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Análise dos parâmetros de estresse oxidativo em espécies vegetais expostas a poluentes ambientais.

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"...the point is not to be right. The point is to make progress,

and one cannot make progress if afraid of to be wrong."

Lucy's Child: The Discovery of a Human Ancestor (1989)

D. Hohanson e J. Shreeve

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Índice

I. Resumo

A poluição ambiental é atualmente um sério problema mundial. Diferentes poluentes são amplamente distribuídos no planeta, o que contribui para a elevação da exposição de animais e vegetais a diversos elementos tóxicos, aumentando a preocupação com relação aos efeitos adversos que os poluentes exercem sobre a saúde humana e ao ambiente. Os metais pesados e os poluentes do ar, como o ozônio, representam os principais contaminantes urbanos e industriais de solo, corpos de água, animais e plantas. O principal mecanismo responsável pela fitotoxicidade tanto dos metais pesados como do ozônio é o aumento nos níveis de espécies ativas de oxigênio que a presença desses poluentes gera. No presente trabalho a exposição controlada de diferentes cultivares de *Phaseolus vulgaris* a ozônio revelou que a diferença de sensibilidade entre as cultivares está associada à capacidade de aumentar a atividade da catalase, evitando a geração de danos oxidativos aos lipídios no tecido foliar. A exposição de propágulos de *Avicennia marina* a Zinco alterou o status oxidativo das plantas, aumentando a atividade da glutationa peroxidase e os níveis de lipoperoxidação. Em campo, observou-se que apenas a atividade da mesma enzima teve correlação com os níveis de metais foliares (Zinco, Cobre e Chumbo), sugerindo a utilidade da atividade dessa enzima em estudos de bioindicação.

Abstract

Environmental pollution is a worldwide problem. Different pollutants are widely distributed on the planet, increasing the exposure of animals and plants to various toxic compounds which increases the concern about the adverse effects to human and environment health. Heavy metals and air pollutants, such as ozone, are the major contaminants of urban and industrial soil, water, animals and plants. The main mechanism responsible for metals and ozone phytotoxicity is the increased generation of reactive oxygen species. In the present study the controlled exposure of different *Phaseolus vulgaris* to ozone revealed that the difference on the varieties' sensitivity is associated with the capacity of increasing catalase activity, thus avoiding oxidative damage to lipids. The exposure of *Avicennia marina* seedlings to Zinc changed the oxidative status of these plants, which increased activity of glutathione peroxidase and levels of lipid peroxidation. Under field conditions, we observed that glutathione peroxidase activity was correlated with the levels of leaf metals (Zinc, Copper and Lead), suggesting the utility of this enzyme activity for bioindication purposes.

II. Siglas e abreviações

O_3 – ozônio troposférico

EAO – espécies ativas de oxigênio

$O_2^{\bullet -}$ - ânion superóxido

$HO^{\bullet}$ - radical hidroxil

H_2O_2 - peróxido de hidrogênio

SOD - superóxido dismutase

CAT - catalase

GR - glutationa redutase

GPX - glutationa peroxidase

APX - ascorbato peroxidase

GSH – glutationa reduzida

GSSG – glutationa oxidada

AOT_{40} – concentração acumulada de ozônio acima de 40 ppb

PEPc - fosfoenolpiruvato carboxilase

III. Introdução

Poluição por metais pesados e ozônio

A poluição ambiental caracteriza-se como um sério problema mundial, de forma que diferentes poluentes encontram-se atualmente distribuídos em diferentes regiões do planeta. Estes surgem como resultado das atividades humanas, uma vez que o aumento populacional registrado nos últimos anos e a maior demanda por recursos tem impulsionado a industrialização da sociedade, contribuindo para a elevação da exposição de animais e vegetais a diversos elementos tóxicos, aumentado assim a preocupação com relação aos efeitos adversos que os poluentes podem exercer sobre a saúde humana e ao ambiente. Entre estes, os metais pesados e os poluentes do ar, como o ozônio, representam os principais contaminantes urbanos e industriais de solo, corpos de água, animais e plantas (Altshuller 1987; Hüttermann et al. 2004).

Mundialmente, o monitoramento dos diferentes contaminantes ainda é considerado restrito, limitando-se às áreas metropolitanas ou aos pólos industriais, contribuindo assim para a escassez de informações acerca dos efeitos nocivos desencadeados pela ação dos contaminantes. Nesse contexto, os organismos vegetais têm servido como importante modelo de estudos sobre as conseqüências da exposição a contaminantes, bem como dos efeitos bioquímicos e moleculares desencadeados pela presença de poluentes nos sistemas vegetais. O fato de serem organismos sedentários torna as espécies vegetais vulneráveis à variação dos poluentes no meio (Feder 1978). Assim, as plantas devem se adaptar às condições impostas para sua sobrevivência, o que resulta no aparecimento de uma gama de mecanismos de defesa e tolerância a contaminantes externos.

Os metais pesados são elementos químicos que ocorrem naturalmente em concentrações traço na crosta terrestre. Entretanto, a quantidade de diferentes metais pesados tem aumentado em diversos ambientes principalmente devido a atividades antropogênicas (Hüttermann et al. 2004). Dentre os metais pesados o Zinco, o Cobre e o Chumbo são considerados tóxicos e prejudiciais ao desenvolvimento das espécies vegetais quando em concentrações elevadas.

Amplamente distribuído na natureza, o Zinco está presente nos solos e como elemento essencial para nutrição das plantas, tendo papel nas funções enzimáticas, estruturais e regulatórias em diferentes sistemas biológicos. Em regiões não poluídas as concentrações de Zinco nos solos variam entre 10 – 30 μg/g. O acúmulo de Zinco no solo ocorre preferencialmente em áreas próximas a fontes industriais, como siderúrgicas e fábricas de telhas e utensílios. Metal de ampla distribuição na crosta terrestre, que apresenta amplo emprego industrial e doméstico, o Cobre é encontrado nos solos em concentrações normais que oscilam entre 10 – 80 μg/g. Como micronutriente essencial o Cobre atua principalmente como cofator ennzimático. O Chumbo ocorre em concentrações normais nos solos que variam entre 15 – 25 μg/g. Caracteriza-se como o principal contaminante de efluentes de indústrias petroquímicas e siderúrgicas (Hagemeyer 2004).

Nas plantas os metais são preferencialmente absorvidos pelas raízes, sendo essa absorção influenciada por fatores como a concentração dos metais no solo e sua biodisponibilidade, sendo esta modulada pelo pH, níveis de matéria orgânica, temperatura, potencial redox e a presença de outros elementos (Greger 2004). A maior parte dos metais absorvidos fica retida no sistema radicular, porém uma pequena parte pode chegar até o xilema via apoplasto ou simplasto, sendo que normalmente apenas uma pequena porção dos metais absorvidos é translocada para as partes aéreas da planta. Quando translocados via xilema, a maioria dos metais necessita estar na forma complexada, a exemplo do Zinco que é

transportado quelado a ácidos orgânicos e do Cobre que forma complexos com aminoácidos (Greger 2004).

O ozônio troposférico (O_3) caracteriza-se como um dos mais importantes poluentes atmosféricos, tendo origem na dissociação fotoquímica dos óxidos de nitrogênio e hidrocarbonetos pela ação da radiação ultravioleta. Proveniente da queima incompleta dos combustíveis nos motores de veículos e fábricas, os poluentes percussores do O_3 ocorrem principalmente em áreas industriais e urbanas, porém sua presença pode se estender até áreas rurais e florestas, atingindo níveis tóxicos. O O_3 penetra nas plantas através dos estômatos localizados na superfície foliar. Uma vez dentro das folhas o O_3 interfere nos processos fisiológicos, metabólicos e celulares das plantas, especialmente devido à produção e acúmulo de EAO (Sharma e Davis 1997).

Poluentes ambientais e espécies ativas de oxigênio em plantas

As EAO apresentam papel fundamental na sinalização celular, e níveis basais de EAO são necessários para a manutenção da viabilidade celular, pois regulam os processos de desenvolvimento celular (Apel e Hirt 2004). Entretanto, concentrações elevadas de EAO no meio intracelular desencadeiam o estresse oxidativo, o que resulta na inibição do crescimento celular, apoptose e necrose (Pryor et al. 2006). O desbalanço entre a produção e eliminação das EAO tem sido apontada como um dos efeitos primários da exposição de plantas a agentes abióticos, como os metais pesados e o ozônio. Metais como Cobre apresentam potencial redox ativo, o que torna estes metais capazes de gerarem diretamente EAO especialmente através das reações de Fenton e Haber-Weiss (Srivastava et al. 2006). O mesmo não ocorre com Zinco e Chumbo, uma vez que ambos não são caracterizados como metais oxi-redutores. Assim os sintomas de estresse oxidativo gerado pela presença destes metais surgem como

conseqüência da interferência destes sobre componentes de rotas metabólicas importantes, como a fosforilação oxidativa nas mitocôndrias e a fotossíntese nos cloroplastos (Flowers et al. 2007; Verma e Dubey 2003). Estudos demonstram que a síntese de clorofila encontra-se diminuída em diferentes espécies vegetais em decorrência da toxicidade de metais como Cádmio, Cobre, Chumbo, Níquel, Mercúrio e Zinco, o que resulta em uma diminuição na taxa de fotossíntese (Mysliwa-Kurdziel et al. 2004). Além disto demonstrou-se que o excesso de metais modifica a fluidez das membranas plasmáticas, causando alteração na conformação e atividade de enzimas localizadas nas membranas (Rama Devi e Prasad 2004). Alguns metais podem ainda alterar a composição dos lipídios de membrana, diminuindo a capacidade das células em manter o balanço iônico e aumentando o vazamento de eletrólitos (Rama Devi e Prasad 2004).

No caso do O_3 os mecanismos responsáveis pela sua fitotoxicidade residem no fato de ser um potente agente oxidante, capaz de ser convertido no espaço intracelular a EAO como ânion superóxido ($O_2^{\bullet -}$), radical hidroxil ($HO^{\bullet}$) e peróxido de hidrogênio (H_2O_2) (Sharma e Davis 1997); (Iriti e Faoro 2008). Em meio aquoso, a reação espontânea de conversão do O_3 a $HO^{\bullet}$ é acelerada pela presença de Fe^{2+} e favorecida por pH alcalino (Pell et al. 1997). Pryor e colaboradores (1991) demonstram ainda que, uma vez dentro das células vegetais, o O_3 reage com ácidos graxos instaurados produzindo H_2O_2, o que contribui para a reação em cadeia da oxidação dos lipídios de membrana.

Os efeitos adversos da ação destes poluentes têm sido muito relatados em diversos estudos realizados nos últimos anos. Entretanto, muitos trabalhos com plantas ainda concentram suas avaliações apenas no impacto que estes poluentes têm sobre os efeitos fisiológicos e morfológicos das plantas, como alteração de biomassa e diminuição da taxa reprodutiva. Estudos que relatam os efeitos negativos da ação de diferentes metais (Arsênico, Cádmio, Mercúrio, Níquel, Alumínio) indicam que estes diminuem ou inibem o crescimento

de raízes e partes aéreas (Chaoui et al. 1997; Shaw et al. 2004). Ainda, entre as injúrias graves desencadeadas pela presença dos metais observam-se as alterações estruturais que ocorrem nas folhas. Sinais visíveis da intoxicação por metais como Zinco, Selênio, Cobre e Manganês são especialmente observados na superfície foliar sob a forma de pontos escuros ou da mudança de cor nas folhas, tornando-as amareladas (Barceló e Poschenrieder 2004; Shaw et al. 2004)

A exposição ao O_3 causa redução na produção de biomassa afetando também o crescimento e metabolismo de órgãos como as raízes e caules (Black et al. 2007; Cano et al. 2007). Danifica ainda a estrutura foliar provocando o aparecimento de sinais evidentes da toxicidade deste gás como amarelamento das folhas em ambas as faces e o curvamento das mesmas, dificultando assim a fotossíntese e contribuindo para a senescência foliar (Calatayud et al. 2007; Iglesias et al. 2006; Sharma e Davis 1997).

Em plantas, a geração de EAO ocorre preferencialmente nas mitocôndrias, cloroplastos e peroxissomos (Foyer e Noctor 2008; Navrot et al. 2007). Os mecanismos de defesa antioxidantes envolvem uma gama de componentes, entre eles enzimas e metabólitos antioxidantes (Figura 1). Estudos recentes têm evidenciado que a geração de EAO apresenta um papel fundamental na toxicidade dos metais e do O_3 (Gratão et al. 2005; Mittler 2002; Sharma e Davis 1997), uma vez que diferentes parâmetros de estresse oxidativo têm mostrado alteração quando espécies vegetais crescem na presença destes agentes poluidores.

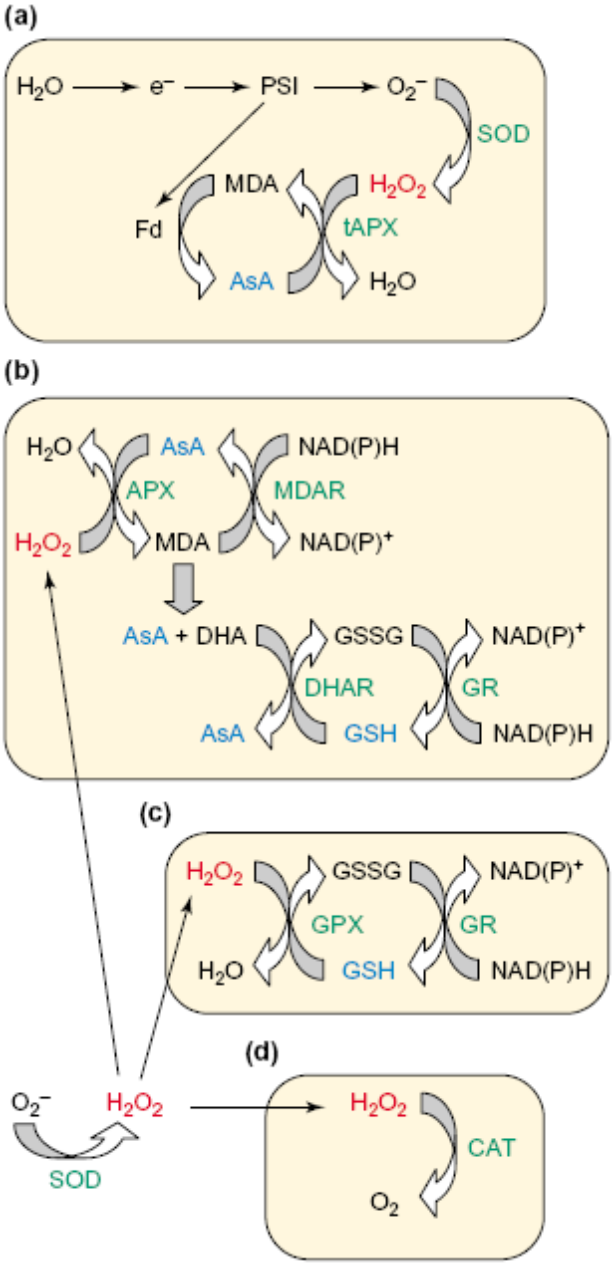

Figura 1. Rotas envolvidas na degradação de EAO em plantas. (a) O ciclo água-água nos cloroplastos que inclui a superóxido dismutase (SOD). (b) O ciclo do ascorbato-glutationa localizado nos cloroplastos, citosol, mitocôndrias, apoplasto e peroxissomos. (c) O ciclo da glutationa peroxidase (GPX). (d) Catalase (CAT), localizada nos peroxissomos. Siglas: AsA, ascorbato; MDA, monodehidroascorbato; DHA, dehidroascorbato; Fd, ferrodoxina; PSI, fotossistema I; APX, ascorbato peroxidase; tAPX, APX acoplada ao tilacóide; MDAR, MDA redutase; DHAR, DHA redutase; GSSG, glutationa oxidada; GSH, glutationa reduzida; GR, glutationa redutase. Retirado de Mittler (2002).

Dentre os danos oxidativos a biomoléculas mais freqüentemente relatadas está o aumento da peroxidação lipídica, observado tanto em células das folhas como das raízes em diferentes espécies vegetais (Mishra et al. 2006; Pell et al. 1997; Pryor et al. 1991; Sandmann e Boger 1980). A fitotoxicidade de ambos contaminantes envolve ainda a modulação da atividade de diversas enzimas antioxidantes. Gratão e colaboradores (2005) em uma ampla revisão sobre as alterações oxidativas mediadas pela ação de metais em plantas, descreve a alteração na atividade de enzimas antioxidantes como superóxido dismutase (SOD), catalase (CAT), glutationa redutase (GR), glutationa peroxidase (GPX) e ascorbato peroxidase (APX). Tanto a modulação da atividade como a alteração no padrão da expressão dessas enzimas têm sido observada em diferentes espécies vegetais expostas a metais como Cádmio, Zinco e Cobre (Chaoui et al. 1997; Cuypers et al. 2002; Mishra et al. 2006; Prasad et al. 1999).

Da mesma maneira, diversas pesquisas com plantas observam que a exposição ao O_3 modula a atividade das principais enzimas antioxidantes (Schraudner et al. 1997). Pell e colaboradores (1997) relatam que evidências bioquímicas e moleculares dão suporte a idéia de que dentre os mecanismos de reparo e proteção das células vegetais contra o ataque oxidativo gerado pelo O_3 está o aumento da expressão e atividade de enzimas antioxidantes como a glutatione-S-transferase, responsável pela detoxificação dos produtos de lipoperoxidação. Os autores relatam ainda que tal modulação na expressão gênica e na atividade enzimática ocorre também para outras enzimas antioxidantes importantes, como SOD, APX e GR, minimizando assim os efeitos negativos do O_3 (Schraudner et al. 1997; Sharma e Davis 1994).

Além de a atividade e expressão das principais enzimas antioxidantes, ambos agentes abióticos causam também alterações severas nos níveis dos metabólicos antioxidantes não enzimáticos, entre eles a glutationa oxidada (GSSG) e reduzida (GSH), o ascorbato, o α-tocoferol, os compostos fenólicos e o β-caroteno (Gratão et al. 2005). O ciclo do ascorbato-

glutationa é descrito por diversos autores como a principal rota de detoxificação de EAO em plantas. A ampla distribuição dos componentes do ciclo nas diferentes organelas das células vegetais (cloroplastos, citosol, mitocôndria, apoplasto e peroxissomos) e o fato da APX apresentar alta afinidade pelo H_2O_2, sugerem que o ciclo do ascorbato-glutationa desempenha um papel crucial no controle da concentração de EAO nos compartimentos onde se encontra presente (Halliwell e Gutteridge 2007; Mittler 2002). O ciclo é o principal responsável pela manutenção das concentrações ótimas entre a forma oxidada e a forma reduzida tanto da glutationa como do ascorbato (Apel e Hirt 2004; Mittler 2002). Alguns metais de transição são capazes de afetar drasticamente o *pool* de GSH total. Estudos recentes relatam que em determinadas espécies vegetais observa-se um desbalanço nos níveis de GSSH/GSH quando estas crescem na presença de metais como Cádmio, Cobre e Zinco (Cuypers et al. 2000; De Vos et al. 1992; Mishra et al. 2006). Em algumas plantas a diminuição nos níveis da GSH está associada à síntese de fitoquelatinas, moléculas derivadas da polimerização enzimática de unidades de glutationa que tem como função ligar-se a metais como o Cádmio, evitando assim a interação do metal com qualquer biomolécula (Prasad 2004). Atualmente se sabe que a GSH é a molécula percussora para síntese das fitoquelatinas, e que a fitoquelatina sintase é a enzima responsável por catalisar a conversão da GSH em fitoquelatina (Gratão et al. 2005; Prasad 2004). No caso do O_3, a variação nos níveis de GSH a ascorbato totais pode surgir como resultado do aumento na geração de EAO, fazendo com que a modulação da atividade de enzimas antioxidantes como APX e GPX altere as proporções das concentrações destes antioxidantes não-enzimáticos (Mahalingam et al. 2006). Alguns trabalhos mostram que a susceptibilidade diferencial que existe entre plantas resistentes e sensíveis ao O_3 é dependente das concentrações relativas de ascorbato e GSH, sugerindo a existência de correlação entre o aumento nos níveis destas defesas antioxidantes e adaptação a fatores abióticos que induzem aumento na produção de EAO (Sharma e Davis 1997). O aumento na tolerância ao O_3 pode

ser também atribuído ao aumento nos níveis de ascorbato, uma vez que plantas com maior concentração de ascorbato foliar apresentam maior capacidade de inativar o O_3 que penetra as folhas já no nível da parede celular, diminuindo assim a quantidade de O_3 disponível para reagir com a membrana celular (Kanofsky e Sima 1995).

Parâmetros de estresse oxidativo como biomarcadores

No entanto, para a maioria das espécies vegetais a análise integrada dos aspectos fisiológicos e bioquímicos relacionados à toxicidade destes contaminantes ainda é limitada. Sabe-se que os efeitos moleculares e celulares desencadeados pela exposição desses poluentes antecedem o aparecimento de danos visíveis, como alteração da coloração foliar e diminuição da biomassa, apontadas como um dos sintomas mais característicos de espécies vegetais intoxicadas por metais pesados e O_3. Assim, as alterações bioquímicas e moleculares decorrentes da ação tóxica de contaminantes oferecem evidências precoces dos efeitos negativos da exposição a contaminantes. De fato, a detecção precoce de uma exposição perigosa pode diminuir significativamente a ocorrência de alterações fisiológicas e estruturais dos organismos vegetais (Ernst e Peterson 1994).

Parâmetros biológicos que podem estar alterados como conseqüência da interação entre agentes poluidores e o organismo podem então ser usados como Indicadores Biológicos ou Biomarcadores. Entretanto é necessário que exista correlação entre a intensidade da exposição e o efeito biológico desencadeado pela ação do contaminante. O biomarcador compreende toda substância ou seu produto de biotransformação, assim como qualquer alteração bioquímica precoce, cuja determinação nos fluidos biológicos e tecidos avalie a intensidade da exposição. A utilização de biomarcadores fornece simultaneamente informações sobre a exposição a poluentes e os efeitos adversos conseqüentes desta

exposição. Neste contexto, os parâmetros de estresse oxidativo podem ser considerados excelentes biomarcadores de exposição a agentes poluidores como metais pesados e ozônio, uma vez que a fitotoxicidade destes está associada ao aumento na geração de EAO e estresse oxidativo. Apesar do recente interesse na busca por biomarcadores e o crescente número de trabalhos que descrevem as correlações entre exposição a poluentes e a geração de EAO, ainda são necessários estudos acerca da relação causa-efeito e dose-efeito na avaliação do uso e validação do status oxidativo de espécies vegetais como biomarcadores de exposição a poluentes com metais e ozônio (Ferrat et al. 2003).

IV. Objetivos

Geral

O presente trabalho teve como objetivo principal avaliar a as alterações nos parâmetros de estresse oxidativo em duas espécies vegetais distintas (*Avicennia marina* e *Phaseolus vulgaris* L.) submetidas à exposição, respectivamente, a metais pesados (Zinco, Cobre e Chumbo) e ozônio.

Específicos

- Avaliar as respostas oxidativas de diferentes cultivares de *P. vulgaris* expostas a ozônio;
- Comparar as diferenças dos parâmetros oxidativos obtidos para quatro cultivares brasileiras de *P. vulgaris* (*Fepagro 26, Guapo Brilhante, Irai* e *Macotaço*) com a cultivar reconhecidamente sensível ao ozônio *Pinto 111*.
- Avaliar as alterações no sistema antioxidante relacionado ao metabolismo da glutationa (GSH e GPX) e os níveis de lipoperoxidação de folhas *A. marina* expostas a Zinco em condições controladas de laboratório;
- Determinar o potencial dos parâmetros de estresse oxidativo estudados como biomarcadores de exposição a Zinco, avaliando a existência de correlação entre os parâmetros estudos e a alteração na biomassa total dos propágulos de *A. marina*;
- Comparar os resultados obtidos para a variação no status oxidativo em laboratório com os resultados obtidos para as mesmas análises em folhas coletadas em locais contaminados e não-contaminados por metais pesados;

- Avaliar se existe correlação entre os níveis de metais acumulados nas folhas de *A. marina* que crescem em locais contaminados e as mudanças no oxidativas do sistema glutationa e os níveis de lipoperoxidação.

Parte 2. Resultados

Capítulo I.

Ozone exposure differentially affects oxidative stress parameters in distinct *Phaseolus vulgaris* L. varieties

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Abstract: Different oxidative stress parameters of four Brazilian varieties of Phaseolus vulgaris (Fepagro 26, Guapo Brilhante, Iraí and Macotaço) exposed to toxic O3 concentrations were compared with the well-established bioindicator common bean variety Pinto 111. Analysis of catalase activity, lipoperoxidation and non-enzymatic antioxidant levels demonstrated that the varieties presented different susceptibilities to O3 toxicity. Results indicated that Fepagro 26 and Guapo Brilhante increased the levels of leaf lipid peroxidation in response to O3 exposure, while catalase activity was not changed in these two varieties. On the opposite, Iraí, Macotaço and Pinto 111 varieties presented a different pattern of lipoperoxidation and CAT activation by O3 exposure, clearly indicating a relationship between CAT activation and resistance to lipoperoxidation in the response to O3. Analysis of the total non-enzymatic antioxidant defenses levels demonstrated that, although O3 exposure decreased the non-enzymatic antioxidant defense in Iraí, O3 was not able to induce lipoperoxidation in the leaves of this variety, as observed with the Fepagro 26 variety. Based on the oxidative parameters analyzed Fepagro 26 variety was the most O3-sensitive variety. Our results suggest that analysis of the oxidative parameters of varieties with known variations of susceptibility to O3 exposure may provide more precise protocols for early detection of O3 toxicity.

Ozone exposure differentially affects oxidative stress parameters in distinct *Phaseolus vulgaris* L. varieties

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ABSTRACT

Different oxidative stress parameters of four Brazilian varieties of *Phaseolus vulgaris* (*Fepagro 26, Guapo Brilhante, Iraí and Macotaço*) exposed to toxic O_3 concentrations were compared with the well-established bioindicator common bean variety *Pinto 111*. Analysis of catalase activity, lipoperoxidation and non-enzymatic antioxidant levels demonstrated that the varieties presented different susceptibilities to O_3 toxicity. Results indicated that *Fepagro 26* and *Guapo Brilhante* increased the levels of leaf lipid peroxidation in response to O_3 exposure, while catalase activity was not changed in these two varieties. On the opposite, *Iraí*, *Macotaço* and *Pinto 111* varieties presented a different pattern of lipoperoxidation and CAT activation by O_3 exposure, clearly indicating a relationship between CAT activation and resistance to lipoperoxidation in the response to O_3. Analysis of the total non-enzymatic antioxidant defenses levels demonstrated that, although O_3 exposure decreased the non-enzymatic antioxidant defense in *Iraí*, O_3 was not able to induce lipoperoxidation in the leaves of this variety, as observed with the *Fepagro 26* variety. Based on the oxidative parameters analyzed *Fepagro 26* variety was the most O_3-sensitive variety. Our results suggest that analysis of the oxidative parameters of varieties with known variations of susceptibility to O_3 exposure may provide more precise protocols for early detection of O_3 toxicity.

INTRODUCTION

Interest in the pollutant ozone (O_3) and in its toxic effects on living organisms has increased in the last decades as a result of the increase in its ground-level concentration (Altshuller, 1987). The damaging effects of O_3 are observed in both animal and plant tissues, representing a potential threat to urban populations and also to crop production and the survival of native species in fragile natural ecosystems (Biswas et al, 2008; Blomberg et al, 1999). Previous studies on plants revealed that both physiological and biological parameters are negatively affected by O_3 exposure (Schraudner et al, 1997; Iriti and Faoro, 2008). O_3-treated plants usually present severe biomass reduction and foliar injury (Iglesias et al, 2006; Black et al, 2007). Besides, laboratory experiments showed that chlorophyll and carotenoid leaf content decrease as the O_3 concentration increases. Also, reductions in stomatal conductance, net photosynthetic CO_2 assimilation, and carboxylation efficiency have all been associated with O_3 exposure, compromising the photosynthesis process (Iglesias et al, 2006; Leitao et al, 2008).

Because of its strong oxidizing potential, O_3 is capable of reacting with several biomolecules, including lipids, proteins, and nucleic acids (Halliwell and Gutterridge, 2007). Exposure to O_3 may perturb the equilibrium between production and scavenging of reactive oxygen species (ROS) within plant tissues and its toxicity is largely enhanced by the spontaneous hydroxyl radical (−OH) generation in aqueous solution, strongly accelerated by Fe^{2+} and favored at alkaline pH (Halliwell and Gutterridge, 2007; Pryor, 1992). In cell membranes, polyunsaturated fatty acids represent the primary target for ozone, stimulating lipid peroxidation and impairing membrane fluidity (Pryor and Church, 1991). For instance, acute O_3 fumigation of different *Medicago truncatula* accessions resulted in increased lipid

peroxidation levels, and highly significant interactions between O_3 -induced oxidative damages and lipid peroxidation levels could be observed (Puckette et al, 2007).

Either physiological or biochemical parameters can be employed to address which species are resistant or sensitive to O_3 toxicity, and both can be useful to outline which species are most suitable to be used as indicators of O_3 exposure (Cano et al, 2007; Torres et al, 2007). Growth reductions and visible symptoms resulting from O_3 toxicity in plants can be both regarded as consequence of physiological and biochemical responses to the stress imposed by this pollutant (Calatayud et al, 2007). Attempts have been made to investigate how early the effects of O_3 exposure on physiological parameters are related to biochemical modifications, which could provide a fast and reliable protocol for detection of O_3-related stress before the appearance of visible symptoms of injury (Ernst and Peterson, 1994). In this regard, the use of common bean (*Phaseolus vulgaris*) as a bioindicator organism has been suggested as a promising strategy to assess O_3 toxicity in closed and open environments, due to many practical reasons related to management and costs (Burkey et al, 2005; Feder, 1978)

In the present work we evaluated different oxidative parameters of four Brazilian varieties of *P. vulgaris* (*Fepagro 26, Guapo Brilhante, Iraí and Macotaço*) exposed to toxic O_3 concentrations, and compared them with the well-known O_3-sensitive common bean variety *Pinto 111* (Arndt et al., 1987). The aim of this study was to compare the sensitivity of these five *P. vulgaris* varieties on the basis of their oxidative responses to acute-ozone exposure. Our results demonstrate that different varieties of *P. vulgaris* present different susceptibilities to O_3 toxicity, and that this probably results from the differential responses of the antioxidant defense system to the exposure to O_3 in each variety. As the search for sensitive plant species for bioindication purposes is imperative, since the signs of biochemical and physiological damage in these plants are manifested earlier than visible symptoms, our

data may be helpful in the further development of bioindication protocols for O_3 toxicity using different varieties *P. vulgaris.*

MATERIAL AND METHODS

2.1. *Plant material and growing conditions*

Seeds of five different varieties of *Phaseolus vulgaris* L. (*Fepagro 26, Guapo Brilhante, Iraí, Macotaço and Pinto 111*) were germinated in 2 liter pots containing 2:1:1 of coarse vermiculite, washed coarse sand and peat. The plants were grown under controlled conditions in a glasshouse, and irrigated once a day with 100 mL of water. One day before the ozone treatment the plants were irrigated with 100 mL of Vitaplan Nutriverde 13-13-15 fertilizer (Nutriplan Products Company, Paraná, Brazil). Eight days after the seeds were planted, ten *P. vulgaris* seedlings from each variety were selected for the experiment. Five seedlings from each variety were subjected to ozone exposure (n=5), and five seedlings were used as control (n=5).

2.2. *Ozone exposure*

Open top chambers were used to expose *P. vulgaris* seedlings to O_3 according to Heagle et al (1973). Ozone concentrations at the chambers were calculated as the accumulated hourly ozone exposure over 40 nl l^{-1} (AOT_{40}) during daylight hours. In this work plants were exposed to an AOT_{40} of 212 ppb h between 10:00 AM and 4:00 PM during one week. Ozone was generated using an electric discharge ozonizer (ozone generator GHR150B, OZ Engenharia, Porto Alegre, Brazil), and added to ambient air. Control plants were maintained in ambient ozone conditions. During the exposure period all seedlings were irrigated with 5 liters of tap water.

At the end of the exposure period control and treated seedlings were harvested, weighted, and measured. The second leaf pair from each plant was sampled. Leaf samples were weighted and immediately stored at -80°C for biochemical analysis.

2.3. *Lipid peroxidation levels assessment*

Lipid peroxidation levels were measured as the amount of thiobarbituric acid-reactive substances (TBARS) reaction as described by Draper and Hadley, 1990. Leaf tissue (0.5 g) was homogenized in 2 mL of 15% trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 x *g* for 10 min. The supernatant was collected and incubated with 0.67% TBA for 30 min at 100ºC. After cooling, the absorbance was read at 535 nm. An absorption coefficient of $1.56\times105M^{-1}$ cm^{-1} was used to calculate the amount of TBARS.

2.4. *Catalase enzyme activity*

Extracts for determination of catalase (CAT, EC 1.11.1.6) activity were prepared from 0.5g of leaf tissue, which was homogenized in 2 mL of ice-cold buffer, containing 50mM phosphate buffer (pH 7.0). Homogenates were centrifuged at 5 000 x *g* for 10 min, and the supernatant fraction was used for the assay. Catalase activity was determined by monitoring the rate of decrease in H_2O_2 absorbance at 240 nm, according to the method of Aebi (1984). The specificity of this method is based in a peculiar property of this enzyme: catalase presents a much higher *V*max value for H_2O_2 than glutathione peroxidase or any other H_2O_2 -metabolizing enzyme in mammalian and plant cells. Thus, at the concentration of H_2O_2 used in the assay buffer (10 mM), catalase presents a high rate of H_2O_2 removal, while any other H_2O_2 -metabolizing enyzme, including glutathione peroxidase, is completely saturated.

2.5. *Total radical-trapping antioxidant potential*

The non-enzymatic antioxidant cellular defenses were analyzed by the total radical-trapping antioxidant potential (TRAP) assay, which determines the non-enzymatic antioxidant capacity of the sample, as previously described (Wayner et al., 1985). Briefly, the reaction was initiated by injecting luminol and AAPH (2,2-azobis[2-methylpropionamidine]dihydrochloride) – a free radical source that produces peroxyl radical at a constant rate – in glycine buffer (0.1 M, pH 8.6), resulting in a steady luminescent emission; 0.5 g of frozen leaf was homogenized in 2 ml of PBS buffer in a mortar and pestle. The samples were centrifuged, and the supernatant was used for TRAP analysis. 10 µL of diluted (10x) homogenized leaf samples were mixed to glycine buffer in the reaction vial. The decrease in luminescence was monitored in a liquid scintillation counter for 60 min after the addition of the sample homogenates. Values obtained from the chemiluminescence assay were transformed in percentage, and the area under the curve was calculated considering the percentage values and were compared to the control.

2.6. *Statistical analysis*

Data are presented as mean ± S.E.M. Differences between control and exposed plants, within each variety, were analyzed using the Student's *t* - test, considering $p < 0.05$ as significant.

RESULTS

According to our results, O_3 exposure led to a 2-fold increase in the lipoperoxidation of *Fepagro 26* seedlings leaf while exposed *Guapo Brilhante* seedlings leaf lipoperoxidation was increased by approximately 30% (Fig.1). The three other varieties (*Iraí*, *Macotaço* and

Pinto 111) did not present any differences in TBARS levels, indicating that these varieties were possibly resistant to lipoperoxidation in response to O_3 treatment.

Catalase (CAT) activity was found to be significantly increased in leaves from three different *P. vulgaris* varieties (*Iraí*, *Macotaço* and *Pinto 111*) when compared to control leaves (Fig.2). The levels of CAT activity on exposed seedlings were increased, respectively, 2.6-fold in *Macotaço*, 2.5-fold in *Iraí*, and 1.9-fold in *Pinto 111* leaves. *Fepagro 26* and *Guapo Brilhante* seedlings did not present significant changes on CAT activity in relation to their respective controls when exposed to O_3.

The TRAP assay was used to assess whether the exposed seedlings had variations on the leaf levels of non-enzymatic antioxidant defenses (Fig. 3). According to our results, leaves from *Fepagro 26* and *Iraí* exposed seedlings increased the emission of TRAP chemiluminescence, indicating a decrease in the total non-enzymatic antioxidant defense content induced by O_3 exposure. Moreover, no significant differences between control and exposed *Macotaço*, *Guapo Brilhante* and *Pinto 111* seedlings on the levels of non-enzymatic antioxidant defenses were observed, indicating that O_3 exposure did not affect the non-enzymatic antioxidant defenses on these three varieties.

DISCUSSION

It is widely known that O_3 is a potent oxidizing agent. Ozone is rapidly degraded into various ROS at the cellular level (Kanofsky and Sima, 1995), which may act either as key signaling molecules (at low-physiological concentrations) or as cytotoxic agents, at higher concentrations (Apel and Hirt, 2004). The data obtained in this work indicate that the five *P.*

vulgaris varieties exposed to an AOT_{40} of 212 ppb h of O_3 during one week exhibited different strategies to cope with O_3 toxicity. The analysis of the results showed that all five varieties presented altered leaf biochemistry as a result of O_3 presence, indicating that it is likely that ROS production in these plants increased in response to O_3 treatment.

Among the several antioxidant enzymes, CAT, together with superoxide dismutase, represents the primary enzymatic defense against ROS. CAT is an intracellular enzyme responsible for the dismutation of H_2O_2 to water and oxygen (Halliwell and Gutterridge, 2007). In plant leaves the major site of CAT activity are the peroxisomes, where CAT detoxifies the H_2O_2 produced by photorespiration (Bartosz, 2005). Although it is well known that O_3 exposure to plants leads to ROS production, there are no previous reports showing that CAT activity is affected by O_3 in the *Irai, Macotaça* and *Pinto 111* varieties. Here, we observed that *Fepagro 26* and *Guapo Brilhante* seedlings presented increased levels of leaf lipid peroxidation, indicating that O_3 exposure was harmful to these two plant varieties; besides, CAT activity was not changed in these two varieties. Concomitantly, the other three varieties (*Iraí*, *Macotaço* and *Pinto 111*) presented an opposed pattern of lipoperoxidation and CAT activation by O_3, indicating a relationship between CAT activation and resistance to lipoperoxidation in the response to O_3 exposure. Besides, these results suggest that differences in the susceptibility to O_3-induced lipoperoxidation observed in different varieties of *P. vulgaris* are probably due to the absence of CAT activation when some varieties are exposed to specific O_3 levels. In cell membranes, polyunsaturated fatty acids represent the primary target of O_3, which stimulate lipid peroxidation and impair the membrane fluidity (Iriti and Faoro, 2008). Together with these reports, our data suggest that CAT is also essential for protection against ROS generated by O_3 exposure in plant cells.

TRAP analysis revealed that *Fepagro 26* and *Iraí* O_3-exposed plants had decreased the levels of total non-enzymatic antioxidant defenses in leaves, suggesting that O_3 exposure does

not only alter the enzymatic antioxidant system (CAT), but it also influences the levels of non-enzymatic antioxidants, such as glutathione (GSH) and ascorbate (AsA). Both AsA and GSH are important non-enzymatic antioxidants and are ubiquitously distributed in plant cells (Iriti and Faoro, 2008). Previous studies revealed that O_3-stressed plants present both increased levels of ROS and decreased concentrations of GSH and AsA, indicating a correlation between O_3-induced ROS production and consumption of non-enzymatic antioxidants in plant cells (Mahalingam et al, 2006). On the other hand, although O_3 exposure decreased the non-enzymatic antioxidant defense in *Iraí* seedlings, O_3 was not able to induce lipoperoxidation in the leaves of this variety, as observed with the *Fepagro 26* variety. It is possible that the resistance to O_3-induced oxidative damage in *Iraí* leaves is due to the enhancement on CAT activity observed in this variety, which reinforces our suggestion that this enzyme plays a major role in the resistance to O_3 toxicity in these bean varieties.

Efforts to identify ozone-sensitive plant species for bioindication purposes have increased in the last years in attempt to quickly assess the the presence of hazardous concentrations of this air pollutant. *Pinto 111* is considered to be O_3-sensitive *P. vulgaris* variety and it is a well-known air pollution bioindicator species (Arndt et al, 1987; Feder, 1978). However, the single use of this non-native variety may become expensive when large scale bioindication surveys are planned. Besides, the current assessment of O_3 toxicity is mostly based on long-time exposure and detection of later parameters of toxicity, such as evaluation of plant biomass, foliar injury and reproductive parameters (Buekey et al, 2005; Feder, 1978). Our results suggest that *Fepagro 26* was the most O_3-sensitive variety, based on oxidative parameters. This idea is corroborated by data from Clebsch et al. (2008) who found that *Fepagro 26* instantaneous net assimilation and carboxylation efficiency of photosynthesis were equally reduced by O_3 exposure (personal communication).The evaluation of biochemical parameters in plants for toxicity assessment is advantageous over other

parameters (such as morphological and biomass evaluation), as biochemical modifications take place very early in time scale compared to other important physiological modifications. In this regard, oxidative stress parameters are probably the most rapidly to be affected by most pollutants and xenobiotics, and are also very accessible for laboratory detection. Taking all that into account, we conclude that the use oxidative parameters of different varieties of *P. vulgaris* that present known variations in their resistance to O_3-induced oxidative stress may be helpful in developing more precise protocols for early detection of O_3 toxicity.

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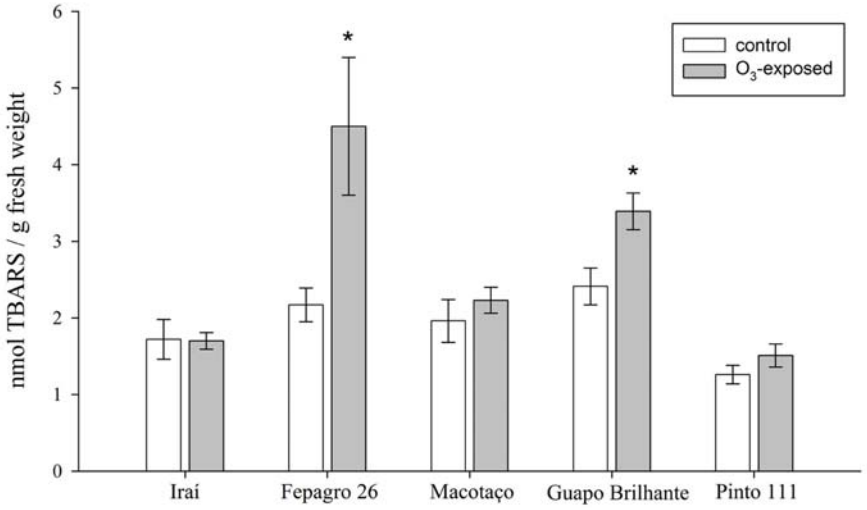
FIGURE LEGENDS

Figure 1. Lipid peroxidation levels (TBARS) on O_3-exposed and control leaves from five different varieties of *P. vulgaris*. * indicates statistical significant differences at $p < 0.05$ between control and O_3-exposed plants, as analyzed by *t*-test. Values represent mean ± SEM (n=5).

Figure 2. Catalase activity on O_3-exposed and control leaves from five different varieties of *P. vulgaris*. * indicates statistical significant differences at $p < 0.05$ between control and O_3-exposed plants, as analyzed by *t*-test. Values represent mean ± SEM (n=5).

Figure 3. Non-enzymatic antioxidant cellular defenses levels on O_3-exposed and control leaves the five different varieties of *P. vulgaris*. * indicates statistical significant differences at $p < 0.05$ between control and O_3-exposed plants, as analyzed by *t*-test. Values represent mean ± SEM (n=5).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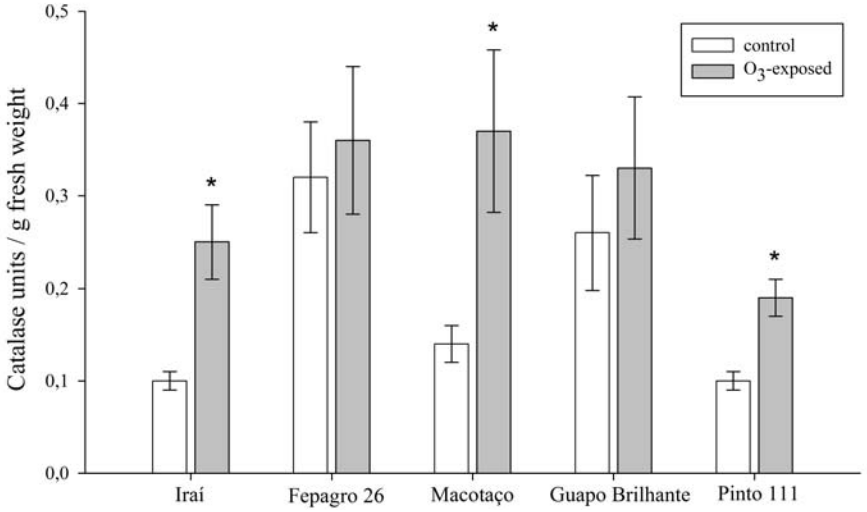
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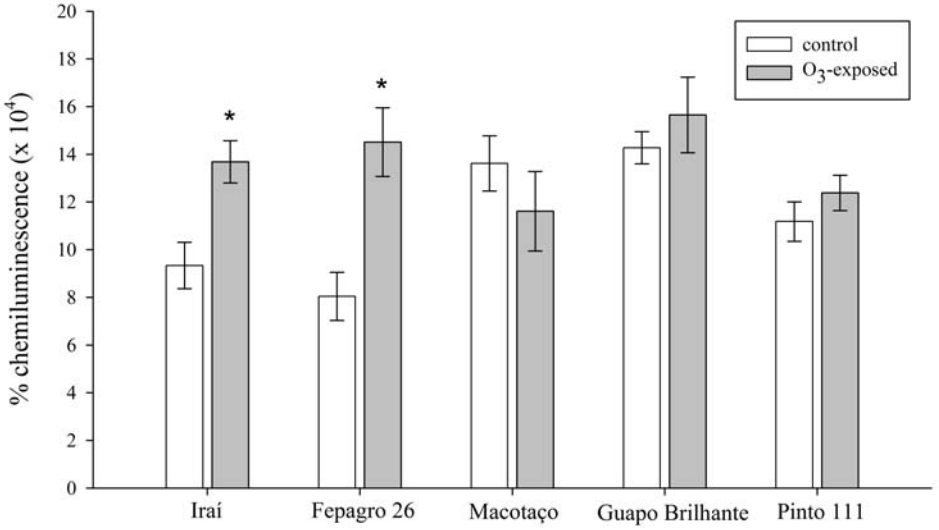
Pinto 111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Iraí
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Guapo Brilhante
Pinto 111

Capítulo II.

The glutathione antioxidant system as a biomarker suite for the assessment of heavy metal exposure and effect in the grey mangrove, *Avicennia marina* (Forsk.) Vierh

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The glutathione antioxidant system as a biomarker suite for the assessment of heavy metal exposure and effect in the grey mangrove, *Avicennia marina* (Forsk.) Vierh

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ABSTRACT

Alterations in the glutathione antioxidant system and lipid peroxidation in *Avicennia marina* were studied under laboratory and field conditions. The activity of glutathione peroxidase (GPx) was found to respond to Zn exposure, and a significant positive relationship between leaf Zn concentration and GPx activity was observed after 96 h and 8 weeks. Lipid hydroperoxides increased proportionally with increasing leaf Zn concentration after 2 and 8 weeks, while no changes in total glutathione were observed. Induction of GPx at 96 h predicted effects at the individual level at a later time interval (reduced biomass at 8 weeks). Results from the field revealed that increasing leaf metal concentration (Zn, Cu or Pb) produced a proportional increase in GPx activity whereas lipid hydroperoxides and total glutathione were not affected. The utility of GPx as an early warning biomarker is suggested, since GPx activity increases in a dose-dependant fashion in response to accumulated leaf metals, and is predictive of later effects on growth.
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1. Introduction

In contrast to the measurement of accumulated contaminants in tissues, or accumulative indication, biomarkers provide the possibility of simultaneously assessing exposure and the potential impact of contaminant loads on the health of organisms (Ferrat et al., 2003). A biomarker may be defined as a variation(s) in molecular, biochemical and/or cellular components induced by exposure to one or more chemical pollutants, which can be measured in biological tissues and fluids (Depledge et al., 1995). Biochemical endpoints measured in tissue samples may provide reliable evidence of not only contaminant exposure, but also biological effect. Biochemical alterations may, in turn, enable an early recognition of contaminant-induced stress in a dose and/or time dependent manner and may be predictive of subsequent effects at the organism level or higher (Ernst and Peterson, 1994).

Mangroves are integral primary producers in estuarine systems, and provide habitat and breeding grounds for many commercial fish species (Laegdsgraad and Johnson, 1995). Despite these ecological attributes, the mangrove environment is often subject to industrial effluents and waste discharges, domestic and agricultural runoff, solid waste dumping and contaminated leachate (Defew et al., 2005; MacFarlane and Burchett, 2001). Heavy metals are common pollutants in urban aquatic ecosystems and are one of the main anthropogenic toxic compounds found in polluted mangrove locales, due to their affinity to, and immobilization within, anaerobic sediments (Harbison, 1986). The metals most often elevated in contaminated estuarine sediments, and thus of greatest ecotoxicological concern, are zinc (Zn), copper (Cu) and lead (Pb) (Machado et al., 2002; Defew et al., 2005).

For the grey mangrove *Avicennia marina*, which is considered a metal tolerant species, microelements such as Zn and Cu usually exhibit some mobility to aerial portions of the plant, while non-essential metals (Pb) show minimal translocation and are mainly accumulated in root tissue (MacFarlane and Burchett, 1999; MacFarlane et al., 2003). Laboratory and field studies on metal exposure to *A. marina* have revealed that Zn and Cu exhibit significant uptake, limited translocation and toxic effects including negative impacts on growth and total inhibition of emergence (500 μg/g for Zn and 800 μg/g for Cu) (MacFarlane and Burchett, 2002). Lead accumulation in root tissue is lower than that of other metals and this metal is largely excluded from leaf tissue (at Pb sediment concentrations of up to 400 μg/g), resulting in little negative effects on growth (MacFarlane and Burchett, 2002).

Furthermore, metals such as Zn, Cu and Pb are known to be pro-oxidants and accelerate the production of reactive oxygen species (ROS) in plants in direct relationship with accumulated dose (Ferrat et al., 2003; Prasad et al., 1999; Fatima and Ahmad, 2005; Drazkiewicz et al., 2004). Copper is a redox active metal and produces

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reactive oxygen species (ROS), including hydroxyl radicals from superoxide and hydrogen peroxide upon Cu uptake via metal-dependent Haber-Weiss or Fenton reactions (Srivastava et al., 2006). However, Zn and Pb are not oxido-reducing metals, thus oxidative stress is indirectly induced as a consequence of toxicity to metabolic pathways and/or membrane associated electron transport processes (Verma and Dubey, 2003). Superoxide radicals are converted to hydroperoxides via the action of superoxide dismutase (Dixit et al., 2001). The glutathione antioxidant system is one of numerous protective mechanisms against hydroperoxides. Glutathione peroxidase (GPx) (EC 1.11.1.9) is an antioxidant enzyme that catalyses the reduction of hydroperoxides employing the glutathione (GSH) pool, thereby protecting the cell against oxidative damage (Halliwell and Gutteridge, 1999). However, metal-induced generation of ROS and depletion of oxidative defence components may lead to cellular damage such as lipid peroxidation (Ferrat et al., 2003; Prasad et al., 1999). Thus, alterations of antioxidant components (including enzymatic and non-enzymatic mechanisms) and oxidative damage (including lipid peroxidation) reflect a calibrated metabolic response which could potentially be exploited as a sensitive and reliable early-warning biomarker for metal exposure and toxicity (Meers et al., 2005; Ferrat et al., 2003).

Most laboratory studies with plant models measure accumulation of metals and biomarker responses, yet only a limited subset have linked these biochemical changes to biological consequences at the individual level in terms of time and/or concentration dependant effects with endpoints such as mortality, physiological responses and growth reductions (Stegeman et al., 1992). Linking early warning biomarkers of metal toxicity in *Avicennia* to productivity/biomass endpoints at the individual level may increase their predictive value in assessing long-term impact and thus utility for anticipatory environmental monitoring initiatives. Furthermore, little work internationally has been performed under realistic field conditions exploring the effects of accumulated sediment borne metals on biochemical processes in plant tissues and assessment of their suitability as potential biomarkers for metal stress (Halbrook et al., 1993; Moustakas et al., 1997). Before biomarkers may become effective legislative and management tools, it is necessary to establish linear cause–effect relationships between exposure to metals, accumulation (dose) and biomarker responses, along with a demonstrated link between the biomarker(s) and later biological effects at the individual level.

The current study represents one of the first integrated assessments of changes in glutathione biochemistry and oxidative damage to lipids in response to metallic exposure under both laboratory and field conditions in the estuarine plant model *A. marina*. Thus, the aims of this study have been to determine:

- environmentally relevant exposure–dose relationships of Zn in leaf tissue of *A. marina* under laboratory conditions;
- if alterations in the glutathione antioxidant system and oxidative damage reflect accumulated leaf Zn patterns in a linear fashion in *A. marina* under laboratory conditions;
- whether early biomarker induction correlates with physiological effects (biomass) at the individual level at later time intervals (early warning utility);
- whether accumulated leaf metals correlate with induced changes in the glutathione antioxidant system and lipid peroxidation levels in situ under realistic field conditions.

2. Materials and methods

2.1. Laboratory-based zinc exposure trials

Mature *A. marina* propagules were collected from an uncontaminated field site (Kooragang Island, Newcastle, Australia). Propagules were grown in hydroponic culture for 7 months under glasshouse conditions in forestry tubes containing washed river sand which were placed in 20% seawater. Ninety similar seedlings were randomly allocated to 30 plastic containers containing 3 L of 20% seawater (three seedlings per container). Zinc was added to water as $ZnCl_2$ (BDH Chemicals, Poole, England) at concentrations of 0, 50, 100, 200, 400 and 800 μg Zn/ml, and the seedlings were sampled at 96 h, 2 weeks and 8 weeks (five replicates of each treatment). At each harvest period, one seedling was taken from each treatment replicate ($n = 5$). The whole plant was weighed and washed with distilled water. The second leaf pair of each seedling was taken and discs cut for determination of antioxidant response and oxidative damage; these were stored at −80 °C. The remaining parts of the same leaves were oven dried at 60 °C and stored for heavy metal analysis.

2.2. Field-based study

2.2.1. Localities and sampling

Eight field locations across two estuaries were selected for sampling in order to establish an environmentally relevant sediment metal contaminant gradient from relatively uncontaminated to highly contaminated. Lake Macquarie is a highly urbanised estuarine lake in New South Wales, Australia (33°05′S, 151°34′E) with a long history of significant inputs of heavy metals including Cd, Cu, Pb, Zn, Se and As (Batley, 1987; Doyle et al., 2003). Field sampling was conducted in September 2006, at four locations: Wyee Bay (WB) adjacent to a coal-fired power station; Cockle Creek (CC) adjacent to a Pb/Zn Smelter, an urban mangrove location, Dora Creek (DC); and a relatively undisturbed mangrove forest, Swansea (SS) (Fig. 1).

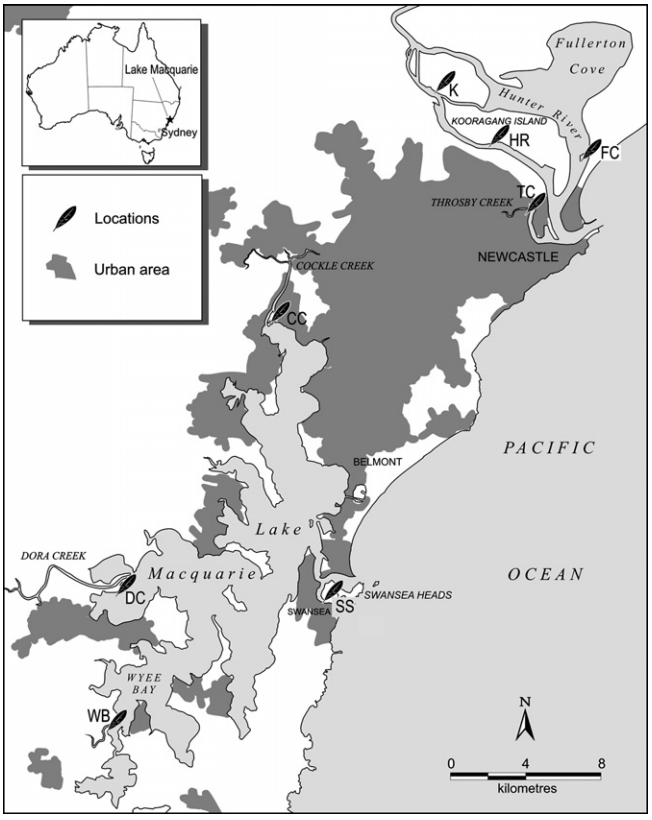

Fig. 1. Locality map of sampling locations in the Hunter River Estuary (32°50′S, 151°45′E) and Lake Macquarie (33°05′S, 151°34′E), New South Wales, Australia.

The Hunter River Estuary is located in New South Wales, Australia (32°50′S, 151°45′E) and site of the largest industrial coal exporting harbour in the Southern Hemisphere. Four further locations were selected to include a range of sediment metal loadings based on historic data from Birch et al. (1997). Field sampling was conducted in September 2006 at Throsby Creek (TC) with urban runoff, stormwater and light industrial inputs; Hunter River (HR) adjacent to a decommissioned steel production facility; Kooragang Island (K), an estuarine rehabilitation site; and Fullerton Cove (FC), a relatively unimpacted estuarine bay (Fig. 1).

2.2.2. Leaf and sediment collection

Five pooled leaf samples were taken within each location. Each sample was a composite of leaves from three individual *A. marina* trees between 2 m and 5 m apart (15 individuals in total for each location). From each mature tree, two leaves of the second whorl from the lateral meristem were collected from each of three branches at 1 m height. Leaves from each tree were combined to obtain a composite sample. Leaves were stored on ice and transported to the laboratory, where the leaves were washed with distilled water. Leaf samples were prepared in the same way as for the laboratory studies. Corresponding soil samples from rooting zone (sampling depth approximately 10–20 cm) were collected at the same locations as leaf samples. These samples were stored in polyethylene bags and transferred to the laboratory, where they were air-dried at room temperature.

2.3. Sample preparation for oxidative stress assays

Frozen leaf discs were ground in liquid nitrogen with a mortar and pestle. For glutathione and lipid peroxidation assays, 150 mg of frozen leaf powder was homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 1mM EDTA (Ajax Finechem, Seven Hills, Australia). The homogenate was centrifuged at 10,000*g* at 4 °C for 15 min. To perform the glutathione peroxidase assay, 150 mg frozen leaf powder was homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.5) (Sigma–Aldrich, St. Louis, USA) containing 5 mM EDTA.

2.3.1. Glutathione assay

The glutathione assay kit utilizes the enzymatic recycling of GSH and GSTNB, the compound produced when the sulphydryl group of GSH reacts with DTNB (5,5′-dithiobis-2-nitrobenzoic acid, Ellman's reagent). It measures absorbance of TNB (5-thio-2-nitrobenzoic acid) which is produced concomitantly with GSH from GSTNB by glutathione reductase. Any GSSG (the oxidized form of glutathione) present in the sample is converted to GSH by the glutathione reductase included in the kit and then forms part of the GSTNB–TNB/GSH cycle; thus the assay reflects total glutathione content in the sample. Homogenised samples were deproteinated with MPA (metaphosphoric acid) (BDH Chemicals, Poole, England) to stop interference due to protein sulfhydryl groups. For determination of the glutathione content, TEAM reagent (triethanolamine) (BDH Chemicals, Poole, England) was added followed by freshly prepared assay cocktail (MES buffer, cofactor mixture, enzyme mixture, water and DTNB). An Ultramark Microplate Imaging System (Biorad) was used to measure the absorbance (405 nm) at 5 min intervals for 30 min, starting within 2 min after adding assay mixture to the first well. Total glutathione was expressed in μmol/g wet weight.

2.3.2. Glutathione peroxidase assay

The glutathione peroxidase (GPx) assay kit was used to determine the levels of glutathione-dependent peroxidase in *A. marina* leaves. The enzyme catalyses the reduction of hydroperoxides, including hydrogen peroxide (H_2O_2), using reduced glutathione as the electron donor. The assay kit measures GPx activity indirectly by a coupled reaction with glutathione reductase in conjunction with oxidation of NADPH, which decreases absorbance at 340 nm. When GPx activity is rate limiting, this decrease in absorbance is directly proportional to GPx activity in the sample. An Ultramark Microplate Imaging System (Biorad) set at 340 nm, was used to measure the linear rate of reaction (ΔA/min) over 5 min. GPx activity is expressed in nmol/min/g wet weight.

2.3.3. Lipid hydroperoxide assay

The lipid hydroperoxide assay kit was used to directly measure lipid hydroperoxides, utilizing their instability and propensity to react with ferrous ions to produce ferric ions which are detected using thiocyanate ion as the chromogen. This provides a more reliable measurement of oxidative damage to lipids than the traditional quantification of lipid peroxidation byproducts (malondialdehyde (MDA) and 4-hydroxy-2(*E*)-nonenal (4-HNE)). The lipid hydroperoxides were extracted into chloroform (Selby Scientific, Clayton, Australia) to avoid overestimation due to endogenous ferric ions in the samples before performing the assay, following the kit protocol. A UV/Vis Spectrophotometer (LW-UV-200-RS, LW Scientific) was used to measure the absorbance at 500 nm. Lipid hydroperoxide concentration was expressed in nmol/g wet weight.

2.4. Heavy metal analysis of leaf tissue and sediment

About 250 mg of oven dried *A. marina* leaf tissue was digested sequentially on a DigiPrep heating block (Choice Analytical, Thornleigh) in concentrated nitric acid (100 °C) (Ajax Finechem, Seven Hills, Australia) and hydrogen peroxide (65 °C) (Merck (Kisyth, Australia), after the method of Krishnamurty et al. (1976). Samples were made to volume (40 mL), filtered (0.45 μm), and metal analysis was carried out on the digests using air/acetylene atomic absorption spectroscopy (AAS; Varian AA-1275, Australia). The standard solutions were matrix matched and an international certified reference material (DC 73349/Bush Branches and Leaves, NCS Beijing) was used to assess percentage of recovery of metals (Zinc recovery = 84%. Copper recovery = 92%. Lead recovery = 103%).

Extractable (soluble) metals in sediment samples were analysed according to Duinker et al. (1974), shaking air dried soil samples (4 g) with 0.1 N HCl (Merck, Kisyth, Australia) at a sample to extractant ratio of 1:10 (w/v) for 16 h. These samples were centrifuged (3000*g* for 3 min), filtered (0.45 μm) and the concentration of metals determined by atomic absorption spectroscopy (AAS; Varian AA-1275, Australia).

2.5. Statistical analysis

Relationships between accumulated metal concentration in leaf tissue, individual sediment metals, glutathione biochemistry and oxidative damage in leaf tissue were assessed using bivariate regression analyses, including linear and exponential models, within SigmaPlot software version 10.0 (Systat Software Inc.). We chose to assess linear relationships for field data based on mean values because it is not possible to reliably pair individual sediment samples with individual pooled leaf samples (biological replicates) within a location as their spatial locations were not recorded.

To evaluate the contribution of accumulated leaf metal on glutathione biochemistry under field conditions, all leaf metals and GPx activity values were added to forward stepwise multiple linear regressions. Forward stepwise linear regression identifies which accumulated leaf metal(s) best predicts changes in GPx activity, by successively adding each independent variable to the model based upon an *F* ratio greater than 1. The weighting of independent variables was assessed by standardised beta coefficients and

significant semi-partial correlations. For forward stepwise multiple linear regressions, independent variables examined with exponential accumulation relationships were log transformed $\ln(x+1)$ prior to analysis.

Differences among locations in sediment and leaf metals as well as glutathione biochemistry and oxidative damage in leaf tissue were analysed employing one-way ANOVA. Statistically significant differences between groups ($p < 0.05$) were assessed via the Tukey's honestly significant difference test. All these analyses were performed within SPSS 14.0 for Windows (SPSS® Inc.)

3. Results

3.1. Zinc exposure and leaf accumulation patterns

No significant relationship between Zn exposure and leaf Zn concentration was observed after 96 h. After 2 weeks however, the Zn concentration in leaves increased in a linear fashion with exposure concentration ($R^2 = 0.91$, $p < 0.01$). Seedlings grown in the presence of 100 μg Zn/ml and above for 2 weeks, presented a 117 ± 8.2% to 151 ± 13.7% increase in leaf Zn content compared to control plants harvested at the same period. A significant linear relationship between Zn exposure and leaf Zn concentration was still observed after 8 weeks ($R^2 = 0.96$, $p < 0.01$). Similarly, after 8 weeks of exposure, leaf Zn concentrations were 108.4 ± 2.5% to 146 ± 20.8% higher in seedlings treated with 100–400 μg Zn/ml compared to respective control plants (Fig. 2). Accumulation of Zn to leaf tissue equilibrated with Zn water concentration after two weeks, and no significant increases in leaf Zn concentrations were observed after longer exposure periods (8 weeks). Furthermore, seedlings exposed to 800 μg Zn/ml presented severe stress symptoms, including chlorosis, desiccation, blackening of leaf tissue and premature leaf abscission. Plants in this treatment could not be used to assess leaf metal concentrations and biochemical parameters after 8 weeks.

3.2. Leaf accumulated zinc and oxidative stress responses

GPx activity in leaf tissue was found to respond to Zn exposure (Fig. 3a). Ninety-six hours after experimental commencement, patterns of GPx activity reflected accumulated leaf Zn, with a significant positive relationship between Zn concentration in leaf tissue and GPx activity ($R^2 = 0.70$, $p < 0.05$). Seedlings exposed to 100 μg Zn/ml exhibited a 230% increase in enzyme activity (43.7 ± 6.5 nmol/min/g wet weight), while seedlings in the presence of 800 μg Zn/ml showed a 300% increase in GPx activity (48.1 ± 1.2 nmol/min/g wet weight) compared to control seedlings. After 2 weeks exposure, there was no significant linear relationship between GPx activity and leaf Zn concentration, but after 8 weeks of treatment, GPx activity was again significantly associated with leaf tissue Zn concentration, with bivariate regression analysis revealing a positive linear relationship between GPx activity and accumulated leaf Zn ($R^2 = 0.88$, $p < 0.05$). Although GPx activity remained responsive in a dose-dependent fashion after 8 weeks of exposure, enzyme activity was lower than in 96 h treated plants. Seedlings exposed to 100 μg Zn/ml at 8 weeks showed a 140% increase in enzyme activity (21.9 ± 2.8 nmol/min/g wet weight),

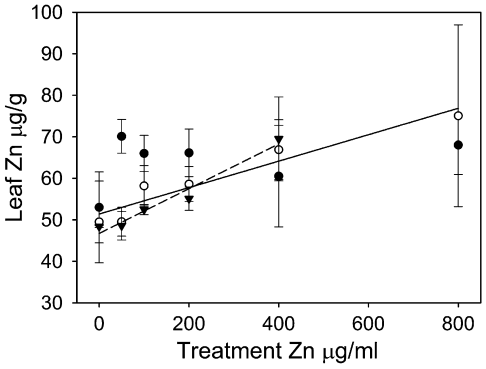

Fig. 2. Zinc concentration in leaf tissue (μg Zn/g dry weight) of 7 month old *A. marina* seedlings grown in hydroponic culture after exposure to dissolved Zn (0–800 μg Zn/mL) for up to 8 weeks prior to harvest. Mean ± SE ($n = 5$). Relationship between dissolved Zn and accumulated leaf Zn at: 96 h (●); 2 weeks, $y = 51.39 + 0.03x$, $R^2 = 0.91$, $p < 0.01$ (○; —); and 8 weeks, $y = 46.75 + 0.05x$, $R^2 = 0.96$, $p < 0.01$ (▼; ---).

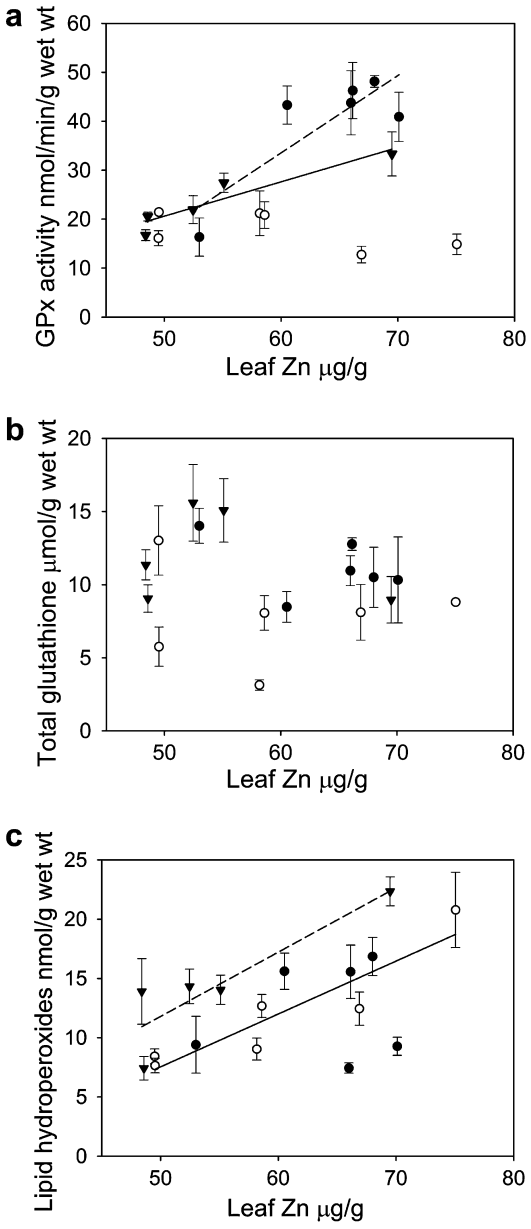

Fig. 3. Relationships between leaf Zn concentration and GPx activity, total glutathione and lipid hydroperoxide concentrations in 7 month old *A. marina* seedlings grown in hydroponic culture and exposed to dissolved Zn (0–800 μg Zn/mL) with temporal assessment of: (a) leaf Zn concentration and GPx activity after 96 h, $y = -61.2 + 1.57x$, $R^2 = 0.70$, $p < 0.05$ (●; —), 2 weeks (○) and 8 weeks, $y = -14.45 + 0.7x$, $R^2 = 0.88$, $p < 0.05$ (▼; —). (b) Zn concentration of leaves and total glutathione concentration after 96 h (●), 2 weeks (○) and 8 weeks (▼). (c) Leaf Zn concentration and lipid hydroperoxides after 96 h (●), 2 weeks, $y = -14.76 + 0.4x$, $R^2 = 0.84$, $p < 0.05$ (○; —) and 8 weeks, $y = -15.32 + 0.5x$, $R^2 = 0.79$, $p < 0.05$ (▼; ---).

while seedlings in the presence of 400 μg Zn/ml exhibited a 210% increase in GPx activity (33.3 ± 4.5 nmol/min/g wet weight) compared to control plants. Levels of total glutathione in leaf tissue did not vary significantly across the treatments, with the values ranging from 3.1 ± 0.3 to 15.6 ± 2.6 μmol/g wet weight. No significant dose-dependent relationships were observed between total glutathione and Zn concentrations in leaf tissue at any time (Fig. 3b).

Significant increases in the levels of lipid peroxidation in leaf tissue were found with exposure to Zn (Fig. 3c). Increasing concentrations of leaf Zn produced a proportional increase in leaf lipid peroxidation content after 2 and 8 weeks of treatment. Bivariate regression revealed a significant relationship between leaf Zn concentration and lipid hydroperoxides at 2 weeks ($R^2 = 0.84$, $p < 0.05$) and 8 weeks ($R^2 = 0.79$, $p < 0.05$). Lipid hydroperoxides were between 13.8 ± 2.7 (low leaf Zn concentration) and 22.3 ± 1.2 nmol/g wet weight (high leaf Zn concentration) higher after 8 weeks exposure than at 96 h, corresponding to ~50% increase in lipid hydroperoxide concentrations across the exposure gradient over time.

3.3. Relationship between GPx activity and final individual biomass

GPx activity presented an early induction (96 h) and a linear relationship with leaf Zn concentration in the laboratory (Fig. 3a), and was the only biomarker exhibiting significant positive relationships with accumulated metals in field trials. For these reasons, bivariate regression analyses between GPx activity and the final seedling biomass were performed to assess if this biochemical parameter predicted effects of accumulated Zn on biomass at later intervals (i.e. 8 weeks) (Fig. 4). Suppression of seedling growth and a dose-dependent biomass reduction due to Zn exposure was evident only after 8 weeks. Plants exposed to 400 μg Zn/ml (the highest exposure where seedlings survived) had a 28% lower final biomass compared to control plants. Increased GPx activity at 96 h predicted this later decrease in seedling biomass (8 weeks) with bivariate linear regression revealing a significant negative relationship between these parameters ($R^2 = 0.80$, $p < 0.05$). Similarly, GPx activity at 8 weeks exhibited a significant negative relationship with reduced biomass at this time ($R^2 = 0.88$, $p < 0.05$).

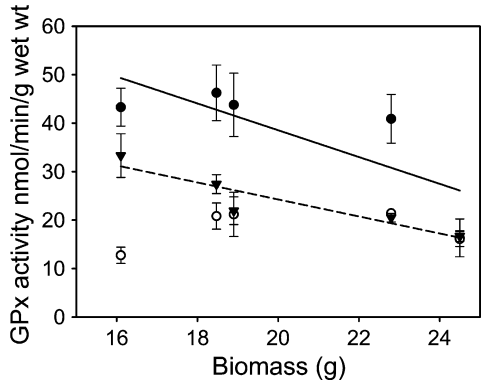

Fig. 4. Relationship between final biomass (g wet weight) at 8 weeks and temporal responses in GPx activity for *A. marina* seedlings after exposure to dissolved Zn (0–800 μg Zn/mL) for: 96 h, $y = 5.28 - 3.48\times$, $R^2 = 0.80$, $p < 0.05$ (●; —); 2 weeks (○); and 8 weeks, $y = 8.18 - 4.83\times$, $R^2 = 0.88$, $p < 0.05$ (▼; ---).

3.4. Field-based assessment of oxidative stress biomarkers

Soluble sediment Zn concentrations ranged from approximately 6 (Dora Creek) to 447 μg Zn/g (Cockle Creek) among sampling locations across both estuaries (Table 1). Zinc concentration in leaf tissue ranged from 10.7 to 28.5 μg/g. Comparing the levels of soluble (bioavailable) Zn in sediment with established sediment quality guidelines (ANZECC and ARMCANZ, 2000), Zn concentrations were found to exceed the criterion values for biological effects (ERL, effects range low, adverse biological effects for 10% of species) in estuarine sediments (Zn = 200 μg/g; ANZECC and ARMCANZ, 2000; Long et al., 1995) at Cockle Creek 2.2-fold. Bivariate regression showed a significant positive linear relationship between soluble Zn in sediment and Zn concentration in leaf tissue ($R^2 = 0.57$, $p < 0.05$, indicating accumulation (albeit restricted) of Zn from root to leaf tissue (Fig. 5a).

Sediment soluble Cu concentrations were significantly elevated at Cockle Creek only (65.4 μg/g), being in the region of the low criterion values for biological effects ERL for Cu (65 μg/g; ANZECC and ARMCANZ, 2000; Long et al., 1995) (Table 1). Copper concentration in leaf tissue increased to approximately 18 μg Cu/g at 11 μg/g sediment soluble Cu; further increases in soluble sediment Cu concentration resulted in little increase in Cu accumulation by leaves (Fig. 5b). Bivariate regression revealed a significant sigmoidal relationship between the soluble Cu concentration of sediment and leaf Cu concentration, implying Cu regulation to leaf tissue ($R^2 = 0.50$, $p < 0.05$).

Soluble sediment Pb concentrations were variable, ranging from 5.3 (Dora Creek) to 360.8 μg Pb/g (Cockle Creek) (Table 1). According to sediment guidelines, Pb sediment concentration at Cockle Creek exceeded the criterion values for biological effects 1.6 times (ERH, effects range high, adverse biological effects on 50% of species) in estuarine sediments (Pb = 220 μg/g; ANZECC and ARMCANZ, 2000; Long et al., 1995). Leaf Pb concentrations were low at all locations, ranging from 1.1 to 5.2 μg/g. Little Pb was accumulated in leaf tissue between 5.8 and 46.4 μg/g soluble Pb in sediment, and only at sediment Pb concentrations above 46.4 μg/g was accumulation in leaf tissue evidenced. An exponential regression model was found to best explain the pattern of leaf Pb accumulation, suggesting Pb exclusion from leaf tissue at lower exposure concentrations with unrestricted uptake at higher exposures ($R^2 = 0.80$, $p < 0.01$) (Fig. 5c).

Bivariate linear regression analysis showed a significant positive linear relationship between the concentrations of accumulated Zn, Cu and Pb in leaf tissue and the GPx activity ($R^2 = 0.67$, $p < 0.05$; $R^2 = 0.80$, $p < 0.01$; $R^2 = 0.50$, $p < 0.05$, respectively) (Fig. 6a). Forward stepwise multiple linear regression revealed that a

Table 1
Soluble (bioavailable) metal concentrations of sediment (μg/g dry weight) from the Hunter River Estuary and Lake Macquarie, NSW, Australia , 2006

	Hunter River Estuary				Lake Macquarie				
	Fullerton Cove (FC)	Throsby Creek (TC)	Kooragang Island (K)	Hunter River (HR)	Dora Creek (DC)	Cockle Creek (CC)	Wyee Bay (WB)	Swansea (SS)	ANOVA *F*
Zn μg/g	70.3 ± 4.2^{ab}	91.4 ± 7.3^{ab}	128.8 ± 11.1^{a}	109.8 ± 0.6^{ab}	6.7 ± 1.4^{b}	446.7 ± 74.0^{c}	43.3 ± 4.6^{ab}	45.1 ± 6.9^{ab}	22.50*
Cu μg/g	3.4 ± 0.7^{a}	21.4 ± 1.6^{a}	10.8 ± 0.9^{a}	21.1 ± 2.5^{a}	1.8 ± 0.3^{a}	65.4 ± 19.8^{b}	4.3 ± 1.4^{a}	8.8 ± 1.0^{a}	8.05*
Pb μg/g	5.8 ± 0.8^{a}	42.7 ± 2.6^{a}	23.8 ± 5.4^{a}	46.4 ± 0.9^{a}	5.3 ± 1.1^{a}	360.8 ± 86.2^{b}	7.2 ± 1.1^{a}	13.7 ± 3.7^{a}	13.22*

Mean values ± standard error ($n = 5$). Results of a one way ANOVA,* = significant difference at $p < 0.05$. Locations identified as similar, according to Tukey's HSD multiple comparison, are linked by identical letters for each metal separately.

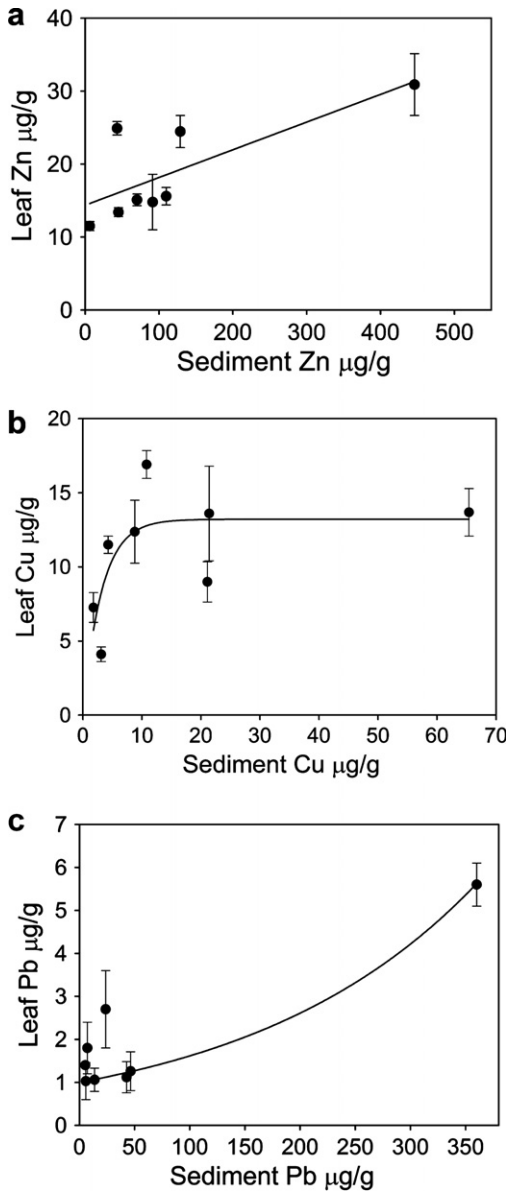

Fig. 5. Relationships between soluble (bioavailable) metal concentrations in sediment and leaf metal concentration. (a) Zn, $y = 14.36 + 0.03x$, $R^2 = 0.57$, $p < 0.05$; (b) Cu, $y = 13.21(1 - \exp(-0.31x))$, $R^2 = 0.50$, $p < 0.05$; (c) Pb, $y = \exp(0.0048x)$, $R^2 = 0.80$, $p < 0.01$.

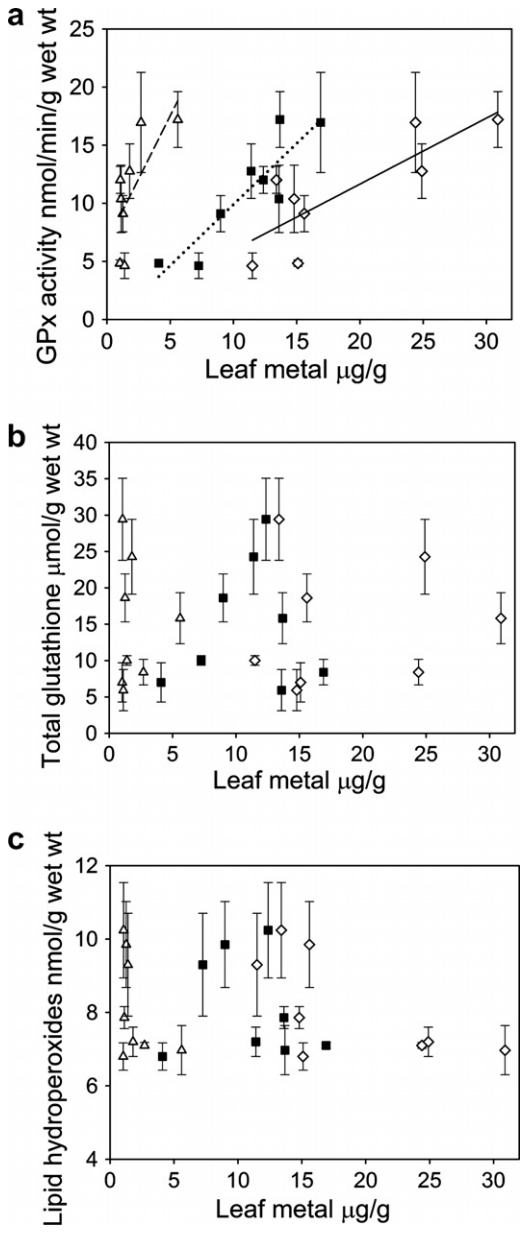

Fig. 6. Biochemical leaf responses and leaf metal concentrations: (a) GPx activity and Zn, $y = 0.28 + 0.56x$, $R^2 = 0.67$, $p < 0.05$ (◇; —); Cu, $y = -0.61 + 1.05x$, $R^2 = 0.80$, $p < 0.01$ (■; ●●●); Pb, $y = 6.63 + 2.17x$, $R^2 = 0.50$, $p < 0.05$ (△; ---). (b) Total glutathione and Zn (◇); Cu (■) and Pb (△). (c) Lipid hydroperoxides and Zn (◇); Cu (■) and Pb (△).

combination of the more mobile metals Zn and Cu contributed to maximize the variance explained for GPx activity ($R^2 = 0.94$; $p < 0.01$; significant semi-partial correlations: Zn, 0.51 and Cu, 0.60) (Table 2).

Although the concentrations of total glutathione varied among locations, no significant relationships between leaf metal accumulation patterns and total glutathione concentrations were observed (Fig. 6b). Lipid hydroperoxide concentrations did not vary greatly among sampling locations. There was no significant dose-dependent response for the concentration of lipid hydroperoxides with leaf metals among locations (Fig. 6c).

4. Discussion

Our current laboratory-based experiments showed that *A. marina* seedlings accumulated Zn in leaf tissue in a dose-dependent fashion after 2 and 8 weeks of Zn treatment, while an exposure time of 96 h was insufficient to detect a dose-dependent leaf Zn accumulation response (Fig. 2). Previous investigations revealed that Zn uptake by *A. marina* plants is high, and the metal is accumulated in a linear fashion in root tissue across a range of sediment Zn concentrations. Zinc translocation to leaf tissue exhibits a dose-dependent relationship with both root and sediment Zn concentrations (MacFarlane and Burchett, 1999, 2002; MacFarlane et al., 2003). In the present study we observed that, after 2 weeks and beyond, equilibration of Zn uptake occurs at any particular Zn exposure concentration up to 400 µg/mL. Higher concentrations of Zn exposure result in mortality at later time intervals (800 µg/mL at 8 weeks).

In contrast, leaf tissue GPx activity in 96 h treated plants increased linearly with accumulated leaf Zn concentration at 96 h, indicating that it is likely that ROS production in these plants in-

Table 2
Significant regression model best describing relationships between accumulated leaf metals and GPx activity in *A. marina* under field conditions, using forward stepwise multiple linear regression

	Intercept	Leaf Zn (μg/g) Beta coefficient	Leaf Cu (μg/g) Beta coefficient	Leaf Pb (μg/g) Beta coefficient	df	*F*	*p*	R^2
GPx activity (nmol/min/ g wet wt)	−13.14	0.51*	0.60*	−0.41	2,5	42.94	<0.01	0.94

Regression models are presented with standardised beta coefficients for each independent variable to assess their contribution to the overall relationship. The adjusted determination coefficient (R^2) and significance level (p) are also displayed.
* Denotes significant semi-partial correlations.

creased in response to accumulated Zn (Fig. 3a). Glutathione peroxidase activity in 96 h treated seedlings was higher when compared to plants exposed for 2 and 8 weeks, suggesting that Zn-mediated oxidative stress was initiated within a few days of metal exposure, yet response adaptation occurred over time, in spite of similar Zn leaf concentrations. Nevertheless, the stimulation of this enzymatic antioxidant system seems to be an important and sustained defense mechanism, as after 8 weeks of Zn treatment, leaf tissue GPx activity still exhibited a positive relationship with leaf Zn accumulation. Indeed numerous past studies indicate that increases in GPx activity in leaf tissue occurs in plants exposed to metals (e.g. Se and Cu), under both laboratory and field conditions (Hartikainen et al., 2000; Boojar and Goodarzi, 2007). Further, Fatima and Ahmad (2005) observed significant increases in both GPx and APx (ascorbate peroxidase) activities in onions (*Allium cepa*) exposed to similar concentrations of metals (Hg, Pb, Cr, Cu, Zn or Cd at 200–800 μg/ml), suggesting that the elevated activity of both enzymes was a result of metal-induced free radical generation. However, Dixit et al. (2001) reported that GPx activity in pea plants (*Pisum sativum*) exposed to Cd (4 and 40 μM for 7 days) decreased in root tissue but remained almost unaltered in leaves. Similarly, Baccouch et al. (2001) showed that although maize plants (*Zea mays*) exposed to Ni (250 μM $NiCl_2$ for 5 days) accumulated the metal in roots (~4000 μg Ni/g), GPx activity in this tissue was unaltered compared to controls. Temporal variation in GPx activity may occur with chronic exposure to metals. Hartikainen et al. (2000) observed that GPx activity was significantly enhanced in ryegrass (*Lolium perenne*) shoots exposed to Se (1–10 μg/g) after 40 days, but chronic metal exposure decreased enzyme activity. After 69 days of metal treatment, the levels of GPx activity at these exposures decreased significantly compared to the first yield, indicating that a GPx adaptation response is possible over longer exposure periods. Similarly, relative reductions in GPx over time in our own experiments concur with a proposed temporal mechanism of biomarker response to contaminant exposure, namely that if exposure is prolonged, the induced biological response at a particular exposure concentration may decline (adaptation). This is perhaps observed because alternative detoxification/depuration mechanisms may be induced in order to restore homeostasis (Wu et al., 2005).

Collectively, it seems that modulation of GPx activity in response to heavy metal exposure is diverse, and depends on the metal in question, the species and plant tissue examined, other co-occurring antioxidant defense (e.g. peroxidase activity in *A. marina*; MacFarlane and Burchett, 2001) and metal detoxification mechanisms (e.g. phytochelatins and vacuolar compartmentalisation; Dixit et al., 2001) and as our current findings suggest, the duration of metal exposure. Despite this, in *A. marina* GPx activity increases with leaf Zn concentration, and although GPx levels at any particular exposure concentration decreased over time, the relative positive linear response across the exposure gradient was maintained for 8 weeks, suggesting appropriate biomarker utility for GPx activity. The absence of a relationship between leaf Zn levels and GPx activity after 2 weeks does not necessarily limit the use of GPx as a biomarker, but rather suggests that the magnitude of this biological response may vary dependent upon the experimental window examined. Temporal changes in the relationship between accumulated metal and GPx responses from our laboratory-based experiments indicate that temporal replication and sampling frequency are important future considerations when assessing responses to metal exposure employing GPx activity. For contaminant monitoring purposes, our results suggest that sampling should occur at least at 2–3 intervals in time, these being more than 2 weeks apart to avoid drawing erroneous conclusions from an unrepresentative time-point(s).

Despite this, our field experiments demonstrated that GPx activity was strongly co-related with accumulated metals, regardless of the many potential confounding variables under realistic environmental scenarios. Positive linear relationships between GPx activity and leaf Zn, Cu and Pb levels were observed (Fig. 6a), with accumulated Zn and Cu being the metals with the strongest relationship (Table 2), indicating that the combined phytotoxic effect of the most mobile (and essential) metals (Cu and Zn) best reflected the observed changes in GPx induction. For many mangrove species, essential metals (Cu and Zn) show limited translocation from root to leaf tissue (Lacerda, 1998). Zinc is a necessary trace metal for protein metabolism, gene expression and structural and functional membrane integrity (Cakmak, 2000). Copper acts as a structural element in a variety of mitochondrial and chloroplast enzymes, and is a required component of enzymes related to photosynthetic electron transport (Yruela, 2005). Lead, conversely, is a non-essential metal, is largely excluded from acropetal translocation, and at elevated concentrations may be toxic to estuarine plants (Sharma and Dubey, 2005).

Several metal ions (Zn, Cu, Pb, Se and Cd) are known (via ROS generation) to cause peroxidation of plasma membrane and chloroplast membrane lipids, modifying membrane properties with subsequent negative effects on plant growth (Halliwell and Gutteridge, 1999; Devi and Prasad, 2004). Glutathione, facilitated by GPx, is preferentially active in reducing hydroperoxides responsible for lipid peroxidation (Eshdat et al., 1997). The glutathione antioxidant defense induction appeared adequate to avoid oxidative damage at 96 h, since no relationship between lipid hydroperoxides and leaf Zn concentration was observed at this time (Fig. 3c). After 2 weeks of treatment, lipid hydroperoxides increased in a dose-dependent fashion with increasing leaf Zn, again showing a positive linear relationship after 8 weeks of treatment, indicating that the *A. marina* glutathione antioxidant defense system was perhaps inadequate to stave off the oxidative stress elicited by Zn at later intervals. The increased generation of lipids with oxidative damage in metal-exposed plants has been extensively reported (Sandmann and Boger, 1980; Chaoui et al., 1997; Dixit et al., 2001; Devi and Prasad, 2004; Mishra et al., 2006). Similar to our laboratory results, chronic exposure to metal ions produces lipid peroxidation, with observed increases in the activity of antioxidant enzymes unable to adequately facilitate the reduction of excessive ROS (Prasad et al., 1999; Hartikainen et al., 2000; Mishra et al., 2006). Under field conditions however, oxidative damage to lipids in *A. marina*

leaves did not vary across locations, and there was no evidence of significant increases in lipid hydroperoxides in leaves with elevated metal concentration (Zn, Cu and Pb) (Fig. 6c). The concentrations of leaf metals observed in field plants were lower than in plants grown under laboratory conditions (Figs. 2 and 5a) (for Cu and Pb see MacFarlane and Burchett, 2001; MacFarlane, 2002). Thus perhaps the concentration of accumulated leaf metals from field locations examined did not exceed the leaves' capacity to alleviate oxidative lipid damage. Moreover, in contrast to the short-term exposure experiment conducted in the laboratory, results from the field reflect effects over chronic exposure scenarios. The relationship between leaf Zn levels and increased lipid peroxidation found under laboratory conditions (Fig. 3c) may reflect an earlier (2 and 8 weeks) biochemical response to relatively high heavy metal stress.

Despite the elevated GPx activity and oxidative damage evidenced at later time intervals, the total glutathione pool remained relatively unaffected by Zn exposure (Fig. 3b). Similar to laboratory results, in leaves from field-grown trees the glutathione pool remained unchanged across locations, with no obvious relationship between Zn, Cu and Pb in leaves and total glutathione concentration (Fig. 6b). Glutathione is the major non-protein thiol in plants involved in metal stress tolerance via a number of mechanisms (e.g. direct antioxidant metabolite, cytosolic metal chelator and the precursor of phytochelatin synthesis) (Foyer et al., 1997; Crawford et al., 2000; Prasad, 2004). Metal exposure thus represents a situation of high glutathione demand (Mendoza-Cózatl and Moreno-Sánchez, 2006; Mishra et al., 2006). Several earlier studies have demonstrated that in metal-stressed plants the levels of GSH may decrease with an increase in ambient metal concentration, although other studies have reported no change in GSH content (Gupta et al., 1998; Prasad et al., 1999; Dixit et al., 2001; Mishra et al., 2006). Our controlled Zn exposure experiment revealed that, despite constant glutathione concentrations, the total available glutathione pool was perhaps insufficient to stave-off oxidative damage in the short term and/or at higher exposure concentrations, resulting in increased lipid peroxidation. As our glutathione assay measured total glutathione (GSH and GSSG), further studies are necessary to elucidate the dynamics of glutathione cycling in Zn-stressed *A. marina* plants, since an unchanged total glutathione concentration resulting in higher levels of lipid peroxidation, may also imply that the reduction rate of the oxidised GSSG by glutathione reductase is insufficient to meet ROS demands in these metal-stressed plants.

It is often assumed, though rarely explicitly tested, that an accumulated pollutant acts first at the biochemical level, and that linked responses are later reflected at higher levels of biological organisation (biomarker early warning utility). Zinc treated seedlings were relatively resistant to Zn toxicity, and only after 8 weeks a relative reduction in final plant biomass was observed among treatments. Relative increases in GPx activity at 96 h were strongly associated with decreasing seedling biomass at 8 weeks (Fig. 4), suggesting that the activity of this antioxidant component is indeed predictive of effects at the individual level at later time intervals within the temporal exposure regime assessed. Further, a similar negative relationship between GPx increases and decreases in biomass at 8 weeks suggests the response is maintained over time (within the experimental window examined). A significant relationship between GPx and biomass in response to Zn accumulation suggests that GPx may be predictive of effects at the individual level in chronic metal exposures scenarios and predict further negative effects at later time intervals in life history, though this remains to be assessed.

Taken together, laboratory and field-based results showed that the glutathione-dependent enzyme GPx in *A. marina* leaf tissue is sensitive to metal loadings, especially Zn and Cu in combination. Metal-induced GPx activity also predicted short-term dose-dependent Zn toxicity, as evidenced by biomass reduction and increase in lipid peroxidation under controlled laboratory conditions. Reductions in plant biomass and increased mortality due to metal toxicity may have implications for primary productivity and carbon export (litterfall and detritus) to adjacent dependent estuarine systems. Overall, the results from controlled laboratory experiments, coupled with field based findings, indicate that GPx activity may be a sensitive biomarker of elevated metal exposure and bioaccumulation, and be predictive of later biological effects at the individual level, with lipid hydroperoxides as a secondary marker for prolonged exposure to high contaminant concentrations.

However, further research is required to explore temporal variability on the oxidative stress parameters studied here, i.e. greater temporal replication in the laboratory and field is still required to establish the reliability of the biochemical changes observed. Biomarkers must exhibit causal links between biochemical changes and subsequent effects at the individual level or higher, and maintain a concentration-dependent effect over time. Further research on the long-term sub-lethal effects of heavy metals at higher levels of biological organisation such as the population and their relationship to glutathione antioxidant biomarker suite responses are also important for biomarker validation and utility.

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Parte 3.

Discussão

A exposição de plantas tanto a metais pesados (Zinco, Cobre e Chumbo) como ao ozônio foi capaz de induzir alterações no status oxidativo do tecido foliar de duas espécies vegetais diferentes, comprovando o fato de que a presença destes poluentes afeta o equilíbrio entre a produção e degradação das EAO, o que acaba por resultar na oxidação de biomoléculas, comprometendo o metabolismo celular e a fisiologia dos organismos vegetais.

A análise dos parâmetros de estresse oxidativo resultantes da exposição controlada de diferentes cultivares de *P. vulgaris* (*Fepagro 26, Guapo Brilhante, Iraí, Macotaço* e *Pinto 111*) ao O_3 revelou que as diferenças na susceptibilidade dessas cultivares à toxicidade desse poluente atmosférico residem no fato da existência de diferenças no metabolismo oxidativo. Diferentes cultivares de *P. vulagaris* têm sido estudados na busca por plantas que apresentem potencial bioindicador cada vez mais sensível ao O_3. Os principais resultados desses estudos são obtidos através da análise de parâmetros fisiológicos, especialmente redução da biomassa e aparecimento de clorose foliar (Bergweiler et al. 2008; Burkey et al. 2005). Entretanto, estas respostas fisiológicas resultam das alterações bioquímicas, e essas antecedem os danos macroestruturas visíveis.

A exposição ao O_3 (AOT_{40} de 212 ppb h) durante o período de uma semana aumentou significativamente os níveis de lipoperoxidação em duas cultivares, *Fepagro 26* e *Guapo Brilhante*. Ainda, a análise da atividade da CAT revelou que apenas para essas cultivares não houve aumento na atividade da principal enzima envolvida na degradação do H_2O_2. As cultivares *Iraí, Macotaço* e *Pinto 111* aumentaram a atividade da CAT quando expostas ao O_3, o que provavelmente contribuiu para que estas cultivares não alterassem os níveis de danos

oxidativos a lipídios. O ensaio para avaliar os níveis totais das defesas antioxidante não-enzimáticas no tecido foliar revelou que as cultivares *Fepagro 26* e *Irai* diminuem os níveis de defesas antioxidantes não-enzimáticas em resposta à exposição ao O_3.

Os resultados sugerem que a *Fepagro 26* é a cultivar mais sensível aos danos oxidativos causados pelo O_3. Clebsch (2008) demonstrou que esta cultivar apresenta um decréscimo significativo na taxa de assimilação líquida de CO_2, redução essa que pode ser explicada pela limitação no processo de carboxilação, o que está associado ao decréscimo na velocidade máxima de carboxilação da Rubisco e na eficiência da carboxilação. O ozônio é conhecido por afetar o processo fotossintético, sendo que a redução na eficiência de carboxilação esta associada a prejuízos causados na fotossíntese. Leitao e colaboradores (2008) recentemente demonstraram que a exposição de plantas de *P. vulgaris* a ATO_{40} de122 nL L^{-1} h de O_3 diminui a atividade da Rubisco, mas aumenta a atividade da fosfoenolpiruvato carboxilase (PEPc). Os autores comprovam que esta redução na atividade da Rubisco foi acompanhada da diminuição na quantidade de ambas subunidades da enzima e de modificações oxidativas da menor subunidade. Nas folhas primárias, estes efeitos são acompanhados da diminuição no conteúdo de pigmentos fotossintetizantes (clorofila *a* e *b*, e carotenóides). Este conjunto de eventos metabólicos desencadeados pela presença do O_3 tem correlação com a aceleração da morte celular programada. O aumento nos níveis de lipoperoxidação observados no cultivar *Fepagro 26* pode explicar o efeito negativo sobre a taxa de carboxilação, uma vez que a desestruturação da membrana dos cloroplastos inviabiliza a manutenção de processos metabólicos essenciais.

Considerada como uma das principais moléculas antioxidantes não-enzimáticas da maioria das células aeróbicas, a GSH desempenha importante papel na regulação do status redox intracelular, na conjugação de metabólitos, na detoxificação de xenobióticos e nas reações de sinalização celular relacionadas a respostas adaptativas (Noctor et al. 2002). O

ascorbato é um antioxidante primário chave em células vegetais. Reage com EAO como o $HO^{\bullet}$ e $O_2^{\bullet -}$ e é a principal molécula antioxidante presente no apoplasto (Chen and Gallie 2004; Mittler 2002). Considerando a relevância destes dois antioxidantes não-enzimáticos na defesa contra EAO, os dados obtidos na análise dos níveis de defesas antioxidantes não-enzimáticos nos levam a supor que a diminuição observada pode estar associada a um decréscimo nas concentrações de GSH e ascorbato. Alguns autores afirmam ser o ascorbato um metabólito relacionado à primeira linha defesa não-enzimática de plantas, e que o aumento nas vias de reciclagem do ascorbato oxidado para o reduzido confere grande tolerância a plantas expostas ao O_3 (Chen and Gallie 2004). O fato da cultivar *Fepagro 26* apresentar uma diminuição nos níveis de defesas antioxidantes não-enzimáticas quando submetida a presença do O_3, aliado ao aumento na oxidação de lipídios, reforça a idéia de que dentre todas cultivares estudadas a *Fepagro 26* é a mais suscetível ao estresse desencadeado pelo O_3. Como já relatado para outras espécies vegetais sensíveis a este poluente, as ações coordenadas que resultam no aumentam a produção de EAO e inibem as respostas das defesas antioxidantes acabam por acionar as rotas de sinalização que desencadeiam a morte celular programada (Apel and Hirt 2004; Mahalingam et al. 2006; Pasqualini et al. 2007). Dessa maneira, comparada com a cultivar *Pinto 111*, reconhecida por ser sensível ao ozônio, a cultivar *Fepagro 26* apresenta ter grande potencial como espécie bioindicadora de exposição ao O_3, fato esse que pode ser comprovado pela análise dos parâmetros de estresse oxidativo aqui apresentados.

A toxicidade de diversos metais pesados encontra-se claramente associada à incapacidade dos sistemas de defesas antioxidantes de espécies vegetais em evitar o surgimento e a propagação do dano lipídico (Chaoui et al. 1997; Rama Devi and Prasad 2004; Verma and Dubey 2003). A presença de metais nas plantas não só altera a estrutura lipídica das membranas devido ao aumento de EAO geradas por reações catalisadas por metais, como também afeta as rotas de biossíntese de lipídios (Prasad et al. 1999). A exposição dos

propágulos de *A. marina* a Zinco aumenta os níveis de lipoperoxidação após 2 semanas, em uma relação que mostrou ser dependente da concentração do metal acumulado nas folhas. A análise do material de campo revelou que as folhas coletadas em locais poluídos não apresentaram aumento na concentração de lipoperóxidos, apesar da evidente presença de metais nestas folhas. Assim, em campo observa-se que não houve correlação entre os níveis de metais foliares acumulados e a indução de dano oxidativo a lipídios, ao contrário dos dados obtidos em laboratório. Tal resultado pode ser tanto explicado pelo fato de que em campo as concentrações de metais nas folhas foram menores do que as concentrações observadas em laboratório, como devido ao fato de que as plantas em campo estão adaptadas ao estresse por metais, evitando a indução de lipoperoxidação nas mesmas concentrações de metais que seriam tóxicas para as demais plantas. Diferentemente dos ensaios de exposição em laboratório, em campo observa-se o efeito crônico da exposição a metais. Desse modo, pode-se inferir que o aumento nos níveis de lipoperoxidação observados na exposição controlada de *A. marina* a Zinco caracteriza-se como uma resposta bioquímica que ocorre em estágio inicial da exposição a concentrações relativamente elevadas de Zinco.

O aumento da atividade da GPX também serve como indicativo de que o Zinco está afetando o metabolismo oxidativo das plantas expostas. A modulação na atividade da enzima inicia-se após 96h de exposição e apresenta ser dose-dependente. A análise dos resultados sugere que o estresse imposto pelo metal tem início em um espaço de tempo curto, e que o aumento na atividade da enzima é mantido ao longo de 8 semanas de tratamento. Apesar do aumento observado na atividade da GPX, os níveis de GSH total (GSH e GSSH) não apresentaram ter relação nem com as concentrações de Zinco utilizadas, nem com o tempo de exposição. Os dados obtidos em campo sugerem que a GPX desempenha um papel fundamental na proteção antioxidante enzimática em plantas que crescem sob a influência de metais como Zinco, Cobre e Chumbo, uma vez que a atividade da enzima foi aumentada nas

folhas com maior concentração de metais. A análise integrada dos resultados ainda mostra que a presença conjunta de Zinco e Cobre no tecido foliar contribui para a amplificação da resposta obtida na modulação da GPX. Assim como se observou no laboratório, os níveis totais de GSH não apresentaram variação e nem correlação com as concentrações de metais foliares.

Além de ser um importante tampão redox encontrado nos diferentes sistemas biológicos, a GSH apresenta um papel fundamental na proteção de tecidos vegetais contra a toxicidade dos metais. A detoxificação de metais envolve a síntese de peptídeos capazes de quelar metais, as chamadas Fitoquelatinas (Gratão et al. 2005). A análise da estrutura química das Fitoquelatinas revelou que a GSH atua como molécula precursora na síntese destes quelantes, que são compostos pela união de glutamato, cisteína e glicina [$(\gamma\text{-Glu-Cys})_n$ – Gly] nas proporções que variam de 2:2:1 a 11:11:1 (Prasad 2004). Alguns autores afirmam que a relevância destes polipeptídeos em proteger as plantas da toxicidade dos metais pode ser evidenciada no fato de que em plantas resistentes a metais as concentrações de GSH não diminuem quando ocorre a síntese de Fitoquelatinas, ao passo que em espécies não-tolerantes a síntese das Fitoquelatinas diminui o *pool* de GSH, comprometendo as defesas antioxidantes e aumentando a suscetibilidade das células vegetais ao estresse oxidativo (De Vos et al. 1992). Considerando assim a importância da GSH na defesa das células contra as EAO geradas pela presença de metais, os ensaios de exposição a Zinco no laboratório revelaram que a manutenção dos níveis constantes de GSH pode ter contribuído para o aumento nos danos aos lipídios, e que talvez a dinâmica de reciclagem da GSH e GSSH esteja afetada nas folhas de *A. marina* que apresentam elevadas concentrações de Zinco. Ainda, o fato das Fitoquelatinas estarem intimamente associadas à tolerância de espécies vegetais a metais evidencia a necessidade de mais estudos sobre a existência de correlação entre a síntese dessas moléculas e o perfil nos níveis de GSH observados em plantas de *A. marina* expostas a Zinco.

A avaliação da exposição, associada aos conhecimentos sobre os efeitos na integridade dos organismos vegetais e os limites considerados seguros para exposição a contaminantes, permite estabelecer as prioridades e as formas de intervenção necessárias para proteger uma população dos riscos da exposição a agentes abióticos tóxicos. O uso de parâmetros biológicos, bioquímicos e moleculares de exposição tem como com a finalidade avaliar a exposição a poluentes, estimando a existência de riscos às populações expostas (Amorin 2003). Nesse contexto, a detecção precoce de uma exposição perigosa pode diminuir significativamente a ocorrência de efeitos adversos à fisiologia das plantas e à estrutura das comunidades vegetais.

Os biomarcadores de exposição têm como finalidade confirmar e avaliar a exposição individual ou de um grupo, para uma substância em particular, buscando estabelecer uma ligação entre as respostas fisiológicas e a quantificação da exposição em um curto espaço de tempo (Peakall 1994). Dessa maneira, os biomarcadores devem ir além dos parâmetros visíveis, possibilitando o diagnóstico precoce da presença de contaminantes ambientais em uma relação dose e tempo-dependente e anterior aos danos estruturais visíveis (Ernst and Peterson 1994). A análise integrada dos resultados obtidos em laboratório e em campo mostra que a atividade da GPX em folhas de *A. marina* é sensível à presença de metais, em especial o Zinco e o Cobre. Em condições controladas de estudo a alteração na atividade dessa enzima ocorreu antes do surgimento de danos macreostruturais a plantas de *A. marina*, os quais foram observados na redução de biomassa. A existência de correlação negativa entra o aumento da atividade de GPX e a redução na biomassa sugere que a mudança na atividade dessa enzima antioxidante no tecido foliar prediz a toxicidade do Zinco em um período de tempo curto e de modo a ser dependente da concentração desse metal.

Conclusões

A partir dos dados obtidos na presente dissertação, concluí-se:

- A análise das respostas oxidativas das distintas cultivares de *P. vulgaris* expostas ao ozônio revelou que as diferenças nos parâmetros avaliados estão relacionadas com a sensibilidade das cultivares à toxicidade desse poluente.
- A diminuição nos níveis de defesas antioxidantes não-enzimáticas de plantas de *P. vulgaris* expostas ao O_3, e o aumento nos níveis de lipoperoxição, indicam que dentre as cultivares estudadas a *Fepagro 26* é a mais suscetível ao O_3.
- Comparada com a cultivar *Pinto 111*, reconhecida como um genótipo sensível ao ozônio, a cultivar *Fepagro 26* apresenta grande potencial como genótipo bioindicador de exposição ao O_3.
- A exposição controlada de *A. marina* a Zinco afeta tanto o sistema antioxidante dependente de glutationa (GSH e GPX), como também aumenta os níveis de lipoperoxidação em uma relação dose-dependente com a concentração do metal no tecido foliar.
- A existência de correlação entre aumento da atividade da GPX depois de 96 horas de exposição e a diminuição na biomassa total dos propágulos de *A. marina* após 8 semanas sugere que a alteração na atividade da enzima antecede os efeitos negativos visíveis do Zinco, indicando a possibilidade do uso desse parâmetro como bioindicador da exposição a Zinco.
- Os resultados obtidos em campo demonstraram que a atividade da GPX foi o único parâmetro analisado que mostrou ter correlação com a concentração de metais na folhas de *A. marina*, corroborando os resultados de laboratório e o potencial da atividade da GPX em estudos de bioindicação.

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