MARCELO PEDROSA GOMES

Trace-elements-tolerance in seeds and seedlings of Brazilian Savanna species is modulated by antioxidant systems

Lavras –MG

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Tolerância a elementos-traço em sementes e plântulas de especies do Cerrado brasileiro é modulada pelos sistemas antioxidantes

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia, área de concentração Fisiologia Vegetal, para obtenção do título de doutor.

Prof. Dr. Luiz Roberto Guimaraes Guilherme

Orientador

Co-Orientadores

Profa. Dra. Ângela Maria Soares

Profa. Dra. Queila de Souza Garcia

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MARCELO PEDROSA GOMES

Trace-elements-tolerance in seeds and seedlings of Brazilian Savanna species is modulated by antioxidant systems (Tolerância a elementos-traço em sementes e plântulas de espécies do Cerrado brasileiro é modulada pelos sistemas antioxidantes)

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia, área de concentração Fisiologia Vegetal, para obtenção do título de doutor.

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Prof. Dra. Ângela Maria Soares	UFLA
Prof. Antonio Chalfun Júnior, PhD	UFLA
Prof. Dr. Jose Donizeti Alves	UFLA
Profa. Dra. Luzia Valentina Modolo	UFMG
Profa. Dra. Queila de Souza Garcia	UFMG

Prof. Dr. Luiz Roberto Guimaraes Guilherme

Orientador

Lavras –MG

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To the old, new and future lovers;
Pour des nouveaux amis et expériences;
A Ângela e Queila
Aos meus pais, motivo pelo qual posso crer em mim
DEDICO

"É melhor tentar e falhar, que preocupar-se em ver a vida passar. É melhor tentar, ainda que em vão, que sentar-se fazendo nada até o final. Eu prefiro na chuva caminhar que em dias tristes em casa me esconder. Prefiro ser feliz, embora louco, que em conformidade viver."

Martim Luther King

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RESUMO

O uso de espécies arbóreas nativas em programas de fitorremediação e de recuperação de áreas contaminadas por elementos-traço atualmente tem sido reconhecido como uma alternativa positiva frente a estes processos. Neste contexto, é importante compreender a tolerância de espécies com potencial fitorremediador a este tipo de contaminante. Primeiramente, foram estudados os efeitos de arsênio (As - 0, 10, 50, 100 mg L^{-1}), cádmio (Cd - 0, 0,1, 0,3, 0,7, 1,1 mg L^{-1}) e zinco (Zn - 0, 50, 80, 120, 200 mg de L^{-1}) na germinação de sementes de quatro espécies arbóreas do Cerrado (*Anadenanthera peregrina*, *Cecropia hololeuca*, *Handroantrus serratifolius* e *Myracrodruon urundeuva*). Verificou-se uma forte relação entre o sucesso de germinação de *A. peregrina* e *M. urundeuva* na presença de As e Zn com a atividades de enzimas antioxidantes. Então, a tolerância a As e Zn foi avaliada em plântulas de *A. peregrina* e *M. urundeuva*, respectivamente. Plantas de *A. peregina* expostas ao As mostraram diminuição da absorção de P. Portanto, foram estudados os efeitos do P na tolerância ao As em plantas de *A. peregrina*. A adição de P aumentou a capacidade de fitorremediação de As em *A. peregrina* aumentando o acúmulo de As nos tecidos vegetais, ao mesmo tempo em que alivia o estresse oxidativo induzido pelo metalóide. Finalmente, foram investigados os mecanismos de tolerância de Zn de *M. urundeuva*. Verificou-se que doses de Zn superiores a 80 mg kg^{-1} foram fitoestáticas, e que a toxicidade de Zn nestas condições foram impostas pelo estresse oxidativo causado por acúmulo de H_2O_2 e sua relacionada peroxidação lipídica. A tolerância ao Zn em *M. urundeuva* está ligada à atividade de sistemas antioxidantes nas folhas que é modulada por esse metal.

Palavras-chave: Arsênio. Enzimas antioxidants. Peroxidação de lipídios. Zinco.

ABSTRACT

The use of native tree species in phytoremediation projects and in programs to recuperate trace-element-contaminated sites has been recognized as a viable strategy. In this context, it is important to understand the trace-element-tolerance of potential phytoremediator species. Firstly, the effects of arsenic (As - 0, 10, 50, 100 mg L^{-1}), cadmium (Cd - 0, 0.1, 0.3, 0.7, 1.1 mg L^{-1}), and zinc (Zn - 0, 50, 80, 120, 200 mg L^{-1}) on the seed germination of four Brazilian Savanna tree species (*Anadenanthera peregrina*, *Cecropia hololeuca*, *Handroantrus serratifolius*, and *Myracrodruon urundeuva*) were evaluated. We also examined the relationships between seed germination success of *A. peregrina* and *M. urundeuva* under As and Zn stress in terms of antioxidant enzymes activities. Due to the observed natural occurrence of *A. peregrina* and *M. urundeuva* seedlings in contaminated areas, their respective As and Zn tolerance were studied. Our nutritional studies of *A. peregrina* plants exposed to As showed that high As levels in substrate implicated in a decrease of P uptake by plants. We therefore evaluated the effects of P on the As-tolerance of *A. peregrina* seedlings. The addition of P increased the As-phytoremediation capacity of *A. peregrina* by increasing As accumulation while also alleviating As-induced oxidative stress. We also investigated the Zn-tolerance mechanisms of *M. urundeuva*. Zn concentrations greater than 80 mg kg^{-1} were observed to be phytostatic, and Zn toxicity under these conditions was imposed by oxidative stress caused by H_2O_2 accumulation and related lipid peroxidation. Zn tolerance in *M. urundeuva* was linked to the activity of antioxidant systems in their leaves that are modulated by this metal.

Keywords: **A**rsenic. Antioxidant enzymes. Lipid peroxidatin. Zinc.

SUMÁRIO

1. GENERAL INTRODUCTION

Although some trace elements such Cu, Zn, and Ni are essential nutrients, plants are often exposed to phytotoxic levels of these elements (as well as to non-essential trace elements) as a consequence of human activities (LI et al., 2005). Considerable losses of plant productivity and hazardous health effects in humans can be linked to trace element contamination of soils and water. Exposure to these toxic elements can intensify the production of reactive oxygen species (ROS) commonly observed in plant cells (GRATÃO et al., 2005). Some of those ROS species are highly toxic and must be detoxified by cellular machinery to guarantee plant survival and growth (GRATÃO et al., 2005).

Different plant organs show different responses to abiotic stress (such as trace element toxicity) during their development, and much attention is now being focused on seed responses. Seed coats provide some protection against trace element stress prior to germination, but they rupture or become more permeable during germination (KRANNE; COLVILLE, 2011). Metal(loid)s may affect seed germination through their general toxicity or by inhibiting water uptake (KRANNE; COLVILLE, 2011). A number of studies have shown that trace element exposure resulted in concentration-dependent reductions in seed germination in many different species (LEFÈVRE et al., 2009; MADZHUGINA et al., 2008; OZDENER; KUTBAY, 2009; STREET et al., 2007) – which has been attributed to induced oxidative stress (KRANNE; COLVILLE, 2011). While these studies have highlighted both inter- and intraspecific variations in seed germination rates, the limited data currently available suggest that the seeds of metal(loid)-tolerant plants can generally germinate even when exposed to high trace element concentrations, and that essential micronutrients (such as Zn)

cause damage only at relatively high concentrations (MAHESHWARI; DUBEY, 2008).

Toxic concentrations of trace elements are known to produce growth reductions and nutritional disturbances and to affect the photosynthetic rates of seedlings by interfering with photosystem and pigment biosynthesis (CUYPERS et al., 2001; EBBS; KOCHIAN, 1997; LEFÈVRE et al., 2009). Trace element toxicity has also been attributed to the overprodution of ROS (MEHARG; HARTLEY-WHITAKER, 2002) that cause oxidative damage to biomolecules and eventual cell death (GUNES et al., 2007). Plants have evolved mechanisms to protect cells and subcellular systems from the effects of ROS (e.g., superoxide radicals, hydroxyl radicals, and hydrogen peroxide) through the synthesis of enzymatic and non-enzymatic antioxidants (GUNES et al., 2007), and the following pathway of H_2O_2 production and destruction has been proposed: SODs catalyze the conversion of highly reactive superoxides to H_2O_2, and these molecules are in turn degraded by CAT, APX, and GPX; APX reduces H_2O_2 into water using ascorbate (AsA) as the electron donor, and the resulting dehydroascorbate (GSH) is cycled back to ascorbate using reduced glutathione (GSH) as the electron donor; the oxidized glutathione (GSSG) formed is converted back to GSH by NAD(P)H-dependent GR; and GPX acts on H_2O_2 to form GSSH, which is then further reduced to GSH by GR (GUNES et al., 2009).

Our view of the role of reactive oxygen species (ROS) has greatly evolved in the light of recent studies. The derivatives of the reduction of oxygen as a superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$), or singlet oxygen ($^1O_2^*$), were initially viewed as hazardous compounds, but they are now recognized as being important in the biological processes of plants. ROS appear to be central factors in plant adaptations to biotic and abiotic stress, exacerbating cell damage as well as signaling the activation of defense responses

(MILLER et al., 2008; SHETTY et al., 2008). Although high concentrations of ROS can lead to phytotoxicity, relatively low levels can function as signaling molecules under conditions of abiotic stress (GILL; TUTEJA, 2010; MILLER et al., 2008). ROS functions, their production sites in plants, their antioxidant scavenging defense functions in response to abiotic stress conditions (MILLER et al., 2008; GILL; TUTEJA, 2010), their signaling functions, and their roles in plant growth and development are now quite well documented (AHMAD et al., 2008; DEL RIO et al., 2006; GAPPER; DOLAN, 2006; TANOU et al., 2010) – but little is currently known about ROS signaling pathways or their biological roles in seeds.

The Brazilian Savanna ("Cerrado") is considered a conservation hotspot (Myers et al. 2000) but is threatened by many human activities – including intense mining activities that can release large amounts of toxic elements into the environment (SOARES; PORTO. 2007) and contaminate the soil (ALVAREZ-ROGEL et al., 2004). The use of native tree species in phytoremediation and in trace element recovery efforts at contaminated sites is becoming more frequent (MARQUES, 2000), and as these programs are directed at recovering vegetation structure (with the priority use of native species) it will be important to understand trace element tolerance in potential phytoremediator species. As such, the present study evaluated the physiological responses of seeds and seedlings of Brazilian Savanna species to trace-metals in order to identify possible tolerant species and elucidate the underlying physiological mechanisms of their tolerance.

2. CONCLUSION

We examined the As and Zn-tolerance of *M. urundeuva* and *A. peregrina* seeds respectively, and investigated the seed germination success of these species under As and Zn stress in terms of their antioxidant enzymes activities, including glutathione reductase (GR) and, especially, ascorbate peroxidase (APX).

Arsenic was observed to impact the nutritional status of *A. peregrina* seedlings, especially in terms of decreasing phosphate nutrition, and As phytotoxicity in this species was found to be due to lipid peroxidation but not hydrogen peroxide (H_2O_2) accumulation. The addition of P to the growthsubstrate increased the As-phytoremediation capacity of *A. peregrina* by increasing As accumulation and alleviating As-induced oxidative stress. Added P increased the activity of important ROS-scavenging enzymes (catalase [CAT] and APX) that help prevent lipid peroxidation in leaves.

Zinc doses greater than 80 mg Zn kg^{-1} were found to be phytotoxic, but not lethal, to *M. urundeuva* seedlings, and its toxicity under these conditions was caused by oxidative stress resulting from H_2O_2 accumulation and related lipid peroxidation. Zn tolerance in this species is linked to the activities of antioxidant systems in their leaves that are modulated by this trace metal.

In summary, we conclude that As and Zn tolerance in the seeds and seedling of the two Brazilian Savanna tree species studied (*A. peregrina* and *M. urundeuva*) is modulated by their antioxidant system activities, and APX was seen to be a central factor mediating seed and seedling tolerance. Additional molecular studies (such as genomic silencing) will almost certainly contribute to

our better understanding of the specific role of APX in trace element tolerance in these species.

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ARTIGO 1 - Toxic trace elements effects on seed germination of four Brazilian Savanna tree species

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RESEARCH NOTE

Toxic heavy metal effects on seed germination of Brazilian Savanna tree species

D.M. Duarte[1], M.P. Gomes[2], L.C. Barreto[1], M.T Matheus[1,3], Q.S. Garcia[1,*]

[1]*Universidade Federal de Minas Gerais, Laboratório de Fisiologia Vegetal, Departamento de Botânica, Laboratório de Fisiologia Vegetal, Caixa Postal 486, 31.270-970, Belo Horizonte, MG, Brazil.*

[2]*Universidade Federal de Lavras, Departamento de Biologia, Campus UFLA, 37200-000, Lavras, MG, Brazil.*

[3]*Universidade Federal do Espírito Santo, Departamento de Engenharia Florestal, Alto Universitário, Caixa Postal 16, Centro, 29.500-000, Alegre, ES, Brazil.*

Summary

The toxic effects of arsenic (0, 10, 50, 100 mg m-3), cadmium (0, 0.1, 0.3, 0.7, 1.1 mg m-3) and zinc (0, 50, 80, 120, 200 mg m-3) on the seed germination of Brazilian Savanna tree species was studied (*Anadenanthera*

* Corresponding author. Email: marcelopgom@yahoo.com

peregrina, *Cecropia sp.*, *Handroantrus serratifolius*, and *Myracrodruon urundeuva)*. Seeds differed in their abilities to germinate under trace element stress. The deleterious effects of As were quite notable. We verified a possible As-tolerance of *M. urundeuva* seeds at concentrations up to 10 mg m-3, and unlike the other species tested, this plant was highly sensitive to Cd. Zn had negative effects on seed germination even at low doses (except in *A. peregrina*). Germination induction at the highest Cd doses and with 50 mg m-3 Zn was observed in *Cecropia sp.* and *A. peregrina* seeds respectively.

Keywords: Cerrado, germination percentage, germination speed index, mining areas, phytoremediation, recovery programs

Experimental and Discussion

Environmental contamination by trace elements is now a global concern. The Brazilian Savanna ("Cerrado"), although be considered conservation hotspot (Myers et al. 2000), continues to be threatened by human activities that release large amounts of these elements into the environment (Soares and Porto 2007), including intense mining activities that frequently contaminated the soils with trace elements (Alvarez-Rogel et al. 2004).

Seeds are generally well-protected against most stress factors (Li et al. 2005), however seed germination in soils that have been contaminated with trace elements is not fully understood (Hsu and Chou 1992). The use of native tree species in phytoremediation and in recovering programs of trace element-contaminated sites is becoming more popular (Marques 2000), and as these programs are directed at recovery the vegetation structure (with the priority use of native species) it is important to understand the influence of trace elements on the germination of potential phytoremediator species. This study evaluated the

effects of trace elements on the germination of four tree species native to the Brazilian Savanna.

Plant species were chosen according their natural occurrence on trace element contaminated sites in the Brazilian savanna (*Anadenanthera peregrina* (L) Speng (Fabaceae), *Cecropia* sp (Urticaceae), *Handroantrus serratifolius* (Vahl) S. Grose (Bignoniaceae), and *Myracrodruon urundeuva* (Allemão) Engl (Anacardiaceae)). Stock solutions of arsenic (Na_2HAsO_4) were prepared containing 8000 mg m^{-3} of As, as well as cadmium ($CdSO_4$) and zinc ($ZnSO_4$) solutions containing 1000 mg m^{-3} each; these were diluted to final concentrations of As (0, 10, 50, 100 mg m^{-3}), Cd (0, 0.1, 0.3, 0.7, 1.1 mg m^{-3}), and Zn (0, 50, 80, 120, 200 mg m^{-3}). Metal doses were based on their occurrence in Brazilian Savanna soils (Marques et al. 2002) including at contaminated sites. Due to insufficient quantities of *H. serratifolius* seeds, it was decided to test them only with the most harmful metals.

Each germination box with filter paper (Whatman No. 1) received 25 seeds and 20 mL of the different As, Cd and Zn treatment solutions at the concentrations described above or with deionized water (controls) with four replicates of each metal treatment. The experiment was conducted in a growth chamber at 25°C, with a 12h photoperiod (30 μmol m^{-2} s^{-1}). The seeds were observed each day for germination, and were considered to have germinated when radicule emerged about 0.2 cm. The germination speed index (GSI) was obtained according to the formula proposed by Maguire (1962).

The results of the germination tests were expressed in percentages and were submitted to normality (Shapiro-Wilk) and homogeneity (Brown-Forsythe) tests using the JMP software program (SAS Institute Ins.). The data was expressed as the averages of four replicates, and were examined using analysis of variance also run using the JMP software program (SAS Institute Ins.).

Arsenic at all concentrations inhibited the germination of *A. peregrina* and *H. serratifolius* (Figure 1). Arsenic concentrations of 10 mg m^{-3} decreased *Cecropia sp.* germination percentages ($P < 0.05$) but not those of *M. urundeuva*; the germinations of both species were inhibited at doses of 50 mg m^{-3} and higher (Figure 1). Seeds of *Cecropia sp.* treated with 10 mg m^{-3} As showed decreased GSI in relation to the control ($P < 0.05$) (Figure 1).

No effects ($P > 0.05$) of Cd were observed on the germination percentages of *H. serratifolius* (Figure 1). While the highest Cd dose (1.1 mg m^{-3}) slightly stimulated the seed germination percentage of *Cecropia sp.*, it decreased germination in *A. peregrina* ($P < 0.05$) (Figure 1). The germination percentages of *M. urundeuva* seeds were negatively affected by Cd (Figure 1). The GSI of *Cecropia sp.* decreased independent of the Cd dosage, but Cd only affected the GSI of *A. peregrina* seeds at the highest dose ($P < 0.05$) (Figure 1).

Were observed marked deleterious effects of Zn on final seed germination in *Cecropia sp.* and on *M. urundeuva* ($P < 0.05$) even at the lowest dose of Zn (50 mg m^{-3}) (Figure 1). Decreases in GSI were noted in *M. urundeuva* seeds in the presence of Zn, with the lowest values being seen at Zn concentrations of 80 mg m^{-3} or more (Figure 1). The germination percentages of *A. peregrina* seeds were highest at 50 mg m^{-3}, but decreased at Zn concentrations above 80 mg m^{-3} (Figure 1). The GSI of *M. urundeuva* decreased with exposure to Zn, having the lowest values at the highest zinc doses (120 and 200 mg m^{-3}) ($P < 0.05$) (Figure 1).

We verified species-specific and, occasionally, dose-dependent effects of different trace elements (As, Cd and Zn) on the germination percentages and GSI of Brazilian Savanna tree species. Although there is limited evidence for influxes of metals into intact mature seeds through their seed coats (Kranner and

Colville 2011), numerous authors have reported trace element influences on germination processes (as revised by Kranner and Colville 2011).

Our study indicated elevated phytotoxicity of As in seed germination processes of studied species. There have been few reports concerning the effects of As on seed germination. Decreases (Abedin and Meharg 2002; Chun-Xi et al. 2007; Liu et al. 2007) as well slight stimulations of germination (Li et al. 2007) have been reported in As-treated seeds. Due to its chemical similarity to phosphorous (P), As (as arsenate, one of its inorganic forms - As(V)) is of particular concern in Brazilian soils. Soil management procedures to correct the pH and the application of organic matter (practices commonly used in recovery programs) may release As (in addition to P) in the soil and thus make it bioavailable. As(V) is taken up through the same mechanisms as P in plant tissues (Meharg and Hatley-Whitaker 2002) and they compete for uptake and adsorption sites (Fitz and Wendel 2002). Inside the plant cell, As(V) replaces P on ATP, forming an unstable ADP-As complex that halts cell energy flow (Meharg and Hatley-Whitaker 2002). As germination is intrinsically related to energy demands by seed embryos, As exposure will lead to germination inhibition (and GSI decreases) as was seen in all of the species at higher As levels. Seed germination of *M. urundeuva* and *Cecropia sp.* at lower doses of As was interesting since As can be adsorbed onto soil particle surfaces, leaving relatively lower concentrations of this trace element in the soil solution (Oken and Hossner 1996), and so, these both species should be interesting on As-site recovering programs.

Cadmium effects on seed germination were reviewed in depth by Kranner and Colville (2011), and increases as well as decreases in the germination of Cd-treated seeds have been reported. In most studies, however, the Cd doses used were very high and did not correspond to actual

concentrations of this metal found in most Brazilian Savanna soils. Street et al. (2007) reported decreases in the germination of *Eucomis autumnalis* (Asparagaceae) seeds at cadmium doses from 0.3 to approximately 1.2 mg m^{-3} (as we observed in *M. urundeuva*) while no effects were seen with *Merwilla natalensis* (Hyacinthaceae) seeds (as we observed with *H. serratifolius*). Similarly, we did not observe any negative impacts of cadmium on the germination percentages of *A. peregrina* except at the highest dose. The lower observed germination sensitivity to Cd can be explained by the capacity of some plants to accumulate this metal (Barceló et al. 1988) and by the nature of their seed coats (Carlson et al. 1991; Munzuronglu and Geckil 2002) and their ability to function as Cd influx barriers (Fernandes and Henriques 1991; Kranner and Coville 2011). Additionally, the effects of Cd are complex and apparently depend on metal concentrations, the plant species examined, and the treatment period (Sandalio et al. 2001), and none of these processes are well documented (Mihoub et al. 2005). According our results, *H. serratifolius* and *Cecropia sp.* may prove useful in recovery programs in areas contaminated with Cd.

Decrease in GSI was seen with *Cecropia sp.* when exposed to Cd (Figure 1), which may be associated with the effects of Cd on energy reserve mobilization (Mihoub et al. 2005) and metabolic disturbances (Bansal et al. 2000; 2002) since this trace element mimics other plant nutrients such as Ca (Clemens *et al*., 1998; Nelson, 1986) and Mg (Baszynski et al. 1980) and can interfere with their mobilization. Cd has been reported to cause alterations in the activities of several enzymes (including α and β amylases) as well as interfere with respiratory activity (Chugh and Sawhney 1996) – which will compromise reserve mobilization and embryo growth (Kabata-Pendias and Pendias 2000).

Although it is a micronutrient, Zn behaved like a hazardous metal in the germination tests of most of the species studied (Figure 1). A review by Kranner

and Colville (2011) noted that Zn influences on germination are dependent on the plant species involved and the Zn doses used. Although micronutrients such as Zn have been reported to negatively impact germination only at relatively high concentrations (Maheshwari and Dubey 2008), we noted germination inhibition in *Cecropia sp.* and decreases in the germination rate of *M. urundeuva*, reinforcing the inter- and intra-specific nature of seed germination responses to metal stress (Kranner and Colville 2011). Zn phytotoxicity (as well as that of other trace elements) may be related to the generation of reactive oxygen species (ROS) (Schutzendübel and Polle 2002; Sharma and Dietz 2009). ROS can react with nucleic acids (affecting the genetic material of the embryo) and/or seed storage compounds (such as proteins and lipids) and therefore compromise germination (Kranner and Colville 2011). The decreases in GSI may be also related to oxidative stress generated by ROS production.

Unlike the other species tested, seed germination in *A. peregrina* demonstrated Zn-tolerance up to 80 mg m^{-3} and, similar to the behavior of *Cecropia sp.* at the highest Cd-doses, *A. peregrina* seeds showed increased germination percentages at 50 mg m^{-3} Zn (Figure 1). Thus, this species can be considered the most suitable for recovery of Zn-contaminated sites. Trace element stimulation of germination at low concentrations has been reported in a number of studies (Kjaer et al. 1998; Li et al. 2007; Lefevre et al. 2009) and has been attributed to the overproduction of ROS and reactive nitrogen species (RNS) – resulting in slightly enhanced levels of oxidative stress that apparently stimulate germination (Lefevre et al. 2009). Germination requires strictly regulated concentrations of ROS (Bailly et al. 2008), which is intrinsically related to metal tolerance. Trace element tolerance in general has been associated with mechanisms of chelation, sequestration, and oxidative stress avoidance. Ferritins and metallothioneins are the main metal chelators in seeds

(Kranner and Colville 2011), and phytic acid has also been described as being able to chelate nutrient cations such as Zn (Doria et al. 2009). Biochemical antioxidant systems may also be induced through trace element exposure, maintaining intracellular redox homeostasis and preventing the accumulation of toxic ROS, while also allowing ROS-mediated signaling (Foyer and Noctor 2009).

Our results should contribute to selecting species that can be used in phytoremediation programs and to improve our comprehension of the effects of metal stress on seed germination in Brazilian Savanna tree species.

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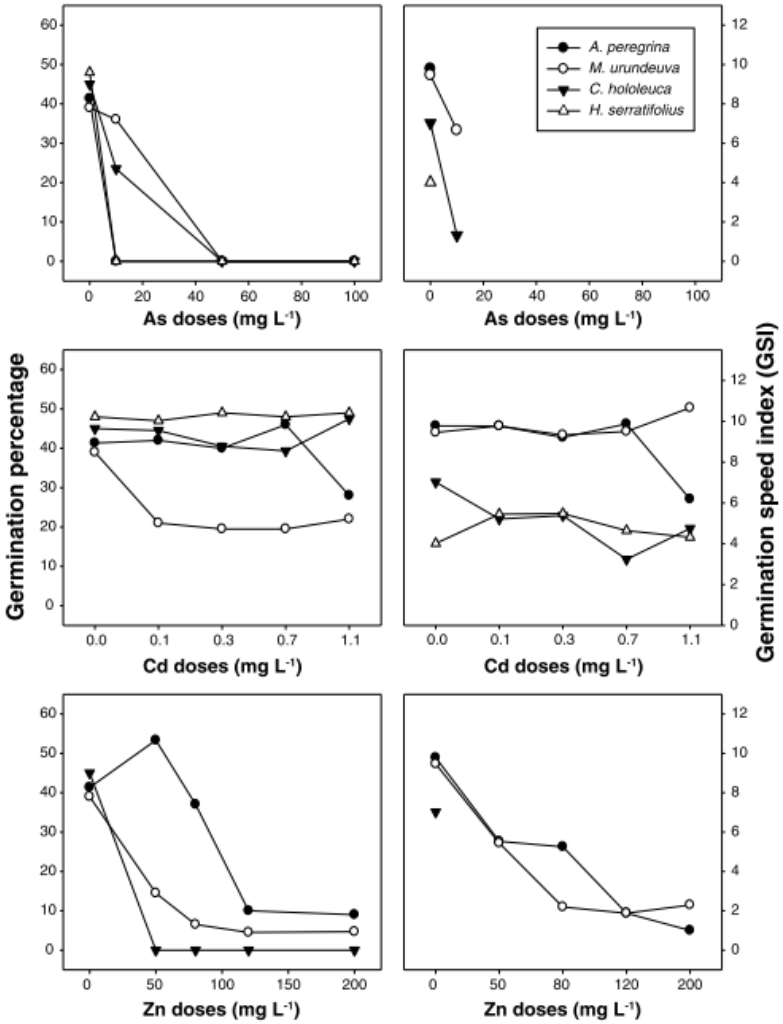

Figure 1. Final germination percentages and germination speed index (GSI) of *Anadenanthera peregrina*, *Myracrodruon urundeuva*, *Cecropia* sp and *Handroantrus serratifolius* seeds (12-h photoperiod at 25°C), in different concentrations of As (0, 10, 50, 100 mg m^{-3}), Cd (0, 0.1, 0.3, 0.7, 1.1 mg m^{-3}), and Zn (0, 50, 80, 120, 200 mg m^{-3}). Data correspond to mean ± SE of four replicates with 25 seeds to each treatment.

ARTIGO 2 – The system modulating ROS content in germinating seeds of two Brazilian savanna tree species exposed to As and Zn

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ORIGINAL PAPER

The system modulating ROS content in germinating seeds of two Brazilian savanna tree species exposed to As and Zn

M. P. Gomes[1,2], M. M. L. C. Carneiro[2], C. O. G. Nogueira[2], A. M. Soares[2] & Q. S. Garcia[3]

[1] Université du Québec à Montréal, Institut des Sciences de l'environnement, Succ. Centre-Ville, C.P. 8888, H3C 3P8, Montréal, Québec, Canada.

[2] Universidade Federal de Lavras (UFLA), Departamento de Biologia, Campus UFLA, C.P. 3037, 37200-000, Lavras, MG, Brazil.

[3] Universidade Federal de Minas Gerais (UFMG), Instituto de Ciências Biológicas, Departamento de Botânica, Avenida Antonio Carlos, 6627, Pampulha, C.P. 486, 31.270-970, Belo Horizonte, MG, Brazil.

Correspondence: M. P. Gomes, Université du Québec à Montréal, Institut des Sciences de l'environnement, Succ. Centre-Ville, C.P. 8888, H3C 3P8, Montréal, Québec, Canada.

E-mail: marcelopgom@yahoo.com.br Tel.: +1 514 987 3000 #3489. Fax. : +1 514 987 4647.

Abstract

The effects of increasing arsenic (0, 10, 50, 100 mg L^{-1}) and zinc (0, 50, 80, 120, 200 mg L^{-1}) doses on germination and oxidative stress markers (H_2O_2, MDA, SOD, CAT, APX, and GR) were examined in two Brazilian savanna tree species (*Anadenanthera peregrina* and *Myracrodruon urundeuva*) commonly used to remediate contaminated soils. The deleterious effects of As and Zn on seed germination were due to As- and Zn-induced H_2O_2 accumulation and inhibition of APX and GR activities, which lead to oxidative damage by lipid peroxidation. SOD and CAT did not show any As and Zn-induced inhibition of their activities as was seen with APX and GR. We investigated the close relationships between seed germination success under As and Zn stress in terms of GR and, especially, APX activities. Increased germination of *A. peregrina* seeds exposed to 50 mg L^{-1} Zn was related to increased APX activity, and germination in the presence of As (10 mg L^{-1}) was observed only in *M. urundeuva* seeds that demonstrated increased APX activity. All the treatments of both species in which germination decreased or was inhibited showed decreases in APX activity. *A. peregrina* seeds showed higher Zn-tolerance than *M. urundeuva*, while the reverse was observed with arsenic up to exposures of 10 mg L^{-1}.

Key-words: arsenic; enzymatic antioxidant systems; lipid peroxidation; zinc

Introduction

Although some trace elements such Cu, Zn, and Ni are essential nutrients, plants can be exposed to phytotoxic levels of these metals (as well as to non-essential trace elements) as a consequence of human activities (Li *et al.* 2005). Different plant organs show different responses to abiotic stress (such as

trace element toxicity) during their development, and much attention is now being focused on seed responses.

Seed coats provide some protection against metal(loid) stress prior to germination, but they eventually crack or become more permeable during germination (Kranner & Colville 2011). Trace elements may affect seed germination through their general toxicity or by inhibiting water uptake, and a number of studies have shown that metal(loid) exposure causes concentration-dependent reductions in seed germination in many species (Lefèvre *et al*. 2009; Madzhugina *et al*. 2008; Ozdener & Kutbay 2009; Street *et al*. 2007). While these studies have highlighted inter- and intra-specific variations in seed germination, the limited data currently available suggest that the seeds of metal(loid)-tolerant plants can generally germinate even when exposed to high metal(loid) concentrations, and that essential micronutrients (such as Zn) cause damage only at relatively high concentrations (Maheshwari & Dubey 2008).

ROS are usually considered toxic molecules as their accumulation can lead to cell damage and alterations of seed development or germination (Bailly, 2004), although ROS have now been recognized as important molecules in plant biological processes and their signaling roles in seed germination and dormancy control have been well documented. The germination phase *sensu stricto* involves the activation of a regulatory system controlled by intrinsic and extrinsic factors, ROS generation and accumulation increases in various species (Bailly et al., 2008). In addition to their recognized roles in endosperm weakening, mobilization of seed reserves, protection against pathogens, and programmed cell death, ROS have been proposed as messengers or transmitters of environmental cues during seed germination (Bailly et al., 2008). The generation of ROS in stressful germination situations (excessive heat, cold, or UV, or hypoxia stresses) would prevent radicle protrusion, and the role of ROS

would therefore reside at the interface between signaling and deleterious effects (Bailly et al., 2008).

Increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been observed in plants challenged with trace elements, resulting in slightly enhanced levels of oxidative stress (Lefèvre *et al*. 2009). The reactivation of mitochondrial metabolism that occurs after imbibition during the germination of quiescent seeds produces ROS (Pergo & Ishii-Iwamoto 2011), and the capacity of seeds to control ROS levels during germination could aid in neutralizing oxidative stress generated by the presence of trace elements. Plant antioxidative defenses fall into two general categories: 1) low molecular weight antioxidants including lipid-soluble membrane-associated antioxidants (e.g., α-tocopherol and β-carotene) and water-soluble reductants (e.g., glutathione–GSH and ascorbate), and; 2) enzymatic antioxidants (e.g., superoxide dismutase-SOD, catalase–CAT, guaiacol peroxidase-GPX, ascorbate peroxidase-APX) (Cao *et al*. 2004). The role of SOD in antioxidative defenses is to eliminate reactive oxygen species (ROS) that generate H_2O_2, with the resulting H_2O_2 being removed by CAT, APX, and GPX enzymes (Cao *et al*. 2004). Changes in antioxidant systems have been reported in seedlings following germination and growth in the presence of metal(loid)s (Kranner & Colville 2011), although information about seed antioxidant systems in response to trace elements during germination is scarce.

Soil contamination by trace elements has been rapidly increasing in Brazil, particularly in the Cerrado (Brazilian savanna) biome where ore deposits are abundant. *Myracrodruon urundeuva* and *Anadenanthera peregrina,* native species of widespread occurrence in this biome, have been found growing in soils with toxic concentrations of zinc (Zn) and arsenic (As) (Gomes 2011), and have therefore been indicated for use in recovery programs. No information was

encountered in the literature concerning the influence of As and Zn on seed germination in these species. We hypothesized the importance of antioxidant enzyme system activation during the germination of seeds exposed to As and Zn as this is a common physiological response to the presence of toxic elements (Oracz *et al.* 2007; Kranner & Colville 2011). It has likewise been reported that despite antioxidant system activation, ROS accumulation under phytotoxic conditions results in decreases in seed germinability and gradual losses of seed vigor (Oracz *et al* 2007). As such, ROS accumulation and related oxidative damage were investigated by evaluating seed H_2O_2 contents and lipid peroxidation. Specifically, this study sought to obtain new insights into the roles of ROS and antioxidant defense systems during seed germination in the presence of As and Zn by investigating: (i) seed germination responses of *M. urundeuva* and *A. peregrina* exposed to increasing As and Zn doses; and (ii) changes in their antioxidant enzymes, H_2O_2 contents, and As and Zn-induced oxidative damage during this process.

Materials and Methods

Seed Germination and Initial Growth

Seeds of *Anadenanthera peregrina* var. *falcata* (L.) Speg. (Fabaceae) and *Myracrodruon urundeuva* Fr. Allem. (Anacardiaceae) were acquired from the Seed Laboratory of the Companhia Energética de Minas Gerais (CEMIG, Belo Horizonte, Brazil). The seeds were stored in plastic bags at 30% relative humidity for 6 months until use. After surface sterilization in a 5% sodium hypochlorite solution for 5 minutes, the seeds were thoroughly rinsed with deionized water. As *M. urundeuva* seeds demonstrate physical dormancy, they were submitted to mechanical scarification using sandpaper. Arsenic (Na_2HAsO_4) and zinc ($ZnSO_4$) solutions were prepared containing 8000 and

1000 mg L^{-1} of these elements respectively. Different concentrations (0, 10, 50, 100 mg L^{-1}) of As and (0, 50, 80, 120, 200 mg L^{-1}) Zn were prepared by further dilutions of the stock solutions. The element doses used were based on concentrations encountered in Cerrado soils, including in contaminated areas (Gomes 2011).

Germination boxes were lined with filter paper (Whatman No. 1) and washed with deionized water and each received 50 seeds and 20 mL of the treatment solution. The treatments consisted of a control (deionized water) and the different As and Zn concentrations as described above. The germination boxes were arranged in a completely randomized order, and each treatment involved four replicates of 50 seeds per germination boxes. The experiments were conducted in a growth chamber at 30°C, under a 12-hour photoperiod (30 μmol m^{-2} s^{-1}, Philips T2 40W/33 lamp). The seeds were observed each day for signs of germination. Seeds were considered germinated when the radicle had emerged about 0.2 cm. The tests were terminated when there was no further germination after five consecutive days from seed treated with As or Zn.

Biochemical measurements

Biochemical measurements were made on whole seeds after 1, 3 and 5 days of treatment with the As and Zn solutions; the radicles of the germinated seeds were included in these assays at days 3 and 5.

Hydrogen peroxide contents of seeds were determined following the method of O'Kane *et al*. (1996). Seeds were ground with a mortar and pestle in liquid nitrogen. Samples were homogenized with 10 ml of perchloric acid 0.2 M and were centrifuged for 15 min at 11,000×g at 4°C. The supernatant was neutralized to pH 7.5 with KOH 4 M, and then centrifuged at 5,000×g for 3 min at at 4°C. The obtained supernatant was used for spectrophotometric determination of H_2O_2. The reaction started with the addition of 20 μl (0.25 U)

of horseradish peroxidase to the reaction mixture contained 50 µl of the collected supernatant, 12.5 mM of dimethylaminobenzoic acid and 1.25 mM of 3-methyl-2-benzothiazolidone hydrazone (final volume of 1.5 ml). The H_2O_2 content were measured by reading absorbance at 590 nm after 5 min at 25°C and comparing with the absorbance obtained with known amounts of H_2O_2.

Oxidative damage was determined by four lipid peroxidation based on the production of 2-thiobarbituric acid-reactive metabolites (particularly malondialdehyde – MDA), following the methods of Heath & Packer (1968) and Buege & Aust (1978). MDA measurements were performed as in Hodges et al. (1999), taking into account the presence of any possible interfering compounds in the assays of 2-thiobarbituric acid (TBA)-reactive substances. Approximately 0.6 g (fresh weight) of seeds were extracted with 3.0 ml of 96% (v v^{-1}) ethanol, and an equal volume of 20% TCA containing 0.5% TBA (w v^{-1}) was subsequently added to the homogenate. The mixture was heated at 95°C for 30 min., centrifuged, and the absorbance of the supernatant measured at 532 nm; non-specific absorbance was read at 600 nm and subsequently subtracted. MDA concentrations were expressed as nmol g^{-1} of seed fresh weight.

Antioxidant enzymes were extracted according Pergo & Ishii-Iwamoto (2011). Seed samples that had been sent out to germinate for 1, 3 and 5 days were transferred to a mortar and thoroughly mixed with 2.0–4.0 ml of an extraction medium containing: for catalase – 67 mM K-phosphate (pH 7.0) and 2% PVP (w v^{-1}); for ascorbate peroxidase and glutathione reductase – 50 mM K-phosphate (pH 7.0), 1 mM EDTA, 1 mM ascorbate, and 2% PVP (w v^{-1}); and for superoxide dismutase – 50 mM K-phosphate (pH 7.8), 1.0 mM EDTA, 1% PVP (w v^{-1}), and 0.1% Triton X-100 (v v^{-1}). Extracts were centrifuged for 20 min at 4000 rpm at 4°C. The supernatants were decanted and used as enzyme sources.

Catalase activity was measured in media containing 67 mM K-phosphate (pH 7.0), 10 mM H_2O_2, and 0.1–0.4 mg of the appropriate enzyme extract, and the consumption of H_2O_2 was monitored at 240 nm (ε, 0.036 mM^{-1} cm^{-1}) (Aebi 1984). Ascorbate peroxidase activity was measured in media containing 50 mM K-phosphate (pH 7.0), 0.5 mM ascorbate, 2 mM H_2O_2, and 0.1–0.4 mg of the appropriate enzyme extract, and ascorbate oxidation was measured at 290 nm (ε, 2.8 mM^{-1} cm^{-1}) (Nakano & Asada 1981). Glutathione reductase activity was measured in media containing 50 mM K-phosphate (pH 8.0), 2 mM EDTA, 0.5 mM GSSG, 0.15 mM NADPH, and 0.1–0.4 mg of the appropriate enzyme extract, and the rate of NADPH oxidation was monitored at 340 nm (ε, 6.2 mM^{-1} cm^{-1}) (Foyer & Halliwell 1976). Superoxide dismutase activity was measured according to Giannopolitis & Ries (1977) in media containing 50 mM K-phosphate (pH 7.8) 6.5 mM methionine, 150 µM nitro blue tetrazolium NBT, 4 µM riboflavin, and 0.02–0.1 mg of the appropriate enzyme extract. The reaction was started by illuminating the medium (20 W) for 20 min at 30ºC. One unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of the (NBT) photoreduction rate as read at 560 nm; the results were expressed as U of SOD mg $protein^{-1}$.

Statistical Analyses

The results were expressed as the averages of five replicates. For germination tests, the results were expressed as percentages and submitted to normality (Shapiro-Wilk) and homogeneity (Brown-Forsythe) testing using JMP software (*SAS Institute Ins.*). The oxidative damage and antioxidant enzyme data were statistically evaluated using two-way analysis of variance run on the SAS software program (*SAS Institute Ins.*). Means were compared using the Scott-Knott multiple range test at a 5% level of probability. Correlation analyses were also performed to test for relationships between the variables.

Results

Germination

Arsenic completely inhibited seed germination in *A. peregrina* at all concentrations tested (Fig. 1). The germination of *M. urundeuva* was not inhibited ($P \leq 0.05$) in the presence of the lower dose of As (10 mg L^{-1}) (Fig.1) although germination was completely inhibited at doses of 50 mg L^{-1} and higher.

Regarding Zn, *A. peregrina* seed germination percentages decreased at treatment levels above 80 ml Zn L^{-1} (Fig. 1) but increased at 50 mg L^{-1} Zn; the germination process was delayed in the presence of this metal.

The germination percentage of *M. urundeuva* decreased severely at Zn doses of 80 mg L^{-1} and higher, and the germination process was delayed in the presence of this metal.

H_2O_2 contents

The H_2O_2 contents of As-treated seeds were quite high (Table 1, Fig. 2) in comparison to the control. *M. urundeuva* seeds exposed to 10 mg As L^{-1} had H_2O_2 contents similar to those of the control at the first evaluation (Fig. 2); H_2O_2 concentrations later increased in these seeds, reaching concentrations similar to those of the highest As doses in the final evaluation (Fig. 2).

H_2O_2 contents were also found to be high in seeds exposed to Zn (Table 1, Fig.2). The H_2O_2 contents of *A. peregrina* seeds exposed to 50 and 80 mg Zn L^{-1} decreased as exposure time increased (being lower on the third day of evaluation than on the first), while the opposite effect was observed in seeds exposed to the highest Zn doses. *M. urundeuva* seeds exposed to low Zn concentrations showed slightly increased H_2O_2 content in relation to the controls in the first evaluation, followed by accentuated increases at later times.

Lipid peroxidation

Lipid peroxidation (MDA content) increased as As doses and time of exposure increased, and was greater in *A. peregrina* than *M. urundeuva* (Table 1, $P > 0.05$). A peculiar response was observed at the exposure concentration of 10 mg As L^{-1}, with a marked increase in lipid peroxidation being observed only after the third day of exposure (T_1=130.94, T_3=137.71, T_5=144.91; F=4.73**; $T_{1,3,5}$= evaluation times), especially in *M. urundeuva* seeds (Fig. 2).

Lipid peroxidation also increased as Zn doses and the times of exposure increased (but no significant interactions were observed between Zn doses and time, $P > 0.05$) (Table 1). MDA contents were higher in *M. urundeuva* than in *A. peregrina* seeds at Zn doses above 80 mg L^{-1} ($P < 0.01$).

Antioxidant enzymes

SOD activity was greater in both *M. urundeuva* and *A. peregrina* seeds in the presence of both As and Zn than in the controls, but consistently higher in the former species than the latter (Table 1, Fig. 2 and 3). SOD activity increased as exposure time to As and Zn increased in comparison to the control (Fig 3 and 4).

Similar to SOD, CAT activity was higher than the control in the presence of As and Zn and increased over time (Table 1, Fig. 3 and 4). CAT activity was greater in *A. peregrina* than in *M. urundeuva* seeds exposed to As and Zn (F=0.01^{ns} for control seeds).

APX activity decreased in the presence of higher As doses and was higher at the last two evaluation times (Table 1). *M. urundeuva* seeds showed higher APX activity than *A. peregrina* seeds ($P < 0.001$) in treatments of 0 and 10 mg As L^{-1}. Additionally, the seeds of the former species showed the highest APX activity in seeds treated with 10 mg As L^{-1} (Table 1, Fig. 4). Higher APX activity was observed in a *A. peregrina* in the 50 and 80 mg L^{-1} treatments, while

APX activity in *M. urundeuva* decreased in seeds exposed to doses ≥ 80 mg Zn L^{-1} (Fig. 3 and 4).

GR demonstrated similar activity to APX, except that it was not statistically affected by exposure time (Table 1, Figs. 3 and 4).

Discussion

The species studied here showed different responses to exposure to the two elements examined. The inhibition of seed germination in *A. peregrina* in the presence of As may be the result of reductions in seed water uptake or direct phytotoxic effects of this metalloid (Kranner & Colville 2011). *M. urundeuva* seeds demonstrated As-tolerance up to 10 mg L^{-1}, with higher doses inhibiting germination.

There have been relatively few reports discerning the effects of As on seed germination. Decreases (Abedin & Meharg 2002; Chun-Xi *et al*. 2007; Liu *et al*. 2007) as well slight stimulations of germination (Li *et al*. 2007) have been reported in As-treated seeds. As replaces P on ATP inside the plant cell, forming an unstable complex ADP-As that halts cell energy flow (Meharg & Hartley-Whitaker 2002). Because germination is intrinsically related to the energy demands of the seed embryo, As may interrupt the energetic processes – leading to the germination inhibition seen in both species at high As exposure levels.

Germination decreases with higher doses of Zn, and germination delays at low doses were seen in both *A. peregrina* and *M. urundeuva* seeds. Essential micronutrients such as Zn are normally only toxic at relatively high concentrations (Maheshwari & Dubey 2008). Induced reactive oxygen species (ROS) generation has been reported under metal(loid) stress (Schützendübel & Polle 2002; Sharma & Dietz 2009). Depending on their ability to regulate internal levels of ROS (and so to tolerate oxidative stress), these compounds (i.e.

H_2O_2) as well as the reactive nitrogen species (RNS) induced by exposure to Zn may be deleterious (as seen at 50 mg L^{-1} Zn in *M. urundeuva*) or slightly stimulatory (as seen at 50 mg L^{-1} Zn in *A. peregrina*) to germination percentages and velocities. Unregulated ROS molecules can chemically react with nucleic acids and affect the genetic code of the embryo as well as with seed storage compounds such as proteins and lipids – compromising the germination process (Kranner & Colville 2011). Slightly enhanced levels of oxidative stress, however, have been observed to stimulate germination (Lefèvre *et al*. 2009) as germination processes are strictly regulated by ROS concentrations (Bailly *et al*. 2008), which, in turn, are intrinsically related to metal(loid) tolerance.

Toxic metal(loid) interference in seed imbibition has been widely reported (Ahsan *et al*. 2007; Kuriakose & Prasad 2008; Li *et al*. 2005; Siddiqui *et al*. 2009) and oxidative stress mediated by toxic elements exposure will increase ROS production (Kranner & Colville 2011) – with losses of seed viability being attributed to increased lipid peroxidation mediated by ROS (Hendry 1993). We observed increased H_2O_2 content and lipid peroxidation in all of the As and Zn treatments (Table 1; Fig. 2 and 3), indicating that As and Zn had direct effects on seed germination by increasing their oxidative stress. Additionally, H_2O_2 content and lipid peroxidation was higher in *A. peregrina* and in *M. urundeuva* seeds in the presence of As and Zn, respectively, and lower germination was observed in these cases - which indicates increased H_2O_2 accumulation and related lipid peroxidation activities in the presence of the most hazardous element for each species. *M. urundeuva* showed less H_2O_2 content and lipid peroxidation than *A. peregrina* seeds (in which no germination was verified) in the presence of As. Moreover, increased H_2O_2 content and lipid peroxidation coincided with the cessation of germination of *M. urundeuva* seeds treated with 10 mg As L^{-1} (3 days after incubation). Likewise, *M. urundeuva*

showed higher H_2O_2 content and lipid peroxidation rates than *A. peregrina* in the presence of Zn, which also coincided with decreasing seed germination (at Zn doses above 80 mg L^{-1}). Decreasing H_2O_2 contents in *A. peregrina* seeds exposed to 50 and 80 mg Zn L^{-1} over time are also related to their relatively unchanged lipid peroxidation activities, and reflected in their increased germination percentages when compared to others Zn treatments. The increased H_2O_2 contents of seeds seen just 24 hours after imbibition indicates that As and Zn-induced ROS production occurs early, during seed imbibition, as was reported by Oracz *et al*. (2007) in mustard seeds exposed to sunflower phytotoxins.

Lipid peroxidation is one of the major effects of oxidative stress, leading to the deterioration of polyunsaturated fatty acids and severe damage to cell membranes (Oracz *et al*. 2007), as has been reported in many plant species following exposure to trace elements (Çavuşoğlu *et al*. 2009; Lin *et al*. 2009; Pandey *et al*. 2009; Tiwari *et al*. 2009; Wang *et al*. 2008). In the present study, we observed that As and Zn impose precocious oxidative stress through H_2O_2 accumulation and associated membrane damage (by lipid peroxidation) that can delay or inhibit seed germination. The accumulation of lipid peroxidation products such as malondialdehyde (MDA) and thiobarbituric acid reactive substance (TBARS) has frequently been observed as a consequence of exposing seeds to metal(loid)s, and concentration-dependent increases in lipid peroxidation have likewise been reported (Li *et al*., 2007).

Strictly regulated concentrations of ROS are currently viewed as being essential for germination (oxidative window) (Bailly *et al*. 2008), and the higher germination percentage of *A. peregrina* seeds treated with 50 mg Zn L^{-1} may be attributable to the enhanced level of oxidative status through ROS generation (these seeds showed higher H_2O_2 contents in relation to the controls) which,

according to Lefèvre *et al*. (2009), can stimulate germination. ROS have been recognized as important signaling molecules during seed germination. Seed germination in sunflowers (Orack *et al*., 2009) and in *Arabidopsis thaliana* (Leymarie *et al*., 2012) has been associated with ROS accumulation (superoxide and H_2O_2) in their embryonic axes and radicles respectively. Likewise, Leymarie *et al*. (2012) reported the existence of an efficient cell-to-cell ROS-propagation system in germinating seeds. Increased NADPH-oxidase activity (a superoxide radical source) likewise (Müller *et al*. 2009; Orack *et al*., 2009) reinforces the role of ROS in seed germination. Slight stimulations of germination by low concentrations of Cd (Lefèvre *et al*. 2009), As (Li *et al*. 2007), and Cu (Kjaer *et al*. 1998) have been reported. Metal(loid) influences on seed germination are closely related to the stimulation/inhibition of antioxidant enzymes, reinforcing the dependence of germination on fine-turned signaling networks that involve both pro- and anti-oxidants (Bailly *et al*. 2008).

Cellular antioxidant systems maintain intracellular redox homeostasis, preventing the accumulation of toxic ROS while allowing ROS-mediated signaling to occur (Foyer & Noctor 2009). We observed a strong relationship between germination percentages and antioxidant system levels (Fig. 1 to 3). The higher SOD activity levels are in accordance with the increases in H_2O_2 levels and related lipid peroxidation observed in the presence of As and Zn – and higher SOD activities were observed as a result of increased ROS production. At 10 mg As L^{-1}, SOD activity was positively related to H_2O_2 content ($r=0.906^{*}$) and MDA content ($r=0.994^{***}$) in *M. urundeuva* seeds at the third day of germination (when maximum germination was observed and did not differ from the control), demonstrating the importance of SOD in the maintenance of ROS scavenging. SOD, the first defense enzyme against oxidative stress (Pompeu *et*

al. 2008), converts O_2^- into H_2O_2, while CAT, APX and GPX detoxify H_2O_2 (Dixit *et al*. 2001; Li *et al*. 2007).

As expected, CAT activity was similar to that of SOD, and both enzymes did not show any kind of As or Zn-inducing inhibition – indicating the importance of the coupled activity of these enzymes in assuring the oxidative status of the seeds during germination, as was suggested by Pergo & Ishii-Iwamoto (2011). On the second day of germination, the CAT activity of *A. peregrina* seeds exposed to 50 mg Zn L^{-1} was positively ($r=0.966^{**}$) associated with their H_2O_2 contents, but this relationship was negative in seeds exposed to 200 mg Zn L^{-1} ($r=-0.973^{***}$). Increasing germination was observed at the same time, demonstrating the importance of CAT in scavenging H_2O_2 produced by SOD and thus favoring seed germination - with increased H_2O_2 contents resulting in increased SOD activity (positive relationship) or decreased H_2O_2 contents due to increased SOD activity (negative relationship). These results also suggest that once exposed to Zn, the induced H_2O_2 accumulation leads to the stimulation of CAT production. The expression and activities of most antioxidant enzymes are stimulated by ROS accumulation (Apel and Hirt *et al*. 2004). Polidoros and Scandalios (1999) demonstrated a direct signaling action of H_2O_2 in regulating antioxidant gene responses in maize. High concentrations of H_2O_2 induced the expression of *Cat1* and *Cat3* genes, while lower doses inhibited them. We observed, however, that CAT stimulation from exposure to toxic concentrations of As or Zn was insufficient to avoid H_2O_2 accumulation and related lipid peroxidation and did not prevent deleterious effects on seed germination. Similar results were reported by Oracz *et al*. (2007) in mustard seeds exposed to sunflower phytotoxins.

An important factor in enzyme activity is substrate affinity. While CAT has low affinity for H_2O_2 (being active only at high peroxide concentrations),

APX and other peroxidases have high substrate affinities and will detoxifying peroxides even at low concentrations (Gechev *et al*. 2006; Jaleel *et al*. 2009).

Considering the germination percentages associated with H_2O_2 content and lipid peroxidation as described above, our study confirmed the importance of APX as a key enzyme in germination as a positive relationship was observed between MDA production and APX activity in the control plants beginning on the first day of evaluation in both species studied ($r=0.934^{*}$ and 0.959^{***} in *A. peregrina* and *M. urundeuva* respectively). APX was especially important under As and Zn stress conditions, as: 1) APX activity increased in *M. urundeuva* seeds exposed to 10 mg As L^{-1} (where maximum germination and slower H_2O_2 accumulation were observed) and a positive relationship was established between MDA production and APX activity in these seeds at the time of the second assessment (third day of germination; r=0.899*). MDA contents and APX activities were also negatively correlated at 50 and 100 mg As L^{-1} when no germination was observed (r=-0.840 and -0.952^{***} respectively); 2) lipid peroxidation as well as SOD and CAT activity in *A. peregrina* seeds treated with 50 and 80 mg L^{-1} of Zn were not statistically different from the controls on the first day, although their H_2O_2 contents and APX activities did increase; at the next evaluation period, increased APX activity was followed by decreasing H_2O_2 content and increased seed germination. At concentrations of 120 and 200 mg Zn L^{-1}, in which very low germination was verified, MDA content was negatively related to APX activity on both the third (r=-0.856 and -0.895, respectively) and fifth days – but only at the highest Zn dose ($r=-0.907^{***}$); likewise, when *M. urundeuva* (which was more prone to Zn toxicity) was exposed to concentrations of 120 and 200 mg Zn L^{-1}, its H_2O_2 content was observed to be negatively related to APX activity on both the third ($r=-0.984^{***}$ and -0.037, respectively) and fifth days ($r=-0.958^{***}$ and -0.997^{***}), and 3) all

treatments which resulted in low or inhibited germination showed decreases in APX activity in both species – indicating a positive relationship between APX activity and germination percentages. Additionally, MDA content and APX activity were negatively correlated in these seeds.

GR activity followed the same pattern as APX in being dramatically reduced by the highest As and Zn concentrations, and significant delays or a lack of germination were observed (Fig. 1, 3 and 4). As with APX, GR activity increased at As and Zn levels that permitted germination (Fig. 1, 3 and 4). As GR and APX are linked by the gluthatione-ascorbate cycle (recycling oxidized ascorbate after catalysis by APX) (Gechev *et al*. 2006), our data suggest that decreased APX and GR activities (or their inhibition) will contribute to increasing H_2O_2 levels in As and Zn treated seeds.

The susceptibility of APX (as well as GR and CAT) to Cd inhibition was reported by Romero-Puertas *et al*. (2002) who noted increases in protein carbonylation targeting the same proteins as those involved in the response to hydrogen peroxide treatment. Protein carbonylation is a common oxidative protein modification in which the side-chains of Lys, Arg, Pro, and Thr are converted to aldehyde or keto groups (Gonçalves *et al*. 2007). Cd was probably causing ROS accumulation that resulted in protein oxidation (Romero-Puertas *et al*. 2002). The same process may have been occurring in the present study, so that when the oxidative status provoked by the presence of Zn or As exceeded the tolerance limits of the two species tested their antioxidant systems were inactivated.

We can conclude that the deleterious effects As and Zn had on seed germination were due to As- and Zn-induced H_2O_2 accumulation and the inhibition of APX and GR activities, which led to oxidative damage by lipid peroxidation. The sensibility of both species is linked to the effectiveness of the

ROS scavenging systems in preventing lipid peroxidation. SOD and CAT activities increased and did not show As or Zn-induced inhibition as was observed with APX and GR. However, APX and GR activities were closely related with seed germination success under As and Zn stress (specially APX activity). *A. peregrina* seeds demonstrated higher Zn-tolerance than *M. urundeuva* seeds, with the reverse situation being observed for As (up to concentrations of 10 mg L^{-1}).

AUTHORS CONTRIBUTION STATEMENT

M.P.G, M.M.L.C and C.O.C.N. performed experiments and analyzed data; M.P.G and Q.S.G designed the experiments and wrote the paper; A.M.S and Q.S.G gave technical support and conceptual advice.

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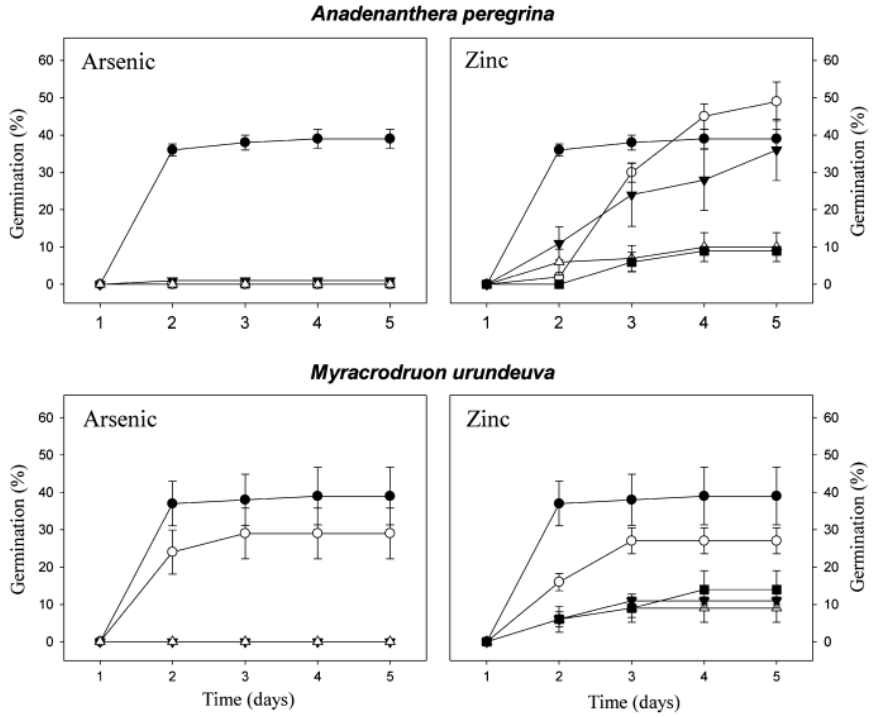

Fig. 1 Germination percentages of Anadenanthera peregrina and Myracrodruon urundeuva seeds exposed to increasing arsenic (0, 10, 50, and 100 mg L^{-1}) and zinc (0, 80, 120, and 200 mg L^{-1}) doses. Values are means ± SE of five replicates. The concentrations of added As were 0 (filled circle), 10 (open circle), 50 (filled inverted triangle), and 100 (open triangle) mg L^{-1}. The concentrations of added Zn were 0 (filled circle), 50 (open circle), 80 (inverted triangle), 120 (open triangle), and 200 (filled square) mg L^{-1}

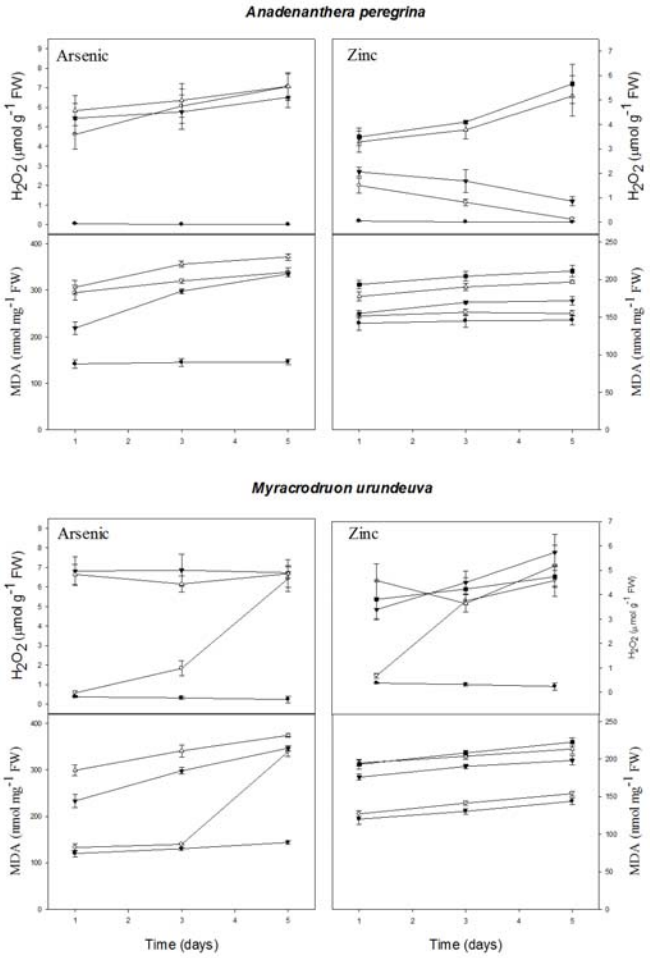

Fig. 2 Time-course of H_2O_2 concentration and lipid peroxidation (MDA) in *Anadenanthera peregrina* and *Myracrodruon urundeuva* seeds exposed to increasing arsenic (0, 10, 50, and 100 mg L^{-1}) and zinc (0, 80, 120, and 200 mg L^{-1}) doses. Values are means ± SE of five replicates. The concentrations of added As were 0 (filled circle), 10 (open circle), 50 (filled inverted triangle), and 100 (open triangle) mg L-1. The levels of added Zn were 0 (filled circle), 50 (open circle), 80 (filled inverted triangle), 120 (open triangle), and 200 (filled square) mg L^{-1}.

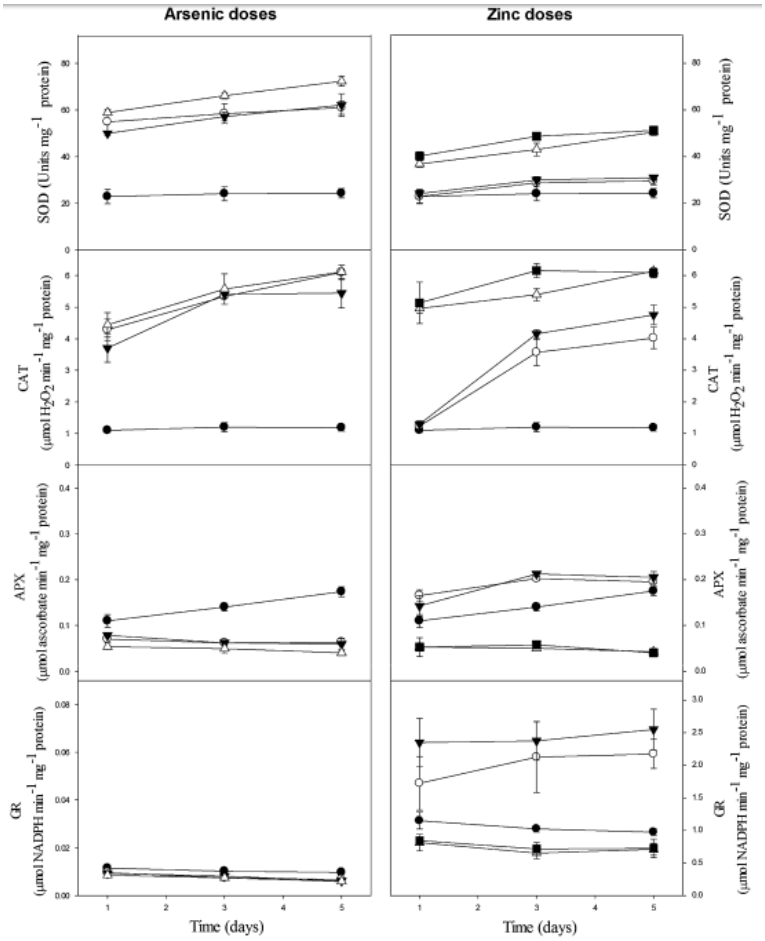

Fig. 3 Time-course of lipid peroxidation (MDA), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activities in *Anadenanthera peregrina* seeds exposed to increasing arsenic ((0, 10, 50, and 100 mg L^{-1}) and zinc (0, 80, 120, and 200 mg L^{-1}) doses. Values are means ± SE of five replicates. The concentrations of added As were 0 (filled circle), 10 (open circle), 50 (filled inverted triangle), and 100 (open triangle) mg L^{-1}. The levels of added Zn were 0 (filled circle), 50 (open circle), 80 (filled inverted triangle), 120 (open triangle), and 200 (filled square) mg L^{-1}.

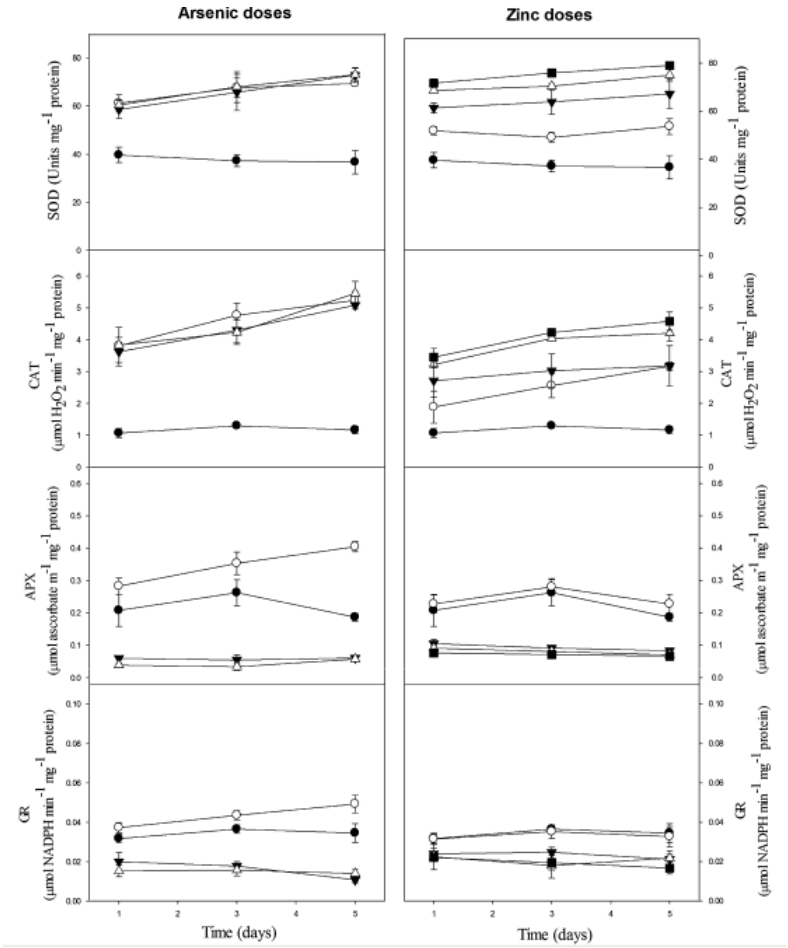

Fig. 4 Time-course of lipid peroxidation (MDA), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activities in *Myracrodruon urundeuva* seeds exposed to increasing arsenic ((0, 10, 50, and 100 mg L^{-1}) and zinc (0, 80, 120, and 200 mg L^{-1}) doses. Values are means ± SE of five replicates. The concentrations of added As were 0 (filled circle), 10 (open circle), 50 (filled inverted triangle), and 100 (open triangle) mg L-1. The levels of added Zn were 0 (filled circle), 50 (open circle), 80 (filled inverted triangle), 120 (open triangle), and 200 (filled square) mg L^{-1}.

Table 1 Results of the two-way ANOVA and Skott-Knott tests for the effects of As/Zn addition (mg L^{-1}) and time of exposure (days) on H_2O_2 (mmol g^{-1} FW), MDA (nmol g^{-1} FW), SOD (U mg^{-1} protein), CAT (μmol H_2O_2 min^{-1} g^{-1} protein), APX (μmol ascorbate m^{-1} mg^{-1} protein) and GR (μmol NADPH min^{-1} mg^{-1} protein) activity in *Anadenanthera peregrina* and *Myracrodruon urundeuva* seeds.

Source of variation	H_2O_2	MDA	SOD	CAT	APX	GR
Arsenate						
As	630.7***	2165.0***	493.8***	842.2***	494.2***	87.2***
Time	34.5***	640.9***	41.2***	131.7***	9.5***	2.5 ns
Species	19.5***	283.7***	123.4***	63.2***	550.2***	585.8***
As × time	17.3***	88.0***	6.7***	13.2***	7.9***	3.0*
As × species	65.9***	228.0***	12.6***	8.8***	336.9***	87.2***
Time × species	1.07 ns	86.8***	0.0 ns	3.8*	5.9**	2.5 ns
As × time × species	5.25***	52.5***	0.4 ns	2.1 ns	14.5***	3.0*
Scott-Knott multiple range test						
As (mg L^{-1})						
0	0.16c	137.8d	30.8c	1.1c	0.18b	0.022b
10	4.25b	260.9c	61.0b	4.9a	0.20a	0.026a
50	6.35a	288.3b	62.0b	4.5b	0.06c	0.013c
100	6.45a	341.3a	66.4a	4.9a	0.04d	0.012c
Time (days)						
1	3.79c	218.4c	50.7c	3.2c	0.11b	0.018
3	4.15b	253.4b	55.5b	4.0b	0.12a	0.020
5	4.96a	299.4a	58.9a	4.4a	0.13a	0.018 ns
Species						
A. peregrina	4.56a	272.7a	51.0b	4.1a	0.07b	0.010b
M. urundeuva	4.04b	241.5b	59.1a	3.6b	0.16a	0.027a
Zinc						
Zn	271.6***	769.4***	586.7***	630.9***	388.6***	80.7***
Time	30.6***	124.7***	52.1***	189.5***	17.2***	0.1 ns
Species	144.6***	13.1***	3198.5***	237.7***	35.8***	427.8***
Zn × time	5.82***	1.4 ns	6.3***	15.7***	4.3***	1.8 ns
Zn × species	41.6***	59.2***	58.6***	42.7***	89.8***	43.9***
Time × species	14.3***	13.9***	10.8***	26.9***	15.5***	0.9 ns
Zn × time × species	17.7***	1.8 ns	0.8 ns	13.3***	6.8***	1.4 ns
Scott-Knott multiple range test						
Zn (mg L^{-1})						
0	0.16d	137.8e	30.8e	1.1e	0.18b	0.022c
50	1.90c	147.5d	39.3d	2.7d	0.21a	0.023b
80	3.03b	176.7c	46.2c	3.1c	0.13c	0.026a
120	4.27a	196.0b	57.3b	4.6b	0.06d	0.014d
200	4.33a	205.4a	61.1a	4.9a	0.06d	0.013d
Time (days)						
1	3.32c	162.9c	44.0c	2.6c	0.12b	0.020
3	2.68b	173.9b	47.1b	3.5b	0.12b	0.020
5	3.22a	181.2a	49.8a	3.8a	0.14a	0.019 ns
Species						
A. peregrina	2.17b	171.0b	33.9b	3.7a	0.12b	0.013b
M. urundeuva	3.31a	174.4a	60.07a	2.9b	0.14a	0.026a

Treatment means from ANOVA. Values followed by the same letter, within the same source of variation, are not significantly different ($P < 0.05$) by the Scott-Knott multiple range test

ns not significant, *F* ratio ($P < 0.05$)

*, ** and *** significant at $P < 0.05$, 0.01 and 0.001, respectively

ARTICLE 3 - The effects of arsenic on the growth and nutritional status of *Anadenanthera peregrina*, a Brazilian savanna tree

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ORIGINAL PAPER

The effects of arsenic on the growth and nutritional status of *Anadenanthera peregrina*, a Brazilian savanna tree

Marcelo Pedrosa Gomes[1,3*], Daniela Moreira Duarte[2,3], Pedro Luis Silva Miranda[2], Leilane Carvalho Barreto[2,3], Miele Tallon Matheus[3,4], and **Queila Souza Garcia[2]**

[1] Université du Québec à Montreal, Institut des Sciences de l'environnement, Succ. Centre-Ville, C.P. 8888, H3C 3P8, Montréal, Québec, Canada.

[2] Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Departamento de Botânica, Avenida Antonio Carlos, 6627, Pampulha, Caixa Postal 486, 31270-970, Belo Horizonte, MG, Brazil

[3] Plant Biology Postgraduate Program- UFMG

[4] Universidade Federal do Espírito Santo, Departamento de Engenharia Florestal, Alto Universitário, Caixa Postal 16, Centro, 29.500-000, Alegre, ES, Brazil

Key words: heavy metal / phytoremediation / contamination / phytotoxicity

Abstract

Anadenanthera peregrina is a Brazilian savanna tree species that occurs naturally in arsenic (As)-contaminated areas, and its As resistance has been associated with arbuscular mycorrhizal fungi (AMF) symbiosis. A plant's ability to survive in stressful environments is correlated with its nutrition status, which can be affected by As uptake. The present study evaluated the influence of As on the concentrations and distribution of nutrients in the roots and shoots of *A. peregrina* grown in the absence of AMF. These plants were grown in substrates spiked with 0, 10, 50 and 100 mg As kg^{-1} for 25 d under greenhouse conditions, and the concentrations of essential macro- (P, K, Ca, Mg, N and S) and micro- (Fe, Mn, Cu, Zn, B and Mo) nutrients in the roots and shoots were then determined. Enhanced As levels increased the concentrations of K, S, and N and decreased P, Ca, Mg and Fe. Although the deleterious effects of As on the plants were striking, the internal As levels were high, which indicated some tissue tolerance of *A. peregrina*.

1 Introduction

Arsenic (As) is ubiquitous in the environment, with natural concentrations generally ranging from 0.1 to 40 mg kg^{-1} in uncontaminated soils (*Kabata-Pendias* and *Adriano*, 1995). In Brazil, especially in the Minas Gerais State (MG), background As soil concentrations are becoming increasingly higher due to mining wastes containing arsenopyrite (FeAsS), whose oxidation results in the mobilization and migration of As into the environment (*Murciego* et al., 2009). We reported soil As concentrations as high as 27.000 mg kg^{-1} in mine spoils at Santa Luzia (MG, Brazil) (*Gomes*, 2011). Due to the carcinogenicity and mutagenicity of this element there is an urgent need to remediate As-contaminated environments (*Liu* et al., 2005).

Common physical or chemical techniques used to remediate soils or sediments polluted by metals are expensive and unsuitable for situations affecting extensive areas (*Dary* et al. 2010), while biotechnological approaches have received a great deal of attention in recent years. Phytoremediation (the use of plants for metal reclamation) has emerged as an environmentally friendly proposal and may be the most cost-effective treatment for metal-polluted soils, especially in cases of extensive pollution (*Dary* et al. 2010). Within this context, plants naturally occurring in contaminated areas may have potential for use in phytoremediation programs.

Arsenic, a non-essential element, interferes with plant metabolism and inhibits plant growth (*Dixon*, 1997) by affecting nutrient uptake and distribution as well as by competing directly with nutrients and/or altering metabolic processes (*Meharg* and *Hartley-Whitaker*, 2002; *Tu* and *Ma*, 2005). Both inorganic forms of As (As[V] and As[III]) are highly toxic to plants (*Quaghebeur* and *Rengel*, 2003), with As(V) being taken up via the phosphate transport systems and therefore competing with P uptake (*Meharg* and *Hatley-Whitaker*, 2002). Within the cell, As(V) replaces the phosphate group in ATP and forms an unstable ADP-As complex, disrupting energy flow (*Ullrich-Eberius* et al., 1989). At low levels, however, arsenate can increase P uptake by plants, possibly by provoking an As-induced physiological P deficiency (*Carbonell* et al., 1998; *Buriló* et al., 1999) as arsenic can substitute for P in specific molecules but cannot perform its biochemical functions (*Tu* and *Ma*, 2005). Arsenic also influences other elements used by plants (*Tu* and *Ma*, 2005), and increasing N, P, K, Ca and Mg concentrations were reported by *Carbonell-Barrachina* et al. (1997) in bean shoots; conversely, *Carbonell-Barrachina* et al. (1994, 1998) noted reductions in B, Cu, Mn, Zn, K, Ca and Mg uptake in tomato plants.

We verified the natural occurrence of *Anadenanthera peregrina* var. *falcata* (an important woody leguminous tree of Brazilian savanna popularly known as "angico-vermelho") in highly As-contaminated soils, which levels ranging from 5 to 576 mg kg^{-1} (*Gomes*, 2011). In spite of the high As levels in these soils, plants appeared to be healthy. However, all samples growing on As-contaminated soil showed arbuscular mycorrhizal fungi (AMF) symbiosis, that according to *Gomes* et al. (2011a) allows the plant survive under the As-stress condition. Plants without AMF symbiosis showed several As-intoxication effects, e.g. growth reduction (*Gomes* et al., 2011a). So, a nutritional study was carried out to investigate the possible As-deleterious effects on nutrient uptake and distribution on *A. peregrina* plants, aiming to explain its sensitivity to the metalloid in AMF symbiosis absence. Moreover, little information has been available until now about how As affects nutrient acquisition and distribution in woody perennial plants. Therefore, our study may contribute to this knowledge.

2 Material and Methods

2.1 Greenhouse experiment

The plant substrate used consisted of a sand and vermiculite mixture (1 : 1 v : v, which was autoclaved at 121°C for 30 min). Arsenic was added to the substrate at 0 (control), 10, 50 and 100 mg of Na_2HAsO_4 kg^{-1} substrate. Arsenic doses were chosen based on the soil As-levels distribution where *A. peregrina* plants were found growing (*Gomes*, 2011). The designated amounts of As and 2.5 g L^{-1} of extended time-release fertilizer with a N-P-K ratio of 10-10-10 (Vida Verde Co., São Miguel) were mixed with the substrate, whose pH (1 : 1 soil : water ratio) was then checked and adjusted to 7.1 $\pm$ 0.1. Firstly, *Anadenanthera peregrina* seeds were germinated in Styrofoam boxes containing vermiculite substrate without As addition, and the seedlings were grown under greenhouse

conditions (15-31 °C; average photosynthetically active radiation 825 μmol m^{-2} s^{-1}) until the stage of first pair of full expanded leaves. Healthy specimens of similar size were selected and transferred to substrates with different As levels where they grew with daily irrigation. Fortnightly, 20 mL of a half-strength Hoagland solution was applied. At 25 d after transplanting, when symptoms of As toxicity were present, plants were harvested and washed thoroughly with tap water. Roots and shoots were separated and then rinsed quickly with 0.1 mol L^{-1} HCl solution followed by several rinses with deionized distilled water. All samples were oven-dried for 3 d at 50-55°C. The plant biomasses were determined and the dried material was ground to a powder with a mortar and pestle for chemical analysis.

2.2 Chemical analyses

Plant samples were digested in a microwave oven (ETHOS 1, Milestone Italy) in 5 mL of concentrated HNO_3 (GR), first at 80°C for 10 min and then at 180°C for 15 min. After digestion, the solutions were cooled and diluted to 50 mL using ultra-pure water. Arsenic concentrations were determined using a graphite furnace atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 400, Norwalk, CT) following USEPA Method 7060A (*Chen* and *Ma*, 1998). Nitrogen and phosphorus concentrations in solution were measured according to *Oliveira* (1986) and *Sarruge* and *Haag* (1974), respectively, sulfur by turbidimetry and K, Ca, Mg, Fe, Mn, Cu, Zn, B, and Mo concentrations were determined using a flame atomic absorption spectrometer (Perkin-Elmer AAnalyst 400, Norwalk, CT). The accuracy of the elemental analysis was checked by carrying a standard reference material (BCR 414 Plankton, Community Bureau of Reference) through digestion and analysis.

2.3 Statistical analyses

The results were expressed as the averages of four replicates. The data were statistically analyzed using one-way analysis of variance run on the SAS software program (*SAS Institute Ins*., 1996). Correlation and regression analyses were also performed to test for relationships between the variables.

3 Results and Discussion

3.1 Plant growth and visual symptoms

Accentuated phytotoxicity symptoms were observed after 25 d of exposure to high As concentrations, with the plants showing wilting, yellowing, and leaf losses. During harvesting, we noted a thickening and blackening of the roots. Biomass production in *A. peregrina* decreased as As concentrations increased (Fig. 1). According the equation of the relationship between dry biomass production and As concentrations (Fig. 1), As critical dose of substrate (CDS – As dose of substrate that causes 10% yield decrease) and the CDS_{50} (As dose of substrate that promotes a decrease of 50% on yield) was approximately 1.8 and 7.6 mg kg^{-1}, respectively. The biomass of plants exposed to 10 mg As kg^{-1} was only 38% of that of the control. At 50 and 100 mg As kg^{-1}, biomass reductions were even greater (~31% of the control). Moreover, Fig. 1 shows a steep decrease up to ~20 mg As kg^{-1}, after which the rate of decrease was diminished (Fig. 1). CDS values were well below those found by *Gomes* et al. (2011a) for *A. peregrina* cultivated at As levels under AMF (*Acaulospora scrobiculata*) presence. Our results indicate that, in absence of mycorrhiza symbiosis, As exposure negatively affects plant growth, even at low concentrations, although from ~20 to 100 mg As kg^{-1} the phytotoxic effects of As on *A. peregrina* biomass production was more or less constant regardless of its exact concentration in the substrate.

Wang et al., (2010) report reduction of 50% of dry biomass production at 0.8 mg As kg^{-1} for rice plants cultivated in nutrient solution. *Singh* et al. (2007) reported a 63% yield decrease for *Phaseolus aureos* yield grown at 0.749 mg As kg^{-1}. In contrast to these species considered As-sensitive, *A. peregrina* seedlings did not show a continuous decrease of yield with increasing As concentrations, which suggest some As tissue-tolerance of this species. Growth reduction is a clear plant response to As phytotoxicity and can be related to the generation of free oxygen species (such as superoxide and hydroxyl radicals, and hydrogen peroxide) that can damage proteins, amino acids, and nucleic acids (*Dat* et al., 2000). Decreases in photosynthetic rates, chlorophyll concentration, and Calvin cycle reactions have also been attributed to As exposure (*Stoeva* et al., 2004) and can lead to plant growth reductions or even death (*Paliouris* and *Hutchinson*, 1991). The occurrence of symptoms such as those seen in the present work may also be associated with multiple deficiencies of several nutrients essential to the formation, expansion and functioning of chloroplasts, and the synthesis of cell-wall material (*Barceló* and *Poschenrieder*, 1992).

The severe growth reduction in the presence of As, is contrary to the natural occurrence of the species in soils where As levels range from 5 to 539 mg kg^{-1} (*Gomes*, 2011). Differences in As forms between the soil, nutrient solution and the substrate used here alter As availability and compartmentation. In addition, AMF colonization was verified in all the root samples of *A. peregrina* collected from As-contaminated areas (*Gomes*, 2011) and the As resistance of this species has been attributed to the symbiotic association (*Gomes* et al., 2011a). AMF-colonized plant did not show growth reduction in the presence of As compared to the plants grown in As absence (*Gomes* et al., 2011a). The AMF are known to affect the physical-chemical characteristics of ions in the soil solution (*Göhre* and *Paszkowski*, 2006) and promote plant

establishment and growth under stressful environmental conditions (*Miller* et al., 1990) such as trace element contamination. Reduction of As phytotoxicity by AMF has been widely discussed and may be attributed to the ability of the fungi to improve and modulate plant nutrition, mainly in relation to P (*Smith* et al., 2010). Accordingly, in the absence of AMF symbiosis, the marked negative effects of As on *A. peregrina* may be associated with changes in the uptake of soil nutrients.

3.2 Arsenic distribution

Arsenic concentrations in plant roots (201 to 676 mg kg^{-1} DW) were always greater than in shoots (87 to 205 mg kg^{-1} DW) at all substrate As concentrations (Fig. 2). Root and shoot As concentrations showed linear increases as substrate As concentrations increased (Fig. 2). We also identified a positive correlation between As concentrations in shoots and roots (r = 0.994**) of plants exposed to higher As levels (Tab. 1). According to the relationship between dry biomass production and shoot As concentrations ($Y = 265.55e^{(-15.77x)}$; $r^2 = 0.84$), the As toxicity critical level ($_{As}$TCL - arsenic-concentration in shoots that causes a 10% yield decrase) was approximately 9 mg kg^{-1} (SDW). This value was greater than that found by *Kabata-Pendias* and *Adriano* (1995) (2 mg kg^{-1} DW for several species), which may indicate a possible As tissue-tolerance of the species studied here.

Plants demonstrate various metal-resistance strategies (*Papazoglou* et al., 2005). Plants using an exclusion strategy reduce their uptake of metals or limit its transport to growing shoots, which reduces the toxic effects of these metal elements in photosynthetic regions. Metal-resistant plant species are frequently used for re-vegetation of bare soils (phytostabilization) where high trace element concentrations otherwise inhibit plant growth (*Papazoglou* et al.,

2005). *A. peregrina* appears to use an As-tolerance strategy and, its relatively high TCL, might therefore be useful in phytostabilization programs. Arsenic uptake is related to metalloid availably, among other factors (*Smith* et al., 2010), which would explain increases in As concentrations in tissues as As substrate-concentrations increase. Unlike P, As generally has low mobility with respect to its translocation from roots to shoots (except among hyperaccumulator plants; *Zhao* et al., 2008). The rapid reduction of arsenate to arsenite in the roots, followed by complexation with thiols (and possible sequestration in the root vacuoles) may explain the observed As transport limitations (*Zhao* et al., 2008).

3.3 Phosphorus distribution

Phosphorus is an important nutrient for energy transfer and protein metabolism in plants (*Marschner*, 1995). Both root (6.2 to 3.8 g kg^{-1} DW) and shoot (1.97 to 1.19 g kg^{-1} DW) P concentrations decreased in *A. peregrina* as As substrate concentrations increased (Fig. 2). These reductions in P accumulation as As concentrations increased were probably due to either As phytotoxicity or competitive uptake (*Wang* et al., 2002). Reductions in P accumulation were also seen in the As-hyperaccumulator plant *Pteris vittata* (*Tu* and *Ma*, 2005) and rice (*Wang* et al. 2010) grown under As. Despite the decreases observed in P concentrations at CDS, the concentrations of this mineral in the roots (5.8 g kg^{-1} DW) and shoots (15.3 g kg^{-1} DW) were comparable to the control plants. So, at these substrate As-concentrations, P uptake may not be limited by As phytotoxicity or chemical competition. At higher As levels the restriction of P uptake may be one of the major factors affecting plant growth.

Significant correlations between P and As concentrations were observed (Tab. 1). Root P concentrations were positively correlated with root As concentrations at high As levels ($r = 0.973^{**}$). At 10 mg As kg^{-1}, root P

concentrations were negatively correlated with shoot As concentrations (r = -0.995**). However, at 50 and 100 mg As kg^{-1} this correlation was positive (r = 0.992* and 0.954* respectively; Tab. 1). Arsenic tissue-tolerance in plants may be partially explained by P/As ratios (*Liu* et al., 2005). Because it is a chemical analogue to P, As may exert its toxicity in plants by interfering with the many physiological functions performed by P (*Meharg* and *Hatley-Whitaker*, 2002), with increasing P/As ratios leading to decreasing As competition for phosphorus binding sites (*Smith* et al., 2010). As was observed in the present study, when exposed to low arsenic levels (10 mg kg^{-1}) P is translocated from the roots to the shoots, as can be seen in the positive correlation between P and As concentrations there (r = 0.915*; Tab. 1). But, when As uptake increased (50 and 100 mg As kg^{-1} treatments), more As was retained in the roots (exclusion strategy) and more P was then needed in the roots than the shoots to maintain viable P/As ratios (*Carbonell* et al., 1998; *Burló* et al., 1999).

3.4 Potassium distribution

The presence of As increased K concentrations in the shoots (12.1 to 22.1 g kg^{-1} DW) but it was still mainly concentrated in the roots (10.8 to 38.0 g kg^{-1} DW; Fig. 2). At CDS, root (13.7 g kg^{-1} DW) and shoot (22.5 g kg^{-1} DW) K concentrations were higher than those of control plants. These data suggest an important role of K and the increase on K uptake may be a mechanism of As resistance by maintenance of plant growth even at low substrate As concentration. A negative correlation between root K and shoot As concentrations was seen at 10 mg As kg^{-1} (r = -0.899*). At 50 mg As kg^{-1}, however, there was a positive correlation between K and As root concentrations (r = 0.890*; Table 1). According to *Mengel* and *Kirkby* (1987), K is preferentially transported to the shoots in plants and has close relationships with

protein synthesis, cytokinin supply, and plant growth, and also serves as a dominant cation for counterbalancing anions in plants (*Marschner*, 1995). At low As levels (10 mg As kg^{-1}), K was mainly transported to the shoots, probably because root As levels were not very high. The higher K concentrations in the roots of plants exposed to As levels higher than 10 mg As kg^{-1} may be attributed to As-depressing effects on root growth.

3.5 Calcium distribution

Calcium concentrations in both roots (27.0 to 19.1 g kg^{-1} DW) and shoots (17.0 to 10.0 g kg^{-1} DW) decreased in the presence of As but Ca was mainly concentrated in the roots (Fig. 2). Calcium is of fundamental importance for maintaining membrane functions, cell-wall integrity (*Tu* and *Ma*, 2005), and plant growth. The greater amounts of Ca found in the roots may be explained due its relative immobility in plants. Ca transport and distribution in plants depends primarily on transpiration rates and the duration of transpiration (*Marschner*, 1995). Lower concentrations of Ca in response to increasing doses of As were also reported in the hyperaccumulator plant *Pteris vitatta* (*Tu* and *Ma*, 2005; *Cao* et al., 2004). According to *Tu* and *Ma* (2005), the fact that increasing As concentrations in shoots decrease Ca concentrations suggests that Ca has a limited role in plant defenses against As toxicity.

3.6 Magnesium distribution

Like Ca, Mg concentrations in both roots (6.2 to 4.0 g kg^{-1} DW) and shoots (7.1 to 3.5 g kg^{-1} DW) decreased in the presence of As (Fig. 2). Mg accumulated principally in roots with increasing As concentrations up to ~27 mg As kg^{-1} soil, beyond which Mg concentrations became higher in the shoots (Fig. 2). Trace elements are known to interfere with chlorophyll biosynthesis (*Cagno*

et al., 1999; *Chugh* and *Sawhney*, 1999; *Horváth* et al., 1996), and as Mg serves as the central atom of the chlorophyll molecule and as a co-factor in many enzymes activating phosphorylation process (*Tu* and *Ma*, 2005), increased Mg transport to shoots may help ensure the maintenance of chlorophyll biosynthesis and avoid further damage to the photosynthetic system. Similar results were observed by *Tu* and *Ma* (2005) and *Cao* et al. (2004).

3.7 Nitrogen and sulfur distribution in plants

Nitrogen and sulfur are important components of amino acids and are biologically ubiquitous elements (*Wiedenhoeft* et al., 2006). Both nutrients were predominantly found in the shoots, but their concentrations increased in the roots of As-treated plants as compared to controls (Fig. 2). The higher concentrations of these two elements in the shoots than in the roots are probably related to the maintenance of protein synthesis, electron transfer in photosynthesis and respiration processes (*Wiedenhoeft* et al., 2006). The addition of As led to increased N (1.2 to 3.1 g kg^{-1} DW) and S (3.2 to 3.4 g kg^{-1} DW) concentrations in the shoots. However, we did not observe significant differences in N and S concentrations in the shoots (1.5 and 3.4 g kg^{-1} DW, respectively) in relation to the control (Fig. 2) at CDS (where minimal stress was occurring).

In response to As-related oxidative stress, plants increase the activities of antioxidant enzymes (e.g. superoxide dismutase, ascorbate peroxidase, catalase; *Cao* et al., 2004; *Hartley-Whitaker* et al., 2001; *Miteva* and *Peycheva*, 1999), which appears to be reflected in increasing demands for nitrogen and sulfur (higher N and S concentrations) in As-exposed plants. A positive correlation was observed between As and N in roots ($r = 0.899^{*}$ and 0.903^{*}) and in shoots ($r = 0.903^{*}$ and 0.892^{*}) of plants grown in substrates containing 50 and

100 mg As kg^{-1}. However, a significant correlation between As and S was seen only in the roots (r = 0.879* and 0.881*). Sulfur is closely linked with phytochelatin metabolism involved in As tolerance (*Hartley-Whitaker* et al., 2001). Glutathione, a sulfur-containing tripeptide thiol with the formula γ-glutamate-cysteine-glycine, and a precursor of phytochelatins (*Rosen*, 2002) is considered to be a very important antioxidant involved in the cellular defense against toxicants (*Scott* et al., 1993). Hence, high As concentrations in roots may result in correspondingly higher S concentrations. The absence of any correlation between S and As concentrations in the shoots may be related to increases in S binding which decreases S translocation from roots to shoots, as was reported by *Gomes* et al. (2011b) for *Salix humboldtiana* growing in trace element-contaminated soils.

3.8 Micronutrient distributions

Micronutrients are important in plant nutrition and are associated with various enzyme systems (*Marschner*, 1995). Arsenic did not influence ($P \geq 5\%$) the concentrations of the micronutrients Cu, Zn, B and Mn (Tab. 2). However, overall Fe tissue concentrations decreased as substrate As concentrations increased (Tab. 2) and were therefore significantly negatively correlated with As concentrations (r = -0.896 to -0.898 for roots and r = -0.898 to -0.954 for shoots). Decreasing Fe concentrations have previously been reported in plant tissues exposed to trace elements (*Gomes* et al., 2011b; *Tu* and *Ma*, 2005; *Sandalio* et al., 2001; *Gussarson* et al., 1996). *Cao* et al. (2004), however, reported increases in plant micronutrient uptakes under As stress.

4 Conclusions

We observed a strong negative influence of As on the growth and nutritional status of *Anadenanthera peregrina* seedlings. Our result showed that in the absence of AMF colonization, seedlings were highly prone to the As-phytotoxic effects, and could live only at concentrations much lower than those observed *in situ*. However, the internal As-toxicity critical levels were high, which indicates some tissue-tolerance. In addition to P, whose role in As resistance is widely known, N and S uptake and distribution may also aid plant growth in the presence of high As concentrations. The concentration of these two elements did not significantly differ from control plants under minimal As stress conditions but were greatly increased in the presence of high As levels.

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Table 1: Linear correlation coefficients (*r*) of arsenic (As) concentrations in the roots and shoots of *Anadenanthera peregrina* in relation to root and shoot dry matter yields and nutrient concentrations.

Variable	Root As			Shoot As		
	10	50	100	10	50	100
	mg (kg substrate)$^{-1}$					
Plant biomass	–0.916*	–0.383	–0.439	–0.628	–0.392	–0.499
Root nutrients						
As				0.274	–0.162	0.994**
P	–0.359	–0.031	0.973**	–0.995**	0.992**	0.954*
K	0.169	0.890*	–0.099	–0.899*	–0.304	–0.017
Ca	–0.247	0.537	0.427	0.831	0.822	0.512
Mg	–0.586	0.384	0.667	–0.339	0.800	0.732
N	–0.587	0.879*	0.891*	–0.578	0.117	–0.748
S	–0.587	0.879*	0.881*	0.01	0.145	–0.312
Fe	–0.765	–0.896*	–0.898*	–0.654	0.345	0.276
Cu	–0.765	0.321	–0.564	–0.562	–0.789	–0.437
Zn	0.324	0.537	0.763	0.634	0.543	0.754
B	0.756	0.231	0.356	–0.568	0.801	–0.756
Mn	–0.123	0.745	–0.123	0.243	0.543	–0.578
Shoot nutrients						
As	0.274	–0.162	0.994*			
P	0.638	0.858	0.093	0.915*	0.125	0.174
K	–0.203	0.754	–0.300	–0.796	–0.464	–0.378
Ca	–0.455	0.398	–0.030	0.730	–0.544	–0.061
Mg	0.761	0.101	–0.460	–0.160	0.717	–0.547
N	–0.245	–0.380	0.140	0.578	0.903*	0.892*
S	–0.452	–0.123	0.049	0.475	0.154	0.650
Fe	0.654	–0.876	–0.543	–0.743	–0.898*	–0.954*
Cu	0.627	0.756	0.111	0.245	0.546	0.806
Zn	0.548	0.645	0.120	–0.676	–0.167	–0.534
B	0.345	0.615	0.783	–0.561	–0.125	0.238
Mn	0.428	0.487	0.654	0.756	–0.453	–0.546

*r0.05 = 0.878; ** r0.01 = 0.959

Table 2: Micronutrient concentrations (Fe, Mn, Cu, Z and B) in the roots and shoots of *Anadenanthera peregrina*.

As concentrations / mg kg^{-1}	Fe	Cu	Zn	B	Mn
Roots					
0	413.1a	15.1	124.8	112.7	68.1
10	374.9ab	11.9	154.7	116.4	54.3
50	291.5b	14.6	167.3	142.8	58.7
100	197.3c	17.3	169.2	136.2	49.9
VC (%)	32.15**	ns	ns	ns	ns
Shoots					
0	532.0a	5.6	132.1	90.1	74.0
10	446.5b	6.4	142.1	95.7	57.8
50	378.1b	6.0	126.2	104.1	65.2
100	345.0c	5.9	158.7	98.3	70.9
VC (%)	11.8*	ns	ns	ns	ns

ns, *, and **: value not significant ($p \geq 5\%$), significant ($p \leq 5\%$), and significant ($p \leq 1\%$) by F test, respectively.

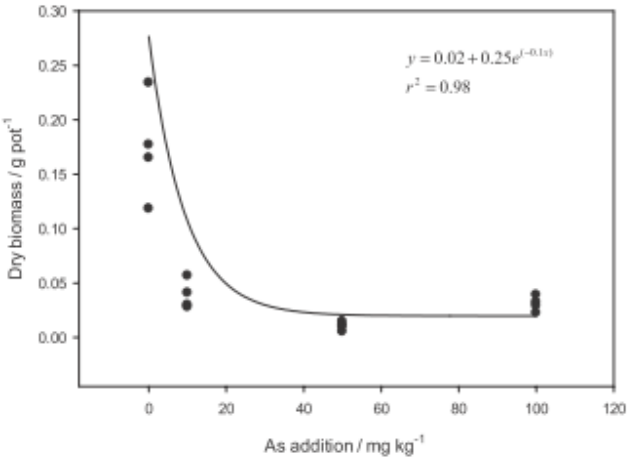

Figure 1: Relationship between dry biomass production and substrate Arsenic (As) concentrations in *Anadenanthera peregrina*. The regression curve refers to the mean values of four replicates.

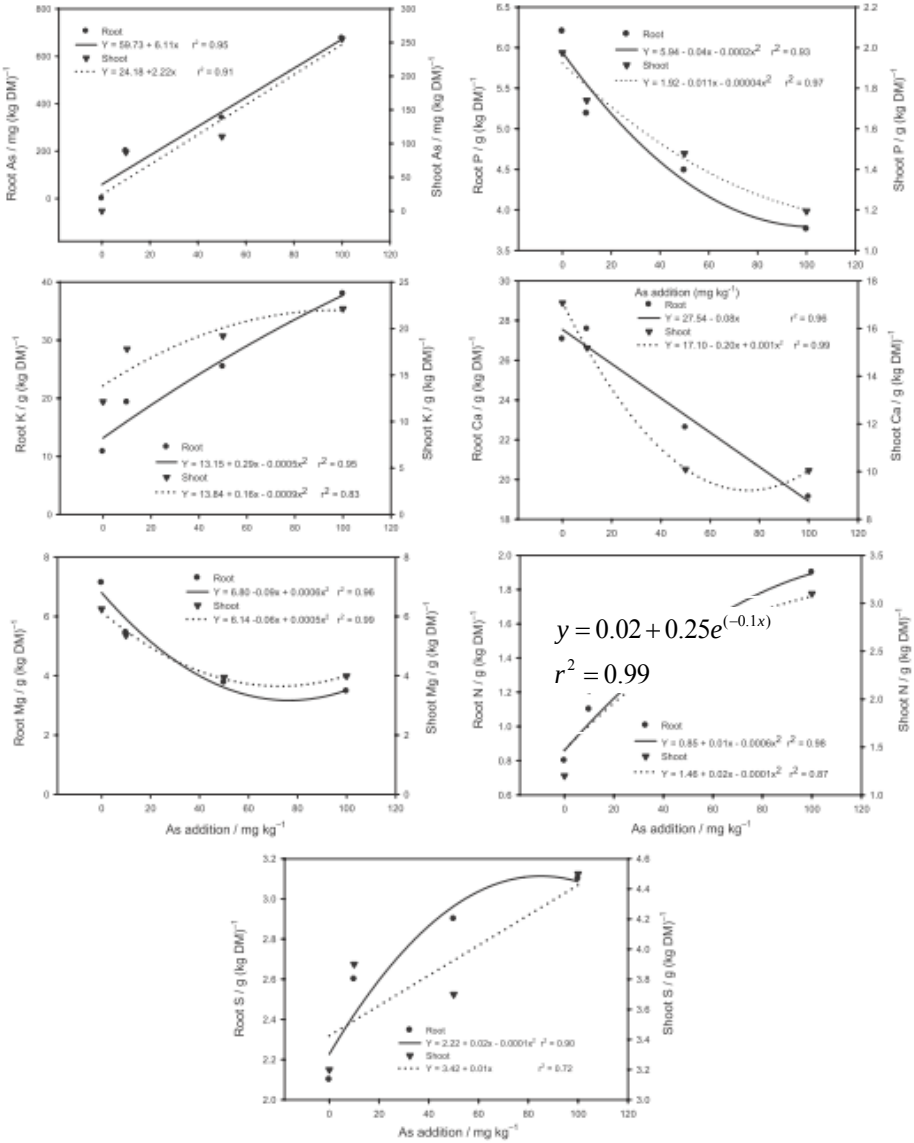

Figure 2: Relationships between As, P, K, Ca, and Mg concentrations in the roots and shoots of *Anadenanthera peregrina* with increasing substrate arsenic (As) concentrations. Values are means of four replicates.

ARTICLE 4 - Phosphorus improves arsenic phytoremediation by *Anadenanthera peregrina* by alleviating induced oxidative stress

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ORIGINAL PAPER

Phosphorus improves arsenic phytoremediation by *Anadenanthera peregrina* by alleviating induced oxidative stress

Marcelo Pedrosa Gomes[1,2,*], Marilia Carvalho[2], Geila Santos Carvalho[3], Teresa Cristina Lara Lansa Sa e Melo Marques[3], Queila Souza Garcia[4], Luiz Roberto Guimarães Guilherme[3], Ângela Maria Soares[2]

[1] Université du Québec à Montréal, Institut des Sciences de l'environnement, Succ. Centre-Ville, C.P. 8888, H3C 3P8, Montréal, Québec, Canada. E-mail: marcelopgom@yahoo.com.br

[2] Universidade Federal de Lavras, Departamento de Biologia, Campus UFLA, C.P. 3037, 37200-000, Lavras, MG, Brazil.

[3] Universidade Federal de Lavras, Departamento de Ciências do Solo, Campus UFLA, 37200-000, Lavras, MG, Brazil.

[4] Universidade Federal de Minas Gerais, Departamento de Botânica, C.P. 486, 31270-970, Belo Horizonte, MG, Brazil

P REDUCE As PHYTOTOXICITY IN *Anadenanthera peregrina*

Abstract

Due to similarities in their chemical behaviors, studies examining interactions between arsenic (As) – in special arsenate - and phosphorus (P) are important for better understanding arsenate uptake, toxicity, and accumulation in plants. We evaluated the effects of phosphate addition on plant biomass and on arsenate and

phosphate uptake by *Anadenanthera peregrina,* an important Brazilian savanna legume. Plants were grown for 35 days in substrates that received combinations of 0, 10, 50, and 100 mg kg^{-1} arsenate and 0, 200, and 400 mg kg^{-1} phosphate. The addition of P increased the arsenic-phytoremediation capacity of *A. peregrina* by increasing As accumulation, while also alleviating As-induced oxidative stress. Arsenate phytotoxicity in *A. peregrina* is due to lipid peroxidation, but not hydrogen peroxide accumulation. Added P also increased the activity of important reactive oxygen species-scavenging enzymes (catalase and ascorbate peroxidase) that help prevent lipid peroxidation in leaves. Our findings suggest that applying P represents a feasible strategy for more efficient As phytoremediation using *A. peregrina*.

KEY WORDS antioxidant enzymes, arsenic, fertilization, lipid peroxidation

1. Introduction

Arsenic (As) is a toxic element and increasing environmental As concentrations are of great global concern (Hingston *et al.*, 2001). Increased As levels have been observed in soils worldwide and also in Brazil, especially due to mining activities (Ono *et al.*, 2011; Bundschuh *et al.*, 2012). Both arsenite [As(III)] and arsenate [As(V)] are common in the soils, but studies on As toxicity have largely focused on As(V) because it is the dominant form of As in aerobic soils and is readily absorbed by plants (Gunes *et al.*, 2009).

With the development of phytoremediation technologies for metal-contaminated soil reclamation in Brazil, many studies have been carried out lately (Araújo *et al.*, 2011; Melo *et al.*, 2011; Schneider *et al.*, 2012), yet information concerning the use of native woody species in recovery programs is still limited (Marques *et al.*, 2000). Some of these plants show high heavy metal tolerance and biomass production (Marques *et al.*, 2000) - important characteristics for successful recovery programs of metal(loid) contaminated

sites. The use of legume plants associated with nitrogen fixing bacteria in phytoremediation programs is particularly interesting since anthropogenically polluted soils often contain low levels of organic matter and plant growth is restricted by nitrogen deficiencies (Pajuelo *et al.*, 2009). *Rhizobium*-legume interactions can improve the nitrogen content of soils and facilitate plant growth in the absence of externally supplied nitrogen (Lafuente *et al.*, 2009; Pajuelo *et al*., 2009). Legumes are usually the first colonizers of poor and degraded soils (Lafuente *et al.*, 2009; Pajuelo *et al*., 2009), and *Anadenanthera peregrina* (Fabaceae) has been reported to occur naturally in As-contaminated sites in Brazilian savanna (Gomes, 2011). These plants are often healthy and nodulated (Gomes, 2011). Due to its good adaptation to soils of low fertility and poor physical characteristics, *A. peregrina* have been commonly used in the revegetation of degraded sites (Tótola and Borges, 2000). This is a woody tropical species that shows low initial growth rate (Gross et al., 2004). However, after the first months of growth, biomass production is high and plants can reach up to 20 meters in height (Silva and Barbosa, 2000). These plants are often used as wood product sources, and due to their high exploration, this species was indicated as high priority for *in situ* conservation (Vieira *et al*. 2002).

Arsenic toxicity in plants (as well as toxicity of other heavy metals) has been attributed to the generation of reactive oxygen species (ROS) (Meharg and Hartley-Whitaker, 2002) that cause oxidative damage to biomolecules and eventual cell death (Gunes *et al.*, 2007). Plants have evolved mechanisms to protect cells and subcellular systems from the effects of ROS (i.e. superoxide radicals, hydroxyl radicals, and hydrogen peroxide) by synthesizing enzymatic and non-enzymatic antioxidants (Gunes *et al.*, 2007). Among the enzymatic systems, ROS-scavenging enzymes (such as superoxide dismutase [SOD], catalase [CAT] and ascorbate peroxidase [APX]) are frequently used as

indicators of oxidative stress in plants (Gunes *et al.*, 2009). Another marker of oxidative stress is malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids produced during the peroxidation of membrane lipids (Mittler, 2002). Although changes in these oxidative stress markers have been reported under various conditions of heavy metal stress (Calgaroto *et al*., 2010; Gupta *et al*., 2011; Marques *et al*., 2011), little information is currently available concerning the effects of As on antioxidant enzymes (Gunes *et al*., 2009).

Plant phosphorus (P) nutrition have been described as one of the main factors affecting the ability of plants to survive upon exposure to toxic concentrations of As (Smith *et al*., 2010). Both P and As are Group V_A elements and have similar chemical properties (Tu and Ma, 2003). Arsenate can replace P in biological molecules such as ATP, DNA, and proteins (Meharg, 1994) and interfere with vital biological processes (Stoeva *et al*., 2004; Panda *et al*., 2010). As such, a possible As-tolerance mechanism would involve increased P uptake and the maintenance of higher P/As ratios (Smith *et al*., 2010). Physiological and electrophysiological experiments have shown that As(V) competes with phosphate (PO_4^-) for uptake (Esteban *et al*., 2003; Pickering *et al*., 2000), making it difficult for plants to distinguish between them, so that their uptake is very likely to be competitive (Tu and Ma, 2003). A number of studies have shown that soil P contents are related to As uptake rates and contents in plants (Tu and Ma, 2003; Gunes *et al*., 2009). Both decreased (Meharg and Macnair, 1991) and increased (Tu and Ma, 2003) plant uptake of As and decreased As phytotoxicity with exposure to P have been reported (Meharg and Macnair, 1991; Tu and Ma, 2003; Gunes *et al*., 2009).

In addition to its influence on As uptake by plants, P may also have a partial protective effect against As-induced oxidative stress (Gunes *et al*., 2009) - although there have been very few reports to date concerning this phenomenon.

Gunes *et al*. (2009) reported lowered lipid peroxidation levels in chickpea plants grown in As-contaminated soil when 400 mg P kg^{-1} was supplied. Decreased CAT and APX activity and low non-enzymatic antioxidant system concentrations were also noted by these authors. As such, in addition to the need for more studies concerning As-induced oxidative damage and antioxidant responses (Singh *et al*., 2007), it will also be important to understand how the addition of P affects As behavior and As-induced oxidative stress in plants.

The present study evaluated the effects of arsenate and phosphate on growth, on the uptake of As and P, and on biochemical responses of the Brazilian woody legume *A. peregrina*. Specifically, we investigated the effects of the addition of P on As-induced oxidative damage by evaluating the oxidative stress markers (MDA and hydrogen peroxide [H_2O_2]) and ROS scavenging enzyme activities (SOD, CAT and APX). Additionally, as P fertilization is a common strategy for managing Brazilian savanna soils (due to their low available P), we examined how arsenate and phosphate interact in *A. peregrina* plants with the goal of establishing recommendations for P fertilization to improve plant growth rates and their phytoremediation potential.

2. Materials and Methods

2.1. Greenhouse experiments

The plant substrate used consisted of a sand and vermiculite mixture (1:1 v v^{-1}, autoclaved at 121 °C for 30 min). This substrate was chosen to represent a worst case scenario, i.e., greater availability of contaminants that may happen to occur in soil, given its small capacity to retain anions such as phosphate and arsenate. Arsenate doses were chosen based on levels occurring in soils where *A. peregrina* plants have been found growing (Gomes, 2011) and are also within the range of Brazilian guideline values, which have set a soil

guideline value of 15 mg As kg^{-1} soil as a screening level based on ecological risk assessment, with intervention values set as 35 mg As kg^{-1} soil for agricultural, 55 mg As kg^{-1} soil for residential, and 150 mg As kg^{-1} soil for industrial areas (CONAMA, 2009). Phosphate doses were chosen according to common fertilization practices in Brazilian savanna soils (Lopes, 2004). Arsenate was added as Na_2HAsO_4 and phosphate as KH_2PO_4, respectively. The desired amounts of these compounds were mixed with the substrate to obtain final doses of 0, 10, 50 and 100 mg As kg^{-1} substrate and 0, 200 and 400 mg P kg^{-1} substrate. Then, substrate pH (1:1 soil water $ratio^{-1}$) was checked and adjusted (by manual addition of 1 M HC1 or 1 M NaOH) to 6.7±0.1 as the pH verified in the As-contaminated soil where plants have been found growing (Gomes, 2011). Seeds of *Anadenanthera peregrina* var. falcata (L.) Speg. (Fabaceae) were acquired from the Seed Laboratory of the Companhia Energética de Minas Gerais (CEMIG, Belo Horizonte, Brazil) and after surface sterilization (5% sodium hypochlorite solution for 5 minutes), the seeds were washed thoroughly with deionized water. Seeds were germinated in Styrofoam boxes containing vermiculite substrate without As and the seedlings grew under greenhouse conditions (temperature 15-31 °C; average photosynthetically active radiation 825 μmol m^{-2} s^{-1}) until the development of their first pair of full expanded leaves. These healthy plants were then transplanted to pots with the prepared substrates with daily watering; 20 mL of half-strength Hoagland solution was applied every two weeks. A total of 12 pots per treatment were used in a completely randomized experimental design, and plants were kept to grow under greenhouse conditions (as described above). Thirty-five days after transplanting (when symptoms of As toxicity were makeable) the plants were harvested, washed thoroughly with tap water, divided into root and shoot fractions, and the fresh biomass was measured. Then, samples were placed in

paper bags and dried in an air circulation oven at 55 °C to a constant weight to determine dry biomass production.

2.2. Chemical analyses

Plant (~0.1 g) samples were digested in a microwave oven (MARS-5®, CEM) in 5 mL of concentrated HNO_3 (GR), using USEPA Method 3051A at 175 °C for 10 min. After digestion, the solutions were cooled, filtered through Whatman nº 40 filter paper and brought to a volume of 10 mL with ultra pure water. The filtered extracts were preserved under refrigeration (4°C) until analysis. Arsenic concentrations were determined using a graphite furnace atomic absorption spectrophotometer (Perkin-Elmer Analyst 800, Norwalk, CT). Phosphorus concentrations in solution were measured according to Sarruge and Haag (1974). The accuracy of the elemental analysis for As was checked by carrying a standard reference material through digestion and analysis. Each batch analysis contained a reference material (BCR 414 Plankton, Community Bureau of Reference) with known levels of As and one blank sample for quality control. The As recovery concentration value in the reference material was around 84%.

2.3. Oxidative responses and antioxidant enzymes

Oxidative responses (H_2O_2 production and lipid peroxidation) and antioxidant enzymes (SOD, CAT and APX) were examined in three plants per treatment. After harvesting, samples of the first and second fully expanded leaves were stored at -40 °C and subsequently used for biochemical studies and assessments of oxidative damage.

Hydrogen peroxide content was measured followed the method of Velikova et al., (2000). H_2O_2 content was determined using an extinction coefficient (e) of 0.28 mM^{-1} cm^{-1} and was expressed as nmol g^{-1} FW. Oxidative

damage was estimated in terms of lipid peroxidation following the methodologies of Heath and Packer (1968) and Buege and Aust (1978). Measurements of MDA were performed as in Hodges et al. (1999). Antioxidant enzymes were extracted by macerating 0.2 g of fresh leaves in 800 µl of an extraction buffer containing 100 mmol L^{-1} potassium phosphate buffer (pH 7.8), 100 mmol L^{-1} EDTA, and 1 mmol L^{-1} L-ascorbic acid. The protein contents of all of the samples were determined using the Bradford method. Superoxide dismutase (SOD; EC 1.15.1.1) activity was performed as described by Beyer and Fridovich (1987). Catalase (CAT; EC1.11.1.6) activity was assayed following the method of Kraus et al. (1995) (with minor alterations as described by Azevedo et al. [1998]) by determining the rate of conversion of H_2O_2 to O_2. Total ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured according to Nakano and Asada (1981).

2.4 Statistical analyses

The results were expressed as the averages of three replicates. The data were statistically evaluated using two-way analysis of variance run on the SAS software program (*SAS Institute Ins.*). Means were separated using the Scott-Knott multiple range test at 5% level of probability.

3. Results

3.1. Plant growth and As toxicity

Arsenate toxicity symptoms began to develop in *A. peregrina* plants 22 days after transplanting in the absence of P treatments, and after about 30-32 days in the presence of P. Browning of the leaf blades and necrotic spots on older leaves, followed by leaf senescence, were observed. At harvest, thickening and browning of the roots was observed, mainly at higher As additions, but to a lesser degree in the P addition treatments.

Both fresh and dry biomass productions of roots and shoots showed similar responses to As and P addition (Table 1). While biomass production decreased as As exposure increased, it increased with increasing P addition (Table 1 and Fig. 1). Significant interactions between As and P additions were observed only for fresh biomass (Table 1). Phosphorus addition increased fresh biomass production of the plants exposed to As (Fig. 1).

3.2. As and P nutrition and As accumulation

Regardless of As/P additions, As concentration were always higher in roots than in shoots (Fig. 2). We noted increases in As concentrations in both roots and shoots as As and P additions increased (Table 1). For plant As concentrations, significant interactions were observed between As and P additions (P>0.001). Phosphorus addition increased tissue As concentrations (Fig. 2). Similar to As, P concentrations were higher in roots than shoots (Fig. 3), and P concentrations increased as As and P additions increased (Table 1). However, for P concentrations, no interactions were verified between As and P additions (P<0.05; Table 1).

Phosphorus application increased As accumulation (As content x biomass production) (Table 2), especially at the highest As exposure level (100 mg kg^{-1}). The addition of P resulted in 222-258 μg As $plant^{-1}$, with increases of 148-188% in total plant As accumulation in the 200 and 400 mg P kg^{-1} treatments in relation to plants without any additional P (89.5 μg As $plant^{-1}$).

3.3. Oxidative damage and antioxidant enzyme activities

Lipid peroxidation (MDA concentration) and hydrogen peroxide (H_2O_2) concentrations as well as enzymatic (SOD, CAT and APX) activity are presented in Table 3. As x P interactions had no significant effect on MDA concentrations (P<0.05). However, MDA content significantly increased with

increasing levels of As application, and was significantly lower when P was provided.

The interactions between As x P additions only had significant effects on the H_2O_2 concentrations of *A. peregrina* leaves. The application of P caused decreased H_2O_2 concentrations in the absence of As. Additionally, the H_2O_2 concentrations were significantly lower in the highest P treatment (400 mg kg^{-1}), especially at the highest As concentration (100 mg kg^{-1}),

There was a progressive increase in SOD activity with increasing As and P additions. CAT activity decreased with exposure to As and increased with increasing additions of P. APX activity, in turn, decreased with higher As exposure (50 and 100 mg kg^{-1}) and increased with P application.

4. Discussion

Biomass is a key factor in phytoremediation practices and is also a general measurement of plant health (Tu and Ma, 2003). Previous experiments demonstrated that *A. peregrina* plants were very susceptible to As phytotoxicity and showed decreased biomass production and marked symptoms of As intoxication (Gomes, 2011). The present study, however, demonstrated As-phytotoxicity alleviation by P, as this element increased both fresh and dry biomass production (Table 1) and delayed visual intoxication symptoms in relation to plants grown in the presence of As without P fertilization. Arsenic concentrations are not the only factor that can affect plant growth and the development of toxicity symptoms, as they can be affected by the mineral nutritional status of the plant (Gulz *et al*. 2005). As such, early symptoms of poor plant growth without the addition of P may have been associated with low P nutritional status. Additionally, differences in plant maturity may have affected As concentrations in the shoots when toxicity symptoms developed

(Ultra *et al*., 2007). Plants with higher maturity stage had higher capability for metal(loids) absorption as they had larger biomass which could be associated with larger number of active absorption sites (Sen *et al*., 1987). The increase in metal(loid) accumulation by plant tissues of different maturity may also be due to increased permeability and metabolic activities associated with increasing age (Choo *et al*., 2006) and, mature tissues may possesses structural differences related to metal(loid) accumulation as verified by Lavid *et al*. (2001).

A number of studies have shown favorable effects of P on plant growth in the presence of As (Tu and Ma, 2003; Ultra *et al*., 2007; Gunes *et al*., 2009), which have been attributed to the higher P nutrition of plants exposed to P fertilization (Sneller *et al*., 1999) and their higher P/As ratios. Arsenic phytotoxicity is related to the ability of As to replace P in biological sites, so that higher P/As ratios resulting in milder As intoxication symptoms in plants (Smith *et al*., 2010). However, as revised by Smith et al. (2010), As-tolerant plants may take up P by high-affinity transport with a higher selectivity against As(V), than in the non-tolerant plants and so, maintaining P/As ratio considerably high. On the other hand, the application of P also enhanced As concentrations in plants (Table 1) – but despite higher As concentration in their tissues, plants fertilized with P showed less As-induced biomass decreases. Phosphorus fertilization also increased root and frond biomass production in the hyperaccumulator Chinese brake fern (Tu and Ma, 2003). However, plant growth promotion by P was accompanied by decreasing As concentrations in plant tissues due to the dilution effects of greater biomass production (Tu and Ma, 2003). Our results, however, show increases in both plant biomass and As concentrations (Table 1), which indicated increased As uptake in P fertilized plants. Additionally, the application of P increased total As accumulation by *A. peregrina*, regardless of the arsenate exposure levels (Table 2). Plant As accumulation takes both arsenate uptake and

plant biomass accumulation into consideration, providing better indications of P effects on As phytoextraction (Tu and Ma, 2003). Therefore, in addition to promoting plant growth, P increases the As-phytoremediation ability of *A. peregrina* plants - an important finding for optimizing its application in environmental recovery programs.

Increased as well as decreased As concentrations in plants in response to P addition have been reported (Tu and Ma, 2003; Gunes *et al*., 2009). Because As and P compete with each other for soil adsorption sites, As or P addition may result in reductions of soil adsorption and increases in their solution concentrations and their availability to plants (Livesey and Huang, 1981; Manning and Goldberg, 1996; Smith *et al*., 2002). Moreover, depending on soil condition and/or relative P/As levels, P may either reduce As concentrations in the plant through competition and/or enhanced plant growth by alleviating As phytotoxicity, or increase As uptake by the plant and hence its phytotoxicity (Creger and Peryea, 1994; Tu and Ma, 2003). P has been reported to suppress plant As uptake in hydroponic systems (Tu and Ma, 2003) due to a greater affinity of membrane transport systems for P than As (although they are generally the same for both elements) (Meharg *et al*., 1994), so that the effects of P on plant As uptake can depend on the specific growing conditions (Tu and Ma, 2003).

P distribution in *A. peregrina* was similar to that of non-hyperaccumulator plants, with greater concentrations of P in the roots than in the above-ground biomass (Table 1) (Tu and Ma, 2003). The P/As influence on P nutrition can vary. While P concentrations were not influenced by P addition in *Pteris vitatta* (Tu and Ma, 2003), increasing P concentrations were observed in chickpea plants as P addition increased (Gunes *et al*., 2009). Arsenic addition did not affect the P concentrations of chickpea plants, but decreased P

concentrations in *P. vitatta*. In the present study, both P and As additions increased plant P concentrations (Table 1). Higher availability of P may lead to higher P uptake by plants, and higher plant As concentrations (due to increased As additions) may enhance P demand to maintain viable P/As ratios (and/or may be due to As-induced increases in physiological P requirements) (Carbonell *et al*., 1998; Burló *et al*., 1999). At the very least, the uptake of P in the presence of As can lead to high internal P concentrations - which would be expected to down-regulate the P/As transporter and prevent further As uptake (Meharg and Hartley-Whitaker, 2002).

Metal(loid) toxicity has been related to induced oxidative stress, and severe lipid peroxidation induced by As has been reported in plants (Hartley-Whitaker *et al*., 2001; Srivastava *et al*., 2005; Stoeva *et al*., 2005; Singh *et al*., 2007). Lipid peroxidation indicates membrane damage resulting from exposure to ROS (Montillet *et al*., 2005), such as high levels of H_2O_2 (Mittler, 2002). In the present study, MDA content was used as a biomarker for lipid peroxidation, and the application of As increased plant MDA concentrations (Table 3) and As-increased lipid peroxidation was followed by shoot growth reduction. The application of P, in turn, decreased lipid peroxidation and increased shoot growth – so that As toxicity in *A. peregrina* plants is apparently due to As-induced lipid peroxidation, which is alleviated by P addition.

P-alleviated oxidative damage was also seen in the reduced H_2O_2 contents of plants, mainly at the highest As exposure (100 mg kg^{-1}) (Table 3). The accumulation of H_2O_2 in plants may lead to uncontrolled oxidation and free radical chain reactions, which will result in oxidative stress in the plants (Srivastava *et al*., 2005). H_2O_2 accumulation was not observed, however, even with added As, thus As-induced oxidative stress was caused by lipid peroxidation but not by H_2O_2 accumulation (Table 3).

Altered activities of antioxidant systems are frequently used as indicators of oxidative stress in plants (Mittler, 2002), and increased SOD activity in response to arsenic toxicity was observed in the present work and by earlier researchers (Hartley-Whitaker *et al*., 2001; Srivastava *et al*., 2005). SODs are known to be major O_2^- scavengers and provide a first line of defense against cell injury by environmental stress factors (Gratäo *et al*., 2005). SODs catalyze the conversion of highly reactive superoxides to H_2O_2, which in turn is scavenging by CAT and APX - two enzymes that are also used as markers of oxidative stress (Gunes *et al*., 2009). However, the increase in SOD activity induced by As was not followed by any increase in the activities of CAT and APX (Table 3). Phosphorus addition, on the other hand, resulted in increased CAT and APX activities. Increased SOD activity and decreased CAT and APX activities in plants exposed to As has been reported by Singh *et al*. (2007). APX is mainly located in the chloroplasts (Singh *et al*. 2007), and since As can cause chloroplast ultrastructural damage (affecting their integrity and associated metabolism) (Li *et al*. 2006), decreases in APX activity would be expected in the leaves of plants exposed to As. Arsenic also interferes with enzyme activity and with other proteins by binding to intracellular thiols (-SH) and inactivating them (Meharg and Hartley-Whitaker, 2002). The decreased activities of CAT and APX indicated their inactivation/degeneration due to As-induced oxidative stress (Singh *et al*. 2007).

The results reported here suggest a central role of CAT and APX in preventing oxidative stress in *A. peregrina*: decreased activities of both of these ROS-scavenging enzymes were related to increased lipid peroxidation induced by As, while their increased activities with P addition were related to decreased lipid peroxidation (Table 3) – emphasizing the importance of P in alleviating As-induced oxidative stress.

5. Conclusion

The addition of P increased the arsenic phytoremediation capacity of *Anadenanthera peregrina* plants by increasing As accumulation and alleviating As-induced oxidative stress. Arsenic phytotoxicity in *A. peregrina* is related to lipid peroxidation but not H_2O_2 accumulation, and P addition increased the activity levels of important ROS-scavenging enzymes (CAT and APX) that help prevent lipid peroxidation in leaves. Our findings suggest that the application of P should be a feasible strategy for more efficient As phytoremediation using *A. peregrina* plants.

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Table 1 Results of the two-way ANOVA and Skott-Knott tests for the effects of arsenate and phosphate on biomass accumulation and As and P concentrations in *Anadenanthera peregrina.*

	Fresh biomass (g)		Dry biomass (g)		As concentration (μg g^{-1} dry matter)		P concentration (mg g^{-1} dry matter)	
Source of variation	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
ANOVA *F* values								
As addition	638.6***	604.0***[a]	597.5***	623.9***	11514.6***	4082.6***	68.2***	403.1***
P addition	124.1***	120.7***	278.0***	273.5***	118.5***	115.3***	17.3**	294.1***
As x P additions	7.6***	7.1***	1.4NS	1.1NS	37.8***	22.2***	1.8NS	2.4NS
Scott-Knott multiple range test								
As addition (mg kg^{-1})								
0	0.50a[b]	1.26a	0.36a	0.99a	0.4d	0.5d	4.5d	1.3d
10	0.28b	0.71b	0.21b	0.57b	167.1c	96.1c	4.9c	1.5c
50	0.25c	0.63c	0.18c	0.48c	437.9b	216.3b	5.3b	1.9b
100	0.20d	0.52d	0.14d	0.40d	609.3a	239.8a	5.9a	2.1a
P addition (mg kg^{-1})								
0	0.25c	0.64c	0.16c	0.44c	281.5c	121.6c	4.8b	1.4c
200	0.32b	0.81b	0.24b	0.66b	300.3b	138.4b	5.3a	1.7b
400	0.35a	0.89a	0.27a	0.73a	329.0a	154.1a	5.3a	2.0a

[a]NS – not significant F ratio ($P < 0.05$), *, ** and *** significant at $P < 0.05$, 0.01 and 0.001, respectively.

[b]Treatment means from ANOVA. Values followed by the same letter, within the same source of variation, are not significantly different ($P < 0.05$) by the Scott–Knott multiple range test.

Table 2 Arsenic accumulation (μg plant^{-1}) and the molar ration of arsenic to phosphorus (P/As) in *A. peregrina* in response to different arsenate and phosphate additions.

Treatments	Roots	Shoots	Total	% of total As accumulation[c]
As_0P_0[a]	0.17 ± 0.1[b]	0.81 ± 0.1	0.88	-
$As_{10}P_0$	24.7 ± 1.8	37.1 ± 3.1	61.9	-
$As_{50}P_0$	48.1 ± 2.4	64.3 ± 2.0	112.4	-
$As_{100}P_0$	45.5 ± 3.0	43.9 ± 1.9	89.5	-
As_0P_{200}	0.08 ± 0.1	0.93 ± 0.1	1.01	-
As_0P_{400}	0.21 ± 0.18	0.56 ± 0.1	0.77	-
$As_{10}P_{200}$	37.6 ± 1.9	57.0 ± 2.3	94.7	53
$As_{10}P_{400}$	45.7 ± 4.6	74.7 ± 5.8	120.4	94
$As_{50}P_{200}$	83.3 ± 3.9	113.7 ± 5.6	197.0	75
$As_{50}P_{400}$	111.4 ± 6.9	146.1 ± 12.4	257.5	147
$As_{100}P_{200}$	106.2 ± 2.7	116.1 ± 6.3	222.4	148
$As_{100}P_{400}$	121.6 ± 4.2	137.0 ± 4.2	258.6	188

[a]As0, As10, As50 and As100 = 0, 10, 50 and 100 mg As kg–1; P0, P200 and P400 = 0, 200 and 400 mg P kg–1, respectively.

[b]Means ± SE (standard error).

[c]percentage increase of total As accumulation in response to P addition.% = AsxPy/AsxP0, where x = 10, 50 or 100 mg As kg–1, and y = 200 or 400 mg P kg–1.

Table 3 Interactive effects of As and P addition on MDA and H_2O_2 concentrations and superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) enzyme activities in *Anadenanthera peregrina* leaves. Values represent the means of 3 replicates. Means for As and P addition are shown under the As x P interaction means. F values for the P x As interaction and As and P addition are shown. Different lowercase letters in each column for As x P interaction, As addition, and P addition represent significant differences at the $P<0.05$ level, based on the Scott-Knott multiple range tests. Different capital letters in the column for As x P interactions represent significant differences of P addition for each As concentration at the $P<0.05$ level based on the Scott-Knott test.

As addition (mg kg^{-1})	P addition (mg kg^{-1})	MDA (nmol g^{-1} FW)	H_2O_2 (nmol g^{-1} FW)	SOD (U mg^{-1} FW)	CAT (μmol H_2O_2 min^{-1} g^{-1}	APX (U mg^{-1} FW)
0	0	138.73	174.3aA	107.41	35.52	109.26
	200	116.39	146.1bB	125.84	39.95	118.46
	400	106.00	139.4bB	137.57	41.17	114.27
10	0	178.26	116.3dNS	153.12	23.09	109.46
	200	144.63	106.1dNS	162.79	29.74	114.12
	400	132.55	119.0dNS	167.95	31.46	119.77
50	0	265.11	128.0cNS	162.72	17.20	96.24
	200	219.70	126.1cNS	167.55	25.92	105.72
	400	205.93	130.3cNS	174.17	29.37	101.81
100	0	276.76	105.1*eA*	171.80	15.57	86.89
	200	230.28	110.5eA	184.84	22.80	100.15
	400	245.01	91.4fB	188.79	26.38	104.33
Lsd, p < 0.05		3.96				
0		120.3d	153.3a	123.6d	38.8a	113.99a
10		151.8c	113.8c	161.2c	28.1b	114.45a
50		230.2b	128.1b	168.1b	24.1c	101.26b
100		250.6a	102.3d	181.8a	21.5d	97.12c
Lsd, p < 0.05		3.85	2.29	1.75	0.56	1.38
	0	214.7a	130.9a	148.7c	22.8c	100.46b
	200	177.7b	122.2b	160.2b	29.6b	109.61a
	400	172.3b	120.0b	167.1a	32.0a	110.04a
Lsd, p < 0.05	3.38	1.98	1.51	0.49	1.20	
F values	As x P addition	1.7^{NS}	7.5^{***}	2.0^{NS}	2.2^{NS}	2.0^{NS}
	As addition	260.1^{***}	91.7^{***}	201.9^{***}	179.0^{***}	40.5^{***}
	P addition	47.6^{***}	8.4^{***}	37.3^{***}	94.1^{***}	20.2^{***}

Significance of ANOVA: NS – not significant F ratio ($P < 0.05$), *, ** and *** significant at $P < 0.05$, 0.01 and 0.001 respectively.

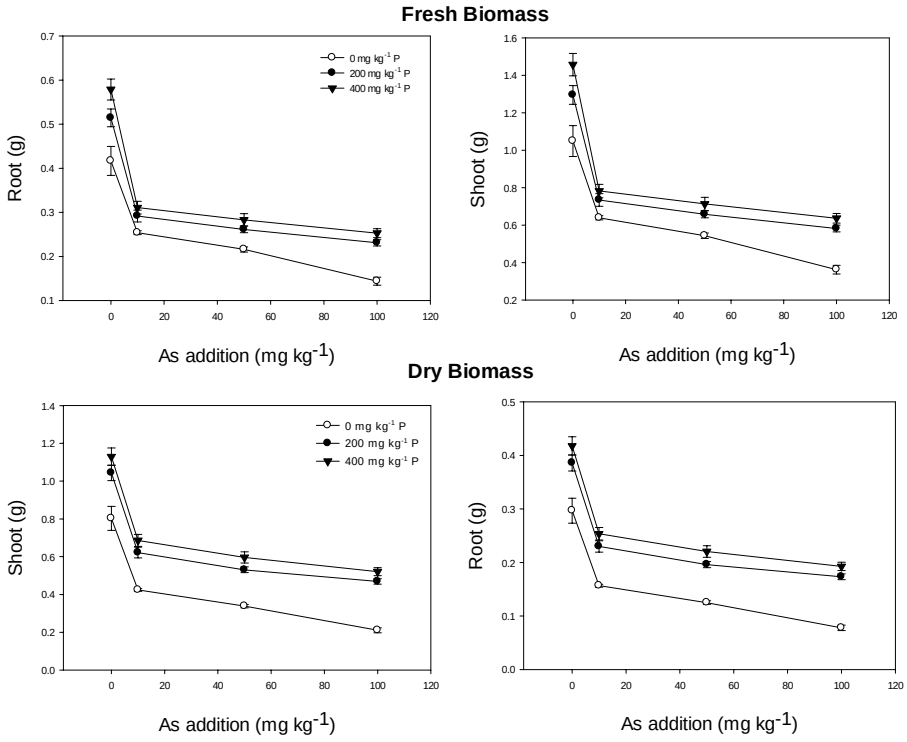

Fig. 1 Effects of different arsenate and phosphate additions on fresh and dry biomass of roots and shoots of *Anadenanthera peregrina* plants grown under greenhouse conditions.

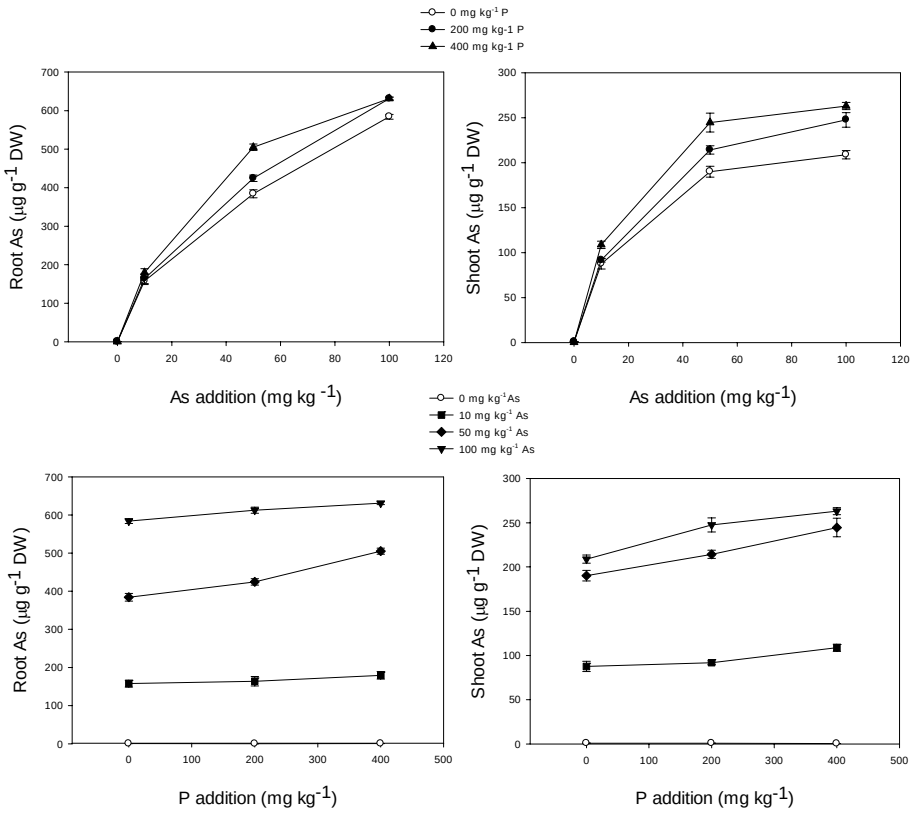

Fig. 2 Effects of different arsenate and phosphate additions on arsenic concentrations in roots and shoots of *Anadenanthera peregrina* plants grown under greenhouse conditions.

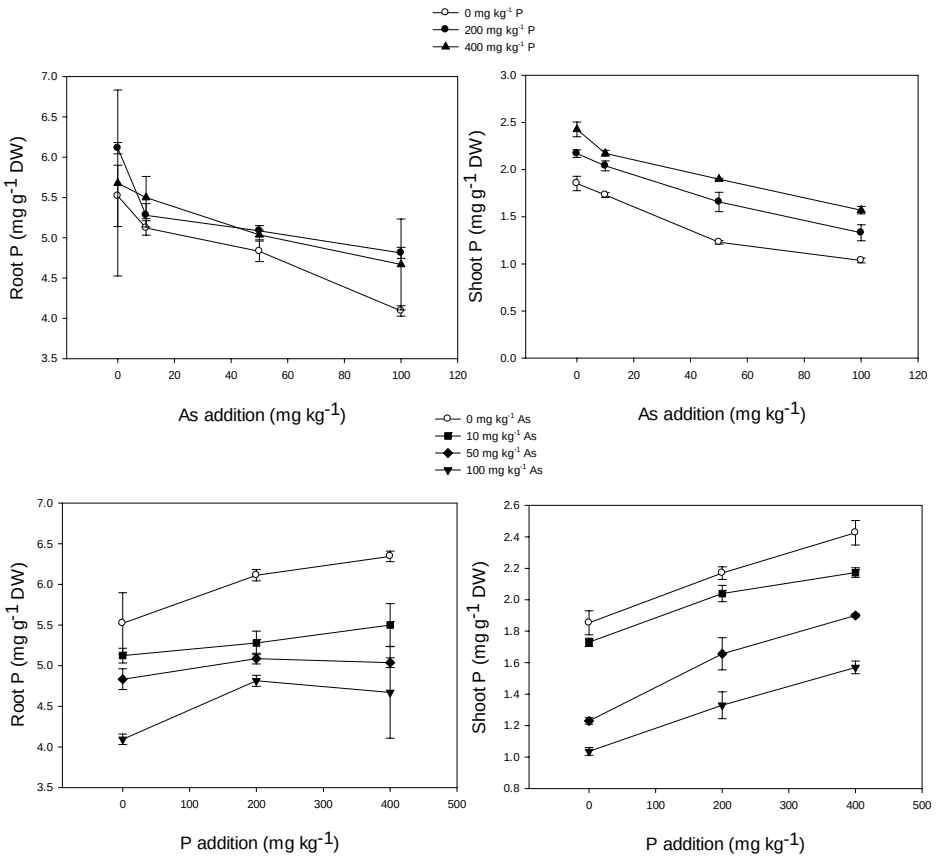

Fig. 3 Effects of different arsenate and phosphate additions on phosphorus concentrations in roots and shoots of *Anadenanthera peregrina* plants grown under greenhouse conditions.

ARTICLE 5 - Zinc tolerance modulation in *Myracrodruon urundeuva* plants

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Zinc tolerance modulation in *Myracrodruon urundeuva* plants

M.P. Gomes[a,b,*], D.M. Duarte[c], M.M.L.C. Carneiro[b], L.C. Barreto[c], M. Carvalho[b], A.M. Soares[b], L.R.G. Guilherme[d], Q.S. Garcia[c]

[a]Université du Québec à Montréal, Institut des Sciences de l'environnement, Succ. Centre-Ville, C.P. 8888, H3C 3P8, Montréal, Québec, Canada.

[b]Universidade Federal de Lavras, Departamento de Biologia, Campus UFLA, C.P. 3037, 37200-000, Lavras, MG, Brazil.

[c]Universidade Federal de Minas Gerais, Departamento de Botânica, C.P. 486, 31270-970, Belo Horizonte, MG, Brazil.

[d]Universidade Federal de Lavras, Departamento de Ciência do Solo, Campus UFLA, 37200-000, Lavras, MG, Brazil.

*Corresponding author. Tel. : +1 514 987 3000 #3489. Fax.: +1 514 987 4647. E-mail address: marcelopgom@yahoo.com.br (M.P. Gomes).

Abstract

We investigated Zn tolerance and related tolerance mechanisms of *Myracrodruon urundeuva* by evaluating the growth (biomass production, pigment content, and photosynthetic activity) and antioxidant systems (redox potential and antioxidant enzyme activities) of seedlings exposed to increasing Zn doses. Plants were grown for 120 days in substrates with 0, 50, 80, 120 and 200 mg Zn kg^{-1} and demonstrated Zn-tolerance. Zn doses greater than 80 mg Zn kg^{-1} were phytotoxic but not lethal, and Zn toxicity under these conditions was imposed by oxidative stress caused by hydrogen peroxide (H_2O_2) accumulation and related lipid peroxidation. Zn tolerance in *M. urundeuva* is linked to the activity of antioxidant systems in their leaves that are modulated by that metal: both superoxide dismutase (SOD) and catalase (CAT) were always higher in the

presence of Zn; lower Zn doses stimulated ascorbate peroxidase (APX) and glutathione reductase (GR) activities, but enzyme activity was inhibited at high doses; APX appeared to be the main peroxidase in H_2O_2 scavenging as stimulated guaiacol peroxidase (GPX) activity was not sufficient to avoid H_2O_2 accumulation at higher Zn doses; the modulation of APX and GR activities was linked to changes in the redox status of leaves.

Keywords: antioxidant, enzymes, glutathione, tolerance, trace element, zinc

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbic acid; CAT, catalase; DHA, dehydroascorbate; DW, dry weight; FW, fresh weight; GPX, guaiacol peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSH, oxidised glutathione; H_2O_2, hydrogen peroxide; O_2^-, superoxide radical; MDA, malondialdehyde; MDHA, monodehydroascorbate; PSII, photosystem II; ROS, reactive oxygen species; SOD, superoxide dismutase.

1. Introduction

Ecosystem contamination by trace elements is a global environmental concern. High soil concentrations of trace elements due to mining activities have been reported in Brazil [1,], leading to searches for technologies that could minimize threats to both human health and ecological systems [1,2]. Considerable interest has been focused on identifying plant species tolerant to elevated levels of trace elements that could be used in phytoremediation programs and on investigating their tolerance mechanisms [3]. High trace element tolerance and biomass production have been identified in a number of native Brazilian species – two important characteristics for their successful use in phytoremediation programs [4].

Myracrodruon urundeuva Fr. Allem. (Anacardiaceae) is a native woody species native to Brazil that occurs naturally in sites contaminated by mining

spoils with Zn levels ranging from ~40 to 250 mg kg^{-1} soil. The natural occurrence of plants in trace element contaminated sites is a strong indication of metal tolerance, and has been used to identify species potentially useful in contaminated soil recovery programs. These plants are also intensively used as wood-product resources and in traditional medicinal practices, and have been included in the official federal list of endangered species (vulnerable category) [6], making their reproduction for soil-recovery programs a desirable combination of both conservation and phytoremediation goals. Due to their longevity and high biomass production, woody species can immobilise large quantities of metals in their internal tissues, thus decreasing the environmental impacts of these elements [5].

Zinc (Zn) is a micronutrient essential for plant growth and development as a component of enzymes and regulatory transcriptional proteins [7] but can become phytotoxic at high concentrations, causing reduced growth, leaf chlorosis, and nutritional disturbances [8]. The deleterious effects of zinc on photosynthesis has been associated with Zn-induced decreases in electron transport yields in photosystem II (PSII) and pigment biosynthesis [9,10] and with Zn- induced oxidative damage [3,11,]. In order to protect themselves from oxidative damage, plants have evolved mechanisms to synthesize both enzymatic (e.g., superoxide dismutase [SOD], catalase [CAT], ascorbate peroxidase [APX], guaiacol peroxidase [GPX], glutathione reductase [GR]) and non-enzymatic antioxidants (i.e., ascorbate and glutathione) [12].

As Zn ions participate in the enzymatic defense systems of plant cells against free radical damage [13] (especially as part of the superoxide radical [O_2^-] CuZnSOD scavenging enzyme) [14], we hypothesized that antioxidant system activation (a common feature in metal-tolerant plants exposed to toxic levels of trace elements [15]) would be important in Zn tolerance. Although plant

antioxidant responses to Zn are not yet well understood, it has been observed that zinc deficiencies (as well as toxic concentrations of this element) decrease the activities of some antioxidant enzymes of the Zn-hyperaccumulator species *Phaseolus vulgaris* [5], and increased exposure time to high zinc doses led to strong increases in ascorbate oxidation in these plants, suggesting that excess zinc can cause oxidative stress [3]. In contrast, Zn was observed to increase antioxidant enzyme activity in *Brassica juncea* [16]. Redox-active metals (i.e., copper) are also known to cause both direct oxidative damage and antioxidative protection [3], although little is known about the role of non-redox active metals (as such Zn) in oxidative pathways. The present study therefore investigated Zn tolerance and related tolerance mechanisms in *M. urundeuva* by evaluating their growth (biomass production, pigment content, and photosynthetic activity) and their antioxidant system activities (redox potential and the antioxidant enzymes SOD, CAT, APX and GR) when exposed to increasing Zn doses.

2. Results

2.1 Zn contents of the leaves and Zn influences on photosynthesis

Leaf Zn concentrations increased as substrate Zn doses increased (Table 1). Zn had both stimulatory and deleterious effects on plant biomass production (Table 1). Both the fresh and dry biomass productions of shoots were higher than the control at 50 an 80 mg Zn kg^{-1} substrate. Only fresh weight decreased at higher zinc doses (120 and 200 mg Zn kg^{-1}) in relation to control plants ($P < 0.05$) (Table 1).

Although affecting fresh weight, Zn did not affect the photochemical efficiency of PSII (Fv/Fm) or the total chlorophyll contents of leaves (Table 1), although increased amounts of carotenoids were seen in the leaves of plants exposed to 120 and 200 mg Zn kg^{-1}.

2.2 Lipid peroxidation and H_2O_2 contents

Lipid peroxidation (MDA concentrations), hydrogen peroxide (H_2O_2) concentrations, and enzymatic (SOD, CAT, APX, GPX, GR) activities are presented in Figure 1. MDA concentrations were greater at higher Zn doses but decreased in the leaves of plants treated with 50 mg Zn kg^{-1} (P>0.05). Similar results were observed in relation to H_2O_2, as concentrations were lower in plants exposed to both 50 and 80 mg Zn kg^{-1}.

2.3 Antioxidant responses of the leaves

SOD and CAT activities were stimulated by the presence of Zn, being higher in treated plants (Fig. 1). APX and GR activities were stimulated in the leaves of plants treated with 50 and 80 mg Zn kg^{-1}, but decreased at Zn doses greater than 80 mg kg^{-1}; GPX activity, in contrast, was stimulated by even the highest Zn doses (Fig. 1).

Total ascorbate concentrations (AsA + DHA) as well as those of its oxidized forms (DHA) increased in leaves of Zn-treated plants, while the concentrations of the reduced form (AsA) decreased (Fig. 2A). The ratios of oxidized to reduced ascorbate concentrations (DHA/AsA) were also greater in Zn-treated plants, and highest at the highest Zn doses (Table 2).

Total (GSH + GSSG) concentrations as well as the quantities of the reduced form of glutathione (GSH) increased in the leaves of plants treated with 50 and 80 mg Zn kg^{-1} (Fig. 2B); GSH decreased in plants exposed to increasing Zn doses (Fig. 2B). The concentrations of the oxidized forms of glutathione were greater in Zn treated plants, and highest in plants exposed to the highest Zn doses (Fig. 2B). The ratios of oxidized to reduced glutathione (GSSG/GSH) were greatest in plants exposed to the highest Zn treatments (Table 2).

3. Discussion

Deleterious effects of Zn were observed in the fresh weight from plants exposed to high Zn doses (Table 1). Seedlings (as used in our study) appeared to

be more sensitive to adverse metal conditions than adult plants [17]. However, considering the long life cycles of trees, the responses of seedlings to toxic metals remains the easiest and most commonly used method of determining a plant's ability to tolerate and survive metal-contamination conditions [18]. In addition to the seedling sensitivity, the higher Zn availability in solution due to the use of the chosen substrate could exacerbate Zn phytotoxicity, for example, turning plants more prone to disturbs in the water status, which would be expressed in decreased fresh weight. However, the maintenance of biomass production (dry weight) even at highest Zn doses, indicate the Zn tolerance of the studied species. Is important to note that, even tolerant plants growing in contaminated sites generally show reduced growth rates as compared to those growing in uncontaminated sites [18]. Furthermore, growth reductions have been described in plants exposed to toxic concentrations of Zn [3,11]. Toxic trace element levels are known to produce negative effects on key metabolic processes coupled to plant growth [11], and Zn can negatively impact photosynthetic processes by inhibiting electron flow on the oxidizing side of PSII (at a site prior to the electron donation site(s) of hydroxylamine and diphenylcarbazide) [9]. In order to study Zn influences on PSII of *M. urundeuva* we investigated changes in chlorophyll fluorescence (Fv/Fm) signals, which are a measure of electron transport yields in PSII. In addition, as trace elements can also impact pigment production [10], we also quantified the chlorophyll and carotenoid contents of the exposed seedlings. We did not observe any negative effects of Zn on either Fv/Fm or the chlorophyll contents of treated plants (Table 1), indicating that Zn has not disturbed the photosynthetic apparatus of *M. urundeuva*. We did note, however, increases in the carotenoid contents of plants exposed to high Zn concentrations (Table 1) (which indicated some oxidative stress), and increased H_2O_2 and lipid peroxidation (MDA) accumulations were

seen in those plants (Fig. 1). These data suggest that Zn toxicity in *M. urundeuva* is due to Zn-induced oxidative stress through hydrogen peroxide accumulation and related lipid peroxidation. The increased carotenoid contents of plants exposed to high Zn doses presumably help protect their photosynthetic systems against Zn-induced oxidative damage – although other cell sites remain targets of reactive oxygen species and ROS-induced damage.

Observations that ROS generators can induce carotenoid accumulation suggest that this carotenogenic response is mediated by ROS [19]. ROS (or their products) may regulate carotenoid content by direct activation of latent biosynthetic enzymes such as glutathione transferase [20] and glutathione reductase [21]. Alternatively, ROS may activate the expression of genes coding for carotenogenic enzymes [22]. Carotenoids are usually involved in the detoxification of oxygen singlets ($^1O_2$) but not other ROS species [23], although ROS can be readily enzymatically converted to oxygen singlets that can subsequently react with carotenoids, thus protecting the plants from oxidative damage [23].

Lipid peroxidation resulting from ROS exposure (such as high levels of H_2O_2) has been shown to disrupt membrane organization and provoke functional losses and modifications of proteins and DNA bases [24]. H_2O_2 accumulation in plants may lead to uncontrolled oxidation and free-radical chain reactions – provoking oxidative stress in plants [25]. Both redox-active (such as Cu and Fe) and non redox-active (such as Zn and Cd) metal ions have been reported to increase lipid peroxidation via ROS generation in plants [11]. Increased lipid peroxidation in bean plants exposed to toxic Zn levels has been attributed to the increased activity of membrane-bound lipoxygenase, which is known to oxidize polyunsaturated fatty acids and produce free radicals [11].

In order to better understand the processes involved in peroxide accumulation (and Zn-induced oxidative damage), we investigated the activities of antioxidant enzymes (SOD, CAT, APX, GPX and GR) and the plant's redox status (metabolites from the ascorbate-glutathione cycle). The following pathway of H_2O_2 production and destruction has been proposed: SODs catalyze the conversion of highly reactive superoxides to H_2O_2 and these molecules are in turn scavenged by CAT, APX, and GPX; APX reduces H_2O_2 into water using ascorbate (AsA) as the electron donor and the resulting dehydroascorbate (GSH) is cycled back to ascorbate using reduced glutathione (GSH) as the electron donor, and the oxidized glutathione (GSSG) formed is converted back to GSH by NAD(P)H-dependent GR; in terms of GPX, this enzyme acts upon H_2O_2 to form GSSH, which is then further reduced to GSH by GR [12,26].

Increased SOD activity was seen in Zn-treated plants (Fig. 1), and the subsequent induction of antioxidant enzyme activity is considered to play an important role in cellular defense strategies against oxidative stress caused by toxic metal concentrations [27]. SOD represents the front line of the antioxidant defense system, and it is a Zn-containing enzyme [12]. The activity of copper-zinc superoxide dismutase (CuZnSOD), the most important isozyme among the superoxide scavenging enzymes [28], is closely related to anti-aging effects and stress resistance in plants [28]; about 85% of the measured SOD activity has been ascribed to CuZnSOD [13]. As a co-factor of CuZnSOD, increased Zn concentrations in leaves may lead to increased activity levels of this enzyme. Qu et al. [29] recently demonstrated that the modulation of CuZnSOD gene expression by salt stress, and increased SOD activity has been reported in plants exposed to trace elements (including Zn) [30].

Similar to SOD, CAT activity was also greater in Zn-treated plants (Fig. 1). Both the inhibition [30] and stimulation [16] of CAT activities were observed

in plants exposed to Zn although according to Weckx and Clijsters [31], CAT has a role in defense mechanisms against oxidative stress in leaves. These conflicting observations regarding CAT activity may be due to differences in the plant organs studied, plant growth conditions, the exposure times and concentrations of the metal utilized, or the plant species examined [27]. Our results indicated that the stimulation of CAT in *M. urundeuva* plants is a probable mechanism of oxidative damage avoidance - as plants with low H_2O_2 accumulations and low related lipid peroxidation (50 and 80 mg Zn l^{-1}) showed correspondingly greater CAT activities (Fig. 1).

APX and GR activities showed similar response patterns to Zn (Fig. 1) with their activities being stimulated at low Zn doses (50 and 80 mg kg^{-1}) but decreasing at higher doses (120 and 200 mg kg^{-1}). GPX activity increased in the leaves of plants treated with increasing Zn concentrations (Fig. 1). GR and APX/GPX are linked to the glutathione-ascorbate cycle (recycling oxidized ascorbate/glutathione after catalysis by APX/GPX) [26]. The observed increase in GPX activity in plants exposed to the highest Zn doses (not seen at lower concentrations) may be a form of compensation (for scavenging H_2O_2) in light of decreasing APX activity. Although its activity increased, GPX was apparently not capable of scavenging sufficient amounts of H_2O_2 to avoid its accumulation and related oxidative damage (lipid peroxidation). GPX activity in bean leaves was not affected by Zn, and the activity of ascorbate-specific peroxidase (APX) has been proposed as the main factor responsible for avoiding metal-induced oxidative stress [11].

H_2O_2 damage to chloroplasts (mainly by APX) is dependent on the action of the ascorbate-glutathione metabolic cycle [11], and we therefore investigated the redox status (AsA and GSH) in *M. urundeuva* leaves to help elucidate Zn inhibition (or stimulation) of antioxidant enzymes. APX uses two

molecules of AsA in the AsA GSH cycle to reduce H_2O_2 to water – with the generation of two molecules of monodehydroascorbate (MDHA), which can then be reduced to AsA by MDHA reductase (using NADPH as electron donors) [32]. If it is not immediately reduced, the short-lifetime MDHA radical is oxidized to dehydroascorbate (DHA). DHA is then reduced to AsA through the action of DHA reductase using GSH, which is generated from oxidized glutathione (GSSG) by NADPH-dependent GR activity [32]. We observed a close relationship between APX and GR activities and ascorbate-glutathione concentrations, as: 1) the reduced form of ascorbate (DHA) was lower and the ratio of oxidized to reduced ascorbate (DHA/AsA) higher in the leaves of plants treated with 120 and 200 mg Zn kg l^{-1} - in contrast to the 50 and 80 mg Zn kg l^{-1} treatments (Table 2); 2) the oxidized form of glutathione (GSSG) and the ratio of oxidized to reduced glutathione (GSSG/GSH) were greater in the leaves of plants exposed to the highest Zn treatments (Table 2); 3) APX and GR activities in these plants were suppressed while GPX activity increased. Reduced GR activity may therefore lead to the accumulation of GSSG, which would help explain the higher GSSG/GSH ratios of leaves in the 120 and 200 mg Zn kg^{-1} treatments. The accumulation of GSSG in these plants is also stimulated by increased GPX activity. Reduced GSH content (also verified in these plants, Fig. 2) leads, in turn, to less reducing power (GSH) to be used in DHA reduction, resulting in the accumulation of the oxidized form of ascorbate (DHA) – and higher DHA/AsA ratios. The starvation of AsA due to GR inhibition may therefore partly explain the observed decrease in APX activity. According to Cuypers et al. [3], GR inhibition (responsible for the reduction of GSSG) led to increases in cell GSSG/GSH ratios – with zinc disturbing the glutathione balance and preventing the cell from maintaining its ascorbate pool in a reduced state. Accordingly, Ding et al. [33] reported that the decreased GR activity

induced by Cd led to a decrease in AsA levels in tobacco plants. These authors also noted that decreasing AsA contents in leaves are associated with increasing MDA contents. Our results, taken together, therefore show that AsA and GSH are directly linked to Zn-tolerance in *M. urundeuva* plants, and that Zn-toxicity in these plants at higher concentrations is related to Zn-induced decreases in APX and GR activity, which leads to H_2O_2 accumulation and related lipid peroxidation.

The most pronounced effect of Zn on cell metabolism is its involvement in protein metabolism [13]. Zn is known to have structural and regulatory roles in many enzymes and proteins directly involved in replication and transcription processes and gene activation [7], although at higher Zn concentration ROS accumulation may lead to protein oxidation [34]. Romero-Puertas et al. [34] reported APX and GR protein carbonylation in plants exposed to Cd – an irreversible oxidative process in which the side-chains of Lys, Arg, Pro, and Thr are converted to aldehyde or keto groups, which appears to contribute to inhibit or impair multiple enzyme systems [35]. The same processes may have been occurring in the present study, so that when H_2O_2 accumulation (provoked by the presence of Zn) exceeded the tolerance limits of *M. urundeuva* seedlings their antioxidant systems (APX and GR) were inactivated.

We conclude that *M. urundeuva* plants show Zn-tolerance and that Zn concentrations greater than 80 mg Zn kg^{-1} become phytotoxic, but not lethal, to those plants. Zn toxicity at high exposures is due to imposed oxidative stress driven by hydrogen peroxide accumulation and related lipid peroxidation. Zn tolerance by *M. urundeuva* is linked to the activities of antioxidant systems in its leaves that are modulated by that metal: both SOD and CAT were always higher in the presence of Zn; low doses of Zn stimulated APX and GR activities, while high doses inhibited those enzymes; APX (and related GR activity) was

identified as the main peroxidase in H_2O_2 scavenging, and even though GPX activity was stimulated it was not sufficient to avoid H_2O_2 accumulation at high Zn levels; modulation of APX and GR activities were also linked to changes in the redox statuses of the leaves.

4. Materials and Methods

4.1 Greenhouse experiments

M. urundeuva plants were grown in sterile vermiculite (previously autoclaved at 121 °C for 30 min). This substrate was chosen to represent a worst-case scenario, i.e., greater availability of contaminants that may happen to occur in soil. Zinc dosages were chosen based on the levels observed in Zn-contaminated soils where *M. urundeuva* plants have been found growing. Zinc was added as $ZnSO_4$, which was mixed with the substrate to obtain final doses of 0, 50, 80, 120 and 200 mg Zn kg^{-1} substrate. The substrate pH (1:1 soil water $ratio^{-1}$) was adjusted to 6.7±0.1 when necessary (to the same pH observed in Zn-contaminated soils where these plants have been found growing) by the manual addition of 1 M HC1 or 1 M NaOH. *Myracrodruon urundeuva* Fr. Allem. (Anacardiaceae) seeds were acquired from the Seed Laboratory of the Companhia Energética de Minas Gerais (CEMIG, Belo Horizonte, Brazil) and were surface-sterilized in 5% sodium hypochlorite solution for 5 minutes and then thoroughly rinsed with deionized water. As *M. urundeuva* seeds show physical dormancy, they were submitted to mechanical scarification using sandpaper. The seeds were sown to germinate in Styrofoam boxes containing a vermiculite substrate (without Zn) and the seedlings grew under greenhouse conditions (temperature 15-31 °C; average photosynthetic active radiation 825 μmol photons m^{-2} s^{-1}) until developing their first pair of fully-expanded leaves (approximately 15 days). Healthy seedlings were then transplanted to pots with the prepared test substrates under greenhouse conditions (as described above)

with daily watering; 20 mL of half-strength Hoagland solution was applied every two weeks. A total of 12 replicates per treatment were used in a completely randomized experimental design. The plants were harvested after 120 days of cultivation, a period that allowed them to produce sufficient biomass for accurate analyses. The harvested plants were thoroughly washed with tap water, divided into root and shoot fractions, and their fresh shoot biomasses measured. The samples were then placed in paper bags and dried in a forced-air circulation oven at 80 °C to a constant weight (to determine dry biomass production).

4.2 Chlorophyll fluorescence and pigment content

Plant chlorophyll fluorescence and pigment content were measured at the end of the experiment period (120 days). The leaves were dark-adapted for 30 min before chlorophyll fluorescence measurements, and the photochemical efficiency of PSII (Fv/Fm) was then measured using a MINI-PAM (pulse-amplitude modulation) fluorometer (WALZ, Effeltrich, Germany). Chlorophyll fluorescence was measured in samples of the first to third fully expanded leaves, for a total of three measurements per plant. These leaves were also used to assay pigment content. Three foliar discs approximately 8.5 mm in diameter were cut from each leaf, and after determining the fresh weighs of the samples, their chlorophyll and carotenoid pigments were extracted in 80% acetone after macerating the discs with a mortar and pestle. The absorption of the extracts at 470 nm, 645 nm, and 662 nm were measured using a Genesys 10UV spectrophotometer. The concentrations (mg/g fresh leaf weight) of total chlorophyll and total carotenoids were then calculated using the equations described by Lichtenthaler and Wellburn [36].

4.3 Chemical analyses

Plant samples (~0.1 g) were digested in 5 mL of concentrated HNO_3 (GR) in a microwave oven (MARS-5®, CEM) at 175 °C for 10 min following USEPA Method 3051A. After digestion, the solutions were cooled, filtered through Whatman nº 40 filter paper, and brought to a final volume of 10 mL with ultra-pure water. The filtered extracts were preserved under refrigeration (4 °C) until analysis. Zinc concentrations were determined using an atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 800, Norwalk, CT). The accuracies of the elemental analyses for Zn were confirmed by carrying standard reference materials through the digestion and analytical processes. Each batch analyzed included reference material (BCR 414 Plankton, Community Bureau of Reference) with known levels of Zn, as well as one blank sample for quality control. Total Zn recovery in the reference material was approximately 80.5%.

4.4 Oxidative responses, antioxidant enzymes and redox potentials

Oxidative responses (H_2O_2 production and lipid peroxidation) as well as antioxidant enzyme (SOD, CAT, APX, GPX and GR), and antioxidant metabolite (total ascobate [AsA + DHA], reduced ascorbate [AsA], dehydroascorbate [DHA], total glutathione [GSH + GSSG], reduced glutathione [GSH], and glutathione disulphide [GSSG]) concentrations were analyzed in four plants per treatment. After harvesting, samples of the first to third fully expanded leaves were stored at -40 °C and subsequently used for biochemical studies and assessments of oxidative damage.

H_2O_2 contents were evaluated following Velikova et al. [37]. Measurements of MDA were performed following Hodges et al. [38]. Antioxidant enzymes were extracted by macerating 0.2 g of fresh leaves in 800 µl of an extraction buffer containing 100 mmol L^{-1} potassium phosphate buffer (pH 7.8), 100 mmol L^{-1} EDTA, 1 mmol L^{-1} L-ascorbic acid and 5% PVP (m/v). The protein contents of all of the samples were determined using the Bradford

method. The activities of superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), guaiacol peroxidase (GPX; E.C. 1.11.1.7) and glutathione reductase (GR; E.C. 1.6.4.2) were measure following the methods described by Marques and Soares [15].

To evaluate redox potentials, 0.2 g of frozen tissue were ground together with inert sand in a mortar and pestle in 5 mL of 6.5 % (w/v) *m*-phosphoric acid containing 1 mmol L^{-1} NaEDTA. Total ascorbate (AsA + DHA) and ascorbate (AsA) contents of the samples were estimated according to Hodges et al. [39]. The amounts of DHA were calculated by the differences between the total and reduced ascorbate concentrations. Total glutathione (GSH + GSSG) and GSSG contents were measured following Anderson [40]. GSH contents were calculated by the differences between total glutathione and GSSG concentrations.

4.5 Statistical analyses

The results were expressed as the averages of four replicates. The data was statistically evaluated using two-way analysis of variance run on the JMP software program (*SAS Institute Ins*.). The means were compared using the Scott-Knott test at a 5% level of probability.

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Table 1. Zn concentrations in leaves (mg kg^{-1} DW) and fresh shoots (FW), dry weight (DW) production (g), quantum yield (Fv/Fm), and total chlorophyll and carotenoid (µg g^{-1} FW) contents in the leaves of *M. urundeuva* seedlings grown on substrates amended with increasing doses of Zn (mg kg^{-1}).

	Zn doses (mg kg^{-1})				
	0	50	80	120	200
Zn concentration in leaves	79.90e	134.74d	207.29c	251.32b	298.96a
Shoot FW	1.60b	1.85a	1.87a	1.12c	0.83c
Shoot DW	0.47b	0.82a	0.75a	0.45b	0.48b
Fv/Fm	0.76	0.76	0.78	0.77	0.78^{ns}
Total chlorophyll	10.58	8.39	10.39	9.93	11.36^{ns}
Carotenoids	1.89b	1.87b	1.76b	2.06a	2.00a

Treatment means from ANOVA. Values followed by the same letter within the same line are not significantly different ($P < 0.05$) by the Scott-Knott multiple range test

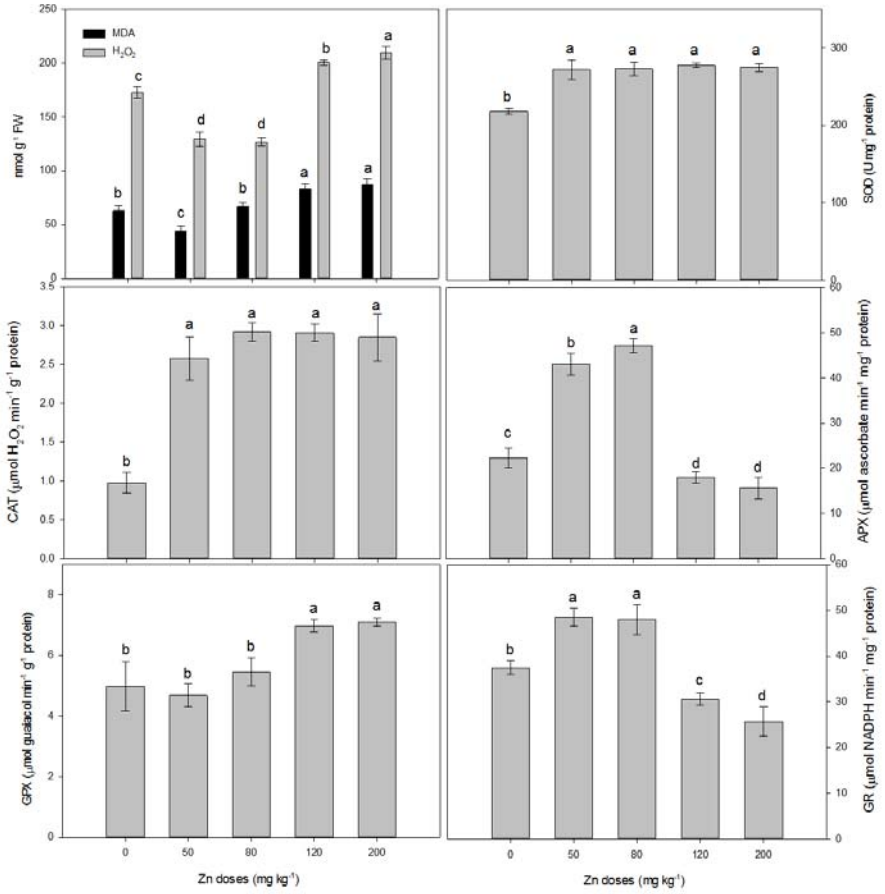

Fig. 1. Lipid peroxidation (MDA contents), H_2O_2 contents, and the activities of SOD, CAT, APX, GPX, and GR in the leaves of *M. urundeuva* seedlings grown on substrates amended with increasing doses of Zn (mg kg^{-1}). Bars indicate the means ± standard errors of four replicates. Means followed by the same letter are not significantly different ($P < 0.05$) by the Scott-Knott multiple range test.

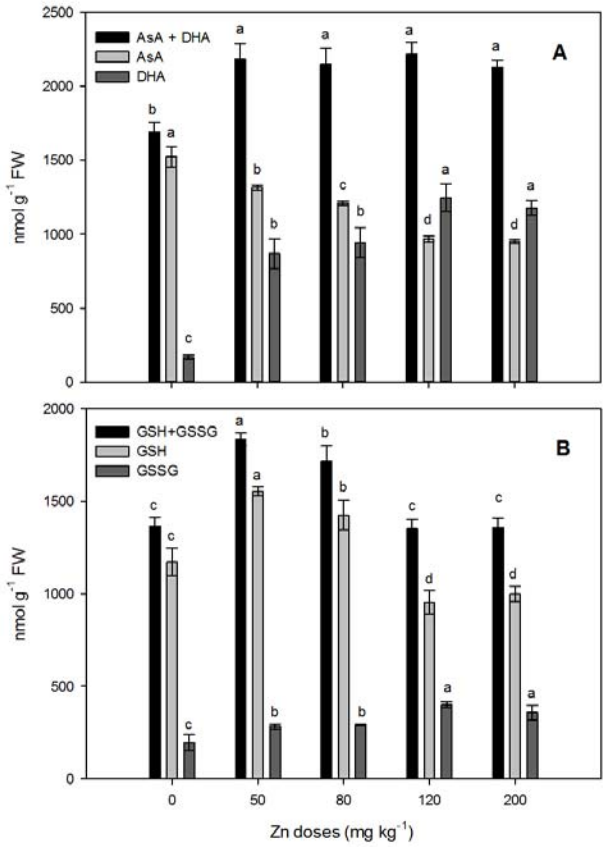

Fig. 2. A- Total (AsA + DHA), reduced (AsA), the oxidized (DHA) form of ascorbate, **B**- total (GSH + GSSG), reduced (GSH), and the oxidized form of glutathione (GSSG) in the leaves of *M. urundeuva* seedlings grown on substrates amended with increasing doses of Zn (mg kg^{-1}). Bars indicate the means ± standard errors of four replicates. Means followed by the same letter are not significantly different ($P < 0.05$) by the Scott-Knott multiple range test.

Table 2. The ratios of oxidized to reduced ascorbate concentrations (DHA/AsA) and The ratios of oxidized to reduced glutathione (GSSG/GSH) in the leaves of *M. urundeuva* seedlings grown on substrates amended with increasing doses of Zn (mg kg^{-1}).

Zn doses (mg kg^{-1})	DHA/AsA		GSSG/GSH	
0	0.11	±0.012	0.16	±0.044
50	0.66	±0.076	0.18	±0.008
80	0.77	±0.081	0.20	±0.011
120	1.24	±0.124	0.08	±0.003
200	1.29	±0.064	0.08	±0.004