content (B) at 10- and 15-days- old cucumber seedlings. Data represent the mean ± S.D. of three independent experiments. *Different from control to $p<0.05$.

Figure 2. Effect of increasing concentration of $HgCl_2$ on the superoxide dismutase activity (A), and –SH groups (B), ascorbic acid (C) carotenoid content (D) of 10- and 15-days- old cucumber seedlings. Data represent the mean ± S.D. of three different experiments. *Different from control to $p<0.05$.

Figure 3. Effect of increasing concentration of $HgCl_2$ on delta-aminolevulinic acid dehydratase activity of 10- and 15-days- old cucumber seedlings. Data represent the mean ± S.D. of three different experiments. *Different from control to $p<0.05$.

Figure 1


(A)
Electrolyte leakage percentage (%)
10 days
15 days
2.50
2.00
1.50
1.00
0.50
0.00
0
0.5
50
250
500
HgCl2 (μM)
(B)
H2O2 (μmol g-1 fresh weight)
10 days
15 days
2.50
2.00
1.50
1.00
0.50
0.00
0
0.5
50
250
500
HgCl2 (μM)

Figures 2

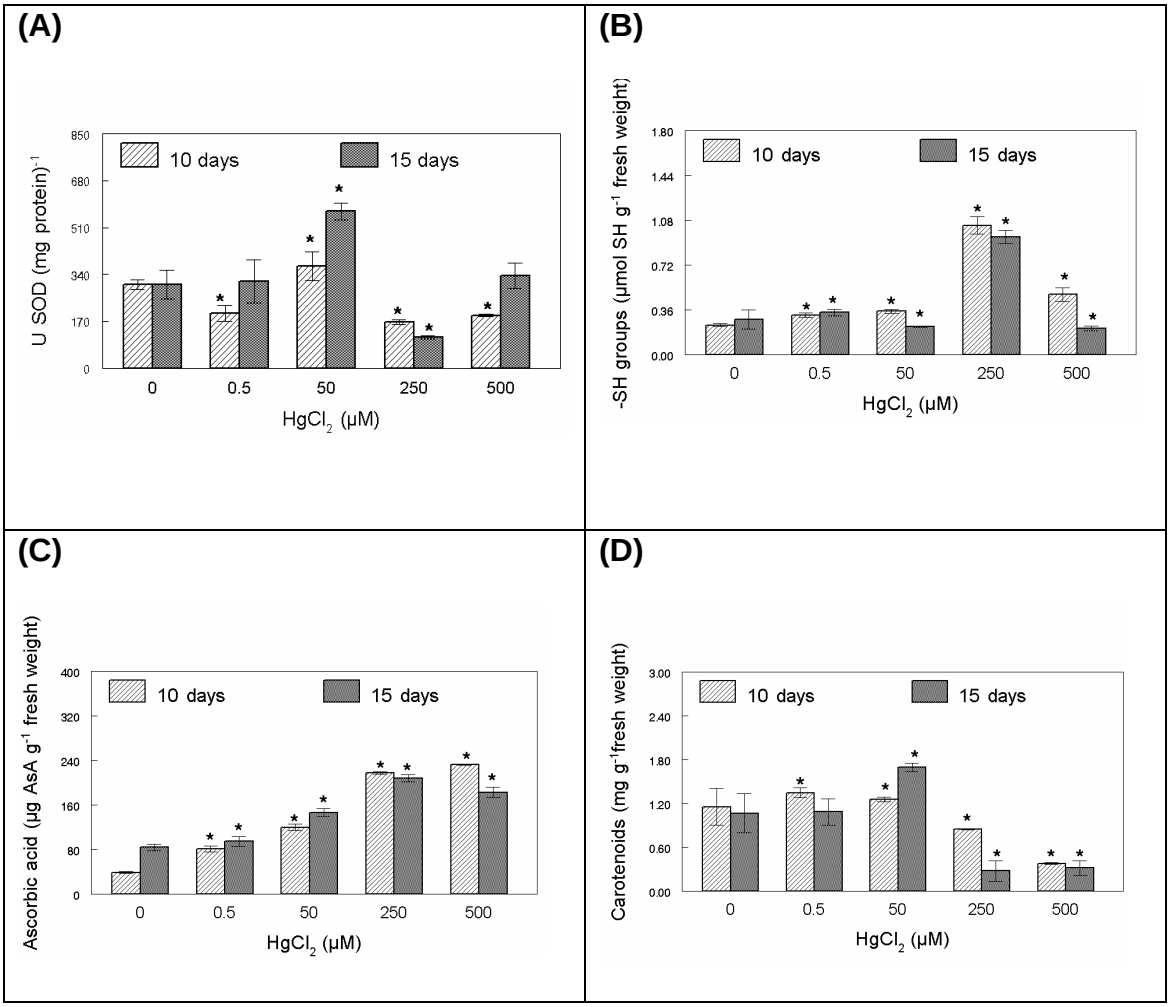
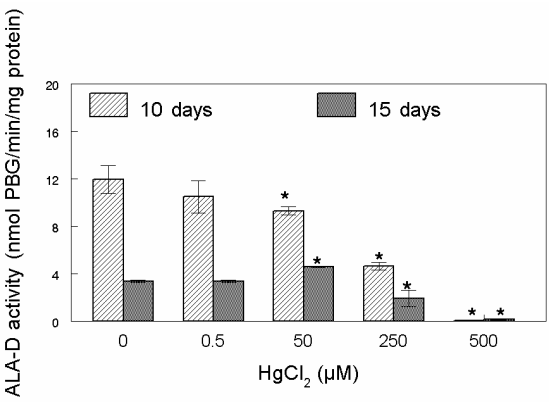

(A)
U SOD (mg protein)-1
850
680
510
340
170
0
10 days
15 days
0
0.5
50
250
500
HgCl2 (µM)
(B)
-SH groups (µmol SH g-1 fresh weight)
1.80
1.44
1.08
0.72
0.36
0.00
10 days
15 days
0
0.5
50
250
500
HgCl2 (µM)
(C)
Ascorbic acid (µg AsA g-1 fresh weight)
400
320
240
160
80
0
10 days
15 days
0
0.5
50
250
500
HgCl2 (µM)
(D)
Carotenoids (mg g-1fresh weight)
3.00
2.40
1.80
1.20
0.60
0.00
10 days
15 days
0
0.5
50
250
500
HgCl2 (µM)

Figure 3


ALA-D activity (nmol PBG/min/mg protein)
20
16
12
8
4
0
10 days
15 days
*
0
0.5
50
250
500
HgCl2 (µM)

4. DISCUSSÃO

A presença de mercúrio alterou o crescimento das plântulas de pepino levando ao dano das raízes, o que segundo Godbold & Huttermann (1986), pode ter conseqüências sérias na absorção de nutrientes e no fornecimento de água para a parte aérea das plântulas em crescimento. Também, concentrações altas de mercúrio aumentaram o peso seco das raízes (Artigo, Fig. 2E), o que de acordo com Arduini et al. (2004) indica a formação de agregados nos tecidos vegetais, através da proliferação anormal das células da raiz induzida pelo mercúrio.

Por outro lado, as concentrações altas de mercúrio reduziram a biomassa da parte aérea, confirmando o poder tóxico desse elemento em plantas (Artigo, Fig. 2F). O aumento do peso fresco das raízes em baixas concentrações de mercúrio (50 μM de $HgCl_2$) está relacionado ao efeito hormético, que representa uma alta compensação devido ao rompimento da homeostase. O efeito hormético é observado em relação a diferentes fatores: compostos químicos orgânicos e inorgânicos, Al, deficiência de um elemento essencial ou estimulação de reações de defesa que levam a ativação geral do metabolismo (CALABRESE, 1999; BARCELÓ & POSCHENRIEDER, 2002; CALABRESE & BLAIN, 2005). Stebbing (1998) relatou que o efeito homético geralmente pode ser definido como um efeito estimulatório que acontece quando concentrações altas de uma substância têm efeitos negativos (por exemplo, inibição de crescimento), e concentrações baixas produzem efeitos positivos (por exemplo, estimulação do crescimento) (CALABRESE & BALDWIN, 2000). Zhang & Tyerman (1999) relataram que o mercúrio inibe a absorção de água via aquaporinas na membrana plasmática em plantas superiores, o que poderia

explicar a redução do peso fresco das plântulas sob o efeito de concentrações altas de mercúrio, observado neste estudo.

O conteúdo de mercúrio nas raízes e cotilédones das plântulas de pepino aumentou com o aumento da concentração deste elemento adicionada ao substrato. As plântulas de pepino acumularam uma maior concentração de mercúrio nas raízes, quando comparada aos cotilédones, indicando que as raízes servem como uma barreira parcial para o transporte de mercúrio até a parte aérea das plântulas (CAVALLINI et al., 1999).

O $HgCl_2$ (0,5 – 500 μM) causou um declínio dependente do tempo e da concentração no conteúdo de clorofila (Artigo, Fig. 2A) em cotilédones. Sabe-se que os íons mercúrio podem substituir íons metálicos nos pigmentos fotossintéticos, causando uma redução nas taxas fotossintéticas (XYLANDER et al., 1996), e, também podem atuar reduzindo os níveis de pigmentos fotossintéticos (clorofila e carotenóides) em um tempo prolongado de exposição. Também, neste estudo, uma diminuição na atividade da enzima ALA-D foi observada em concentrações maiores do que 50 e 250 μM de $HgCl_2$, a 10 e 15 dias de exposição ao metal, respectivamente. Este resultado confirma o poder inibitório do mercúrio sobre a atividade da ALA-D (MORSCH et al., 2002). Entretanto, baixas concentrações (50 μM $HgCl_2$) e exposição por 15 dias, levou a um aumento na atividade da ALA-D (Manuscrito, Fig. 3). Esse efeito pode ser devido a um aumento da síntese de moléculas de enzimas pelo sistema homeostático das plantas, e baixas concentrações do metal não foram suficientes para inibir a enzima. Além disso, nossos resultados demonstram uma relação entre o conteúdo de clorofila e a atividade da ALA-D, indicando

que além da destruição de pigmentos, ocorre síntese diminuída de clorofila através da redução da atividade da ALA-D.

Foi demonstrado neste trabalho, que o mercúrio aumentou os níveis de MDA, de proteínas carboniladas (Artigo, Fig. 2B e 2C) e a porcentagem de vazamento de eletrólitos (ELP) (Manuscrito, Fig. 1A), sendo que esse aumento foi dependente do tempo de exposição e da concentração do metal. Estes aumentos indicam que as plântulas de pepino experimentam dano oxidativo substancial às proteínas e aos lipídios das membranas, o que levou ao vazamento de íons e à redução da permeabilidade da membrana plasmática. Também, a acumulação de grupos carbonil em plântulas de pepino, indica que a quantidade de ROS excedeu a capacidade do sistema de defesa antioxidante.

No presente estudo, um efeito bifásico foi observado para a atividade da catalase aos 10 dias de exposição ao metal, o que também pode ser atribuído à resposta hormética. Este efeito está relacionado a um mecanismo estimulatório em baixas concentrações e uma inibição em altas concentrações de um composto (CALABRESE & BALDWIN, 2000). Além disso, aos 10 dias, o efeito inibitório do mercúrio na atividade da catalase coincidiu com uma diminuição no conteúdo de proteína solúvel. Sabe-se que as altas concentrações de mercúrio podem levar à precipitação de proteínas (PATRA & SHARMA, 2000), reduzindo assim a função de algumas enzimas. A perda da função das proteínas sugere que as defesas antioxidantes estão reduzidas e os níveis de ROS aumentados, levando ao dano das células teciduais. Também, nossos resultados indicam que a exposição ao mercúrio resulta em um aumento no conteúdo de H_2O_2 nas plântulas (Manuscrito, Fig. 1B), que

coincidiu com uma diminuição na atividade da catalase. No entanto, a diminuição da acumulação de H_2O_2 observada a 50 e 250 µM de $HgCl_2$, aos 10 e 15 dias, respectivamente, poderia ser correlacionada com o aumento da atividade da catalase.

O efeito bifásico observado para a atividade da enzima superóxido dismutase (SOD) aos 10 e 15 dias, esteve de acordo com os resultados obtidos em outros estudos (MA, 1998; CHO & PARK, 2000; ISRAR et al., 2006). Concentrações baixas do mercúrio induzem a ativação da SOD devido ao efeito compensatório produzido pelas plântulas em resposta ao metal. Entretanto, altas concentrações do mercúrio inibem a atividade da SOD. Esta inibição está relacionada aos níveis aumentados de H_2O_2, pois, de acordo com Charles & Halliwell (1980) e Bowler et al. (1994), o H_2O_2 pode inativar as enzimas através da oxidação dos seus grupos tióis.

Um declínio nas atividades das enzimas superóxido dismutase, catalase e ascorbato peroxidase em plântulas tratadas com mercúrio sugerem uma possível demora na remoção do $O_2^{\bullet-}$, H_2O_2 e dos peróxidos tóxicos nas células, levando a um aumento na peroxidação lipídica e oxidação protéica.

Em relação aos antioxidantes não enzimáticos, nossos resultados mostraram um aumento no conteúdo de grupos –SH por um curto período de exposição ao mercúrio (Manuscrito, Fig. 2B). O aumento dos grupos –SH leva à inativação da reatividade do mercúrio por mecanismos de detoxificação citoplasmáticos. De acordo com Patra et al. (2004) e Clarkson (1997), o mercúrio possui alta afinidade por grupos -SH, o qual representa um mecanismo de defesa contra o dano celular causado pelo metal. Além disso, com o aumento do tempo de exposição ao mercúrio, foram observados níveis

aumentados de grupos –SH nas concentrações de 0,5 e 250 μM de $HgCl_2$. Por outro lado, nas concentrações de 50 e 500 μM de $HgCl_2$, os níveis de grupos –SH foram reduzidos em plântulas de pepino (Manuscrito, Fig. 2B), demonstrando distúrbios no sistema de defesa antioxidante.

Na presente investigação, os níveis de ASA aumentaram nas amostras de plântulas crescidas em todas as concentrações de mercúrio tanto aos 10 quanto aos 15 dias, evidenciando um mecanismo removedor de ROS ativo. Além disso, um aumento no conteúdo de carotenóides foi encontrado em baixas concentrações de mercúrio (acima de 50 μM de $HgCl_2$), demonstrando também o poder antioxidante dos carotenóides que protege a clorofila contra o ataque dos radicais livres. Contrariamente, acima de 250 μM de $HgCl_2$, foi observado um declínio no conteúdo de carotenóides aos 10 e 15 dias (Manuscrito, Fig. 2D), evidenciando a destruição de pigmentos pelo mercúrio e a redução do sistema de defesa antioxidante nas plântulas.

Portanto, nossos resultados mostraram que altas concentrações de mercúrio induzem estresse oxidativo, evidenciado pela inibição de enzimas antioxidantes, pela redução dos níveis de antioxidantes não enzimáticos (exceto o ácido ascórbico), pela inibição da enzima delta-ALA-D e pela redução no conteúdo de clorofila. Além disso, os níveis elevados de H_2O_2, a peroxidação lipídica, as proteínas oxidadas e a porcentagem de vazamento de eletrólitos indicam o dano às membranas e as proteínas, o que contribui para o vazamento de íons através das membranas celulares. No entanto, em baixas concentrações e durante 10 dias de exposição ao metal, foi observado uma ativação do sistema de defesa antioxidante. Esse fator indica mecanismos de

detoxificação ativos o que poderia estar relacionado com um processo de tolerância das plântulas de *C. sativus* ao mercúrio.

5. CONCLUSÕES

- Concentrações altas de mercúrio inibiram a atividade das enzimas catalase, ascorbato peroxidase e superóxido dismutase, e baixas concentrações do metal (50 μM de $HgCl_2$) exibiram um efeito compensatório em relação as enzimas catalase e superóxido dismutase. O estresse causado pelo mercúrio elevou os níveis de ácido ascórbico, -SH total e carotenóides em plântulas de *C. sativus* durante o período inicial de exposição e em baixas concentrações de $HgCl_2$, sugerindo que o sistema de defesa antioxidante estava ativo em baixos níveis de mercúrio e atuando na remoção das ROS. Níveis altos de mercúrio e um prolongado período de exposição causaram estresse oxidativo em *C. sativus*, indicando que as defesas antioxidantes das plântulas não foi capaz de proteger a toxicidade causada pelo metal.

- O mercúrio aumentou a peroxidação lipídica, o conteúdo de H_2O_2, a oxidação de proteínas e a porcentagem de vazamento de eletrólitos. Isto sugere que o mercúrio possa induzir dano às membranas e às proteínas, levando a perda de íons através das membranas celulares, bem como à diminuição da atividade das enzimas de plântulas de pepino.

- O mercúrio inibiu a atividade da delta-ALA-D, assim como reduziu os níveis de clorofila em plântulas de *C. sativus* durante os 10 e 15 dias de exposição ao metal, indicando que o mercúrio influenciou no processo fotossintético, alterando, consequentemente, o crescimento das plântulas de pepino.

- Níveis altos de mercúrio e um período prolongado de exposição resultaram em efeitos negativos observados no crescimento das plântulas de pepino, pelo qual pode-se concluir que o mercúrio absorvido pelos tecidos produziu efeitos tóxicos no metabolismo das plântulas, levando à perda de matéria seca.

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