NAIARA VIANA CAMPOS

ARSENIC HYPERACCUMULATION IN Pityrogramma calomelanos (L.) LINK (PTERIDACEAE): MORPHOPHYSIOLOGICAL MECHANISMS OF TOLERANCE

Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Post-Graduate Program in Botany for obtention of the degree of Doctor Scientiae.

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Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Post-Graduate Program in Botany for obtention of the degree of Doctor Scientiae.

APPROVED: December 12th of 2014.

Adriano Nunes Nesi	Cléberson Ribeiro
Marcelo Braga Bueno Guerra	Wagner Campos Otoni

Aristéa Alves Azevedo
(Adviser)

This thesis is dedicated...

To my great love, Vitor,
To my parents, Lídia and Oldac,
To all my family and friends.

“Let nothing disturb you,
Let nothing frighten you,
All things are passing away:
God never changes.
Patience obtains all things
Whoever has God lacks nothing;
God alone suffices”.

St. Teresa of Avila

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BIOGRAPHY

NAIARA VIANA CAMPOS, Brazilian, married, daughter of Oldac Campos and Ilídia Vieira Viana Campos, was born in Sete Lagoas-MG, on October 16, 1985.

In March of 2004 initiated an undergraduate course in Biological Sciences at the Universidade Federal de Viçosa. In April of 2006 began the scientific initiation, working for two years in the project "Evaluation of the phytotoxic effects of fluoride in Spondias dulcis Forst F. (Anacardiaceae), a tropical sensitive species". In the third year of scientific initiation developed the project of monograph "Accumulation and phytotoxic effects of fluoride in boldo-gambá and capim-cidreira used for tea". In January of 2009 concluded the Bachelor's and Licentiate's degree in Biological Sciences receiving praise votes for the Biological and Health Sciences Center.

In March of 2009 began the MSc studies in Botany at Plant Biology Department (DBV/UFV). In June of 2010 participated in the "IX Programa de Jóvenes Liberoamericanos – Una inmersión en la realidade política, social y económica de Espanã y la Unión Européa", organized by the Fundación Carolina (Madri/ES). In February of 2011 concluded the dissertation entitled "Effects of arsenic and phosphorus interaction on the arsenic tolerance of two populations of Borreria verticillata (Rubiaceae)".

In November of 2011 initiated the doctorate in Botany (DBV/UFV), giving continuity in the line of research of anatomical and physiological effects of pollutants in plants, and submitting the thesis on the 12th of December of 2014.

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Arsênio (As) é um metaloide amplamente distribuído no meio ambiente. A sua presença em água potável constitui um sério problema de saúde pública em diversos países devido aos seus efeitos mutagênicos e genotóxicos. A descontaminação de águas e solos requer o uso de tecnologias apropriadas de remediação de modo a prevenir ou reduzir os impactos no ecossistema. A utilização de monilófitas hiperacumuladoras de As possibilita o desenvolvimento de programas de fitorremediação menos onerosos e com melhor preservação ambiental. Pteris vittata L. foi a primeira hiperacumuladora descrita e tem sido amplamente estudada. Pityrogramma calomelanos L. (Link) por sua vez é uma hiperacumuladora menos conhecida, naturalmente encontrada em sítios contaminados com As. Esse trabalho teve como objetivo caracterizar as respostas morfoanatômicas e fisiológicas envolvidas no acúmulo e na tolerância ao As em P. calomelanos. Três experimentos foram conduzidos (E1, E2 e E3) utilizando esporófitos em diferentes estágios de desenvolvimento e expostos ao As (E1: 1 mM As; E2-E3: 1, 10, e 30 mM As) durante duas (E2) ou três (E1 and E3) semanas. As plantas foram cultivadas em solução nutritiva de Hoagland (1/2 F) e o As foi adicionado à solução na forma de arsenato de sódio. Plantas crescidas em solução sem adição de As foram usadas como controle. A fluorescência da clorofila a foi avaliada com auxílio de fluorômetro de imagem, Imaging-PAM (E2), ou fluorômetro portátil, Mini-PAM (E3; apenas parâmetros do escuro), e a concentração de clorofila foi estimada usando um medidor de clorofila portátil (SPAD) durante E1. Ao final dos experimentos, foram coletadas amostras para as análises fisiológicas (E1 e E3), microscópicas (E2), químicas (E1-E3) e de microfluorescência de raios-X com energia dispersiva (μ-EDXRF) (E1 e E3). Pityrogramma calomelanos apresentou elevado potencial de absorção, translocação e acúmulo de As. Concentrações de As nas frondes variaram de 3000 a 12000 mg kg^{-1} de massa seca (MS), sendo maiores para as plantas do E3. Em baixa dose de As (1 mM) as plantas acumularam As principalmente nas pinas (75 % arsenito), enquanto estipes e raízes apresentaram conteúdos de As próximas (74 e 95 % arsenato, respectivamente). Em E2, a partição de As foi semelhante a do E1, entretanto, em E3 estipes e raízes

apresentaram concentrações de As tão altas quanto às pinas, em plantas expostas a 10 e 30 mM As. O As reduziu a concentração de P em plantas do E1, mas não alterou naquelas do E2 e E3. Em E1, As reduziu o conteúdo de K, Fe e Mg, bem como a translocação de K e S. A concentração de clorofila não foi alterada em plantas do E1 e E3, entretanto foram observadas diferenças na fluorescência da clorofila a em plantas expostas a 10 e 30 mM As (E2). Plantas apresentaram menor MS de pinas (E2: 30 mM As), e necroses marginais e apicais em frondes mais velhas (E2 e E3: 10 e 30 mM As). A análise anatômica destas plantas revelou o maior acúmulo de fenóis (áreas aparentemente sadias da pina) e colapso de tecidos principalmente na margem de pínulas e próximo às nervuras secundárias (áreas necróticas da pina). O As induziu alterações sutis em raízes (E2), como o escurecimento progressivo, despreendimento de células da coifa lateral aumento da espessura da parede celular e acúmulo de compostos granulares em células corticais de plantas tratadas com 30 mM As. A concentração de As na região apical e marginal das pinas, e ao redor de feixes vasculares, foi confirmada pelas análises de μ- EDXRF. Pityrogramma calomelanos apresentou um eficiente sistema de defesa antioxidante, demonstrado pelo aumento na atividade de enzimas antioxidantes (E1 e E3), de tióis não proteicos (E1), fenóis (E2 e E3), e outras moléculas antioxidantes (E3), especialmente na pina. Em doses elevadas, entretanto, os mecanismos de proteção se tornaram ineficientes ocasionando o aumento da produção de espécies reativas de oxigênio e da peroxidação lipídica na pina (E3), a qual também foi observada em raízes expostas a 1 mM As (E1). Em E3, a acumulação de As reduziu ou manteve a concentração de metabólitos associados ao metabolismo central de carbono (ex. glicose) e aumentou a de aminoácidos e proteínas totais. Aminoácidos envolvidos com o metabolismo de S, fotorrespiração, osmoproteção e síntese de compostos secundários foram os principais alvos do As. A análise conjunta dos resultados mostra que P. calomelanos pode ser usada com sucesso na remediação de sítios com contaminação moderada de As. Plantas com maior biomassa (ex. 5-7 fronds – E3) são preferíveis devido ao seu maior potencial de extração. Experimentos com exposição crônica são necessários para esclarecer os mecanismos dose-dependentes de tolerância ao As, aprimorando o desempenho de P. calomelanos em campo. A remoção da parte aérea das plantas tão logo apareça sintomas de toxidez (necroses e murcha) contribuirá para uma fitorremediação mais eficiente.

ABSTRACT

CAMPOS, Naiara Viana, D.Sc., Universidade Federal de Viçosa, December, 2014. **Arsenic hyperaccumulation in Pityrogramma calomelanos (L.) Link (Pteridaceae): morphophysiological mechanisms of tolerance.** Adviser: Aristéa Alves Azevedo. Co-adviser: Bruno Lemos Batista.

Arsenic (As) is a metalloid ubiquitously distributed in the environment. The presence of As in drinking water is a serious health problem in many countries due to its genotoxic and mutagenic effects. The cleanup of contaminated water and soils requires the use of appropriate remediation techniques to prevent possible impacts on the ecosystems. As-hyperaccumulating ferns provide the possibility of developing cost-effective, and ecofriendly As phytoremediation programs. Pteris vittata L. was the first As hyperaccumulating fern described, and has been widely studied. Pityrogramma calomelanos L. (Link) is a less known hyperaccumulating fern that grows naturally in As-contaminated sites. This study aimed to characterize the morphoanatomical and physiological mechanisms involved in As accumulation and tolerance in P. calomelanos. Three experiments (E1, E2 and E3) were performed using ferns at different stages of development and exposed to As (E1: 1 mM As; E2-E3: 1, 10, and 30 mM As) for two (E2) or three (E1 and E3) weeks. Arsenic was supplied as a solution of sodium arsenate and was added to Hoagland's nutrient solution (1/2 strenght). Plants grown in solution without As were used as control. The chlorophyll (chl) a fluorescence was evaluated by Imaging-PAM fluorometer (E2) and Mini-PAM portable fluorometer (E3; only dark parameters), and the chl content was estimated by portable chlorophyll meter (SPAD) in E1. At the end of the experiments, plants were sampled for physiological (E1 and E3), microscopic (E2), chemical (E1-E3) and micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF) (E1 and E3) analyses. Pityrogramma calomelanos showed a high potential to As uptake, translocation and accumulation. Arsenic concentrations in fronds ranged from 3000 to 12000 mg kg^{-1} dry weight (DW) and was higher in E3. Plants exposed to 1 mM As (E1), ferns accumulated As mainly in pinnae (75 % as arsenite), whereas roots and stipes showed similar As contents (95 % and 74 % as arsenate, respectively). Arsenic partition was similar between E1 and E2, whereas in E3, stipes and roots showed As concentrations higher than those found in pinnae at 10 and 30 mM As in solution. Arsenic reduced the P

concentrations in ferns of E1, but not in those from E2 and E3. In E1, As reduced the content of K, Fe and Mg, and the translocation of K and S. Arsenic did not change chl content in plants from E1 and E3, however, differences were observed in chl a fluorescence of plants exposed to 10 and 30 mM (E2). Ferns showed reduction in DW of pinnae at 30 mM As (E2), and apical and marginal necrosis in old fronds at 10 and 30 mM As (E2 and E3). In an anatomic view, these ferns (E2: 10-30 mM As) showed increase in phenols (healthy pinna areas) and tissue disruption mainly in the pinnule margins and near the secondary veins (necrotic pinna areas). Arsenic induced slight alterations in the radicular system (E2), such as progressive darkening of the root, detachment of border-like cells, increase in cell wall thickness and accumulation of granular compounds in cortical cells at 30 mM As. The µ-EDXRF analysis confirmed that As was preferentially located in apical and marginal regions of pinnules and around the veins. Pityrogramma calomelanos showed an efficient antioxidant defense system, as observed by the increase in the activities of antioxidant enzymes (E1 and E3) and increase in non-protein thiols (E1), phenols (E2 and E3), and other scavenging molecules (E3), especially in pinnae. At extremely high As concentrations (e.g. 30 mM As) these protective mechanisms became inefficient, increasing the concentration of oxygen reactive species and culminating in lipid peroxidation in fronds (E3). Roots exposed to 1 mM As also showed increase in lipid peroxidation. In E3, As accumulation promoted reduction or maintenance of metabolites associated with central carbon metabolism (e.g. glucose) and increased the total concentration of amino acids and proteins. Amino acids up-regulated by As were associated with S metabolism, photorespiration, osmoprotection and secondary metabolite biosynthesis. Altogether, these findings indicate that P. calomelanos can be successfully used for remediation of moderate As-contaminated sites. Adult plants with higher biomass (e.g. 5-7 fronds - E3) are preferable due to their higher potential to extract As from the medium. Experiments with low-dose and long-time exposures (chronic exposure) are needed to clarify the dose-response-mediated mechanisms of As-tolerance in P. calomelanos, in order to improve its performance in the field. The harvesting of plants as soon as the appearing of visual symptoms, such as necrosis and shriveling of young fronds, can also improve the phytoremediation efficiency.

INTRODUCTION

Arsenic (As) is a metalloid that belongs to the group 15 of periodic table of elements, along with nitrogen, phosphorus, antimony, and bismuth. The most common valence states of arsenic are −3, 0, +3 and +5 (Henke, 2009). In its elemental form As is a brittle, grayish crystal that becomes darker (yellow) when exposed to air (Krebs, 2006). According to Krebs (2006) the term 'arsenic' is probably derived either from the Latin word 'arsenicum' or the Greek word 'arsenikon', both meaning a yellow pigment.

Arsenic is widely distributed in the Earth's crust, which contains about 3.4 mg kg^{-1} As (Wedepohl, 1991). It is mostly found in nature in minerals, such as realgar (arsenic monosulfide, AsS), orpiment (arsenic trisulfide, As_2S_3), and arsenopyrite (iron arsenosulfide, FeAsS); and it is also present in the most sulfide ores of other metals (Krebs, 2006; Zhao et al., 2010). Arsenic is naturally released into the environment through activities such as volcanic action, low temperature volatilization, erosion of rocks and forest fires (Tripathi et al., 2007). Anthropogenic sources of arsenic include metal mining and smelting (e.g. copper and gold), pesticide application, coal combustion, wood combustion, and waste incineration (Zhao et al., 2010). The oxidation of arsenosulfides in natural rock formations or mining wastes can release As into ground- and surface-waters and represents a serious risk to human health and the environment (Henke, 2009).

Arsenic is currently the most hazardous substances to human health, according to the Agency for Toxic Substances and Disease Registry (ATSDR, 2013). It is a carcinogen element and the chronic or long-term human intake of toxic inorganic As from drinking water and food may result in arsenicosis (Hughes, 2011; Shankar et al., 2014). Arsenicosis is a common name generally used for As related health problems including skin disorders, skin cancers, internal cancers (bladder, kidney, and lung), cardiovascular and peripheral vascular diseases and possibly diabetes (Sun et al., 2014 for review). Studies suggest that As inhibits cell cycle check point proteins, DNA repair system and DNA methylation, which ultimately lead to the tumor development (Reichard and Puga, 2010; Sinha et al., 2013). A synthetic view of the deleterious As effects in animal cells is shown in the Figure 1.

Elevated concentrations of As in groundwater and other natural waters with geogenic sources occur in many areas around the world, especially in Asia and Americas (Smedley and Kinniburgh, 2002). About 150 million people around the world

are estimated to be affected and new affected areas are continuously discovered (Ravenscroft et al., 2009). Bengal (Bangladesh) and West Bengal (India) are the two major impacted areas, which are dependent on As-contaminated groundwater for drinking purposes (Henke, 2009; Shankar et al., 2014). A global overview of the As-contaminated areas is shown in the Figure 2.

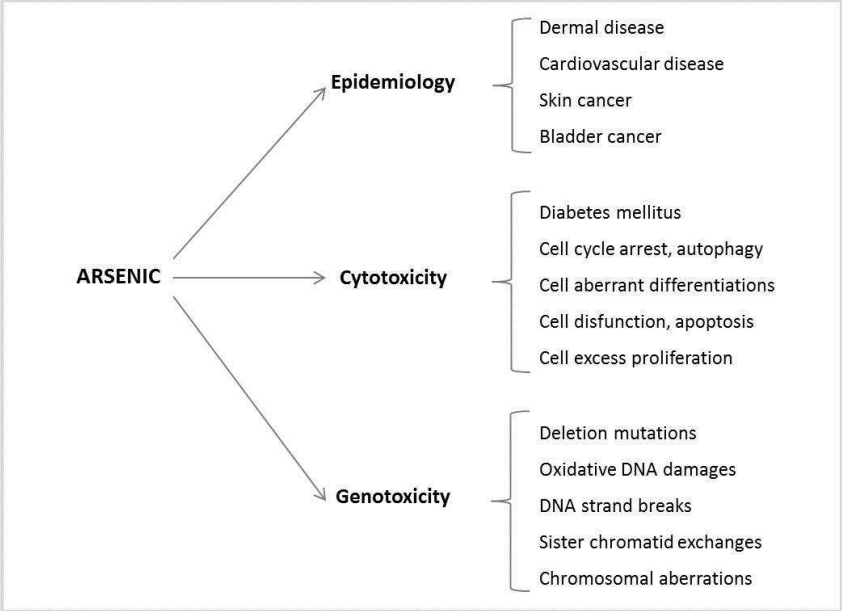

Figure 1: Effects of arsenic toxicity in humans and rats (Sun et al., 2014).

Arsenic exposure in Brazil occurs mainly through industrial wastes and atmospheric emissions (Deschamps et al., 2003). The most As-impacted areas are related to gold mines, mainly located in the Iron Quadrangle region of Minas Gerais that include the districts of Nova Lima, Raposos, Mariana, Ouro Preto and Santa Bárbara (Matschullat et al., 2000; Borba et al., 2003). A study conducted in the districts of Nova Lima and Santa Bárbara showed that twenty per cent of the total sampled population had elevated As concentrations in the urine, indicating that adverse health effects cannot be excluded on a long-term basis (Matschullat et al., 2000). Paracatu city (Minas Gerais state), Ribeira Valley (São Paulo state), and Santana city (Amapá state) have been also reported as As-contaminated regions mainly due mining activities. Additionally, other areas of risk have been appointed including the Brazilian states of Bahia, Mato Grosso, Paraná and Rio Grande do Sul (Murcott, 2012).

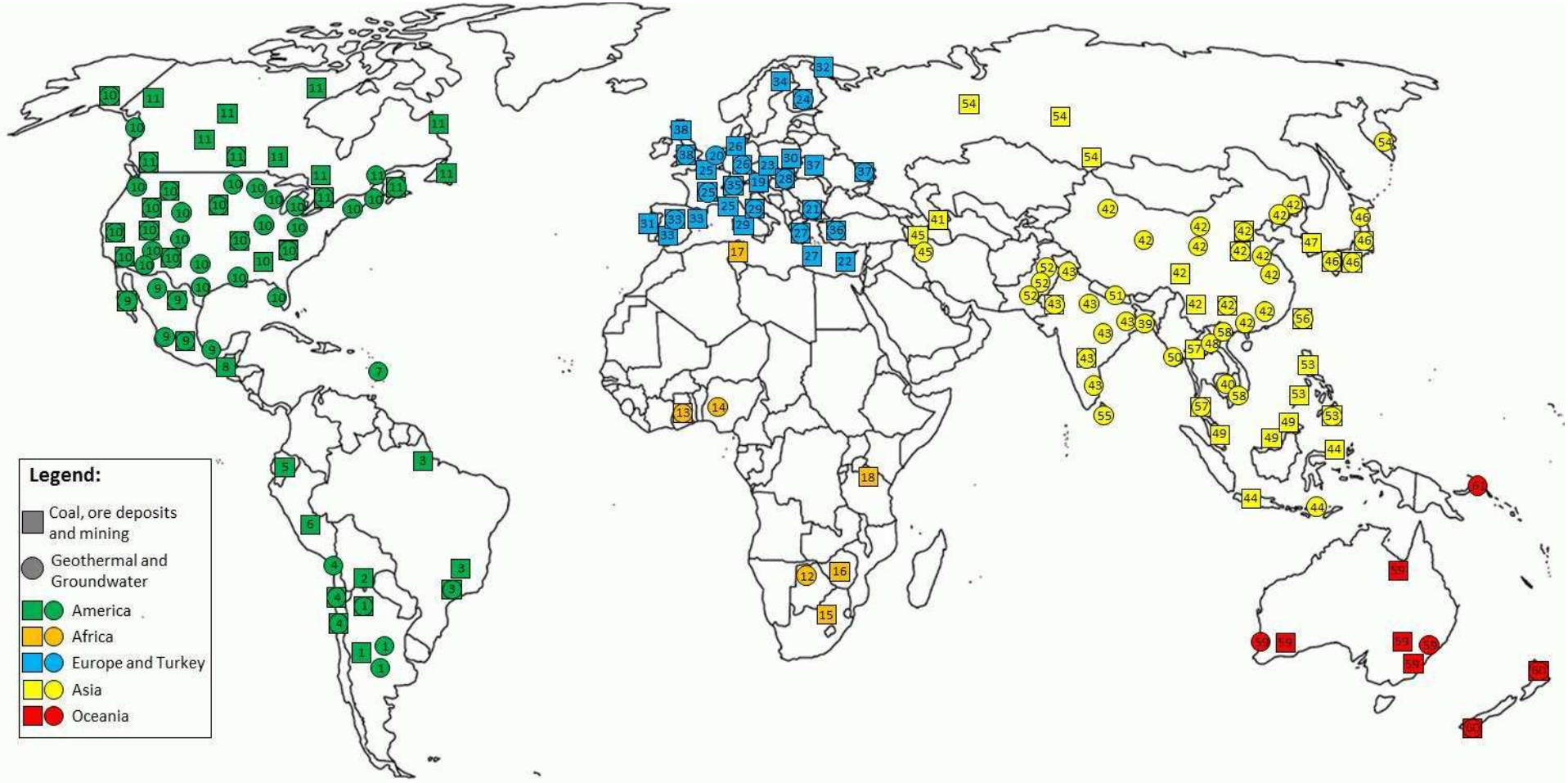

Figure 2. Global overview of the arsenic (As)-impacted areas through coal, ore deposits and mining activities, and geothermal/groundwater contamination (adapted from Henke, 2009). Numbers printed on map are described in the Table 1.

Table 1. List of the global arsenic As-contaminated areas with the correspondent local sources (adapted from Henke, 2009).

America		
Number	**Location**	**Arsenic-bearing medium**
1*	Argentina: Cordoba, Jujay, Salta, Tucumán, Santiago del Estero and La Pampa Province.	Groundwater, ore deposits and mining wastes.
2	Bolivia: Pilcomayo River Basin.	Ore deposits and mining wastes.
3	Brazil: Amapá (Santana area), Minas Gerais (Iron Quadrangle and Paracatu) and São Paulo (Ribeira valley).	Groundwater, ore deposits and mining wastes.
4	Chile: Antofagasta, Loa River, Elqui River watershed and Lluta River Basin.	Geothermal, groundwater, evaporation, ore deposits and mining wastes.
5	Ecuador.	Ore deposits and mining wastes.
6	Peru: Huancavelica district.	Ore deposits and mining wastes.
7	Dominica.	Geothermal.
8	Guatemala: Northwestern.	Ore deposits and mining wastes.
9	Mexico: Baja California Sur, Chihuahua, Guanajuato, Salamanca aquifer, Guerrero, Morelos, Puebla, Hidalgo, Rioverde Basin, La Primavera Geothermal Field, Lagunera Region, Lerma-Chapala Basin, Michoacán, Nuevo Léon, San Luis Potosí, Tabasco.	Geothermal, groundwater, formation waters, smelting, ore deposits and mining wastes.
10	U.S.A: Alabama, Alaska, Appalachia, Arizona, Arkansas, California, Colorado, Connecticut, Florida, Idaho, Illinois, Indiana, Iowa, Kentucky, Louisiana, Massachusetts, Michigan, Minnesota, Missouri, Montana, Nevada, New England, New Hampshire, New Mexico, North Dakota, Ohio, Oklahoma, Oregon, South Carolina, South Dakota, Tennessee-North Carolina, Texas, Utah, Vermont, Virginia, Washington, Wisconsin, Wyoming.	Geothermal, groundwater, pegmatite, sediment pore waters, smelter, evaporation, coal and coal mining, ore deposits and mining wastes.
11	Canada: Alberta, British Columbia, Labrador, Manitoba, New Brunswick, Newfoundland, Yellowknife region, Nova Scotia, Nunavut, Ontario, Quebec, Saskatchewan, Yukon.	Geothermal, groundwater, coal, weathered shale, porewaters, ore deposits and mining wastes.

	Africa	
12	Botswana.	Groundwater.
13	Ghana.	Groundwater, ore deposits and mining wastes.
14	Nigeria.	Groundwater.
15	South Africa.	Ore deposits and mining wastes.
16	Tanzania: Serengeti NP (Orangi River).	Ore deposits and mining wastes.
17	Tunisia.	Ore deposits and mining wastes.
18	Zimbabwe.	Ore deposits and mining wastes.
	Europe	
19	Austria: East Alps.	Ore deposits and mining wastes.
20	Belgium and Netherlands.	Groundwater.
21	Bulgaria: Plovdiv-Assenovgrad area, Southwest.	Geothermal, coal, ore deposits, smelting and mining.
22	Cyprus: Mathiatis Mine.	Ore deposits and mining wastes.
23	Czech Republic: Bohemian Massif, Krusné hory Mountains, Sokolov Basin, Ostrava-Karvina Basin.	Mineralization, coal and coal mining wastes, ore deposits and mining wastes.
24	Finland: Ilomantsi, Kittila, Pirkanmaa, Seinajoki district and Ylojarvi.	Tills, groundwater, ore deposits and mining wastes.
25	France: Corsica, Douai area, Massif Central, Rhône River.	Smelter, geothermal, ore deposits and mining wastes.
26	Germany: Bavaria, Mulde River, Dessau area, Freiberg, Koenigstein Mine, Mansfeld region, Southeast Harz Forelands and Ebersdorf Coal Deposit.	Groundwater, chemical industry, coal deposits and mining wastes, ore deposits and mining wastes.
27	Greece: Central Macedonia, Crete, Elassona Basin, Northwestern, Mygdonia region, Eastern Attica and Santorini.	Groundwater, geothermal, coal and coal mining wastes, ore deposits and mining wastes.
28	Hungary, Romania and Slovakia: Great Hungarian Plain, Mátra Mountains, Pannonian Basin, Danube Basin, Baia Mare Region and Cierna Lehota.	Groundwater, Mining wastes, Mineralized Shales, ore deposits and mining wastes.
29	Italy: Campanion Volcanic Province, Sardinia and Southern Tuscany.	Geothermal, ore deposits and mining wastes.

30	Poland: Sudety Mountains and Lyublin Basin.	Mineralization, coal deposits and mining wastes, ore deposits and mining wastes.
31	Portugal: Castromil gold deposit.	Ore deposits and mining wastes.
32	Russia: Kola Peninsula.	Smelter.
33	Spain: Anllóns River, Asturias and León, Aznalcóllar, Central, Eastern Pyrenees, Madrid Aquifer and Puertollano Basin.	Groundwater, coal and coal mining wastes, ore deposits and mining wastes.
34	Sweden: Kalix River and Bothnian Bay, Kristineberg mining site, Ronnskar Smelter, Adak Mine and Vormbacken River.	Smelter, ore deposits and mining wastes.
35	Switzerland: Alps, Camignolo area and Malcantone watershed.	Geothermal, groundwater, hydrothermal deposits, ore deposits and mining wastes.
36	Turkey: Central Anatolia, Emet, Gediz and Kutahya.	Geothermal, coal, groundwater, volcanics, ore deposits and mining wastes.
37	Ukraine-Russian: Donbas Basin, L´vov-Volynsk Basin.	Groundwater, coal deposits and mining wastes.
38	United Kingdom: Cornwall, Scotland and South Wales.	Groundwater, intrusive igneous rocks, coal, ore deposits, smelting, and mining wastes.
	Asia	
39	Bangladesh.	Groundwater.
40	Cambodia.	Groundwater.
41	Caspian Sea.	Mine runoff and other anthropogenic sources
42	China: Anhui, Beijing, Guangdong, Guizhou, Henan, Hunan, Inner Mongolia, Jilin, Liaoning, Ningxia, Northeast, Qinghai, Shandong, Shanxi, Sichuan, Xinjiang, Yunnan.	Groundwater, coal and coal mining, ore deposits and mining wastes.
43	India: Andhra Pradesh, Assam, Bihar and Jharkhand, Chhattisgarh, Karnataka, Punjab, Haryana, Himachal Pradesh, Rajasthan, Tamil Nadu, Uttar Pradesh and West Bengal.	Groundwater, coal, schist belt, ore deposits and mining wastes.
44	Indonesia: Ngada district (Bejawa geothermal area), North Sulawesi and West Java.	Geothermal, volcanic deposits, ore deposits and mining wastes.
45	Iran: Kurdistan and Zarshuran.	Groundwater, ore deposits and mining wastes.

46	Japan: Ichinokawa Mine, Honshu, Kyushu and Osaka.	Geothermal, groundwater, ore deposits and mining wastes.
47	Korea, South.	Ore deposits and mining wastes.
48	Laos.	Groundwater.
49	Malaysia: Peninsula, Sabah and Sarawak.	Ore deposits and mining wastes.
50	Myanmar: Ayeyarwaddy Division, Bago and Rakhine state.	Groundwater.
51	Nepal: Southern.	Groundwater.
52	Pakistan: Punjab and Sindh.	Groundwater and drinking water.
53	Philippines: Luzon, Mindanao and Palawan.	Geothermal, ore deposits and mining wastes.
54	Russia: Altai Mountains, Kamchatka Peninsula, Kemerovo region and Ural Mountains.	Geothermal, smelting, coal and coal mining, ore deposits and mining wastes.
55	Sri Lanka.	Groundwater.
56	Taiwan.	Groundwater and smelter
57	Thailand: Mae Moh and Nakhon Si Thammarat Province.	Groundwater, coal mining and combustion, ore deposits and mining wastes.
58	Vietnam: Red River region and Mekong River region.	Groundwater.
	Oceania	
59	Australia: New South Wales, Queensland, Western Australia.	Groundwater, smelter and industrial complex, ore deposits and mining wastes.
60	New Zealand: North and South Island.	Geothermal, coal and coal mining, ore deposits and mining wastes.
61	Papua New Guinea: Ambitle Island (Tatum Bay).	Geothermal.

* Numbers on the first column correspond to those indicated on the map (Figure 2).

In response to the widespread As contamination, many governments have instituted regulations on the disposal of arsenic bearing wastes and As emission from ore smelters and coal-combustion. Remediation of As-contaminated soils and waters is needed to protect the biosphere. Conventional physicochemical technologies have been applied to remediate As such as vitrification, soil washing/flushing and precipitation, however, without success (Mondal et al., 2006; Mirza et al., 2014). Most of these methods are found very costly (75 – 500 dollars per ton of soil), and thus low-cost technologies are needed to effectively treat As-contaminated sites (Mirza et al., 2014). Phytoremediation has been suggested as the most cost effective and efficient method for removal or minimization of metal contamination both in soil and water.

Phytoremediation involves the use of plants to remove, transfer, stabilize, and/or degrade contaminants in soil, sediment, and water (Hughes et al., 1997). Different processes can be involved in the phytoremediation of As-contaminated sites: phytostabilization, which refers to the use of plants to minimize As dispersion from soil to water or air; phytoextraction (or phytofiltration), which refers to the use of plants to remove As from soil or water; and phytovolatilization that involves the transformation of As to volatile compounds and their emission from plants (Zhu and Rosen, 2009).

Plants exhibit a wide variation in their response to As. Not considering the As-sensitive, plants can be classified as excluders, accumulators and hyperaccumulators according to their physiological mechanisms of resistance/tolerance (Table 2). Excluders are plants adapted to grow in soils that have high As concentrations, without accumulating it (Moreno-Jiménez et al., 2012). The concentration of As in non-accumulator plants rarely exceeds 2 mg As per kg in aerial parts (Horswell and Speir, 2006). Plants that can accumulate high concentrations of metals in the aboveground biomass (higher than 1000 mg As per kg), without suffering growth constraints, have been referred as hyperaccumulators (Reeves and Baker, 2000; Kramer, 2010). Excepting hyperaccumulators, most plants accumulate As in roots (accumulators).

Arsenic hyperaccumulating plants have been described since the beginning of 21th century, including several Pteris ssp. (Ma et al., 2001; Zhao et al., 2002; Srivastava et al., 2006) and Pityrogramma calomelanos (Visoottiviseth et al., 2002; Kachenko et al, 2007). Pteris vittata was the first hyperaccumulating plant described (Ma et al., 2001) and has been widely studied. Arsenic tolerance mechanism of P. vittata have been reported to involve higher capacity of As translocation and sequestration in

vacuole of leaf cells combined with a higher antioxidant capacity (Lombi et al., 2002; Srivastava et al., 2005; Singh et al., 2006; Zhao et al., 2009).

Table 2. Physiological mechanisms involved in As exclusion, accumulation and hyperaccumulation by plants. Adapted from Moreno-Jiménez et al. (2012).

Process	**Exclusion**	**Accumulation**	**Hyperaccumulation**
Uptake	Transporters with higher affinity for P or Si than for As	Transporters with higher affinity for As than for P or Si	Transporters with higher affinity for As than for P or Si
Root efflux	Intense	Low	Very low
Complexation	Low rates of complexation	High rates of complexation and accumualtion in roots	Low rates of complexation
Xylem transport	Low xylem As concentration	Weakly translocated, mostly as As-SH complexes	Highly translocated, probably as As-SH complexes or free As
Vacuole storage	Low (in roots)	In roots	In shoots

Pityrogramma calomelanos (L.) Link is considered to be of American origin (Wardlaw, 1962) and is now virtually pan-tropical in its distribution. This species is known to accumulate unusually high levels of As, and is commonly found associated with mine sites in Australia, Brazil and Thailand (Visoottiviseth et al., 2002; Melendez et al., 2011; Niazi et al., 2012). Pityrogramma calomelanos has been pointed as a suitable species for phytoextraction/phytoremediation (Jankong et al., 2007), however, the mechanisms responsible for its tolerance remain unknown, as well the As effects on its development.

This study aimed to characterize the morphoanatomical and physiological responses of Pityrogramma calomelanos (L.) Link to arsenic exposure in order to understanding its mechanisms of As-accumulation and tolerance and provide data to improve its features for phytoremediation approaches.

The following chapters presented in this thesis, were edited based on the format requirements of specific scientific journals, and adapted to the norms for elaboration of thesis proposed by the Universidade Federal de Viçosa.

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CAPÍTULO 1: Alterações fisiológicas em pina e raiz de Pityrogramma calomelanos (L.) Link para minimizar efeitos deletérios do arsênio

RESUMO: Hiperacumulação de arsênio (As) tem sido descrita para algumas espécies de Pteridales; entretanto, as bases fisiológicas da hiperacumulação permanecem desconhecidas, especialmente em Pityrogramma calomelanos única hiperacumuladora descrita que não pertence ao gênero Pteris. Com o intuito de aprimorar o entendimento acerca das respostas induzidas por As em diferentes partes da planta, indivíduos de P. calomelanos foram expostos a 1 mM As durante 21 dias e foram subsequentemente comparados com indivíduos do tratamento controle. Plantas tratadas com As apresentaram concentrações médias de 3108, 275, and 283 mg kg^{-1} na matéria seca de pinas, estipes e raízes, respectivamente. Nas pinas, o As foi encontrado principalmente na forma de arsenito, enquanto o arsenato predominou em estipes e raízes. Aumento na atividade da peroxidase do ascorbato (APX) e da peroxidase (POX) e no conteúdo de tióis não-proteicos foi observado nas pinas, as quais não apresentaram sintoma de toxidez. Apesar do aumento da atividade da superóxido dismutase (SOD) e da catalase (CAT), as raízes de plantas tratadas apresentaram menor massa fresca, menores concentrações de P, K, Fe e Mg, e aumento da peroxidação lipídica. Plantas expostas ao As apresentaram, ainda, redução da translocação de K e S. O elevado fator de translocação de As e potencial antioxidante observado para a pina se mostraram essenciais para evitar o aumento da concentração de espécies reativas de oxigênio permitindo o acúmulo crescente de As nas frondes. A hiperacumulação de As em P. calomelanos requer ainda ajustes na nutrição mineral das plantas, em especial em relação às concentrações de K e o P.

Palavras-chave: balanço nutricional; espécies reativas de oxigênio; hiperacumuladora de As; tióis

CHAPTER 1. Physiological changes in the pinna and root of Pityrogramma calomelanos (L.) Link to alleviate arsenic deleterious effects

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ABSTRACT: Arsenic (As) hyperaccumulation has been described in a number of Pteridales. However, the physiological basis of hyperaccumulation remains unclear, especially in non-Pteris species such as Pityrogramma calomelanos. Aiming at a better understanding of As-induced responses in different fern parts, P. calomelanos plants were exposed to 1 mM As for 21 days, and subsequently compared with control plants. Chemical analysis revealed As concentrations in pinnae, stipes, and roots of 3108, 275, and 283 mg kg^{-1} dry weight, respectively. Arsenic was present mainly as arsenite in pinnae and as arsenate in stipes and roots. Increases in ascorbate peroxidase (APX) and peroxidase (POX) activities and non-protein thiol contents were observed in pinnae, without symptoms of As toxicity. Despite increases in superoxide dismutase (SOD) and catalase (CAT) activities, the roots showed reductions in fresh weight and P, K, Fe, and Mg concentrations and an increase in lipid peroxidation. Plants exposed to As showed a reduction in K and S translocation factors. The higher As translocation and antioxidant capacity of the pinnae are essential for quenching oxygen reactive species at lower concentrations, thus enhancing continuous As accumulation in fronds. Arsenic hyperaccumulation in P. calomelanos also requires adjustments in the mineral nutrition of plants, especially with regard to K and P.

Keywords: As-hyperaccumulator; nutrient balance; reactive oxygen species; thiol

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1. Introduction

Hyperaccumulating plants have the ability to accumulate extremely high concentrations of metals or metalloid within their tissues, wherein the threshold concentration used to define a hyperaccumulator depends on the particular element sequestered (van der Ent et al., 2013). Arsenic (As) hyperaccumulating plants must contain at least 1000 mg As kg^{-1} dry weight (DW) (Kramer, 2010). Nominal threshold criteria, however, should be complemented with a suite of characteristics that include a bioconcentration factor > 1, a shoot-to-root metal concentration quotient > 1 and extreme metal tolerance due to effective biochemical detoxification (Baker and Whiting, 2002).

Arsenic hyperaccumulation has been described for a number of Pteris spp., most notably Pteris vittata, which accumulates up to 22630 mg As kg^{-1} DW in the frond (Ma et al., 2001), and other ferns such as Pityrogramma calomelanos, with up to 8350 mg As kg^{-1} DW (Francesconi et al., 2002). Although P. vittata has been widely studied, P. calomelanos remains a lesser-known As-hyperaccumulating fern. Two varieties of P. calomelanos have been recognized: var. calomelanos (Silver back fern), usually referred to only as P. calomelanos, and var. austromericana (Gold dust fern). The Silver back fern has a white indument and is typical of humid tropics, differing from the Gold dust fern, which exhibits a yellow indument and is common in temperate and subtropical environments (Schelpe, 1975; Moran, 1995). The Gold dust fern has also been recognized as an As hyperaccumulator, with As concentrations reaching 5845 mg kg^{-1} DW in its young fronds (Kachenko et al., 2010).

Arsenic hyperaccumulation in ferns is generally accepted as a combination of a high As uptake and translocation, coupled with a high tolerance to the damaging effects of As by means of sequestration at the cellular level and an enhanced antioxidative responses (Srivastava et al., 2005; Singh et al., 2006; Kramer, 2010). Under aerobic conditions, As is taken up mainly as arsenate (As^{+5}) through phosphate transporters in the root plasma membrane (Ullrich-Eberius et al., 1989). Arsenate is further reduced to arsenite (As^{+3}) before sequestration in the vacuoles of frond cells (Hokura et al., 2006). Inorganic arsenic species are highly toxic to plants: arsenite disrupts the sulfhydryl groups of proteins and interferes with enzyme function, whereas arsenate acts as a phosphate analog, disrupting P metabolism (Ullrich-Eberius et al., 1989).

There is considerable evidence that inorganic As exposure results in the generation of reactive oxygen species (ROS) in plants, including superoxide anion, hydroxyl radicals, and hydrogen peroxide (Hartley-Whitaker et al., 2001). Increased levels of ROS induce lipid peroxidation and cause a decrease in biomass and other physiological disorders in non-hyperaccumulating plants (Hartley-Whitaker et al., 2001; Singh et al., 2006; Garg and Singla, 2011; Campos et al., 2014). Pteris vittata can tolerate high tissue As concentrations due to the enhancement of the antioxidant machinery, leading to As detoxification and hyperaccumulation (Srivastava et al., 2005). Antioxidative defense falls into two general classes: (1) low molecular weight antioxidants, which consist of lipid-soluble membrane-associated antioxidants (e.g., α-tocopherol and β-carotene), and the 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 peroxidase POX is another important enzyme induced under stress conditions (Sasaki et al., 2004).

Arsenic hyperaccumulation (absorption, transport, and storage) in P. vittata has been addressed by elsewhere (Tu et al., 2004; Kertulis-Tartar et al., 2005; Hokura et al., 2006; Huang et al., 2007; Wang et al., 2010). However, the physiological bases of As metabolism remain unclear, especially in non-Pteris species, which makes difficult the evolutionary interpretation of the As accumulation and resistance mechanisms in ferns.

In the present research, we evaluated the effects of arsenic on the activities of antioxidant enzymes (APX, CAT, GR, POX, and SOD), acid-soluble thiols, malondialdehyde content and the mineral nutrition of Pityrogramma calomelanos under hydroponic culture. Arsenic speciation analysis was also conducted. The results indicate that As affects nutrient balance in P. calomelanos and that antioxidative systems are differentially involved in fern organs, which can be related to As species partitioning.

2. Materials and methods

2.1. Site characterization and sampling

Plants of Pityrogramma calomelanos were collected nearby a deactivated gold mine site in Nova Lima district, Minas Gerais State, Brazil (19º 59' 00.35" S; 43º 49' 25.41" W) (Figure 1 A-C). Content of available As in this mine soil was previously reported as 118 mg kg^{-1} (Campos et al., 2014). Fertile fronds of P. calomelanos were

collected in paper bags and placed in a desiccator until spores release (Figure 1 D). Spores were separated from the sporangia and stored at 4 ºC for two months.

2.2. In vitro development of gametophytes and sporophytes

Spores were transferred to 1.5 ml microtubes containing 1.0 ml of 70 % ethanol (EtOH) and centrifuged for 1 min at 13000 g. Then, the supernatant was discarded and the spores were treated with 20 % sodium hypochlorite (NaClO, 2 % of active chlorine) for 15 min and centrifuged for 5 min at 13000 g. The spores were washed and resuspended in autoclaved ultrapure water. Aliquots of the spore suspension were inoculated in Petri dishes (200 µl per dish) containing 20 ml of half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The medium was supplemented with Gamborg B5 vitamins (Gamborg et al., 1968; 2.5 ml L^{-1}), sucrose (10 g L^{-1}) and solidified with agar (6.5 g L^{-1}). After spore germination, the gametophytes were transferred to glass jars containing liquid MS medium (1/2 strength) supplemented with Gamborg B5 vitamins (2.5 ml L^{-1}) and sucrose (10 g L^{-1}) (Figure 1 E). The jars were placed on an orbital shaker (110 rpm) in a climate-controlled room with a photoperiod of 16 h of light (100 µmol m^{-2} s^{-1}). After one month, the sporophytes were transferred to jars containing semi-solid MS (3.0 g L^{-1} gelrite) (Figure 1 F).

2.3. Experimental design

In vitro produced sporophytes were carefully removed from the media, washed in tap water and transferred to 200-ml pots containing the commercial substrate Plantmax®. Sporophytes were cultivated in a greenhouse with controlled temperature (23 ºC), under 50 %-shading nylon net (Sombrite®), irrigated daily with water and once a week with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Ferns at the 4-5 frond stage were transferred to hydroponic system with half-strength Hoagland's solution, pH 5.5 and continuous aeration. The ferns were cultivated for 40 days before the beginning of the treatments (Figure 1 G). Then, the ferns (n = 5) were treated with 0 (control) and 1 mM As, supplied as sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$), for 21 days. Arsenic was added to the nutrient solution, which was renewed every four days. Each experimental unit consisted of one 2.2-L pot containing one plant.

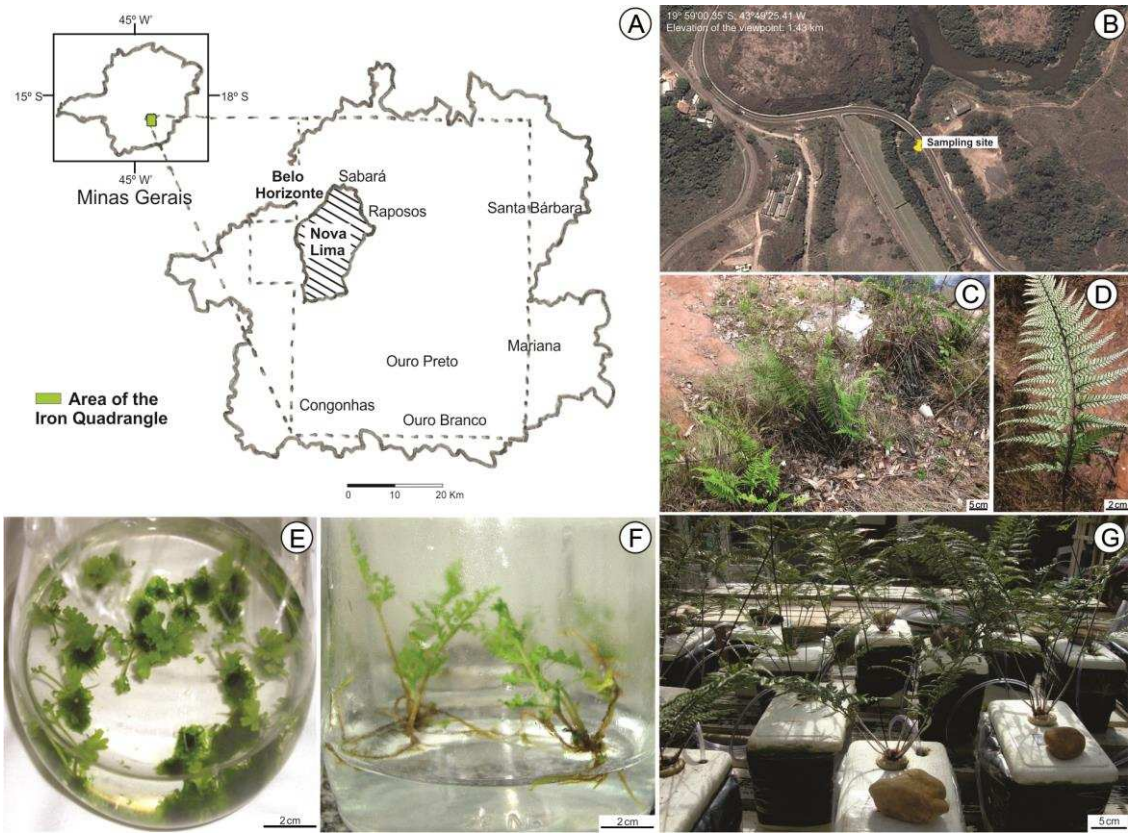

Figure 1. Location map of the sampling area colonized by Pityrogramma calomelanos. Nova Lima district situated in the Iron Quadrangle, Minas Gerais State, Brazil (A). Satellite images of the sampling area by Google Earth (B). Adult individuals of P. calomelanos with fertile fronds (C-D). Gametophyte and sporophyte in vitro development, respectively (E-F). Adult sporophytes in hydroponic system after 40 days of acclimatization (G).

2.4. Growth parameters and chlorophyll content

After 21 days, the ferns were harvested, separated into roots, pinnae and stipe + rachis (referred to here as the stipe), and oven-dried at 60 ºC. The fresh weight (FW) and dry weight (DW) were determined for the pinnae, stipe and roots of each plant. The total FW and DW of the plants were assumed as the sum of the weights of these parts. The moisture content (MC) was given by (FW - DW/ FW × 100%) (Lee and Yu, 2012).

The relative chlorophyll (chl) content was assessed in the pinnae using a SPAD-502 portable chlorophyll meter (Minolta Camera Co., Osaka, Japan). The chl content was estimated at 3, 6, 12, 18, and 21 days after the beginning of As exposure and expressed as the average of three readings for each pinna. On the same days, the

root volume was evaluated by measuring the rise in the water level by immersing the organ in a water-filled graduated cylinder.

2.5. Total arsenic and nutrient determination

The dry matter was milled and powdered with a knife mill to evaluate the total concentrations of As, Ca, Cu, Fe, K, Mg, P, and S. Samples (0.1 g) were extracted with nitro-perchloric solution (3:1) in a digestion block as described by Tedesco et al. (1995). The samples were cooled at room temperature and the volume was completed to 10 ml with ultrapure water and then filtered. The samples were analyzed by inductively coupled plasma-optical emission spectrometry (ICP OES) (Perkin Elmer, Shelton, CT, USA).

The translocation factors (TF) of arsenic and nutrients were calculated as the ratio between As concentration in fronds and roots (Huang et al., 2007).

2.6. Arsenic species determination

Arsenic species were evaluated according to Batista et al. (2011). Samples (n = 3, 0.2 g) were accurately weighed into a 50-ml conical tube, added of 10 ml of 2 % (v/v) nitric acid. The tubes were closed and left at room temperature for 48 hours. The mixture was heated in a water bath at 95 ºC for 2 h, cooled at room temperature, filtered with a 0.20-µm cellulose filter and analyzed using high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS, Perkin Elmer Sciex - Elan DRC II, Framingham, MA, USA). Gallium was used as an internal standard at a final concentration of 5 µg L^{-1}.

2.7. Enzyme extraction and activity assays

Enzyme extracts were obtained according to Carlberg and Mannervik (1985) and Peixoto et al. (1999). Fresh pinna and root samples were ground in liquid nitrogen and homogenized in the following buffer: a) 0.1 M potassium phosphate buffer (KPB, pH 6.8), 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), and 1 % (w/v) polyvinylpolypyrrolidone (PVPP) for superoxide dismutase (SOD, EC 1.15.1.1) and peroxidase (POX, EC 1.11.1.7); b) 0.1 M KPB (pH 6.8), 1 mM EDTA, 1 mM DTT, 0.5 % (w/v) Triton X-20,

and 1% PVPP for catalase (CAT, EC 1.11.1.6) and glutathione reductase (GR, EC 1.6.4.2); c) 0.1 M KPB (pH 6.8), 0.1 mM EDTA, 0.1 mM PMSF, and 1 % (w/v) PVPP for ascorbate peroxidase (APX, EC 1.11.1.11). The homogenate was centrifuged at 13000 g for 15 min at 4 ºC and the supernatant was used as the source of crude enzyme.

Enzyme activities were determined by adding 0.1 ml of the crude enzyme extract to the following media: a) POX, 1.7 ml of a reaction medium consisting of 50 mM M KPB (pH 6.8), 20 mM pyrogallol, and 20 mM H_2O_2; b) CAT, 0.9 ml of a reaction medium consisting of 50 mM KPB (pH 7.0) and 12.5 mM H_2O_2; c) APX, 1.0 ml of a reaction medium consisting of 50 mM KPB (pH 6.0), 0.8 mM ascorbic acid, and 1 mM H_2O_2 (Peixoto et al. 1999); d) GR, 0.9 ml of a reaction medium consisting of 0.1 M KPB (pH 7.5), 1 mM oxidized glutathione (GSSG), and 0.1 mM NADPH (Carlberg and Mannervik, 1985). In all cases, the mixtures were incubated at 30 ºC and the absorbances were measured during the first minute of the reaction. Enzyme activities were estimated using the following molar extinction coefficients: APX (290 nm, ε: 2.8 mM^{-1} cm^{-1}), CAT (240 nm, ε: 36 M^{-1} cm^{-1}), GR (340 nm, ε: 6.22 mM^{-1} cm^{-1}), and POX (420 nm; ε: 2.47 mM^{-1} cm^{-1}).

The SOD activity was determined by adding the enzyme extract to a reaction mixture consisting of 50 mM KPB (pH 7.8), 13 mM methionine, 0.1 mM EDTA, 75 µM nitroblue tetrazolium (NBT) and 2 µM riboflavin. The reaction was carried out in a chamber with a 15-W fluorescent lamp at 25 ºC. After 10 min of illumination, the blue formazan formed was measured at 560 nm (Giannopolitis and Ries, 1977). All rates were corrected for non-enzymatic activity. One unit of SOD activity was defined as the amount of enzyme required to cause a 50 % inhibition of the rate of NBT reduction.

2.8. Protein assay

Soluble protein was estimated using the reagent Coomassie Brilliant Blue G-250 and bovine serum albumin as the standard, according to the method of Bradford (1976).

2.9. Determination of total and non-protein thiols

Total thiols (TT) and non-protein thiols (NPT) were determined with 5,5´-ditiobis-(2-nitrobenzoic acid) (DTNB), according to Sedlak and Lindsay (1968). Freeze-dried pinna and root samples (15 mg) were ground to a fine powder with liquid nitrogen and homogenized in 0.3 ml of cold extraction buffer containing Tris–HCl (0.1 M, pH

8.0), 1 mM Na-EDTA and 1 % (w/v) ascorbic acid. The homogenate was centrifuged for 10 min at 10000 g in a refrigerated centrifuge at 4 °C. For TT analysis, 0.1 ml of the supernatant was mixed with the reaction buffer containing 0.3 ml of KPB (0.2 mM; pH 8.2), 20 µl of DTNB (10 mM) and 1.58 ml of absolute methanol. The samples were incubated for 15 min at 37 °C and the absorbance was measured at 412 nm (Multiskan Spectrum, Spectra 190, Dynex Technologies, USA).

The NPT analysis was carried out using 1.0 ml of the supernatant mixed with 0.2 ml of 50 % trichloroacetic acid (TCA) and 0.8 ml of distilled water. After 1 h in ice bath, the samples were centrifuged for 15 min at 10000 g. Aliquots of 0.4 ml of the supernatant were added to 0.4 M KPB (pH 8.9) and 20 µl of DTNB (10 mM). After 5 min, the absorbance was measured at 412 nm (Multiskan Spectrum, Spectra 190, Dynex Technologies, USA).

The thiol concentration was estimated using the molar extinction coefficient of 13100 mM^{-1} cm^{-1}. The concentration of protein thiols (PT) was calculated by subtracting NPT from TT.

2.10. Lipid peroxidation assay

Lipid peroxidation in the pinna and root samples was determined as described by Heath and Packer (1968). Freeze-dried samples were mixed with 2 ml of TBA reagent (20 % w/v trichloroacetic acid + 0.5 % w/v thiobarbituric acid (TBA)), heated to 95 °C for 30 min, cooled for 15 min, and centrifuged at 10000 g for 15 min. The amount of malondialdehyde (MDA)-TBA complex was measured by its specific absorbance at 532 nm; the nonspecific absorbance at 600 nm was subtracted from 532 nm.

2.11. Statistical analysis

A statistical analysis was performed using the SISVAR software (Ferreira 2011). Means were compared using the Student's t test or Tukey's test at the 0.05 significance level. Graphics were created with SigmaPlot 11.0.

3. Results

3.1. Arsenic accumulation and speciation and its effects on fern nutritional homeostasis

Pityrogramma calomelanos accumulated large amounts of As, with the highest As concentration in pinnae (3108 mg As kg^{-1} DW). Stipes and roots accumulated similar concentrations of As, 275 and 283 mg As kg^{-1} DW, respectively. The As translocation factor (TF-As) was 12.0 (Table 1).

Table 1. Concentrations of arsenic (mg kg^{-1} dry weight, DW), macronutrients (g kg^{-1} DW) and micronutrients (mg kg^{-1} DW), and translocation factors (TF) of these elements in plants of Pityrogramma calomelanos of control and 1 mM As treatments (Treat).

Sample	Treat	As	Ca	K	Mg	P	S	Cu	Fe
Pinna	0 mM	(ND)	7.72^{Aa}	28.3^{Aa}	3.78^{Ba}	15.0^{Aa}	3.16^{Aa}	7.91^{Ba}	551^{Ba}
	1 mM	3108^{A*}	7.26^{Aa}	19.3^{Ab}	3.29^{Ba}	10.4^{Ab}	2.86^{Ba}	4.59^{Ba}	482^{Ba}
Stipe	0 mM	(ND)	2.96^{Ba}	22.3^{Ba}	1.81^{Ca}	6.38^{Ba}	1.05^{Ba}	1.96^{Ba}	161^{Ba}
	1 mM	275^{B}	2.05^{Ba}	16.7^{Ab}	1.47^{Cb}	5.30^{Bb}	0.90^{Ca}	1.87^{Ba}	97^{Bb}
Root	0 mM	(ND)	8.61^{Aa}	19.5^{Ba}	5.46^{Aa}	5.64^{Ba}	3.61^{Aa}	18.9^{Aa}	3988^{Aa}
	1 mM	283^{B}	6.09^{Aa}	22.3^{Aa}	4.98^{Aa}	4.35^{Bb}	4.36^{Aa}	14.8^{Aa}	3182^{Aa}
TF									
Frond/	0 mM	2.36^{b}	1.28^{a}	2.68^{a}	1.05^{a}	3.88^{a}	1.19^{a}	0.63^{a}	0.19^{a}
root	1 mM	12.4^{a}	1.66^{a}	1.71^{b}	0.96^{a}	3.68^{a}	0.88^{b}	0.41^{a}	0.19^{a}

*Means (n = 5) by treatment followed by same letters were not significantly different. Capital letters refer to comparisons between the samples type, for each treatment (Tukey test; $p < 0.05$), and lowercase letters between the treatments, for each sample type (t test; $p < 0.05$). (ND) = non-detected.

The distribution of As species was related to the plant part. Arsenic in pinnae was present mainly as arsenite (75 %), with the remainder as arsenate (Table 2). In contrast, stipes and roots had arsenate as the predominant form (74 and 95 %), with approximately 21 and 5 % of arsenite, respectively. Dimethylarsinate was detected only as a trace constituent in the root and stipe samples. Typical chromatograms obtained for As species in the pinna and root samples are shown in Figure 2. Chromatogram referred to the stipe sample was similar than that obtained for root.

The amounts of nutrients accumulated in pinnae, stipes and roots were compared between the control and As-treated plants. The plants cultivated with As showed 31 %, 17 % and 23 % of reduction in the P concentration of pinnae, stipes and roots, respectively. Arsenic decreased the concentration of K in pinnae and stipes and the concentrations of Fe and Mg in stipes. No changes were observed in the concentrations of Ca, Cu, and S in each fern parts. Plants exposed to As showed reductions of 36 % and 26 % in FT-K and FT-S, respectively (Table 1).

Table 2. Concentration of arsenic species (mg kg^{-1} dry weight) in the pinna, stipe and root of Pityrogramma calomelanos exposed to 0 and 1 mM As.

Sample	Arsenic	AsB	As^{3+}	DMA	MMA	As^{5+}	Sum
Pinna	0 mM	0.01*	0.51^{b}	0.11	(ND)	0.57^{b}	1.20^{b}
	1 mM	(ND)	761^{a}	(ND)	(ND)	258^{a}	1019^{a}
Stipe	0 mM	(ND)	0.02^{b}	0.31^{b}	(ND)	0.10^{b}	0.43^{b}
	1 mM	(ND)	27.5^{a}	6.24^{a}	(ND)	96.3^{a}	130^{a}
Root	0 mM	0.03	0.04^{b}	1.00^{b}	0.07	0.34^{b}	1.47^{b}
	1 mM	(ND)	6.95^{a}	4.70^{a}	(ND)	139^{a}	151^{a}

*Means (n = 3) followed by different letters within the same column, for each sample type, indicate a significant difference (t test; $p < 0.05$). Legend: (As^{5+}) arsenate, (As^{3+}) arsenite, (AsB) Arsenobetaine/non-retained species on the column, (DMA) dimethylarsinate, (MMA) monomethylarsinate, (ND) non-detected.

Comparing the concentrations of nutrients among the plant parts, Cu, Fe, and Mg predominantly accumulated in roots of both treatments, whereas the highest P concentrations were reported in pinnae. The concentrations of Ca and S were always lower in stipes than in pinnae and roots. In control ferns, the concentration of K was higher pinnae than stipes and roots, whereas As-treated ferns showed no difference among fern parts (Table 1).

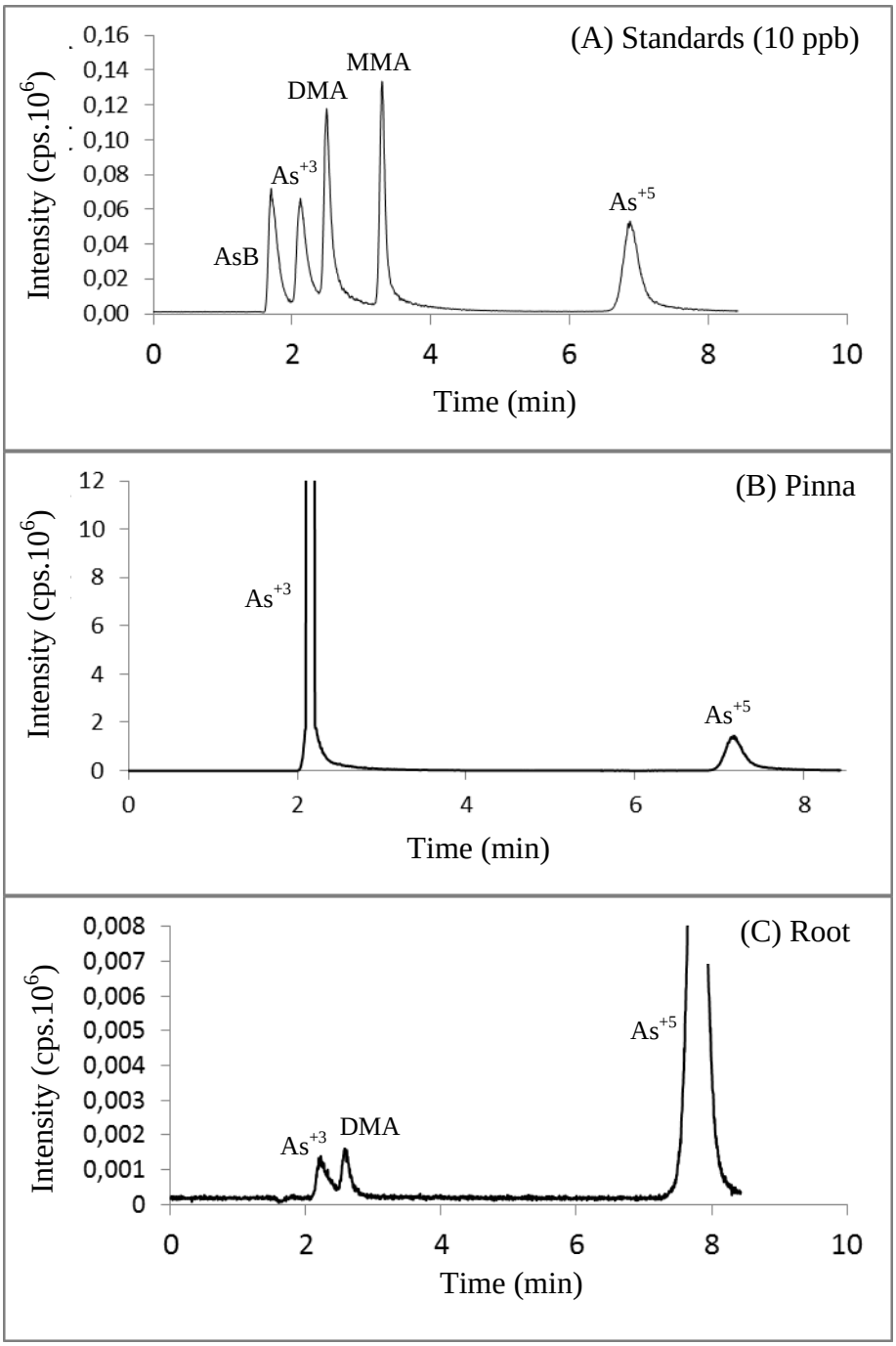

Figure 2. Typical chromatograms obtained for arsenic species in stardard samples (A) in pinna (B), and root (C) samples of Pityrogramma calomelanos after 21 days of exposure to 1 mM As. Legend: arsenobetaine / non-retained species on the column (AsB), arsenite (As^{3+}), dimethylarsinate (DMA), and arsenate (As^{5+}).

3.2. Arsenic effects on growth parameters and chlorophyll content

After 21 days of exposure, there were no visual symptoms of As toxicity in the ferns. However, As induced reductions in the root-FW (17 %), total-FW (17 %) and stipe-DW (21 %) (Table 3). No difference was observed between the MC of control and As-treated plants.

Ferns of the control and As treatments showed similar values of chl content (Figure 3 A) and root volume (Figure 3 B) over the time. The chl content did not change with the time in control and As-treated ferns. A significant increase of 29 % ($p < 0.05$) in the root volume was observed for ferns in both treatments, after three weeks of the beginning of the experiment.

Table 3. Fresh weight (FW), dry weight (DW) and the moisture content (MC) of pinna, stipe, root, and total weight of Pityrogramma calomelanos exposed to 0 and 1 mM As.

	FW (g)		DW (g)		MC (%)	
Sample	0 mM	1 mM	0 mM	1 mM	0 mM	1 mM
Pinna	31.8[a*]	27.0[a]	9.34[a]	7.81[a]	70.5[a]	70.7[a]
Stipe	16.1[a]	13.2[a]	4.70[a]	3.71[b]	70.4[a]	71.8[a]
Root	66.7[a]	55.1[b]	4.39[a]	4.00[a]	93.4[a]	92.7[a]
Total	115[a]	95.3[b]	18.4[a]	15.5[a]	83.9[a]	83.7[a]

*Means (n = 5) followed by different letters within the same line, for each parameter, indicate a significant difference (t test; $p < 0.05$). MC = [(fresh weight - dry weight)/ fresh weight x 100].

3.3. Oxidative damage and antioxidant responses induced by arsenic

The concentration of MDA-TBA complex was used as an indicator of lipid peroxidation. Arsenic induced an increase in MDA-TBA in P. calomelanos roots, though its concentration in pinnae was not changed (Figure 4). The content of MDA-TBA was much higher in pinnae than roots in both treatments.

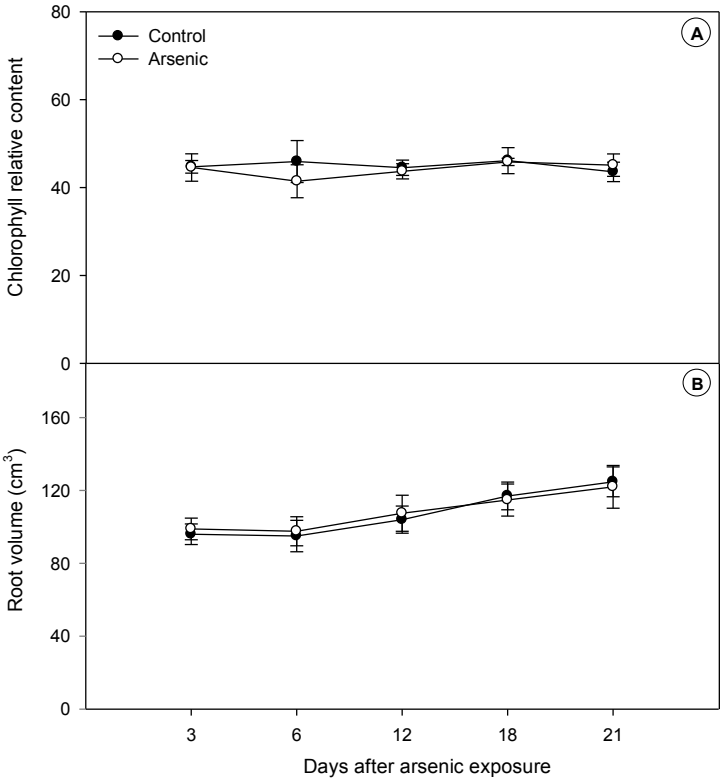

Figure 3. Chlorophyll relative content (A) and root volume (B) of ferns of Pityrogramma calomelanos from control and 1 mM As treatments measured during the exposure time (21 days). Vertical bars represent standart erros (n = 5).

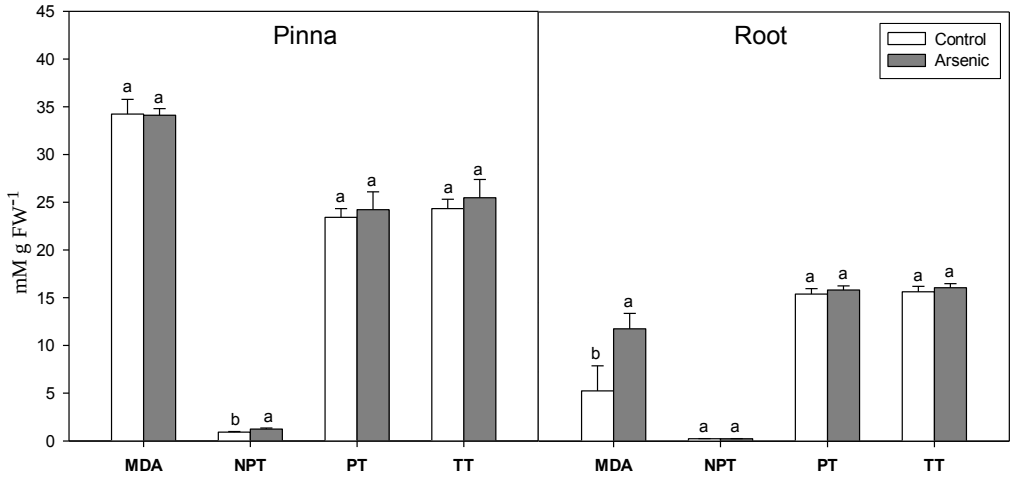

Figure 4. Content of malondialdehyde-thiobarbituric acid complex (MDA-TBA), and total (TT), non-protein (NPT) and protein (PT) thiols in the pinna and root of Pityrogramma calomelanos upon exposure to 0 (control) and 1 mM As after 21 days. Different letters indicate a significant difference between treatments (t test; $p < 0.05$). Vertical bars represent standart erros (n = 5).

Arsenic induced an increase in the concentration of non-protein thiols (NPT) in pinnae, but no change in thiols concentrations was observed in roots of P. calomelanos (Figure 4). The concentrations of total thiols (TT) and NPT were two and five times, respectively, higher in pinnae than in roots of P. calomelanos ($p < 0.05$).

In general, the antioxidant enzymes showed higher activity in pinnae compared to roots, which is in agreement with the higher MDA content observed in the latter. Activity of SOD and CAT increased in roots in response to As exposure, but they were maintained in pinnae. In contrast, APX and POX showed higher activities in response to As only in pinnae. GR activity did not change in either organ (Figure 5).

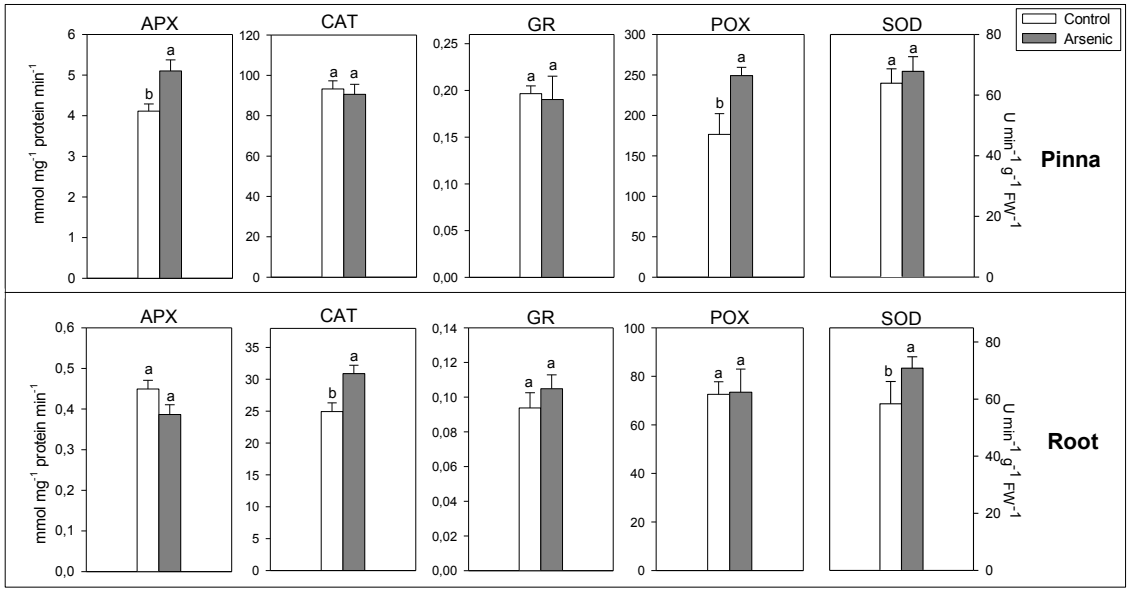

Figure 5. Enzymatic activity of ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), peroxidase (POX), and superoxide dismutase (SOD) in pinna and root of Pityrogramma calomelanos upon exposure to 0 and 1 mM As for 21 days. Different letters indicate a significant difference between treatments (t test; $p < 0.05$). Vertical bars represent standart erros ($n = 5$).

4. Discussion

4.1. Arsenic reduction and translocation leads to arsenic hyperaccumulation

Despite several well-documented examples of As hyperaccumulation and tolerance in Pteris spp., few studies have been performed on Pityrogramma calomelanos. Here, using only 1 mM As in nutrient solution during 21 days of

hydroponic culture, we show that P. calomelanos accumulated As in their pinnae in amounts greater than 3000 mg kg^{-1} dry weight (DW) which represents 85 % of the cumulative total As in ferns. Francesconi et al. (2002) were the first to describe P. calomelanos as an As-hyperaccumulating species, accumulating As mostly in the fronds (from 2760 up to 8350 mg kg^{-1} DW), with roots containing lower As concentration (310 mg kg^{-1} DW). Our results support the distribution of As species reported by these authors, whereby arsenate was the major form of As in roots and stipes (92 % and 75 %, respectively), and arsenite was the predominant form in pinnae (75 %).

Reduction and compartmentalization of As in pinnae are considered to be essential mechanisms in As-hyperaccumulating species, contrasting with plants that only tolerate As through the accumulation of this metalloid in roots (reviewed by Zhao et al., 2009). The specific organ(s) responsible for As reduction is still a matter of debate in the literature. Arsenate reductase activity has been reported in root extracts of Pteris vittata, suggesting that arsenite in the fronds may arise mainly from the reduction of arsenate in the roots (Duan et al., 2005). However, later studies demonstrated that arsenate reduction mostly occurs in the rhizomes and pinnae of P. vittata (Singh and Ma, 2006; Mathews et al., 2010).

The higher percentage of arsenate observed in stipes of P. calomelanos indicate that a low percentage of As was reduced in roots. These findings are in agreement with Kertulis-Tartar et al. (2005), who reported arsenate as the main As species in the xylem sap of P. vittata. Furthermore, X-ray absorption near-edge structure analysis (XANES) has shown that As is found as a mixture of arsenate and arsenite and their proportions in the rachis depend on the analyzed points, whereas it exists as arsenite in the pinna (Hokura et al., 2006).

A low concentration of organic forms of As was found in tissues of P. calomelanos. According to Francesconi et al. (2002), As-methylated forms can be present as trace constituents in fern samples. Small amounts of organic forms, such as monomethylarsonate (MMA), dimethylarsinate (DMA) and arsenosugars have been described in other plant species (Mattusch et al., 2000). However, it remains unclear whether the methylated forms are produced by microorganisms and merely taken up by plants or are endogenously methylated by the plants themselves (Lomax et al., 2012). Indeed, the pathway and enzymology of As methylation in plants have not been fully elucidated (Zhao et al., 2009).

The reduction of arsenate to arsenite and As sequestration in pinnae are essential

processes for As tolerance in hyperaccumulating ferns. The study of As speciation in the xylem sap, along with the stipe and rachis of P. calomelanos, as well as As distribution at subcellular levels would clarify these processes and contribute to the understanding of As metabolism in this species.

4.2. Arsenic affects nutrient concentrations in different organs

Arsenic is a toxic element for plants, and its presence in the soil/water may interfere with the uptake of essential nutrients and plant growth. The majority of studies regarding As effects on plants are mainly focused on phosphorus (P) nutrition due to the competition between arsenate and phosphate for P transporters in the root plasma membrane (Ullrich-Eberius et al., 1989). Arsenic exposure reduced P concentrations in all analyzed organs of P. calomelanos but did not affect FT-P. These findings suggest that P reduction in the frond results mainly from decreased P uptake.

Significant differences in the levels of other macronutrients have been observed in Pteris vittata under As exposure (Cao et al., 2004; Tu and Ma, 2005). However, the effects of arsenic on the mineral nutrition of P. calomelanos have not been reported to date. In this study, As negatively affected the contents of K, Fe, and Mg in P. calomelanos. The decrease of K concentration in stipes and pinnae suggests that As exposure reduced the K translocation, as proved by the lower FT-K of As-treated ferns. Deficiency of K has been demonstrated to protected rice seedling from Cd stress by increasing the antioxidant status (Liu et al., 2013). The reduction of K concentration in fronds of P. calomelanos may have contributed to increased CAT and POX activities ensuring the integrity of lipid membranes under As stress, whereas in roots, the K content was not altered and the MDA content increased. Contrasting results of the As effect on macronutrients concentration were observed by Tu and Ma (2005) in the fronds of P. vittata, however, it seems to be more related to the differences in culture conditions (hydroponic versus soil culture) than differences in species traits.

The decrease of Fe and Mg concentrations in stipes of P. calomelanos, is most likely a consequence of reduced root uptake of these elements, however, only the total concentration of Mg in ferns was significantly altered by As ($p < 0.05$). Plant stressed by lead showed reduction in uptake of Fe and Mg that were associated with metal-induced changes in membrane enzyme activities and membrane structure (Sharma and Dubey, 2005). The increase of lipid peroxidation in roots and the reduction of root-FW

also indicate a possible reduction in nutrient and water uptake. Furthermore, both elements (Fe and Mg) are required for chlorophyll synthesis and carbohydrate metabolism (Robb and Pierpoint, 1983). However, the Fe e Mg concentrations in the pinna were not changed in As-exposed ferns what should be correlated with the absence of effects on chlorophyll content.

Sulfur concentration in roots was not affected by As, which correlates well with the unaltered concentration of soluble thiol compounds in this organ. Sulfur is an essential nutrient in plants, required for the synthesis of amino acids and proteins and is also a precursor for the formation of GSH and other thiols in plants (Wei et al., 2010). Interestingly, As reduced the S translocation in P. calomelanos, without changing S concentration in the pinna, but increased the NPT content in this fern part. Similar results were reported for P. vittata, in which As did not change S concentrations in the ferns but induced GSH synthesis in the fronds (Wei et al., 2010), pointing out that these ferns have complex and well regulated S metabolism .

Very few studies have detailed the effect of As on the mineral nutrition of hyperaccumulating ferns, and the majority of this research was performed in soil systems. The investigation of As interaction with micro- and macronutrients using hydroponic systems can broaden the understanding of the effects of As on metabolism, because it is a more sensitive system that allows a higher availability and rapid uptake of nutrients. In soil systems, a range of variables can interfere with As availability, masking some of the effects on plant nutrition that could be visible in a less complex hydroponic system.

4.3. Roots and pinnae differ in enzymatic and non-enzymatic antioxidant responses to arsenic toxicity

Arsenic has been reported to increase the concentration of reactive oxygen species (ROS), which can affect membrane permeability, enzyme activity, chlorophyll synthesis, photosynthetic reactions and plant biomass (Mascher et al., 2002; Singh et al., 2006). The absence of both visual symptoms of As toxicity and negative effects on chlorophyll content showed that P. calomelanos not only hyperaccumulates As, but also tolerate high internal As concentrations, especially in the fronds. However, P. calomelanos was not able to avoid oxidative damage in roots, once As induced a two-fold increase in root lipid peroxidation and decreased the root fresh weight. However,

the root development was not necessarily affected due to the similar increase in the root volume of control and As-treated ferns.

Other interesting fact is that the activities of the antioxidant enzymes in P. calomelanos were differentially affected by As in pinnae and roots, which can be related to As species partitioning. According to Foyer et al. (1994), there are two pathways for ROS scavenging: SOD/CAT and the ascorbate-glutathione cycle, the latter includes APX, GR, and ascorbate reductases (Foyer and Noctor, 2011). Both SOD and CAT activities increased in roots, whereas APX and POX showed higher activities in pinnae of the As-exposed ferns. SOD is considered the first line of defense against the damage caused by ROS and is responsible for catalyzing the dismutation of highly reactive anion superoxide to oxygen and hydrogen peroxide (Alscher et al., 2002). H_2O_2 produced by SOD was decomposed to water and oxygen, especially by CAT, in roots and by APX and POX in pinnae of P. calomelanos. Gametophytes of P. vittata showed increased level of APX, CAT, GR, GST and POX (Raj et al., 2011), whereas As induced the level of APX, CAT and SOD in fronds and roots of P. vittata (Srivastava et al., 2005). Antioxidant responses to As can vary among hyperaccumulating ferns, but no data have been published to date for P. calomelanos.

Because the activity of antioxidant enzymes was higher in pinnae than roots, we can suggest that the pinnae have higher antioxidant capacity, which explains their not changed MDA content. Nonetheless, the increase in SOD and CAT were insufficient to avoid lipid peroxidation in roots, as indicated by the increase in MDA concentration. Furthermore, an increase in NPT was observed only in pinnae of P. calomelanos under As exposure. Glutathione is the main NPT of the cell and is a non-enzymatic antioxidant that participates of free radical scavenging and modulation of the cellular redox status and thiol-disulfide status of proteins (Cnubben et al., 2001). According to Singh et al. (2006), an As-induced increase in GSH may represent a defense system that is not as direct as the primary defense response, such as vacuole compartmentalization. An increase in the NPT level in pinnae may have contributed to the protection of membranes against free radicals induced by As in these organs.

Glutathione is also a component of the ascorbate-glutathione cycle, which regenerates ascorbate by reducing dehydroascorbate. Glutathione reductase is the complementary enzyme of the ascorbate–glutathione cycle that maintains a high reduced/oxidized glutathione (GSH/ GSSG) ratio for protection against oxidative damage (Noctor and Foyer, 1998). Despite the increased APX activity in pinnae, GR

activity was not affected by As. However, it is well known that ascorbate regeneration may be independent of GSH and occur by other mechanisms depending on ferredoxin or NADPH (Foyer and Noctor, 2011).

Altogether, these results indicate that the root of P. calomelanos is more sensitive to As effects and not a specialized organ for As accumulation in this species. However, the As concentration was much higher in the pinna, and As toxicity in this organ was most likely mitigated by the higher activities of antioxidant enzymes and higher NPT in comparison to the root. These observations reinforce the importance of As translocation and hyperaccumulation in the frond as a mechanism to reduce oxidative damage in the roots.

5. Conclusion

The root-shoot As translocation in Pityrogramma calomelanos is essential to avoid toxic effects in the root, once this organ showed to be more sensitive to the metalloid. The higher capacity of P. calomelanos to sequester arsenite in the pinna and its efficient antioxidant system maintain the reactive oxygen species at a low level, thus enhancing the continuous accumulation of As. Arsenic hyperaccumulation requires also adjustments in the mineral nutrition of ferns, especially with regard to K and P. Molecular investigations are needed to elucidate the evolution of As-tolerance mechanisms in Pteridaceae species, especially with regard to membrane transporters.

6. Acknowledgments

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CAPÍTULO 2: Acúmulo e distribuição espacial de arsênio e fósforo em Pityrogramma calomelanos usando fluorescência de raios-X com energia dispersiva

RESUMO: O acúmulo e distribuição espacial de arsênio (As) e fósforo (P) foram investigados na monilófita hiperacumuladora de As Pityrogramma calomelanos utilizando fluorescência de raios-X com energia dispersiva (µ-EDXRF). Plantas foram cultivadas em solução nutritiva de Hoagland à meia-força contendo 1, 10 ou 30 x 10^{-3} mol L^{-1} As durante três semanas. Plantas cultivadas em solução sem adição de As foram usadas como controle. A análise de µ-EDXRF foi utilizada para mapear As e P em diferentes áreas da pina, e também para determinar a concentração desses elementos em amostras secas de pina, estipe e raiz prensadas na forma de pastilhas. As amostras utilizadas na feitura de pastilhas foram digeridas em microondas, e analisadas em ICP OES para validação do método. Correlações elevadas foram obtidas entre as intensidades de emissão características de raios-X e as frações de massas correspondentes obtidas para cada elemento. Os coeficientes de correlação (r) e a raiz quadrada média dos erros de previção (RMSEP) foram maiores e menores, respectivamente, quando amostras de diferentes partes da planta foram usadas separadamente na construção de modelos de calibração. O As acumulou principalmente na nervura mediana da pina e nas regiões apicais, marginais e nas nervuras secundárias de pínulas de P. calomelanos. O acúmulo de As promoveu alterações na distribuição de P ao longo da pina. A µ-EDXRF mostrou-se uma ferramenta analítica apropriada para mapeamento de As e P em P. calomelanos. Esse método simples e validado pode ser amplamente aplicado em estudos de monitoramento ambiental e de fitorremediação.

Palavras-chave: µ-EDXRF, Bioimagem, arsênio, fósforo, Pityrogramma calomelanos.

CHAPTER 2: Accumulation and spatial distribution of arsenic and phosphorus in Pityrogramma calomelanos by micro-energy dispersive X-ray fluorescence spectrometry

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ABSTRACT: The accumulation and spatial distribution of arsenic (As) and phosphorus (P) in the As-hyperaccumulating fern Pityrogramma calomelanos were investigated using micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF). Ferns were grown in half-strength Hoagland nutrient solution containing 1, 10 or 30 x 10^{-3} mol L^{-1} As during three weeks. Ferns grown in a medium without As addition were used as control. Micro-EDXRF analysis was used to map As and P in different areas of the pinna, and to determine the concentration of these elements in dry pelletized samples from the pinna, stipe and root. A validated microwave-assisted acid digestion followed by ICP OES determination was used as the reference method for As and P determination in fern samples. Strong correlations were found between the X-ray characteristic emission intensities obtained from the analysis of the pelletized samples and the corresponding elemental mass fractions for both elements. Higher linear correlation coefficients (r) and lower root mean square error of prediction (RMSEP) were observed when fern parts were separated into different calibration models. Arsenic accumulated mainly in the pinna midrib, secondary veins, and apical and marginal regions of the pinnule of P. calomelanos, causing alterations in the P distribution along the pinna. Micro-EDXRF analysis showed to be an appropriate analytical tool for mapping As and P in P. calomelanos. By applying this simple and validated method,

important information towards environmental monitoring and phytoremediation studies can also be accessed.

Keywords: μ-EDXRF, Bioimaging, Arsenic, Phosphorus, Pityrogramma calomelanos.

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1. Introduction

Arsenic, recognized as a contaminant of great concern, is ubiquitously distributed in nature, being primarily found as arsenopyrite (FeAsS), the most common arsenic mineral. Parent materials containing arsenopyrite and other As-bearing sulfides are oxidized when exposed to atmospheric oxygen and water and can release this toxic element [2]. This natural phenomenon is called acid mine drainage [3-4] and is the main cause of elevated As levels in natural water reservoirs [2]. Anthropogenic activities such as the intensive use of arsenical pesticides, mining, fossil-fuel burning and the disposal of As-enriched wastes can also substantially enhance the As contamination [5]. High As levels in soil and groundwater can bring deleterious effects to the ecosystem and efforts towards environmental monitoring and remediation programs must be implemented in order to minimize the exposure to the organisms [6-8].

Plants colonizing metal(loid)-contaminated soils are adapted to survive in this stressful environment and are classified into three main categories: metal excluders, indicators and accumulators/hyperaccumulators [9]. The majority of plant species are excluders, which contain low levels of potentially toxic elements in their aerial tissues even when exposed over an extensive concentration range of contaminants; whereas indicators accumulate metal(loid)s into their aboveground biomass reflecting the elemental concentration in the soil. Accumulators/hyperaccumulators plants are those able to increase metal(loid) internal sequestration, translocation and accumulation into their aboveground biomass to levels that far exceed those found in the soil [9, 10].

Arsenic-hyperaccumulating species, e.g. Pteris vittata [11] and Pityrogramma calomelanos [12], differ from As-accumulators by having metalloid concentration higher than 1 % m m^{-1} of dry matter. These plants are especially interesting to be used as sentinel organisms for monitoring and in remediation programs of contaminated sites [13, 14]. Arsenic concentration in leaves of hyperaccumulators can be used to infer about the input of As in the soil/ water by natural/ anthropogenic sources. These plants can also be harvested to reduce potential As contamination thereby limiting the metalloid entry into the food chain, a strategy known as phytoremediation.

In environmental assessment studies, the quantitative determination of trace elements in plant materials has often been performed by atomic absorption and atomic emission spectrometry, which require a prior chemical treatment of the solid samples for the destruction of their organic matrices with mineral acids and hydrogen peroxide

[15, 16]. Micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF) is a fast and non-destructive method that has been successfully used for the determination of macro- and micronutrients in plant materials [17]. The simultaneous multielemental capability of this method combined with its high spatial resolution can be explored as a versatile screening tool for elemental mapping in plants growing in a site under investigation. This method was successfully used for mapping and quantifying Al in hyperaccumulator plants from High Altitude Rocky Complexes [18].

A few studies have addressed the As spatial distribution and its speciation in P. vittata using non-destructive techniques like X-ray absorption near edge spectroscopy (XANES) [19-22]. To the best of the authors' knowledge, only Kachenko et al. [23, 24] carried out As mapping in P. calomelanos (var. austroamericana), but P distribution was not investigated. Arsenate and phosphate competition for membrane transporters have been demonstrated in plants [25], but interference of As in P metabolism of hyperaccumulating ferns remains as a controversial issue. The simultaneous investigation of As and P accumulation in the fronds can provide additional information about the physiological As-tolerance mechanisms that operate in these ferns, contributing for the optimization of phytoremediation programs [26]

In this study, we aimed at the evaluation of the micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF) in the investigation of the accumulation and spatial distribution of As and P in sporophytes of Pityrogramma calomelanos grown in As-enriched solution. We also intended to validate μ-EDXRF method for the quantitative determination of As and P in pelletized powdered fern samples.

2. Material and methods

2.1. Experimental conditions and sampling

Sporophytes of Pityrogramma calomelanos (Pteridaceae) were obtained through in vitro culture and then were cultivated in commercial substrate in a greenhouse, as described in the Chapter 1. Ferns at 4-5 frond stage were transferred to a hydroponic system with half-strength Hoagland nutrient solution [27], pH 5.5, under continuous aeration. After an acclimatization period of 4 weeks, 1×10^{-3} mol L^{-1} As was added in the solution of half of the ferns. Arsenic was supplied as sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$). The remaining ferns were grown in Hoagland nutrition solution [27] without As addition (control treatment). There were five replicates per treatment

and each experimental unit consisted of one 2.2-L pot containing one plant. Plants were exposed during 21 days and the solution was renewed weekly. Similarly, a second experiment was conducted using ferns at the 5-7 frond stage (see Chapter 4) treated with 1, 10 and 30 x 10^{-3} mol L^{-1} As for the same exposure time.

At the end of the experiments, samples of pinnas, stipes, and roots were collected and oven-dried at 60 ºC to constant weight. For elemental mapping, part of the pinna samples was manually pressed between two previously decontaminated glass plates to obtain flat surfaces.

2.2. Determination of arsenic and phosphorus by ICP OES

Dried samples were powdered with a ball mill and sieved through a 200 mesh (74 µm) stainless steel sieve. The sieved material was accurately weighed (ca. 50 mg) in triplicate in closed TFM® digestion vessels (ETHOS 1600, Milestone, Italy). To each vessel were added 5.0 ml of 2.8 mol L^{-1} HNO_3 and 2.0 ml of H_2O_2 30 % w/w. The microwave heating program was applied as follows: step 1: room temperature to 160 °C in 3 min; step 2: no heating for 2 min; step 3: heating to 200 °C in 5 min; step 4: 15 min at 200 °C. The final solutions were transferred to volumetric flasks and diluted to 10 ml with deionized water. The elemental determinations were performed using a dual view ICP OES spectrometer (iCAP 6500 Duo, Thermo Scientific, Waltham, MA, USA) equipped with a cyclonic spray chamber and a PEEK Mira Mist® nebulizer. ICP OES measurement conditions were as follows: generator frequency: 40 MHz; RF applied power: 1.2 kW; sample flow rate = 1.5 ml min^{-1}; argon flow rates: 12, 0.5 and 0.6 L min^{-1} for plasma, auxiliary and nebulizer, respectively; measurement time of 15 s. Arsenic emission line was monitored in the axial viewing mode and P emission line in the radial viewing configuration. The following emission lines were monitored: P I 213.618 and As I 197.262 nm.

2.3. Micro-EDXRF analysis

Micro-EDXRF analysis were performed using a benchtop spectrometer (µEDX-1300, Shimadzu, Kyoto, Japan) coupled with a Rh X-ray tube and a Si(Li) semiconductor detector. Limits of detection (LODs) were estimated using the raw intensity of each Kα peak (P Kα 2.01 keV and As Kα 10.54 keV) from the spectra of 10

different test portions of pelletized pinna samples. Background (BG) data were obtained by subtracting the net intensity (calculated by the equipment software) from the raw data. All BG values were used to calculate the standard deviation.

2.3.1. Pellet preparation and calibration method

Pellets of pinna, stipe, and root samples from ferns exposed to 1, 10 and 30 $x10^{-3}$ mol L^{-1}As, and also control samples from ferns grown in medium without As addition, were prepared applying 10 t cm^{-2} pressure (Perkin Elmer, Waltham, MA, EUA) for 5 min on 0.20 g of powdered plant material in order to produce pellets with approximately 1 mm thickness and 15 mm diameter. Fourteen samples were selected from each fern part (pinna, stipe and root), totalizing 42 pellets.

Three lines, comprising 30 points each, were randomly selected on the surface of the pellet. Each point was analyzed during 10 s and the distance between the points was 100 µm. The detailed operational conditions were described in Table 1.

Table 1. Operational conditions in µ-EDXRF analysis

Instrumental parameters	Operational conditions
Measuring principle	X-ray fluorescence spectrometry
Measuring method	Energy-dispersive X-ray analysis
Working distance (mm)	1.5
Detector	Si(Li) semiconductor
Irradiated diameter (µm)	50
Measurement time (s) for pellet interrogation	300
Analyzed spectra region (keV)	1.00 – 40
Measuring atmosphere	Atmospheric air
X-ray power unit	X-ray tube (Rh target)
Monitored peaks	P Kα 2.01 keV and As Kα 10.54 keV
Channel	Ti-U
Electric voltage (kV)	50
Electric current (µA)	100

Linear regression models were adjusted to correlate As and P mass fractions determined by ICP OES and µ-EDXRF intensity (cps μA^{-1}), as recommended by Guerra et al. [17], using data from all samples and also separately for each fern part.

2.3.2. Microchemical As and P mapping

Qualitative maps

Pinna dried samples from control and As-treated ferns, exposed to 1 x 10^{-3} mol L^{-1}As were used for As and P mapping. Pinna samples were horizontally fixed on pure cellulose supports using adhesive tape. Areas of 4 x 3 mm (40 x 30 measurement points; 100 µm step per point) were selected on the basal portion of the pinna, including the midrib pinna, for As and P mapping. Additionally, the meddle portion of the pinnule and apical portion of the pinna from ferns exposed to 30 x 10^{-3} mol L^{-1}As were selected for As mapping. For qualitative maps, the following peaks were monitored: P Kα 2.01 keV and As Kβ 11.73 keV.

Samples analyzed by µ-EDXRF were photographed in a stereo microscope (SZX7 Olympus) equipped with an EVOLT E-300 Olympus digital camera (Olympus Optical). Figure 1 illustrates the morphological characteristics of P. calomelanos emphasizing the pinna regions analyzed by µ-EDXRF.

Semi-quantitative maps

The apical region of the pinnule (necrotic region) of ferns from the control and 30 x 10^{-3} mol L^{-1}As treatment were selected for semi-quantitative As and P mapping. The samples were prepared as described for the qualitative maps and the operational conditions were the same of the pellet analysis. The linear regression models obtained by the correlation between As and P mass fractions and the corresponding characteristic X-ray intensities of As (Kα peak: 10.54 keV) and P (Kα peak: 2.01 keV) were used to convert cps μA^{-1} into elemental mass fraction data (mg kg^{-1} for As and g kg^{-1} for P), resulting in semi-quantitative maps. The color scales were normalized by replacing the lowest scale point for the As and P limits of detection (LOD). The limits of quantification were also pointed in the scale of each map.

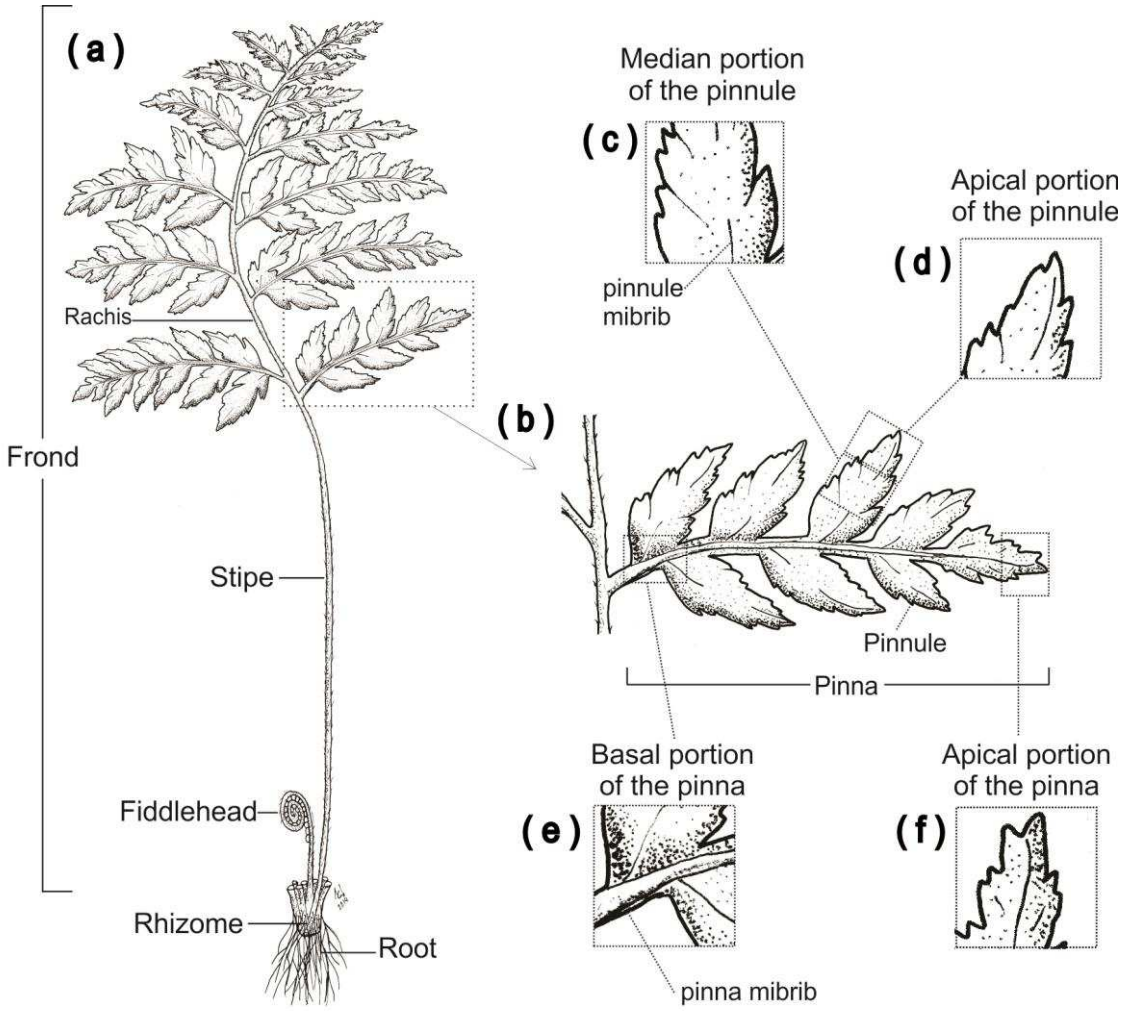

Figure 1. Morphological characteristics of P. calomelanos emphasizing the pinna regions used for arsenic and phosphorus mapping by μ- EDXRF. (a) Sporophyte. (b) Pinna. (c-d) Median and apical regions of the pinnule, respectively. (e-f) Basal and apical regions of the pinna, respectively.

2.4. Statistical analysis

The software Origin v. 8 (Northampton, MA, USA) was used to perform the linear regression analysis. For each linear regression model, the linear correlation coefficient (r) and the root mean square error of prediction (RMSEP) were calculated as described by Guerra et al. [28].

3. Results

3.1. Determination of As and P by ICP OES

The trueness of the proposed method that combines microwave-assisted acid digestion and ICP OES determination of As and P was confirmed by the analysis of the certified reference materials (Table 2).

Table 2. Concentration of arsenic in BCR-060 (Lagarosiphon major), and phosphorus in NIST SRM 1515 (apple leaves) and in NIST SRM 1547 (peach leaves)

Reference material	Element	Concentration unit	Certified	Found (n = 3)
BCR-060	As	mg kg^{-1}	8*	7.5 ± 0.3
NIST SRM 1515	P	g kg^{-1}	1.59 ± 0.11	1.61 ± 0.07
NIST SRM 1547	P	g kg^{-1}	1.37 ± 0.07	1.44 ± 0.02

*Reference value.

Ferns from the control treatments showed As concentrations between 1.6 – 2.7 mg kg^{-1}, in the pinna and stipe, and from 9 – 52 mg kg^{-1} in the roots. Arsenic concentrations in the pinna, stipe and root of P. calomelanos ferns exposed to different As doses ranged from: 580 – 8800, 70 – 6600, and 200 – 7800 mg kg^{-1}, respectively. Higher As concentrations were observed in the pinna from ferns exposed to 10 and 30 x 10^{-3} mol L^{-1}As that presented apical and marginal necrosis in the old fronds. Phosphorus concentration ranged from 3.8 – 11, 2.4 – 4.2, and 3.8 – 6.6 g kg^{-1} in the pinna, stipe and root, respectively. Arsenic promoted the reduction of P concentration in ferns of the first experiment, but did not alter in those from the second experiment (for detailed results see chapters 1 and 4, respectively).

3.2. Micro-EDXRF analysis of pelletized samples

A fragment of a typical μ-EDXRF spectrum obtained from the analysis of a pellet from pinna of P. calomelanos is shown in Figure 2 where are highlighted the following peaks: As Kα 10.54 keV, As Kβ 11.73 keV and P Kα 2.01 keV.

Spectra of pelletized powdered samples of pinnae were used to calculate the limits of detection (LODs) of As and P by μ-EDXRF. The obtained values were 76 mg

kg^{-1} As and 0.6 g kg^{-1} P. Limits of quantification were estimated as three times the obtained LODs.

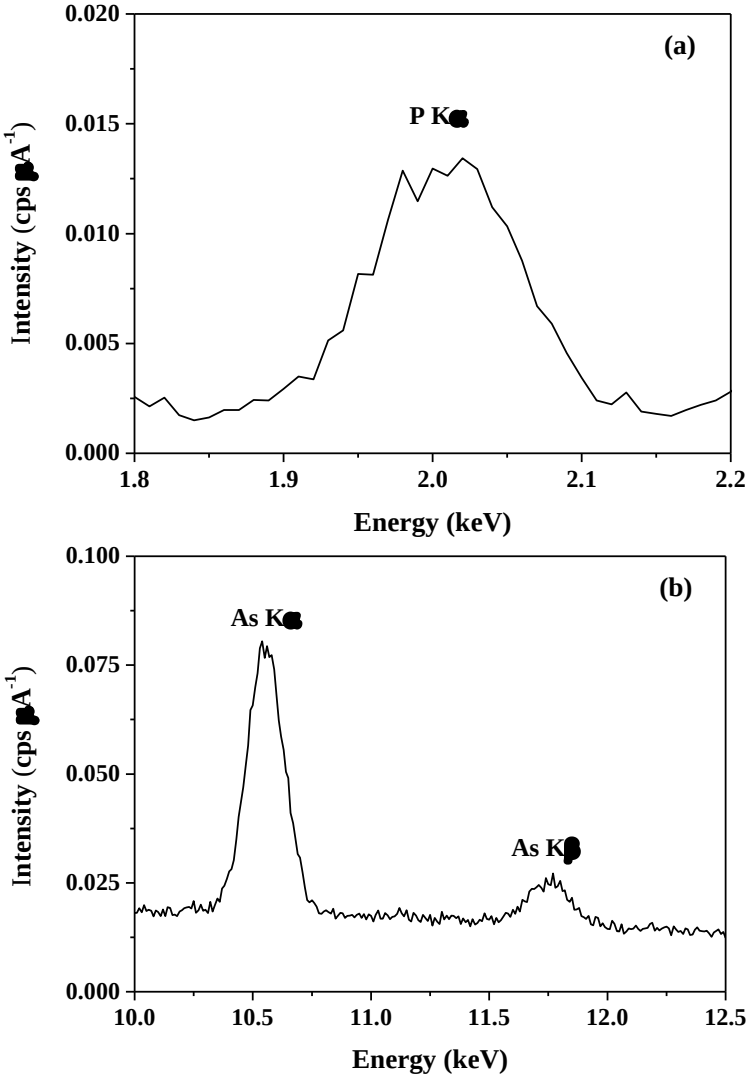

Figure 2. Fragments of typical μ-EDXRF spectrum obtained from the analysis of pellet from pinna of P. calomelanos containing 1600 mg kg^{-1} As and 12 g kg^{-1} P.

Figure 3 shows the linear regression models adjusted for X-ray characteristic emission intensities (As Kα 10.54 keV and P Kα 2.01 keV) and the corresponding elemental mass fractions for As (mg kg^{-1}) and P (g kg^{-1}) obtained from the analysis of pressed pellets of powdered plant material. Higher linear correlation coefficients (r) and lower root mean square error of prediction (RMSEP) were obtained by analyzing samples from different fern parts separately.

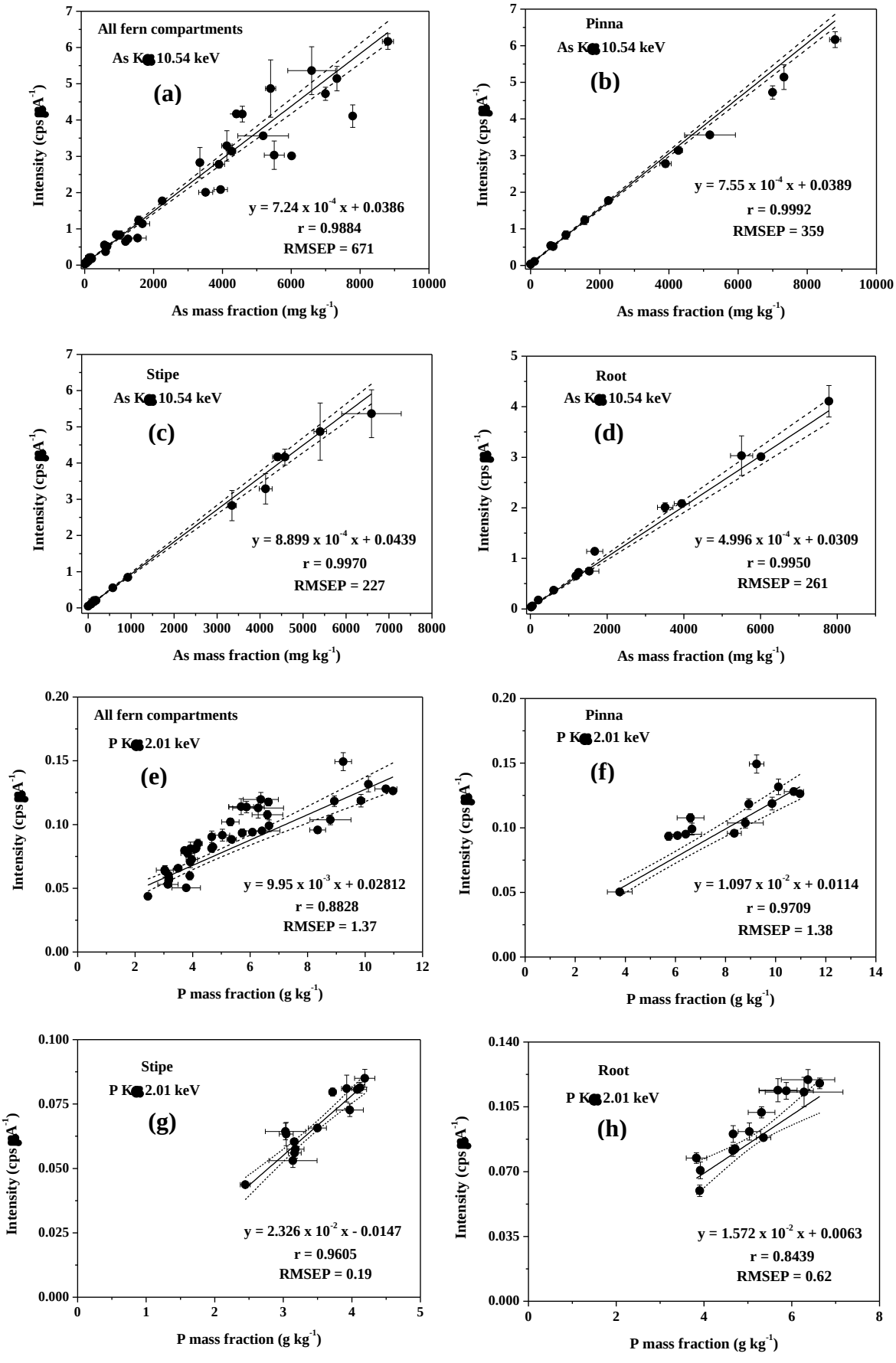
All fern compartments
As Kα 10.54 keV
(a)
y = 7.24 x 10-4 x + 0.0386
r = 0.9884
RMSEP = 671
Intensity (cps μA-1)
As mass fraction (mg kg-1)
Pinna
As Kα 10.54 keV
(b)
y = 7.55 x 10-4 x + 0.0389
r = 0.9992
RMSEP = 359
Intensity (cps μA-1)
As mass fraction (mg kg-1)
Stipe
As Kα 10.54 keV
(c)
y = 8.899 x 10-4 x + 0.0439
r = 0.9970
RMSEP = 227
Intensity (cps μA-1)
As mass fraction (mg kg-1)
Root
As Kα 10.54 keV
(d)
y = 4.996 x 10-4 x + 0.0309
r = 0.9950
RMSEP = 261
Intensity (cps μA-1)
As mass fraction (mg kg-1)
All fern compartments
P Kα 2.01 keV
(e)
y = 9.95 x 10-3 x + 0.02812
r = 0.8828
RMSEP = 1.37
Intensity (cps μA-1)
P mass fraction (g kg-1)
Pinna
P Kα 2.01 keV
(f)
y = 1.097 x 10-2 x + 0.0114
r = 0.9709
RMSEP = 1.38
Intensity (cps μA-1)
P mass fraction (g kg-1)
Stipe
P Kα 2.01 keV
(g)
y = 2.326 x 10-2 x - 0.0147
r = 0.9605
RMSEP = 0.19
Intensity (cps μA-1)
P mass fraction (g kg-1)
Root
P Kα 2.01 keV
(h)
y = 1.572 x 10-2 x + 0.0063
r = 0.8439
RMSEP = 0.62
Intensity (cps μA-1)
P mass fraction (g kg-1)

Figure 3. Linear regression models adjusted to correlate As (a-d) and P (e-h) concentration determined by ICP OES and µ-EDXRF intensity (cps μA^{-1}), using data from all samples and also separately for each fern part (pinna, stipe and root).

Linear correlation coefficients were in general higher for As than for P. Micro-EDXRF measurements (n = 3) presented coefficient of variation from 1.0 to 17 %.

3.3. Microchemical As and P mapping

3.3.1. Qualitative maps

Microchemical qualitative maps were constructed for a preliminary investigation of the arsenic and phosphorus distribution patterns in different regions of the pinna. Arsenic mapping in control samples did not show any pattern of As distribution (Figure 4 A). In the basal portion of pinnae from ferns treated with 1 x 10^{-3} mol L^{-1}As, arsenic was preferentially localized in the pinna midrib, followed by the pinnule midribs (Figure 4 E). The middle portion of pinnules from the 30 x 10^{-3} mol L^{-1}As treated fern also showed a preferential accumulation of As in the vascular regions, following an increasing gradient toward the margin (Figure 5 D). Arsenic concentrated mainly in the pinna tip (necrotic region), including the veins and interveinal regions (Figure 5 B).

Ferns grown in solution without As (control) showed a homogenized distribution of phosphorus in the basal portion of the pinna that includes the midrib pinna, with a few hot spots in each side of the pinnule midrib (Figure 4 C). Ferns treated with 1 x 10^{-3} mol L^{-1}As showed a preferential P accumulation in the pinnule veins, on the basal portion of the pinna, presenting low P concentration in the pinna midrib (Figure 4 F).

3.3.2. Semi-quantitative maps

To evaluate the applicability of µ-EDXRF in the quantitative determination of As and P, the apical region of the pinnule was selected for mapping. Similar as observed for the qualitative maps, As was not detected in control samples, once the signal intensities were below the As LOD (Figure 6 B), even when monitoring the most sensitive characteristic X-ray emission line. Arsenic was mainly accumulated in the pinnule veins and towards the most apical region of the pinnule (Figure 6 E). Average and maximum As concentration estimated by the pinna regression equation were 415 and 1712 mg kg^{-1}.

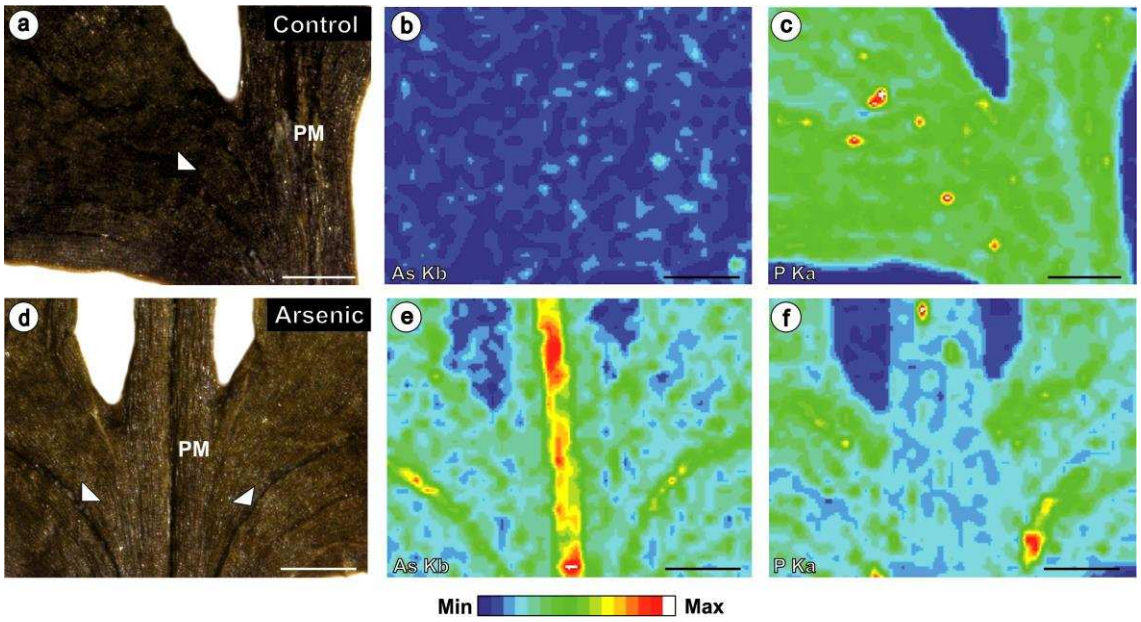

Figure 4. Qualitative μ-EDXRF maps for As and P of the basal region of the pinna of Pityrogramma calomelanos from control ferns (a-c) and ferns exposed to 1 x 10^{-3} mol L^{-1} As (d- f). (a) and (d), Optical micrographs of pinna highlighting the pinna midrib (PM) and pinnule veins (white triangle); (b) and (e), As maps; (c) and (f). P maps. Legend: Min = minimum value; Max = maximum value. Bars length = 0.8 mm.

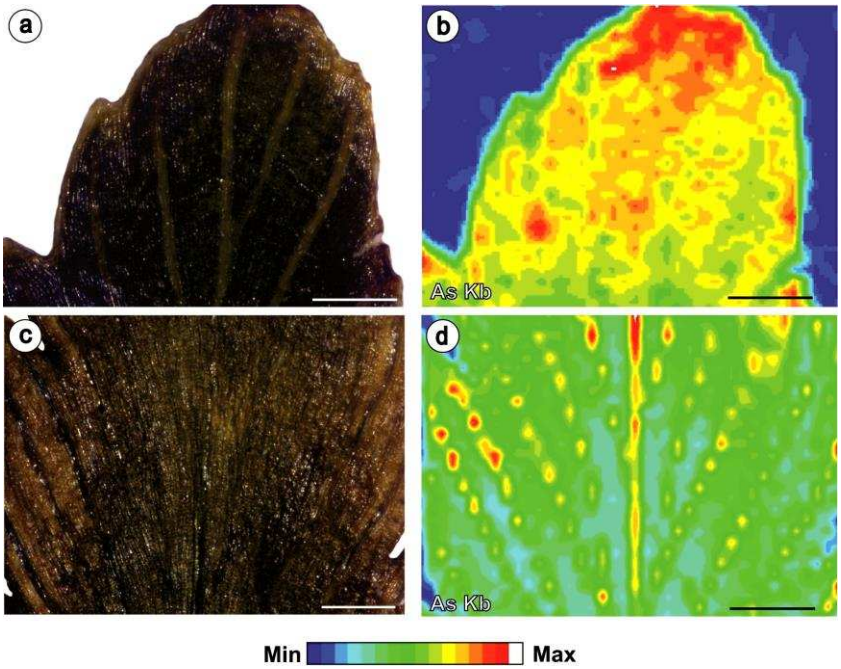

Figure 5. Qualitative μ-EDXRF maps for As of pinnule of Pityrogramma calomelanos ferns exposed to 30 x 10^{-3} mol L^{-1} As. (a) and (c), Optical micrographs of apical and median regions of the pinnule, respectively; (b) and (d), As maps. Legend: Min, minimum value; Max, maximum value. Bars length = 0.4 mm.

Control and As-treated ferns showed a similar P distribution in the pinnule tip. Arsenic was preferentially accumulated around the pinnule veins (Figure 6 C, F). However, P concentrations in control samples were much higher than that reported in As-treated pinna samples. The average and maximum values obtained for P were 11 and 22 g kg $^{-1}$ for the control sample, and 2.2 and 10 g kg $^{-1}$ for the As-treated samples. Phosphorus localization in the pinnule tip presented the same pattern of that observed for arsenic in As-treated samples.

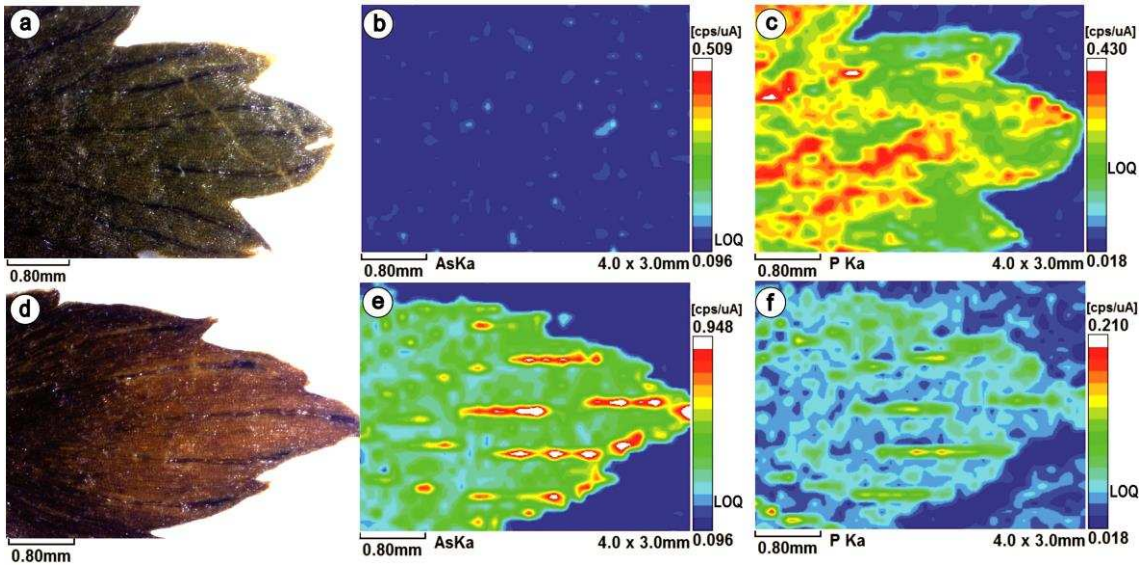

Figure 6. Semi-quantitative µ-EDXRF maps for As and P of pinnule of Pityrogramma calomelanos ferns from control (a-c) and 30 x 10^{-3} mol L^{-1} As (d-f) treatments. (a) and (d), Optical micrographs of the apical region of the pinnule; (b) and (e), As maps; (c) and (f): P maps. Legend: LOQ, limit of quantification.

4. Discussion

4.1. Micro-EDXRF is an appropriate analytical tool for As and P determination in pelletized fern samples

Linear regression models adjusted to correlate ICP OES and µ-EDXRF data showed that both methods matched closely due to the high linear correlation coefficients, as reported for other elements such as for Al in plants from High Altitude Rocky Complexes [18] and for macro- and micronutrients in sugar cane leaves [17]. Better performance of the mathematic modeling was observed when the calibration models were built with pelletized powdered samples from the same ferns parts. This

behavior was confirmed by the better RMSEP and linear correlation coefficients obtained by using this calibration strategy which circumvents matrix effects. RMSEP represents an estimation of the predictive capacity of the calibration models, and lower RMSEP values, for a same data set, indicate higher predictive capacity.

Higher linear correlation coefficients obtained for As (0.99 for all fern parts) than for P (0.84-0.97) can be derived from specific characteristics of these elements, such as the lower atomic number of P. XRF has a lower sensitivity for the detection of low atomic number elements, presenting higher LOD values for these elements [29]. The LOD found for P was in agreement with that obtained in the study of Guerra et al [17] in the analysis of pelletized sugar cane leaves by a validated micro-energy dispersive X-ray fluorescence spectrometry method.

EDXRF-based analytical methods can be used as a quantitative tool in phytoremediation studies, including plant biology research, environmental monitoring and quality control [16]. Gupta et al. [30] have successfully applied the μ-EDXRF analysis to determine non-essential and toxic element concentrations in leaf and root samples of radish collected around an urban waste-dumping site. The results obtained here demonstrates the usefulness of this method to detect bioaccumulation of As in plants, especially those grown in As-enriched soils or irrigated with contaminated water. Arsenic uptake and accumulation by crop plants such as rice and vegetables represent an eminent risk for human health.

Additionally, μ-EDXRF has been considered as a fast analytical method that is suitable to be used in the analysis of a large number of samples, because of its multielemental and simultaneous capability, requiring simple sample preparation steps [16]. Nevertheless, it is important to emphasize that this is a matrix-dependent method, and higher accuracy can be obtained when samples and standards have similar matrix composition.

4.2. Advantages and limitations of As and P localization by micro-EDXRF analysis in plants

We also investigated the accumulation and spatial distribution of arsenic and phosphorus in the As-hyperaccumulating fern Pityrogramma calomelanos using micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF). In view of the well-recognized arsenate resemblance to phosphorus [25], we also evaluated the As effects in P distribution in the pinna. The results showed that As is located mainly along the veins

and in the apical and marginal regions of the pinna, in line with previous reports for Pteris vittata [21]. This distribution pattern indicates that As transport from root to shoot is driven by transpiration, similarly to several other elements [31, 32]. Arsenic influence on P distribution was observed to be linked with the pinna regions. In the basal portion of the As-treated pinna, the lower P concentrations were observed in the midrib, which was an As hotspot. On the other hand, overlap of As and P was observed in the pinna tip, the end point of the transpiration stream. This finding suggests that, in the presence of As, P distribution can change along the pinna, which may be associated to As species distribution.

In plants, As is present mainly as its inorganic forms, with arsenate (As^{5+}) and arsenite (As^{3+}) being the predominant species in roots and pinnae of As-hyperaccumulating ferns, respectively [12, 20]. Arsenate is a chemical analog of phosphate and shares with it the Pi transport pathway [25]. Once uptaken by the root, arsenate is partially reduced to arsenite, and both As forms are loaded into vascular tissues for transport into aboveground biomass [24]. In Pteris vittata, arsenate has been demonstrated to be the predominant As specie transported into the xylem sap [33]. Higher As^{5+} concentration in the xylem sap of P. calomelanos may have restricted the P uptake and transport, reducing consequently the P concentration in the pinna midrib. Furthermore, the lower P concentration observed in the necrotic pinnule of ferns exposed to 30 x 10^{-3} mol L^{-1} suggests that As promoted P deficiency in old fronds or enhanced the mobilization of P towards young fronds. The observed overlap between As and P in pinnule tip veins may indicate that in this compartment, the competition between both anions, arsenate and phosphate, cannot be detected. Along the pinna blade, As^{3+} concentration increases from the base to the tip, with arsenite being the main As species found in the pinna tip [22, 24]. Once arsenite is not a P-analog, it does not compete for P transporters [34].

Giving the difference of thickness observed between the pelletized test samples and the dried fern parts, the construction of quantitative maps for As was impaired, because of the higher X-ray characteristic energy of this element (As Kα 10.54 keV). For P, the values obtained in the semi-quantitative maps can be considered appropriate, considering that the ICP OES data represent the medium values obtained for the pinnae of each plant and the P distribution in the pinnule tip is inherently heterogeneous. The five-fold lower X-ray characteristic energy of P (P Kα 2.01 keV), in comparison with As, minimizes the sample thickness effect.

Micro-EDXRF analysis showed to be an appropriate analytical tool for mapping As and P in ferns, however, detailed studies are needed to clarify the exactly physiological mechanisms involved in As and P distribution in pinna tissues of P. calomelanos under As exposure.

5. Conclusions and outlook

This is the first study to propose a validated analytical method using micro-energy dispersive X-ray fluorescence spectrometry for mapping and quantifying As and P simultaneously in Pityrogramma calomelanos, a well-recognized As-hyperaccumulating fern. An appropriate analytical strategy to circumvent matrix effects when analyzing the test samples was to build linear calibration models using pelletized powdered samples from the same ferns parts. By using this simple and validated method, important information towards environmental monitoring and phytoremediation studies can be obtained in a fast and non-destructive way.

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CAPÍTULO 3. Hiperacumulação de arsênio em Pityrogramma calomelanos (L.) Link: características adaptativas para lidar com elevadas concentrações do metaloide

RESUMO: Pityrogramma calomelanos é, particularmente, a única monilófita externa ao gênero Pteris descrita como hiperacumuladora de arsênio (As), para a qual não se conhece as implicações morfofisiológicas da acumulação de As. Nesse estudo, investigaram-se as características anatômicas e fisiológicas que permitem P. calomelanos sobreviver em ambientes com elevadas concentrações do metaloide. Plantas expostas a diferentes concentrações de arsenato em solução nutritiva, durante 21 dias, acumularam concentrações de até 3576 mg kg^{-1} peso seco nas frondes. Em elevadas concentrações do metaloide (10 e 30 mM As), foi observado o decréscimo da fluorescência máxima da clorofila a e o aparecimento de necroses marginais e apicais. As necroses iniciaram em feixes vasculares situados na margem das pínulas e progrediram em direção da endoderme, parênquima clorofiliano e epiderme, seguindo a corrente transpiratória. As alterações anatômicas nas pinas foram precedidas pelo aumento do acúmulo de compostos fenólicos nos tecidos afetados. O As promoveu alterações discretas nas raízes como o desprendimento de células da coifa lateral (‘border-like cells’) e acúmulo de substâncias granulares intensamente coradas em azul de toluidina na camada mais interna do córtex. Em síntese, P. calomelanos apresentou diversas características adaptativas que favorecem sua sobrevivência em sítios contaminados, incluindo: elevada translocação de As para a parte aérea, aumento da dissipação não-fotoquímica de energia, presença e aumento do acúmulo de compostos fenólicos e desprendimento de células da coifa protegendo o meristema apical da raiz.

Palavras-chave: alterações anatômicas; fluorescência da clorofila a; toxidez do metaloide

CHAPTER 3. Arsenic hyperaccumulation in Pityrogramma calomelanos (L.) Link: adaptive traits to cope with higher metalloid concentrations

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ABSTRACT: Pityrogramma calomelanos is interestingly the only non-Pteris arsenic (As)-hyperaccumulating fern for which the morpho-physiological implications of As accumulation are still unknown. We investigated the anatomic and physiological traits that allow P. calomelanos to survive in high As-contaminated environments. In an 21-days hydroponic experiment with varying concentrations of arsenate, P. calomelanos accumulated up to 3576 mg kg^{-1} dry weight in the fronds. At higher As concentrations (10 and 30 mM As), it was observed a decrease of maximum chlorophyll a fluorescence and development of apical and marginal necrosis. Necrosis initiated in the marginal vascular bundles and spread toward endodermal, chlorenchyma and epidermal cells, following the transpiration stream. Anatomic alterations were preceded by increase of phenols content in the affected tissues. Arsenic promoted slight alterations in the roots such as the detachment of border-like cells and accumulation of granular substances in cortical cells. We identified several adaptive traits that allow P. calomelanos to survive in contaminated sites, including: higher root-to-shoot As translocation, increase of the fraction of energy passively dissipated in form of heat and fluorescence, presence and increased accumulation of phenolic compounds and detachment of border-like cells protecting the root tips.

Keywords: metalloid toxicity; anatomic alterations; chlorophyll a fluorescence

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1. Introduction

Hyperaccumulator is a term originally coined to describe the extraordinary accumulation of nickel by plants grown in ultramafic soils [1,2]. The term was recently revised by van der Ent et al. [3] and refers to a species growing in its natural habitat that accumulates unusual high metal(loid) concentrations in the aboveground organs, remaining healthy enough to maintain a self-sustaining population. About 500 species are able to accumulate high amounts of metals such as Cd, Zn and Ni, reaching concentrations that are 100–1000 times higher than those normally found in plants [4].

Arsenic (As) hyperaccumulation was most recently discovered in plants [5]. Interestingly, whereas around 400 species scattered in 40 families can accumulate high amounts of Ni [4], only twelve species with potential to hyperaccumulate As have been described. Most of them belonging to the genus Pteris (Pteridaceae): P. vittata, P. cretica, P. umbrosa, P. longifolia, P. biaurita, P. quadriaurita, P. ryukyuensis, P. aspericaulis, P. fauriei, P. multifida and P. oshimensis [5-9]. However, not all of them have proven ability to hyperaccumulate As in natural conditions. Pityrogramma calomelanos is the single non-Pteris As-hyperaccumulating fern known, and was reported to accumulate As concentrations as high as 8350 mg kg^{-1} in dry weight (DW) in Thailand areas that had episodes of As pollution from mine tailings [10].

Arsenic is a metalloid of great environmental concern due to its detrimental toxicity and abundance [11]. Hyperaccumulation of arsenic (and heavy metals) by plants is a complex phenomenon that involves several steps such as (a) transport of As across the plasma membrane in root cells, (b) xylem loading and translocation, and (c) detoxification and sequestration at the whole plant and cellular levels [12-13]. Arsenate (As^{5+}) is the main As species found in aerobic conditions, and is taken up by plants through root phosphate transporters [14]. In roots of As hyperaccumulators (e.g. Pteris vittata), As^{5+} can be reduced to arsenite (As^{3+}), which is translocated and sequestered as free anion in vacuoles of pinnule cells [15]. Arsenate can be abundant in xylem sap of P. vittata and is probably reduced in the pinna before being stored [16]. However, the specific transporters involved in As translocation in hyperaccumulators are still unknown.

The efficient As translocation from root to shoots and further compartmentalization in vacuoles are key requirements for As hypertolerance that protects the fern development and metabolism. Excessive metal(loid) concentrations can

be very cytotoxic even for hyperaccumulators, resulting in dry mass reduction and physiological damages as lipid oxidation and reduced photosynthetic efficiency as reported for Pteris spp. [17-19]. Arsenic effects on the photosynthesis of P. calomelanos have not been reported, and it is evident that the bipinnatisect fronds represent a physical limitation to measure leaf gas exchange. Chlorophyll a fluorescence imaging can be used as an alternative method to assess As effect in the photosystem II activity of these plants, providing also spatial information about the damages [20].

In non-accumulators, As have been demonstrated to induce moderate to severe anatomical damages, especially in the root, with alterations in the shape and integrity of epidermal and cortical cells and cell wall lignification of endodermal cells [21-23]. Anatomic effects on As hyperaccumulating ferns are poorly understood, especially in comparison with Zn and Cd accumulators, for which there are several studies addressing metal histolocalization and structural/ ultrastructural responses to metal toxicity [24-26]. Arsenic induced morphological effects are related only to Pteris vittata, including damages in the chloroplast membrane structure [27] and increase of root hairs and border-like cells [28]. The knowledge of organ- and tissue-specific As responses can be an indicative of As detoxification pathways in accumulators.

This study aimed to evaluate the morphological responses of P. calomelanos ferns to acute arsenic exposure and the effect of the metalloid on chlorophyll a fluorescence. Arsenic induced morphophysiological alterations combined with the study of As distribution in plant organs can help understand the As detoxification pathways in As accumulators, clarifying the adaptive characteristics that allow P. calomelanos to survive in contaminated sites.

2. Material and methods

2.1. Plant material and growth conditions

Spores of Pityrogramma calomelanos collected from ferns in a deactivated gold mine site in Nova Lima (Minas Gerais State, Brazil) were germinated in vitro in Murashige and Skoog medium (MS) [29] until obtaining of young sporophytes, as described in the Chapter 1. The young sporophytes were then cultivated ex vitro to obtain adult sporophytes. Spores collected from these adult sporophytes were germinated in pots containing yellow latosol to generate gametophytes and new sporophytes, sequentially. The new sporophytes were transferred to commercial

substrate Plantmax®. During all the process, the substrate was maintained humid through daily irrigation with water and twice a week with Hoagland's solution [30]. The ferns were cultivated under Sombrite® 50% net in a greenhouse with controlled temperature of 25 ± 5 ºC.

Ferns at 3-4 fronds stage were transferred to hydroponic system with half-strength Hoagland's solution, pH 5.5 and continuous aeration for one month, before imposing the treatments. The ferns were exposed to As concentrations of 0 (control), 1, 10 and 30 mM As for two weeks. Arsenic was supplied as sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$). Each experimental unit consisted of one 2.2-L pot containing one plant, with four replicates for each treatment. The pH was adjusted to 5.5 every two days.

2.2. Dry weight, arsenic and phosphorus determination in fern tissues

At the end of the experiment, the ferns were harvested, separated into roots, pinnae, and stipe + rachis (referred here simply as stipe), and oven-dried at 60 ºC. The dry weight was obtained for each fern part. Then, the dry matter was powdered with a knife mill to evaluate the As and P concentrations in fern parts. Samples (0.1 g) were decomposed with nitro-perchloric solution (3:1) in a digestion block following the procedure described by Tedesco et al. [31]. The final volume of the extract was made up to 10 ml with ultrapure water and filtered. The samples were analyzed by inductively coupled plasma-optical emission spectrometry (ICP OES) (Perkin Elmer, Shelton, CT, USA).

The As and P concentrations were used to calculate the As/P molar ratio for each fern part. The As bioaccumulation factor (BF_{As}) was calculated as the ratio of frond to medium As concentration and the translocation factors (TF) of As and P as the ratio of frond to root elemental concentration [32].

2.3. Visual and anatomical characterization

At the end of the experiment, the fronds of P. calomelanos were photographed with a digital camera (model Cyber-Shot DSC-W310, Sony Corporation, Japan) for description of visual symptoms. The morphology of root tips, after two weeks of As

exposure, was examined and photographed using a stereo microscope (model SZX7; Olympus, Tokyo, Japan).

The middle pinnae of old fronds were selected for microscopic examination. Samples from the median region of the pinnules, without visual symptoms, and samples near necrotic regions, were collected for structural characterization. Root tips and fragments 3 cm far from the root tip were also collected for characterization of As-induced damages in the roots. The samples were fixed in Karnovsky's solution [33], dehydrated through an ethanol series and embedded in methacrylate resin (Leica Historesin, Nussloch/Heidelberg, Germany). Longitudinal and cross sections (8 μm thick) were obtained using an automatic rotary microtome (Model RM2155; Leica Microsystems Inc., Deerfield, USA) and were stained with toluidine blue (pH 4.0) [34].

To perform histochemical tests of phenolic compounds, samples from the median portion of pinnules were also fixed in a solution of ferrous sulfate in formalin. The samples were dehydrated through an ethanol/butanol series, embedded in Paraplast (Histosec, Merck) [35], and sectioned (10 μm in thickness) using a rotary microtome.

The sections were mounted on slides and coverslip with synthetic resin (Permount, Fisher Scientific, Bridgewater, USA). Photographs were taken using a light microscope (Olympus AX70TRF; Olympus Optical, Tokyo, Japan) coupled with a U-Photo Camera system (Spot Insightcolour 3.2.0; Diagnostic Instruments Inc., New York, USA).

2.4. Chlorophyll fluorescence imaging

The chlorophyll fluorescence was measured in dark acclimated non-detached fronds (30 min) using an Imaging-PAM fluorometer (Heinz Walz, Effeltrich, Germany). The charge-coupled device (CCD) camera had a resolution of 640×480 pixels. The minimal fluorescence (F_0) was measured under a weak modulating light (0.5 μmol m^{-2} s^{-1}) and maximal fluorescence (F_m) was induced by a saturating pulse of light (2400 μmol m^{-2} s^{-1}) supplied for 800 ms. After sample illumination, saturation pulses were applied at 20 s intervals for 100 s to determine the Fm' (light-acclimated maximal fluorescence) and Fs (steady state fluorescence yield). The maximal PSII quantum yield was determined as $F_v/F_m = (F_m–F_0)/F_m$ [36] and the quantum yield of non-regulated non-photochemical energy dissipation in PSII as $Y(NO) = F_s/F_m$ [37].

Analysis of the fluorescence parameters was performed using the software ImaginWin. The pixel value images of the fluorescence variables were displayed using a color code ranging from black (0.0) to purple (1.0).

2.5. Statistical analysis

The experiment was performed in a completely randomized design, with four treatments and four biological repetitions. The data were analyzed using analysis of variance (ANOVA), followed by Tukey test at 5 % significance level. All analyses were performed using the statistics software package R [38]. Graphs were created with SigmaPlot 11.0.

3. Results

3.1. Dry weight, arsenic and phosphorus accumulation by ferns

At the end of the experiment, the dry weight (DW) of pinnae, stipes and root represented 48, 24 and 28 % of total DW of control plants (Figure 1). Similar proportions were observed for As-treated plants, with significant reduction only for the pinnae of 30 mM As treatment, that represented 38 % of the total DW and was 21 % smaller than pinnae DW of control plants.

The As accumulation in Pityrogramma calomelanos increased with the increase of As concentration in the hydroponic solution (Table 1). Ferns exposed to the highest As dose accumulated in average 2826, 1822 and 1641 mg As kg^{-1} DW in pinnae, stipes and roots, respectively. Arsenic accumulation in pinnae did not differ between the treatments 10 and 30 mM As, while As accumulation in roots and stipes did not differ between 1 and 10 mM As.

Arsenic accumulation was always higher in pinnae, while stipes and roots accumulated similar As concentrations (Table 1). The relative percentages of As present in fern parts were estimated as the ratio of each fern part to total plant As concentration. The relative percentages decreased in pinnae (67 to 45 %), and increased in stipes (23 to 29 %) and roots (10 to 26 %), with the increase of As concentration in the solution from 1 to 30 mM As. The relative percentages of As in the fronds (pinnae + stipes) ranged from 90 to 74 %.

The As translocation factor (TF_{As}) was higher in ferns exposed to 1 mM As, but did not differ between ferns exposed to 10 and 30 mM As (Table 1). Ferns exposed to 1 mM As showed As bioaccumulation factor (BF_{As}) six and fifteen times higher than

Tab 1. Concentration of As and P, As/P molar ratio, translocation factor (TF) of As and P, and bioaccumulation factor (BF) of As in Pityrogramma calomelanos plants of the control treatment and exposed to 1, 10 and 30 mM As for two weeks.

Treatment	As (mg kg^{-1})				P (g kg^{-1})				As/P molar ratio			TF		BF
	Pinna	Stipe	Root	Total	Pinna	Stipe	Root	Total	Pinna	Stipe	Root	P	As	As
Control	0.01^{C}	(ND)	(ND)	0.01^{D}	12.7^{Aa}*	12.8^{Aa}	7.08^{Ab}	32.52^{A}	–	–	–	3.60^{A}	–	–
1mM	1715^{Ba}	579^{Bb}	257^{Bb}	2551^{C}	11.6^{Aa}	11.0^{Aa}	7.58^{Ab}	30.15^{A}	0.15^{Ba}	0.05^{Bb}	0.03^{Ab}	2.98^{A}	8.93^{A}	30.6^{A}
10mM	2552^{Aa}	1024^{Bb}	820^{Bb}	4396^{B}	10.8^{Aa}	10.5^{Aa}	7.06^{Aa}	28.40^{A}	0.24^{Aa}	0.10^{ABa}	0.12^{Aa}	3.02^{A}	4.36^{B}	4.77^{B}
30mM	2826^{Aa}	1822^{Ab}	1641^{Ab}	6289^{A}	11.7^{Aa}	8.30^{Aab}	6.64^{Ab}	26.68^{A}	0.24^{Aa}	0.22^{Aa}	0.28^{Aa}	3.01^{A}	2.83^{B}	2.07^{B}

* Means by treatment followed by same letters were not significantly different. Capital letters refer to comparisons between the treatments and small letters between the fern parts, for each treatment (Tukey; $p < 0.05$). (ND) = non-detected.

those treated with 10 and 30mM, respectively. Ferns exposed to 10 and 30 mM presented similar BF_{As}.

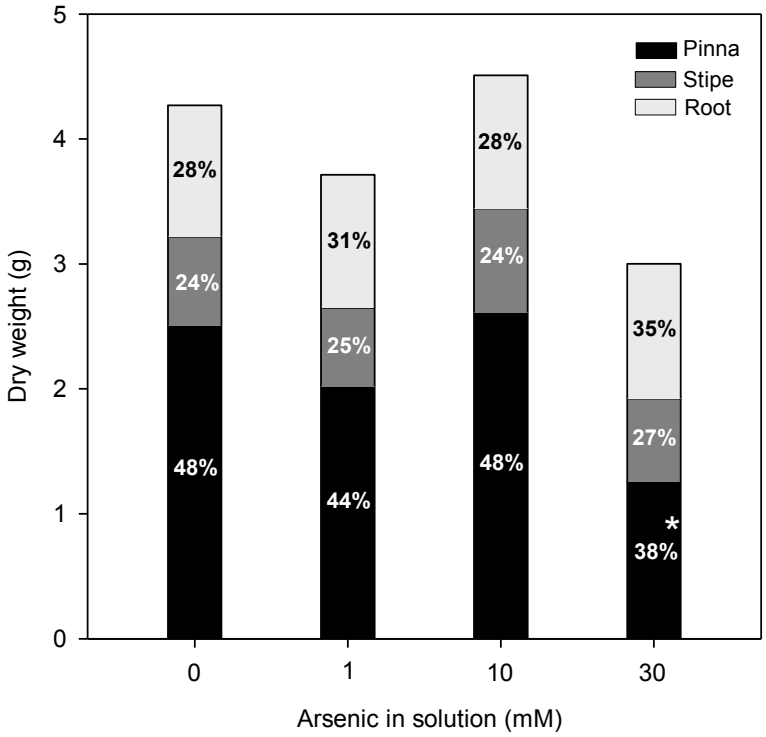

Figure 1. Dry weight of Pityrogramma calomelanos plants from control treatment and exposed to 1, 10 and 30 mM As for two weeks in Hoagland's nutrient solution. Asterisk indicates significant difference between treatments (Tukey; $p < 0.05$).

Arsenic did not change either P concentration in the ferns or the TF_P among the treatments (Table 1). The P concentration was higher in pinnae and stipes than roots, except for ferns exposed to 10 mM As, which did not show differences in P concentration among fern parts. The As/P molar ratio increased in pinnae and stipes with the increase of As concentrations in the solution (Table 1). However, As exposure did not affect the As/P in roots that presented the lowest molar ratio among fern parts.

3.2. Visual and morphological characterization

Fronds of Pityrogramma calomelanos are bi-pinnate with the abaxial surface covered with a white indumentum. Ferns from the control treatment showed healthy pinnae and developed normal new fronds (Figure 2 A-C). Arsenic induced necrosis on the edge and tip of pinnules in mature fronds of plants exposed to 30 mM As, especially

in the most basal pinnae (Figure 2 D-E), and in some individuals of the 10 mM As treatment. The emergence and development of new fronds were not affected in ferns exposed to up to 10 mM As. At 30 mM As, the ferns showed wilting and shriveling of not fully-expanded fronds (Figure 2 F). The necrosis observed in the pinnules was not preceded by chlorosis.

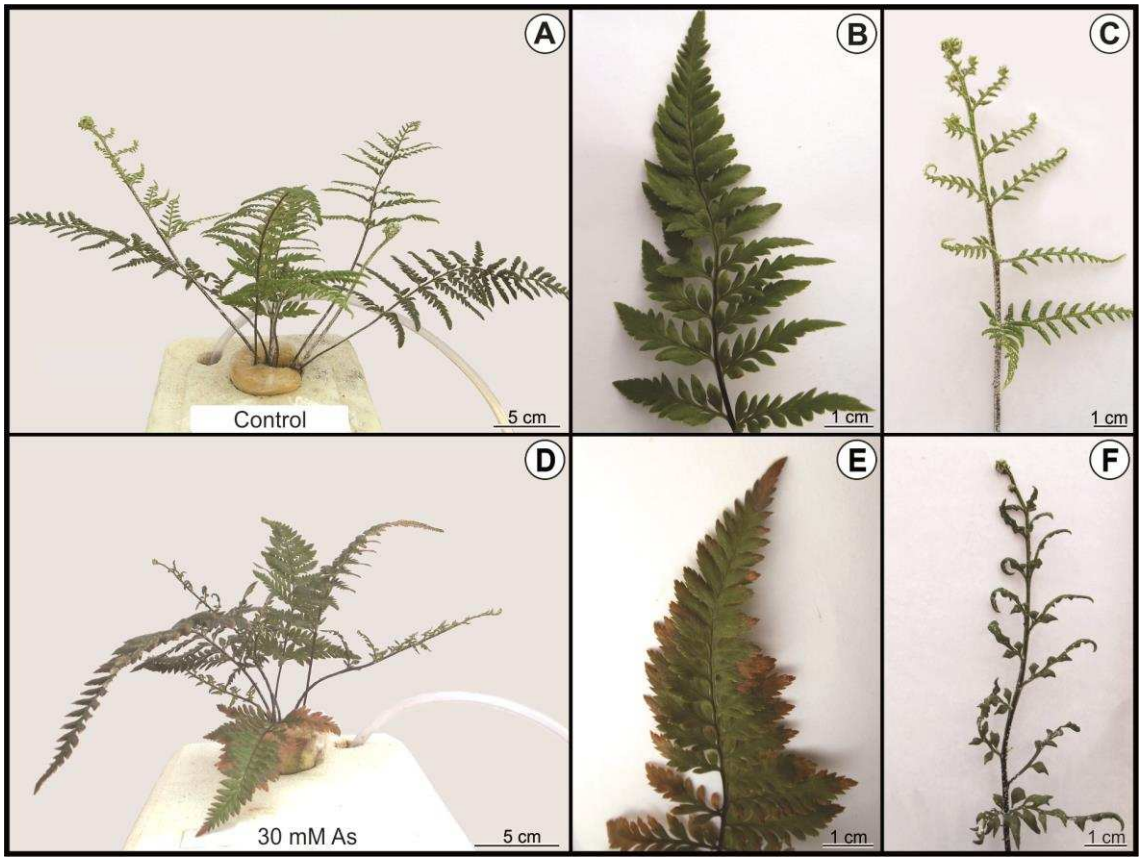

Figure 2. Pityrogramma calomelanos plants from the control (A-C) and two weeks after exposure to 30 mM As (D-F) in Hoagland's nutrient solution. A-C. Visual aspect of ferns from the control treatment. B-C. Healthy old and new fronds, respectively. D. Visual symptoms in ferns treated with 30 mM As. E. Necrosis on margins and tips of pinnules from old fronds. F. Wilting and shriveling of young frond.

In the absence of As, the roots showed a typical whitish color, with evident yellow-greenish root tips (Figure 3 A, E). The border-like cells are normally observed in the root tips of ferns from the control treatment (Figure 3 E). Arsenic induced slight alterations in the root cap morphology of As-treated plants, and the As effects intensified with increasing concentrations in the solution (Figure 3 B-D). Roots of ferns exposed to 10 and 30 mM As showed narrow or swollen regions (Figure 3 C-D) and

detachment of border-like cells (Figure 3 F). Arsenic induced a progressive darkening of the root, especially in the ramification zone (Figure 3 G-I).

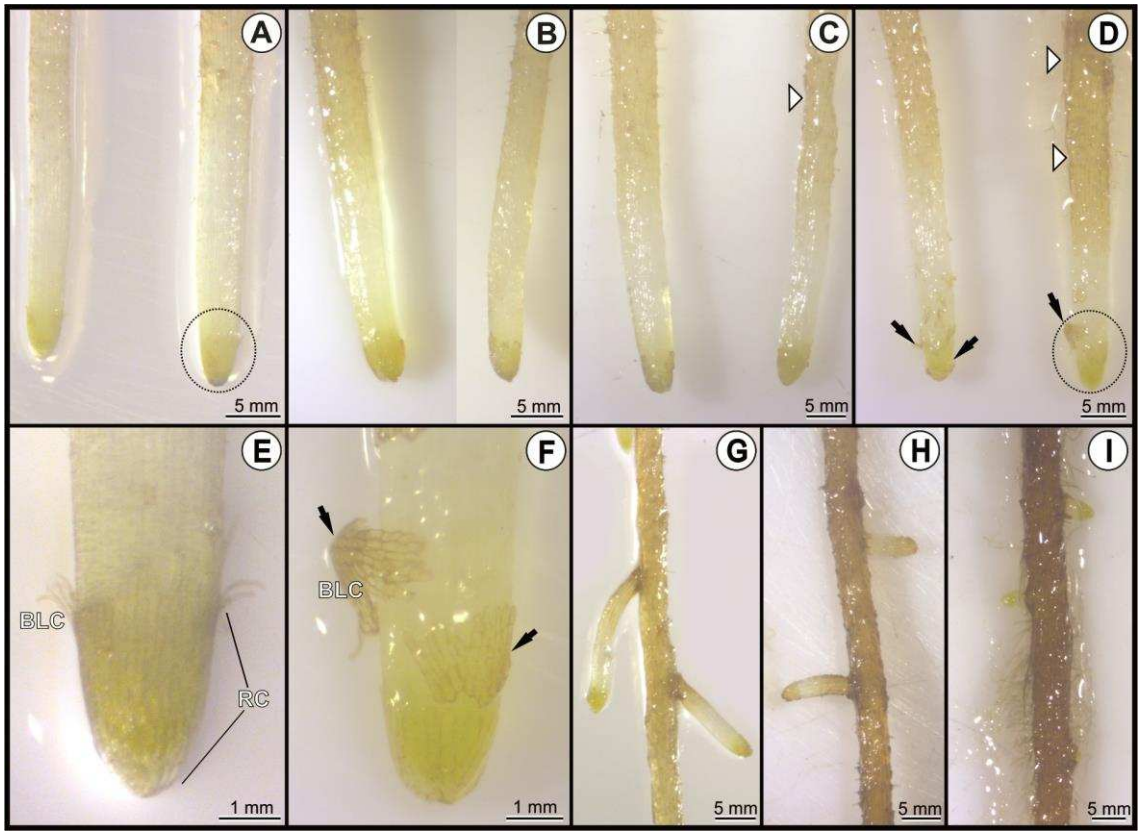

Figure 3. Pityrogramma calomelanos root morphology of control treatment plants (A, E, G) and two weeks after exposure to 1 (B), 10 (C, H) and 30 mM As (D, F, I) in Hoagland's nutrient solution (stereo microscope images). A. Root tip with normal aspect. B-D. Arsenic effects in root tip: deformation zones (white arrowheads) and deteachment of border-like cells (BLC, black arrows). E-F. Details of A and D, respectively, showing the BLC. G-I. Ramification root zone showing a progressive darkening with the increase of As concentration in the solution. RC, root cap.

3.2. Structural characterization in light microscopy

Pinnules of P. calomelanos are hypostomatic and have an uniestratificated epidermis (Figure 4 A, 5 A). Sporangia are distributed along the veins in the abaxial surface (Figure 4 A, F). The mesophyll was considered as dorsiventral, however, the differentiation of a typical palisade parenchyma was not observed. The chlorophyll parenchyma in contact with the epidermis of the adaxial surface is more compact and has cells slightly elongated in the anticlinal plane while those cells in contact with the

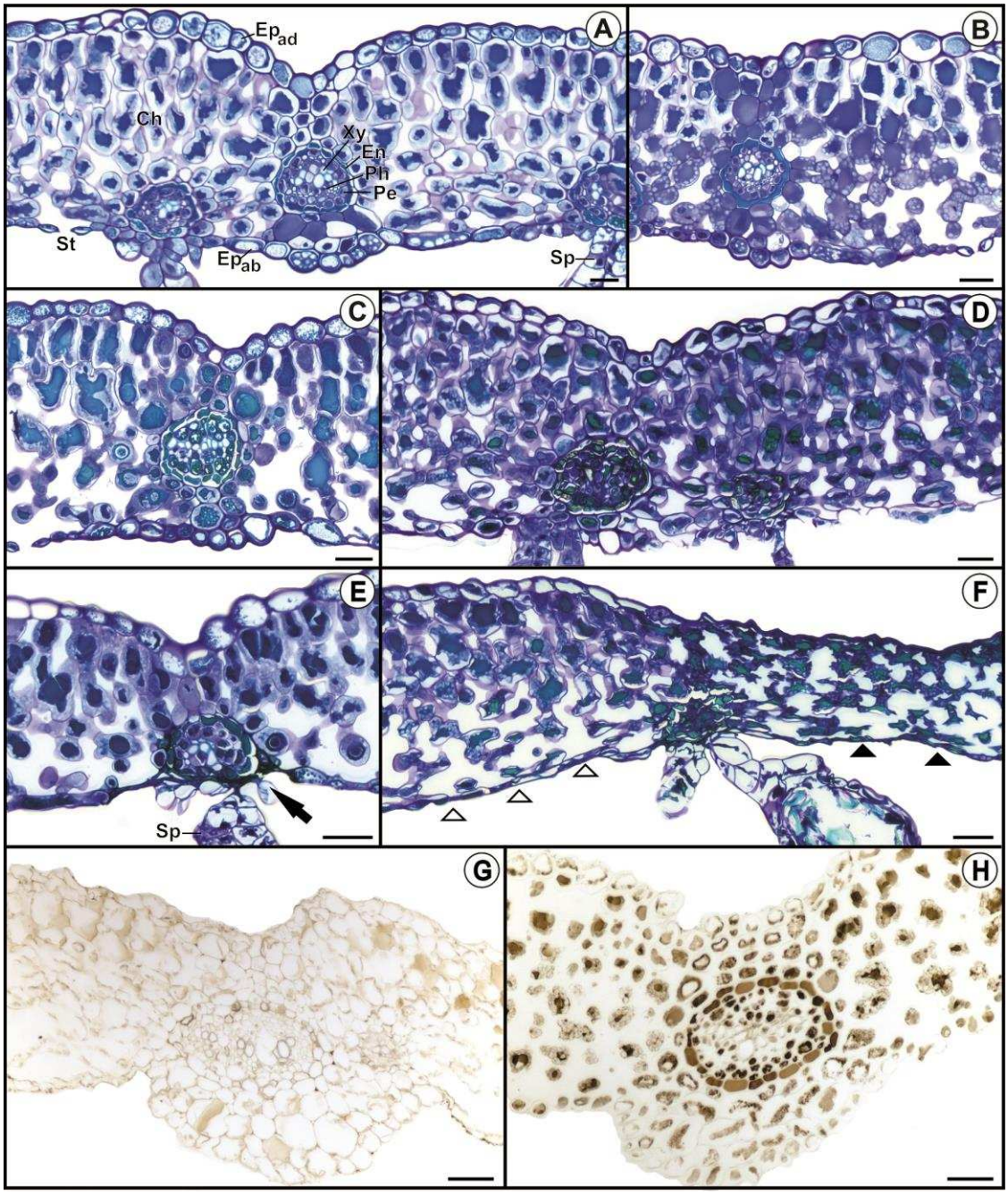

Figure 4. Pinnules of Pityrogramma calomelanos from the control treatment (A, G-H) and two weeks after exposure to 1 (B), 10 (C) and 30 mM As (D-F) in Hoagland's nutrient solution (cross sections). B-D. Pinnule without visual symptoms, showing As-induced increase of phenolic compounds in the mesophyll and vascular bundle. E. Middle portion of the pinnule with phenol accumulation in the basal cells of sporangia and collapse of adjacent epidermal cells (E, black arrow). F. Transition of healthy (white arrowheads) to necrotic mesophyll area (black arrowheads). G-H. Histochemical test for phenol showing absence of color in the negative control (G) and positive result for the mesophyl and epidermal cells in the mibrib (H). Ch, chlorenchyma; Ep, epidermis (ab, abaxial; ad, adaxial); En, endodermis; Pe, pericycle; Ph, phloem; Sp, sporangium; St, stomata. Bars = 50 μm.

abaxial surface were lobed. The midrib pinnae and secondary veins present collateral vascular bundles surrounded by a conspicuous endoderm (Figure 4 A, 5 A). Epidermal and parenchyma cells show intense toluidine blue staining of the vacuolar content that coincides with the phenol localization indicated by specific histochemical test (Figure 4 G-H). Higher amounts of phenols were observed in the endodermis. The pinnule margin forms an acute angle (Figure 5 A), sometimes ending in a more prominent cell.

The pinnules from the 1 mM As treatment did not show anatomical damages induced by As (Figure 4 B, 5 C). Microscopic symptoms of As toxicity were observed in pinnules of ferns exposed to 10 and 30 mM As, in regions with and without visual symptoms. Arsenic induced the increase of green-brownish content mainly in the phloem and associated vascular parenchyma cells of the midrib and secondary veins (Figure 4 C-E; 5 E). At 30 mM As, these compounds also accumulated in sporangia pedicel and in the adjacent epidermal cells (Figure 4 E). Tissue disruption was observed mainly in the pinnule margins and near the secondary veins (Figure 5 E). Arsenic promoted the collapse of epidermal and mesophyll cells, like in vascular tissues (phloem, xylem and vascular parenchyma), pericycle, endodermal, and clorenchyma cells (Figure 5 D-E).

Necrotic regions of the pinnules showed reduction on blade thickness, due to the tissue collapse and increased green-brownish content (Figure 4 F, 5 F). The necrosis started on the pinnule margin and advanced until the middle portion of the pinnule or sometimes spread over all the pinnule area. A transition region between the healthy and affected mesophyll is shown in the Fig. 3 F. Also, hypertrophied cells were observed in the epidermis of the pinnule margin (Figure 5 F) in 30 mM As treatment.

The root apex of P. calomelanos showed the classical pattern of fern species, with a prominent pyramidal apical cell that gives rise to the meristematic cells of protoderm, procambium and ground meristem (Figure 6 A). Each of the different cell files that constitute the root tissues can be traced back to a single initial, configuring a closed meristem. The root cap has 5-6 cell layers, including the border-like cells.

Under As exposure, the root tip showed modifications in the shape and detachment of border-like cells (Figure 6 B-D). At 10 and 30 mM As, it was observed alterations in the shape with protoplast retraction of epidermal cells and hypertrophied cells close to the procambium (Figure 6 C-D).

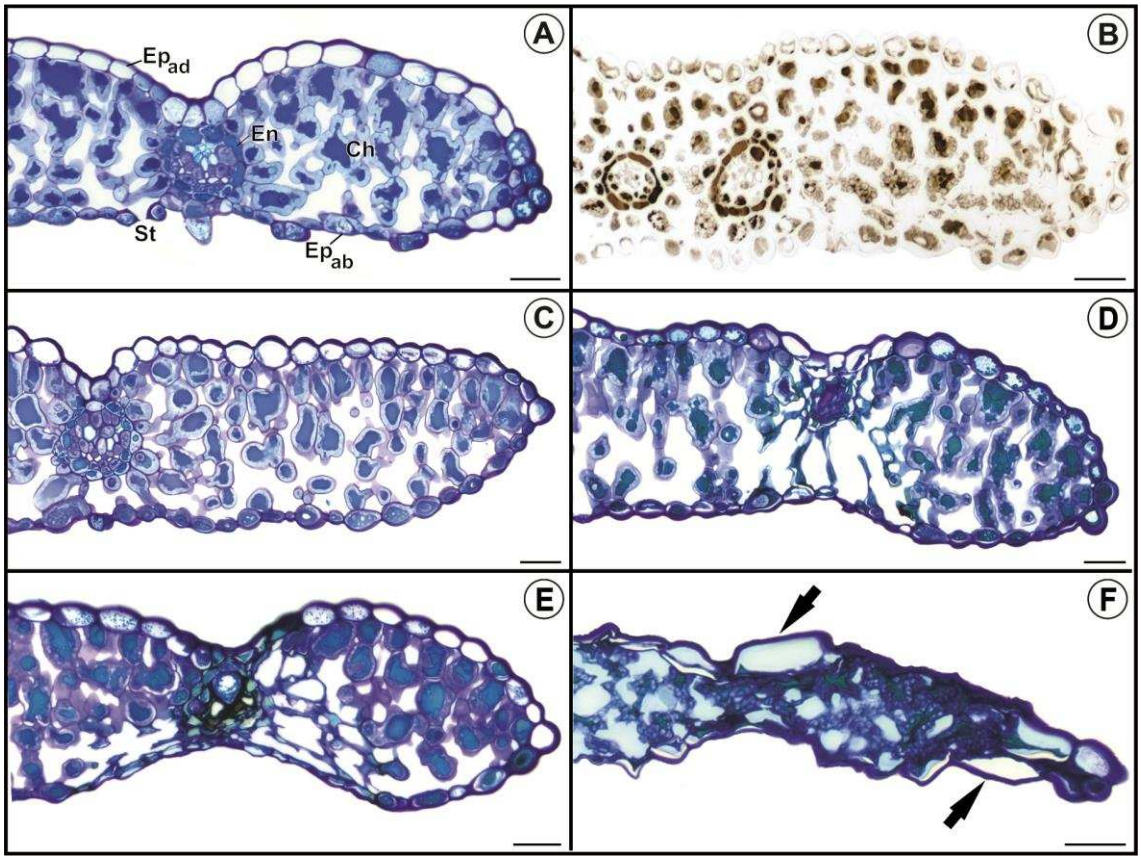

Figure 5. Pinnule margin of Pityrogramma calomelanos from the control treatment (A-B) and two weeks after exposure to 1 (C), 10 (D) and 30 mM As (E-F) in Hoagland's nutrient solution (cross sections). A. Healthy tissues. B. Accumulation of phenols proved by histochemical test for phenol. C-E. Samples without visual symptoms, showing As-induced increase of phenolic compounds and collapse of tissues near secondary veins. F. Necrosis of the pinnule margin, with mesophyll collapse and hypertrophy of epidermal cells (black arrows). Ch, chlorenchyma; En endodermis; Ep, epidermis (ab, abaxial; ad, adaxial); St, stomata. Bars = 50 μm.

Cross roots sections of P. calomelanos show unstratified epidermis, cortex composed of 3-4 layers of parenchyma cells, and vascular cylinder with a diarch xylem. The cortical parenchyma layer adjacent to endodermis has cells with U-shaped cell wall thickening, with endodermal cells showing intensely blue-stained cytoplasm (Figure 6 E). Ferns exposed to 1 and 10 mM As showed no symptoms of As toxicity in root cross sections. At 30 mM As, it was observed an apparently increase in cell wall thickness and accumulation of dark-purple granular compounds, after toluidine blue staining, in cortical cells (Figure 6 F).

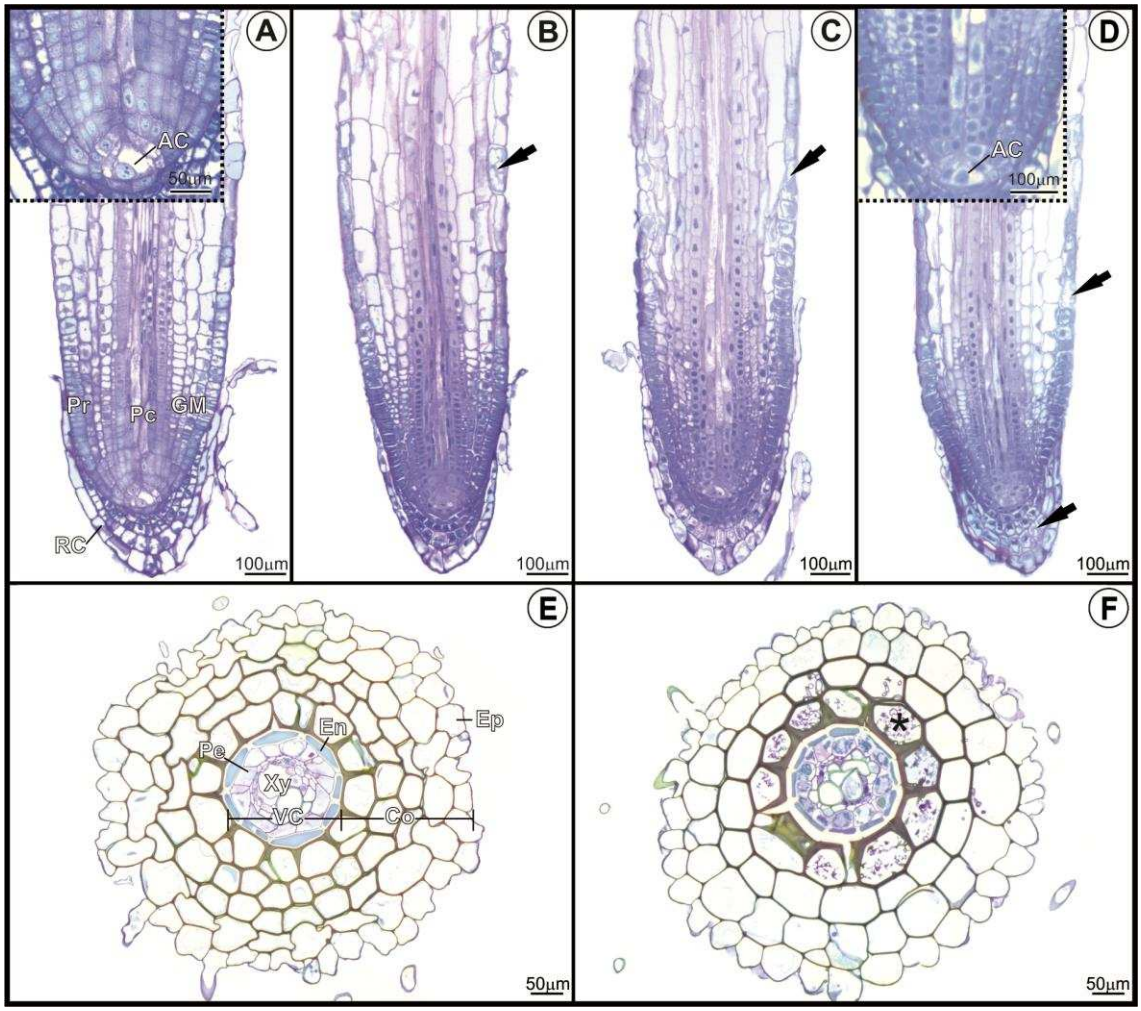

Figure 6. Roots of Pityrogramma calomelanos from the control treatment (A, E) and two weeks after exposure to 1 (B), 10 (C) and 30 mM As (D, F) in Hoagland's nutrient solution. Longitudinal (A-D) and cross (E-F) sections stained with toluidine blue. Arrows indicate protoplast retraction and asterisk indicates the accumulation of dark-purple granular compounds. AC, apical cell; Ep, epidermis; En, endodermis; GM, ground meristem; Pc, procambium; Pe, pericycle; Pr, protoderm; RC, root cap; VC, vascular cylinder; Xy, xylem.

3.3. Chlorophyll a imaging fluorescence analysis

In order to evaluate the As effects on photosynthesis, old fronds were selected for chlorophyll a imaging fluorescence analysis. Ferns exposed to different As doses showed similar fluorescence signals relative to the maximum quantum yield of PSII

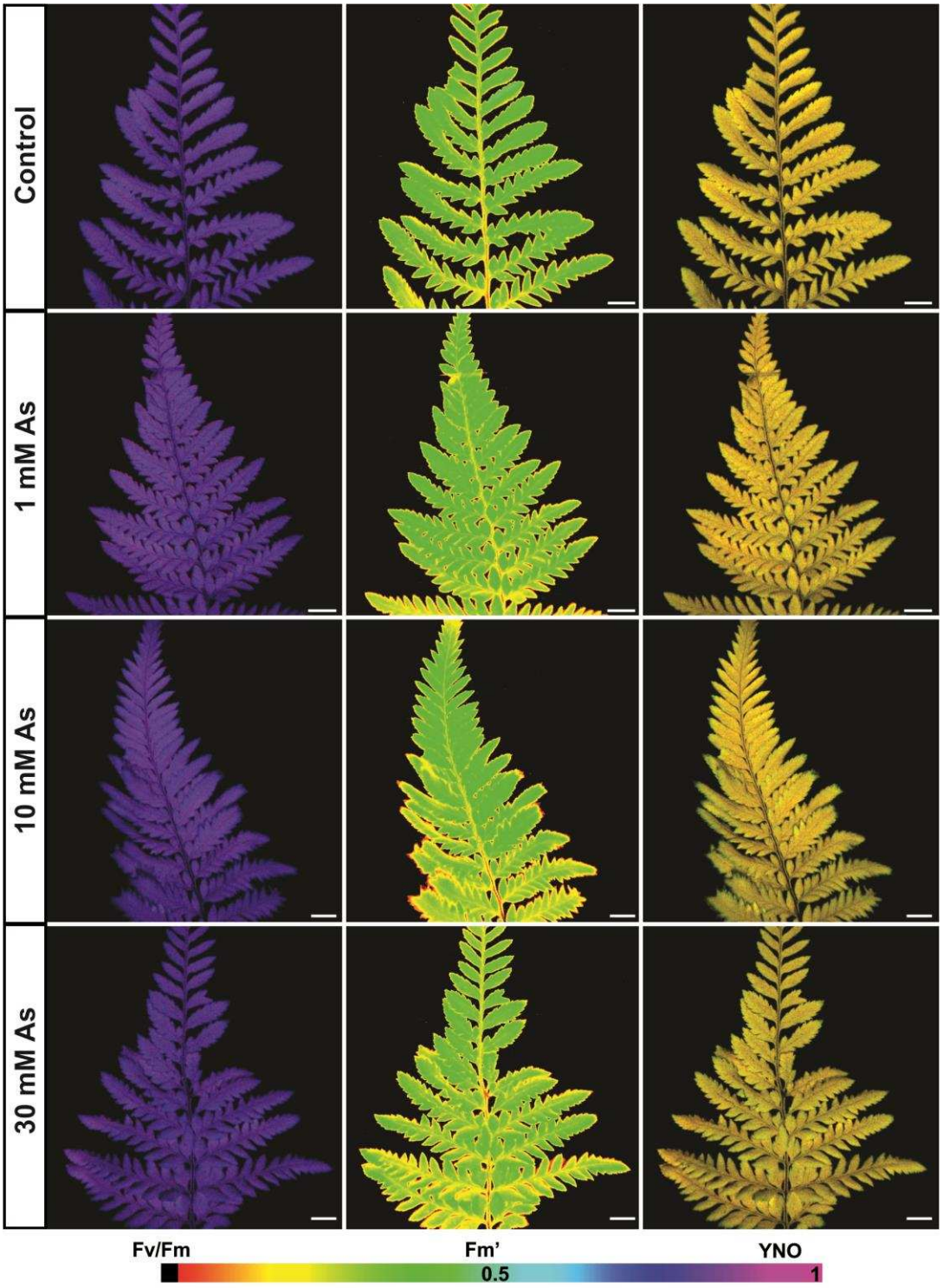

Figure 7. Chlorophyll fluorescence imaging of mature pinnae of Pityrogramma calomelanos plants grown in Hoagland's nutrient solution cointaining 0 (control), 1, 10 and 30 mM As for two weeks. Legend: F_v/F_m, fluorescence signal relative to the light-acclimated maximal fluorescence; $F_{m'}$ (II) effective quantum yield of PSII; Y(NO), non-regulated non-photochemical energy dissipation in PSII. The pixel value display is based on a color code ranging from black (0.0) to purple (1.0). Bars = 1 cm

(F_v/F_m) (Figure 7). However, the images of the Chl fluorescence showed a decrease in the fluorescence signal relative to the light-acclimated maximal fluorescence ($F_{m'}$) in ferns exposed to 10 and 30 mM As. The lower intensity was observed at the tip and edge of the pinnae from mature fronds. A discrete increase in the fluorescence signal relative to the quantum yield of non-regulated non-photochemical energy dissipation in PSII (Y(NO)) was noted for the same regions of the pinnules, especially for the ferns exposed to 10 mM As.

4. Discussion

Pityrogramma calomelanos exposed to 1, 10 and 30 mM As, for two weeks, accumulated concentrations 300 – 900 times higher than those normally achieved in plants (3.6 mg kg^{-1}) [39], as expected for an As-hyperaccumulator [3, 40]. Metal (loid) tolerant plants are classified in two basic groups: excluders, which limit the metal uptake and transport, and accumulators, which concentrate metals in plant parts from low or high background levels [41]. In As-excluders, the suppression of phosphate/arsenate transporters has been reported as a mechanism to limit As uptake [42]. The high As uptake and translocation observed in *P. calomelanos*, even under the highest As concentration in solution, represent, together with the high capacity for As detoxification, a strategy to ensure adequate P supply and avoid As toxicity.

The high As translocation (FT_{As}) and bioaccumulation (BF_{As}) factors, especially at 1 mM As exposure, show that *P. calomelanos* has a highly efficient system of As translocation from root to shoot. After uptake by roots, arsenate is reduced to arsenite (not a P analog), which is the main As form accumulated in aboveground parts of hyperaccumulating ferns [10, 43]. In As hyperaccumulators, the ratios of shoot to root As concentrations is typically 5 – 25 [15]. The FT_{As} observed to ferns exposed to 1 and 10 mM are in agreement with Zhao et al. [15], however, ferns exposed to 30 mM As showed a reduced FT_{As} (< 3). A possible explanation is that under lower As doses, the metalloid can be rapidly transported due to the high availability of membrane transporters, whereas under high As contaminated environment, the FT_{As} can be reduced drastically due to the saturation of As transport and sequestration systems. In this study, the relative percentage of As in fern parts decreased in the fronds (from 90 to 74 %) and increased in the roots (from 10 to 26 %) with the increase of As in solution (from 1 to 30 mM), indicating a restriction of As translocation.

Pityrogramma calomelanos treated with 1 mM As showed no macroscopic alterations in the fronds, whereas, symptoms of As toxicity were observed in fronds of ferns exposed to 10 and 30 mM As, which accumulated in average 3576 and 4648 mg As kg^{-1} DW in the fronds. These findings show that, in these experimental conditions, P. calomelanos can tolerate internal concentrations smaller than 3500 mg As kg^{-1} DW. Arsenic is highly phytotoxic, with toxicity threshold concentrations varying from 0.005 – 0.1 g As kg^{-1} DW in non-hyperaccumulating species [39]. The limit of As tolerance observed in P. calomelanos, which was much higher than that found for non-hyperaccumulators, contrasts with results obtained for Pteris spp. in relative long-term experiments [6, 17, 44]. For example, Pteris vittata growing in medium containing 0.08 – 2 mM As for 18 days accumulated up to 27000 mg kg^{-1} DW, showing visual symptoms only when concentrations in fern exceed 10000 mg kg^{-1} DW [44]. The lower As concentrations in the solution probably contributed to the higher As tolerance and accumulation in P. vittata, in addition to the longer exposure time. Slower increases in As internal concentration give plants more time to protect themselves against As toxic effects, whereas rapid increases overload the plant defense systems. In a previous study P. calomelanos ferns at 4-5 frond stage exposed to 1 mM As for three weeks accumulated up to 3300 mg kg^{-1} DW without shown visual symptoms (Chapter 1).

Arsenic toxicity is usually detected by necrosis of pinnae in the margins and tips of fronds [27, 44]. In P. calomelanos, the necrosis was observed mainly in mature fronds, including fertile and infertile ones, which can be related to As accumulation. Arsenic concentrations have been demonstrated to be higher in old fronds than young fronds in P. vittata [45], maybe due to their longer time of accumulation. Tu et al. [46] also reported that arsenite re-oxidation to arsenate occurs more often in the old fronds than in the young fronds in the same plant, possibly due to a decline in the levels of reductants. A higher percentage of arsenate may have increased the As cytotoxicity in old fronds, once arsenite is the only As form known to be sequestered in vacuoles of pinna cells [12, 47]. The shriveling of not fully-expanded fronds suggests that As promoted the fall in leaf turgor and water potential in young fronds. Shoot tips with leaf primordia have been reported to be more sensitive to water stress than fully-expanded leaves [48], probably due to the lower transpiration rate.

The decrease of maximum chlorophyll fluorescence ($F_{m'}$) in the edge and tip of pinnules, from the basal pinnae of 10 and 30 mM As treatments, is in agreement with the morphological characterization of As toxicity. Reduction on Fm' may suggest

damages on the photosynthesis, especially in the 30 mM As treatment in which was observed the decrease of pinnae dry weigh. The increase of Y(NO) observed in the same regions of these pinnules may indicate the increase of the fraction of energy passively dissipated in form of heat and fluorescence, mainly due to closed PSII reaction centers. The absence of alterations in the chlorophyll fluorescence of pinna exposed to 1 mM As suggest that these plants were not under stress.

The occurrence of necrosis in the apical and marginal region of leaves in response to As exposure is well known [17, 27, 49], however, the microscopic alterations that precede the necrosis are not well understood. The anatomical changes observed in the pinnule of ferns exposed to 10 and 30 mM indicate that As-induced toxic effects initiate in the margin of the pinnules, and spread from the vascular bundle toward the adjacent endodermal, chlorenchyma and epidermal cells. The progress of the symptoms is probably related to As accumulation and distribution along the pinna tissues. According to Hokura et al. [43] and Kachenko et al. [50] As concentrations in the pinnule tip of hyperaccumulating ferns increase from the midrib to the pinnule margin.

As other essential and non-essential elements, arsenic accumulation in leaves seems to be driven mainly by mass flow during transpiration [51-53]. The solutes are transported via xylem vessels and can accumulate at terminal sites of the transpiration stream or be transported to the sink cell via symplastic diffusion [51]. Considering that P. calomelanos presents the venation free with the secondary veins ending near the margin, the highest concentration and As damages are expected to be found in this region. The collapse of cells near the vascular tissues, also indicate that As is efficiently translocate to surrounding mesophyll tissues. Similarly, Chen et al. [54] reported that highest As contents in the midrib of Pteris vittata are found in the cortex tissue adjacent to endodermis and decrease from this tissue to the epidermis. According to Lombi et al. [12] and Indriolo et al. [47], the vacuoles played an important role in As tolerance in Pteris vittata. A similar mechanism seems to operate in P. calomelanos [50], showing the importance of mesophyll cells in As tolerance and accumulation.

The accumulation of excess As in the mesophyll cells may enhance the production of reactive oxygen species (ROS) that can induce peroxidation of lipids, oxidation of proteins and damage to nucleic acids [18, 55-56]. In this study, the accumulation of green-brownish contents, analyzed together with the histochemical results for phenols, indicates the increase of phenolic compounds in mesophyll cells as

the first response to As accumulation in the pinnule. Pityrogramma calomelanos presents a naturally high amount of phenols in pinnae, and the increase in their concentration can represent a mechanism of free radicals detoxification [57-58]. The phenol accumulation at the base of a sporangium indicates these cells as a preferential site of As accumulation, as observed in P. vittata by micro-X-ray fluorescence analysis [43]. The increase of total soluble phenols in pinnae of P. calomelanos exposed to 10 and 30 mM As were confirmed by a further experiment (see Chapter 4). Arsenic-induced of phenolic compounds have been associated with the increase of reactive oxygen species due the scavenging potential of these compounds [58].

Contrasting with As-induced damages in the pinnae, the roots of P. calomelanos showed less As toxic effects. For most of the potentially toxic elements, toxicity is first verified in the root tips in direct contact with the As-enriched solution/soil [59]. In Cajanus cajan, an As sensible species, As reduced the mitotic division index and promoted the retention of root primordia in the cortex [60]. Although alterations in epidermal cells were observed in the root tips of P. calomelanos, the meristematic zone remained morphologically intact after the As treatment, suggesting that the mitotic divisions are not As-sensible in P. calomelanos. The fact of this fern has only a well protect single apical cell, in contrast with a group of meristematic cells as in angiosperm species, can be a differential factor for As tolerance.

The detachment of border-like cells (BLC), cell layers programmed to separate from the root cap has been reported as a mechanism to protect the root tip [61], and probably help ensure the continuity of cell division in P. calomelanos. Border cells can show higher As concentrations and contribute to the plant's ability to tolerate excess As [62]. Induced production of border-like cells was reported for P. vittata exposed to concentrations up to 0.5 mM As [28]. Additionally to the protection supplied by BLC, the rapid transport of the metalloid to the root, and then to the shoot, may have reduced As concentration and its toxicity in the root tips.

The highest root As concentrations were found in ferns exposed to 30 mM As, which showed cell wall thickening and increase of dark-purple granular compounds in the cortical parenchyma cells adjacent to the endodermis. Endodermis has been pointed as a preferential site for As accumulation in roots of non-accumulators [62-63] and also in Pteris vittata [64]. Besides the endodermis, the cortical parenchyma cells can also be important for As sequestration in roots of As-hyperaccumulators exposed to higher As concentrations, reducing As negative effects at photosynthetic sites.

Further studies are necessary to clarify the cellular and subcellular mechanisms of As hypertolerance. The composition and functional role of the vacuole are issues of particular interest, once it has been configured a key organelle involved in intracellular accumulation and detoxification of As in root and pinna cells.

5. Conclusion

In our experimental conditions, Pityrogramma calomelanos showed highly efficient mechanisms of As detoxification at 1 mM As, without visual and microscopic alterations. At higher As doses, 10 and 30 mM As, morphological and physiological injuries were observed in pinnae and roots, following the gradient of As concentrations.

Pityrogramma calomelanos show adaptive traits that allow it to survive in contaminated sites, such as: higher root-to-shoot As translocation, increase of the fraction of energy passively dissipated in form of heat and fluorescence, abundant presence of phenolic compounds, border-like cells that protects the root tips, cell wall thickening and increase of granular substances in endodermal cells. Our data also suggest that As tolerance of P. calomelanos depend not only to the As concentration reached in each organ but also on the rate of As uptake and transport that interfere with the time response of the ferns to the metalloid.

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CAPÍTULO 4: Hiperacumulação de arsênio induz reprogramação metabólica em Pityrogramma calomelanos (L.) Link para evitar o estresse oxidativo

RESUMO: A poluição por arsênio (As) constitui uma preocupação ambiental de grande magnitude em função de sua ampla distribuição geográfica e elevada toxicidez para animais e plantas. Pityrogramma calomelanos é uma das poucas espécies capazes de hiperacumular As, entretanto, os mecanismos fisiológicos envolvidos permanecem amplamente desconhecidos. Esse estudo teve como objetivo investigar os reajustes metabólicos induzidos pelo As em P. calomelanos. Para isso, plantas com 5-7 frondes foram expostas a concentrações crescentes de As em solução nutritiva. Amostras de pina, provenientes de frondes novas, foram utilizadas para análises bioquímicas e do perfil metabólico das plantas em cromatógrafia gasosa com espectrômetro de massa (GC-MS). O As aumentou a concentração total de aminoácidos, proteínas e fenóis solúveis, bem como a atividade de peroxidases e induziu diversos distúrbios ao metabolismo de C e N. A redução de 50 % nos níveis de glicose foi uma das alterações mais acentuadas referentes ao metabolism de C. Diferenças significativas no conteúdo de diversos aminoácidos foram observadas, incluindo aqueles relacionados à síntese de glutationa e fenóis e envolvidos na osmorregulação e fotorrespiração. Aumentos nos níveis de poliaminas e de outros metabólitos também foram reportados. Pityrogramma calomelanos apresentou elevada tolerância ao As, em função do aumento da biossíntese de aminoácidos, e consequentemente de proteínas e de metabólitos antioxidantes, sem sofrer danos significativos no metabolismo central de carbono. Em concentrações muito elevadas de As os mecanismos de proteção podem se tornar ineficientes, levando ao aumento da concentração de espécies reativas de oxigênio e da ocorrência de danos oxidativos.

Palavras-chave: aminoácidos; arsenato; estresse oxidativo; tolerância

CHAPTER 4: Arsenic hyperaccumulation induces metabolic reprogramming in Pityrogramma calomelanos (L.) Link to reduce oxidative stress

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ABSTRACT: Arsenic (As) pollution is a major environmental concern due to its worldwide distribution and high toxicity to animals and plants. Pityrogramma calomelanos is one of the few species able to hyperaccumulate As, however, the mechanisms involved are largely unknown. This study aimed to investigate the metabolic adjustments involved in As tolerance of P. calomelanos. For this purpose, ferns at 5-7 frond stage were exposed to increasing As concentrations. Pinna samples of young fronds were used for biochemical analysis and GC-MS metabolite profile. Arsenic increased the total concentration of amino acids, proteins, soluble phenols, enhanced the activity of peroxidases, and promoted several disturbances in the N and C metabolism. In the C metabolism, the reduction of 50 % of the glucose pool was one of the striking responses to As exposure. Considerable differences in the levels of a wide range of amino acids were observed in As-treated plants, including those related to glutathione and phenols synthesis, and other physiological process as osmoregulation and photorespiration. Increased levels of polyamines and others metabolites were also reported. Pityrogramma calomelanos showed a great tolerance to As because of its capacity to up-regulate biosynthesis of amino acids and, consequently, antioxidant metabolites, without significant damages to the central C metabolism. At extremely high As concentrations, its protective mechanisms may become inefficient to block the production of oxygen reactive species, leading to oxidative damages.

Keywords: amino acids; arsenate; oxidative stress; tolerance

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1. Introduction

Arsenic (As) pollution is a major environmental concern due to its high toxicity to all living organisms and worldwide distribution (Jankong et al., 2007). Despite of its natural occurrence, As concentration in water and soils has been incremented by anthropogenic sources such as mining activities, use of arsenical herbicides and wood preservatives, and irrigation with As-contaminated groundwater (Zhao et al., 2009). Excessive uptake of As by crops represent a food safety problem and much effort has been direct to investigate As accumulation in crop plants (Matschullat, 2000; Garg and Singla, 2011; Lee and Yu, 2012). Human As intake from rice (Oryza sativa) consumption can be substantial because rice is particularly efficient in assimilating As from paddy soils, which become contaminated from the irrigation water (Abedin, 2002; Meharg and Rahman, 2003; Ma et al., 2008).

The most abundant environmental forms of arsenic are arsenate (As^{5+}), which is predominant in aerobic soils, and arsenite (As^{3+}), which is found in anaerobic environments, such as submerged soils (Tripathi et al., 2007). Plants take arsenate and arsenite up via phosphate transporters and aquaglycerol porins, respectively (Ullrich-Eberius et al., 1989; Ma et al., 2008; Zhao et al., 2009). Both inorganic As forms are highly phytotoxic and can potentially cause several physiological changes (Miteva and Merakchiyska, 2002; Stoeva et al., 2005). Toxicity may result from the binding of arsenite to sulphydryl (-SH) groups in proteins, leading to inhibition of activity, or replacement of phosphate by arsenate, disrupting the energy flows in cells (Meharg and Hartley-Whitaker, 2002; Cozzolino et al., 2010). Furthermore, As anions can stimulate the formation of free radicals and reactive oxygen species resulting in oxidative stress (Flora, 1999).

Plants exhibit a wide variation in their response to As. The main strategies adopted by plants able to survive in contaminated As-environments are: reduction of As influx (Meharg and Macnair, 1992; Bleeker et al., 2003), transformation of inorganic As to less toxic organic forms, and complexation and/or sequestration in vacuoles (Hartley-Whitaker et al., 2001). Plants may either rely solely on one strategy or adopt one strategy as the major and others as subsidiary mechanisms to tolerate their As load (Srivastava et al., 2012). Non-hyperaccumulators species decrease arsenate uptake through suppression of the high affinity P/As^{5+} transport system, and the remaining arsenate that enters into roots is reduced to arsenite and complexed with thiol rich

compounds in root cell vacuoles (Hartley-Whitaker et al., 2001; Raab et al., 2005). Contrasting, As-hyperaccumulators show a enhanced arsenate uptake, decreased arsenite-thiol complexation in root cells, and increased As translocation and sequestration as free arsenite in fronds (Lombi et al., 2002; Zhao et al., 2009). This particular strategy has been recently ratified by the discovery of a vacuolar arsenite transporter in Pteris vittata (Indriolo et al., 2010).

Pteris vittata was the first hyperaccumulating plant described (Ma et al., 2001), and to date Pityrogramma calomelanos is the only non-Pteris fern to exhibit this ability (Francesconi et al., 2002; Jankong et al., 2007). Additionally to the high capacity to uptake and accumulate As in their fronds, usually in concentrations higher than 1 % of the total dry mass, efficient enzymatic and non-enzymatic antioxidant systems have been reported for these hyperaccumulators (Cao et al., 2004; Singh et al., 2006). However, the mechanisms underlying As hyperaccumulation by ferns are largely unknown (Xie et al., 2009; Bona et al., 2010).

The recent development of global approaches such as genomics, proteomics and metabolomics opens possibilities for a deeper exploration of the adaptive mechanisms that contribute to metal(loid)s detoxification and tolerance (Villiers et al., 2011; Kumar et al., 2015). Arsenic effects on genome-wide expression have been widely examined in rice revealing the differential expression of genes involved in metal transporters, sulfur metabolism, antioxidant responses and protein degradation (Norton et al., 2008; Chakrabarty et al., 2009; Yu et al., 2012). Arsenic-induced alterations in gene expression and metabolite/protein profiles were also reported in maize (Requejo and Tena, 2006); Arabidopsis thaliana (Abercrombie et al., 2008), Brassica juncea (Srivastava et al., 2009), Agrostis tenuis (Duquesnoy et al., 2009) and Crambe abyssinica (Paulose et al., 2010). Regarding hyperaccumulators, Bona et al. (2010) were the first to investigate the proteins responsive to As stresses using proteome analysis in Pteris vittata. Taken together these results have pointed the carbon, nitrogen and sulfur metabolism pathways as the main As-targets, with differences between the species.

Metabolomics is a well-established technique in biological systems and has been largely applied in toxicology (Wu et al., 2013). The use of this technique to understand how plants uptake and metabolize As can contribute for developing mitigation measures to counter the problem of food chain contamination by As (Zhao et al., 2009). The use of As-hyperaccumulating fern species for remediation of As-contaminated soils have been pointed as a viable alternative to reduce As environmental impacts.

This study aimed to investigate the metabolic adjustments involved in As-hyperaccumulation by P. calomelanos. For this purpose, P. calomelanos ferns were exposed to increasing As concentrations in the nutrient solution in order that the pinna metabolite profile, biochemical analysis and analysis of antioxidant responses would bring out important metabolic biomarkers of As exposure in this potential phytoremediator species.

2. Material and methods

2.1. Reagents

Reagents NAD^+, NADH, $NADP^+$, and NADPH and all enzymes except invertase were purchased from Roche. All other reagents and enzymes used for metabolite analysis were obtained from Sigma-Aldrich.

2.2. Experimental design and sampling

Sporophytes at 5-7 fronds stage and approximately 30 cm in height were obtained as described in the Chapter 3, transferred to hydroponic system with half-strength Hoagland's solution, pH 5.5 and continuous aeration. The ferns were grown for one month before start of the treatments. The ferns (n = 6) were exposed to the arsenic concentrations of 0 (control), 1, 10 and 30 mM for 21 days. Arsenic was supplied as sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) and pH was adjusted to 5.5 every two days. Each experimental unit consisted of a 2.2-L pot containing one plant. The ferns were cultivated under Sombrite® 50 % net in a greenhouse with controlled temperature of 25 ± 5 ºC.

At the end of the experiment, fresh samples of the middle pinnae of fully developed young fronds were collected at midday, quickly freezed in liquid nitrogen and stored at -80 ºC. The ferns were harvested, separated into roots, pinnae, and stipe + rachis (referred here as stipes), and oven-dried at 60 ºC for determination of As and P concentrations.

2.3. Determination of the maximum quantum yield of photosystem II (PSII) and photosynthetic pigments concentrations

After 20 days of As exposure, the maximum quantum yield of photosystem II (PSII) was measured at midday using a modulated light Mini-PAM portable fluorometer (FMS2, Hansatech, Norfolk, UK). Previously dark adapted fronds (30 min) were illuminated with a low-intensity modulated light (0.12 μmol m^{-2} s^{-1}) to obtain the initial fluorescence F_0. The Fm was measured with 0.3-s pulses of a saturating light of 4000 μmol m^{-2} s^{-1}. The fluorescence variable (F_v) was calculated from the difference between Fm and F_0, and maximum quantum yield of PSII by the ratio F_v/F_m.

To determine the chlorophyll content, fresh leaf discs (113 mm^2) were incubated in 5 ml of dimethylsulfoxide (DMSO) and placed in a water bath at 65 ºC overnight. The absorbance of the extracts was measured at 480, 649 and 665 nm using a UV–Vis spectrophotometer. The concentrations of chlorophylls a and b and carotenoids were calculated according to the Wellburn equations (Wellburn, 1994) referred to extraction with dimethylsulfoxide, and expressed as mg g^{-1} of fresh weight (FW).

2.4. Determination of arsenic and phosphorus

Dried samples were powdered with a ball mill and sieved through a 200 mesh (74 μm) stainless steel sieve. The sieved plant material was accurately weighed (ca. 50 mg) in closed TFM® digestion vessels (ETHOS 1600, Milestone, Italy) and added of 5.0 ml of 2.8 mol L^{-1} HNO_3 and 2.0 ml of H_2O_2 30 % v/v. Microwave-assisted digestion and As determination in ICP OES spectrometer (iCAP 6500 Duo, Thermo Scientific, Waltham, MA, USA) were performed as described in Chapter 2.

2.5. Biochemical analysis of metabolites

Fresh samples of pinna (25 mg), from the median portion of the frond without the pinna midrib, were extracted with 250 μl of 98 % (v/v) ethanol at 80 ºC and re-extracted in two subsequent steps with 150 μl of 80 % (v/v) ethanol and 250 μl of 50% (v/v) ethanol at 80 ºC (Fernie et al., 2001; Gibon et al., 2004). The combined supernatants were centrifuged for 5 min at 13000 g. The soluble fraction was used for nitrate and amino acid determinations; whereas the insoluble fraction was used for protein and starch analyses. Quantification of metabolites was performed in a

microplate reader (OptiMax Tunable Microplate Reader), with five repetitions for each treatment.

Nitrate concentration was determined as described by Fritz et al. (2006). The reactions were conducted at 25 ºC in the dark. An aliquot of 5 µl of the extract was added to 0.1 M potassium phosphate (pH 7.5), 0.25 mM NADPH, 0.005 units/ reaction nitrate reductase and 83.5 µl of ultrapure water. After 30 min of incubation, 15 µl of 0.25 mM phenazine methosulfate (PMS) was added to the reaction. The samples were mixed and incubated for 20 min. Then, nitrate detection was triggered with the addition of 60 µl of 1% (w/v) sulfanilamide in 3M phosphoric acid, and 60 µl of 0.02 % (w/v) N-(1-naphtyl)-ethylenediamine dihydrochloride (NNEDA). After 10 min, the optical density (OD) was read at 540 nm. Sample blanks with no nitrate reductase were also analyzed to determinate the nitrite concentrations in the samples.

Amino acid accumulation was determined from 25 µl of ethanoic extract using the acid-ninhydrin reagent method described by Gibon et al. (2004). The extract was reacted with 100 µl of 1% (w/v) ninhydrin reagent in 70 % (v/v) ethanol, 50 µl of 1M citrate buffer and 25 µl of 70 % (v/v) ethanol. The mixture was boiled at 95 ºC for 20 min and the OD was measured at 570 nm. Amino acid content was determined from a standard curve of leucine in the range of 0 – 0.2 mM and expressed as $\mu g\ g^{-1}$ FW.

Starch and proteins were extracted as previously described (Hendriks et al., 2003), with addition of 400 µl of 0.1 M NaOH to the pellet and heating at 95 ºC for 30 min with shaking at 13000 g. After ice cooling, 3 µl of the extract was reacted with Bio-Rad Bradford reagent (Bio-Rad Laboratories), according to the manufacturer's instructions for determination of protein content. The OD was measured at 595 nm. The analysis of starch content was carried out by adding 70 µl of 1M acetic acid to the extract. An aliquot of 40 µl of extract was added to 60 µl of the mix reaction containing 1:10 α-amylase: amyloglucosidase and 0.5 M sodium acetate (pH 4.9). Then, a second reaction was performed using 30 mM HEPES/KOH (pH 7.0), 2.5 mM magnesium chloride, 36 mg ml^{-1} NADP, 60 mg ml^{-1} ATP and 1.5 units Glc6P dehydrogenase grade II. The OD was measured at 570 nm.

2.6. GC-MS metabolite profiling

GC-MS metabolite profiling was assessed as described by Lisec et al. (2006) with adaptations. Aliquots of 25 mg of fresh pinna samples were extracted in 1500 µl of

a water: methanol:chloroform (1: 2.5: 1) solution. Sixty microliters of ribitol (0.2 mg ml^{-1}) was used as internal standard. The samples were vortex mixed for 10 sec, incubated for 30 min at 4 ºC with shaking at 900 rpm, in thermomixer, and then centrifuged for 5 min at 13000 g. The supernatant (1000 µl) was added of 750 µl of ultrapure water and the samples were centrifuged for 15 min at 13000 g. Fifty microliters of the polar phase was dried under vaccum for derivatization. The samples were derivatized with methoxyamine hydrochloride (20 mg ml^{-1} pyridine) and N-metil-N (trimethylsilyl) trifluoracetamide (MSTFA). Extraction and derivatization reagent blanks were also analyzed. A mix of fatty acid methylesters (FAMEs; 0.8 mg ml^{-1} in chloroform) was used as retention time standards.

The GC-MS system consisted of a CTC CombiPAL autosampler, an Agilent 6890N gas chromatograph and a LECO Pegasus III TOF-MS running in EI+ mode. Chromatograms and mass spectra were evaluated using the Chroma TOF 1.0 (Leco, http://www.leco.com/) and Target Search software (Cuadros-Inostroza et al., 2009). Metabolite identification was manually supervised using the mass spectral and retention index collection of the Golm Metabolome Database in comparison to database entries of authentic standards (Kopka et al., 2005; Schauer et al., 2005). Peak heights of the mass fragments were normalized based on the dry weight of the sample and the added amount of an internal standard (ribitol). Data were normalized with respect to the mean response calculated for the control plants.

2.7. Antioxidant activity and cellular damage

The activity of the antioxidant enzymes ascorbate peroxidase (APX, EC 1.11.1.11) and peroxidase (POX, EC 1.11.1.7) was determined in fresh pinna samples as described in Chapter 1. These enzymes were selected according to previous results obtained for P. calomelanos exposed to 1 mM As. The results were expressed as mmol mg^{-1} protein min^{-1}.

Total soluble phenol (TSP) content in pinnae was evaluated using the Folin-Ciocalteu's reagent assay, according to the method of Singleton et al. (1999), with adaptations. TSP content was extracted with 2 ml of 80 % methanol, and the extracts were centrifuged at 13000 g at 4 ºC, for 15 min. The reaction mixture was made by mixing 10 µL of the extract with 950 µL of 10 % Folin-Ciocalteu's reagent. After 10 min, 950 µL of 7.5 % Na_2CO_3 was added to the reaction mixture. The extract was

maintained at 45 ºC for 45 min and centrifuged at 13000 g at 4 ºC for 3 min. The absorbance was measured at 725 nm using an Elisa reader (Multiskan GO, Thermo Scientific, Waltham, EUA). The results were expressed as grams of tannic acid equivalents per kilogram of fresh weight tissue (g TAE kg^{-1} FW).

Accumulation of thiobarbituric acid-reactive substances (TBARs) in pinnae was used to evaluate cellular damages induced by As. TBARs concentration was determined as described by Heath and Packer (1968). Freeze dried samples were mixed with 2 ml of TBA reagent (20 % w/v trichloroacetic acid + 0.5 % w/v thiobarbituric acid), heated to 95 °C for 30 min, cooled for 15 min, and centrifuged at 10000 g for 15 min. The amount of TBARs was measured by subtracting the non-specific absorbance at 600 nm from its specific absorbance at 532 nm.

2.8. Statistical analysis

The experiment was performed in a completely randomized design. Data from metabolite profile were compared by the Student's t-test at 5 % significance level using Microsoft Excel. All other data were compared by the Tukey test at 5 % significance level and the analyses were performed using the statistics software package R (R Development Core Team, 2006). Graphs were designed with SigmaPlot 11.0.

3. Results

After three weeks of arsenic exposure, P. calomelanos plants accumulated the highest As concentrations in fronds and roots, ranging from 2729 to 6454 in pinnae, from 803 to 5658 in stipes, and from 931 to 5488 mg kg^{-1} dry weight (DW) in roots (Fig. 1). Arsenic concentrations in fern parts increased with the increase of As in the solution, except for pinnae at the dose of 10 mM, which did not differ from the treatments with 1 and 30 mM As. Arsenic distribution in fern parts was affected by increasing As doses. Pinnae showed the highest As concentration among fern parts at 1 mM As, whereas at the doses of 10 and 30 mM, As distributed equally among fern parts.

Considering the well-known chemical resemblance of arsenate and phosphate, the P concentration was also determined in fern parts. Arsenic did not affect the P concentration in pinnae, stipes and roots. In control plants, the highest P concentration was observed in pinnae, and P content was higher in roots than stipes. Under As

exposure, ferns showed the same trend of P distribution, however, significant differences were observed only comparing stipes with pinnae and roots at 1 and 30 mM As.

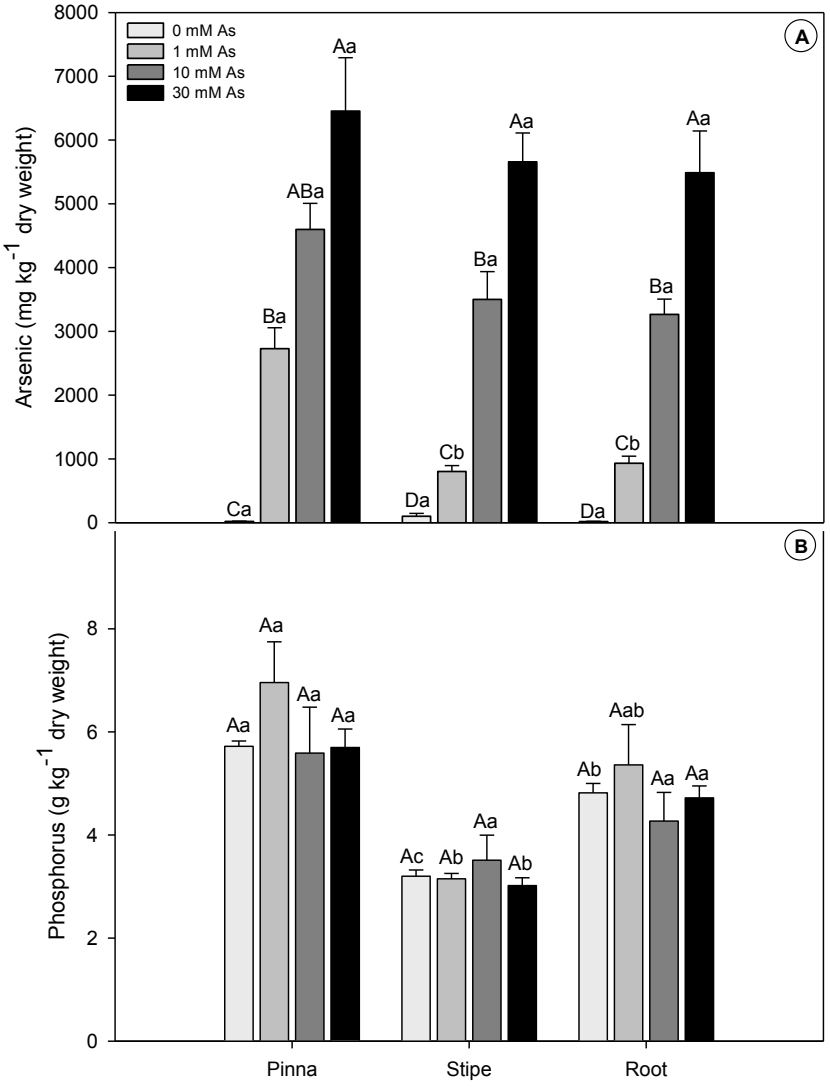

Fig.1. Arsenic (A) and phosphorus (P) concentrations in the pinna of Pityrogramma calomelanos plants grown in Hoagland's nutrient solution cointaining 0 (control), 1, 10 and 30 mM As for three weeks. Values presented are means ± standard error (SE). Means (n = 5) followed by same letters were not significantly different. Capital letters refer to comparisons among the treatments and small letters among the fern parts, for each treatment (Tukey's test; $p < 0.05$).

Symptoms of As stress, such as necrosis in the margin and tip of old fronds, were observed in ferns exposed to 10 and 30 mM (as reported in Chapter 3). However,

the fully-expanded young fronds showed no symptoms of As toxicity and were chosen for maximum quantum yield of PSII (F_v/F_m) measurement and metabolite analysis.

Despite of the higher As accumulation in P. calomelanos fronds, the As treatment did not affected F_v/F_m, and values ranged from 0.827 to 0.838 (Supplemental Table SI). Additionally, no differences were observed on photosynthetic pigment concentrations (chl a, chl b and carotenoids), as well on total chl concentration and chl a/chl b ratio among the treatments (Fig. 2). A significant As effect on nitrogen metabolism was observed. Arsenic increased the total concentration of amino acids and proteins, especially at the doses of 10 and 30 mM As, with a non-significant increase of nitrate concentration. Starch concentration in pinna samples was similar among the treatments (Fig. 2).

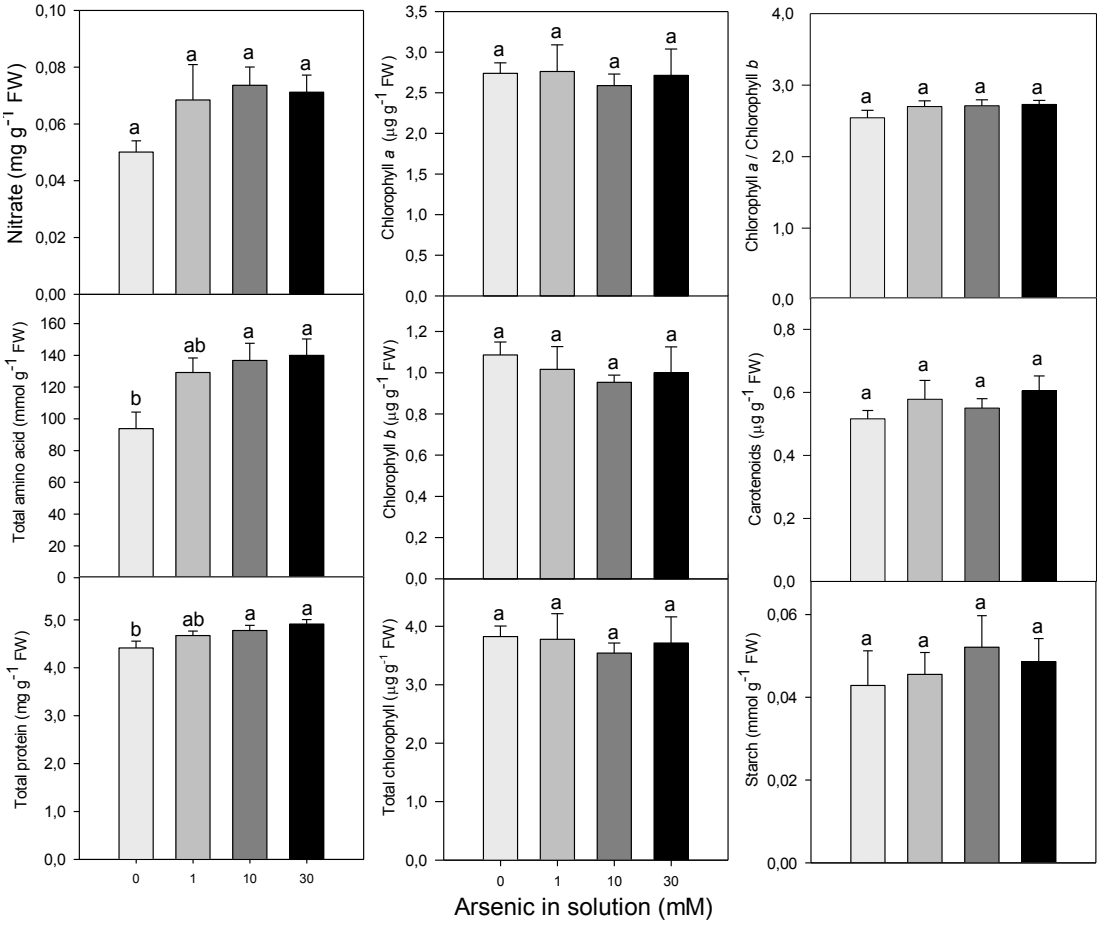

Fig. 2. Arsenic (As) effects on nitrogen metabolism (nitrate, amino acid, protein), photosynthetic pigments, and starch concentration of Pityrogramma calomelanos plants grown in Hoagland's nutrient solution cointaining 0 (control), 1, 10 and 30 mM As for three weeks. Values presented are means ± SE. Means followed by the same letters did not differ by the Tukey's test ($p < 0.05$).

Metabolite profile of pinna samples resulted in 94 successfully annotated compounds, including 25 amino acids, 15 organic acids, 16 sugars (including triose phosphates), 6 fatty acids, 4 sugar alcohols, 3 polyamines and 25 others metabolites of primary and secondary metabolisms. To provide an easy overview of the effects of As accumulation in frond tissues, the major metabolic changes were synthesized in a schematic figure wherein they are mapped onto metabolic pathways (Fig. 3). The full data set is presented in the Supplemental Table SII.

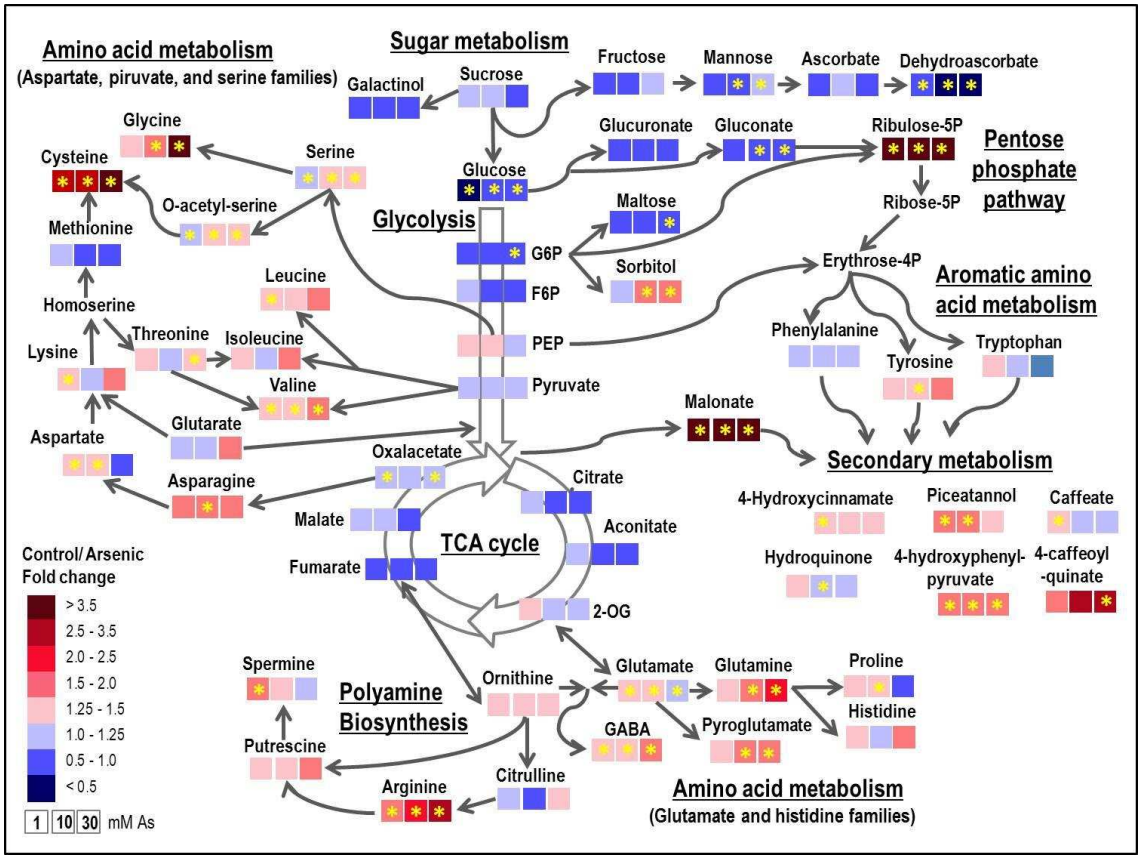

Fig. 3. Schematic summary of the major metabolic alterations in Pityrogramma calomelanos pinnae in response to arsenic (As) treatments (1, 10 and 30 mM). Ferns were grown in Hoagland's nutrient solution (pH 5.5) and exposed to As for three weeks. Data are normalized with respect to the mean response calculated for the control plants; values presented are means of six biological replicates. The asterisks indicate means considered significantly different from the control ($p < 0.05$) by the Student's t tests. Legend: 2-OG, 2-oxoglutarate; F6G, fructose-6-phosphate; G6P, glucose-6-phosphate; GABA, 4-Aminobutyric acid. PEP, phosphoenolpyruvate; TCA cycle, tricarboxylic acid cycle.

Arsenic treatment promoted several disturbances in the nitrogen and carbon metabolism of P. calomelanos. In the C metabolism, the reduction of 50 % of the glucose pool was one of the striking responses to As exposure. With the exception of lactose that increased in plants of the 10 mM As treatment, the level of sugars and triose phosphates were always lower or equal among As-treated and control plants, with significant reduction of glucose-6-phosphate and maltose (at 30 mM As), and mannose (at 10 and 30 mM As). Similar to the sugar pools, the levels of organic acids associated with the glycolytic pathway and the tricarboxylic acid (TCA) cycle were, in general, similar to those observed for control plants, with the exception of the oxaloacetate that reduced in plants exposed to 1 mM As. Also, at 10 and 30 mM As, it was observed 30 % reduction of the gluconate pool.

Considerable differences in the levels of a wide range of amino acids were observed in As-treated plants. Arginine, cysteine, glutamate, O-acetyl-serine, serine and valine were always higher in plants exposed to As than those of the control; whereas asparagine, aspartate, glutamine, glycine, leucine, lysine, proline, pyroglutamate, threonine, and tyrosine increased in at least one of the As doses. Larger differences were observed for glycine (1.6 – 4.2 fold higher), cysteine (2.6 – 3.6), arginine (2.0 – 2.8) and O-acetyl-serine (1.3 – 2.8). Among the amino acids, methionine and tryptophan showed a clear trend of reduction with increasing As concentrations in the solution. GABA (4-aminobutyric acid), an non-peptide amino acid involved in glutamate metabolism, was 1.3 fold higher in the 1 and 10 mM As treatments and 1.8 higher in plants exposed to 30 mM As.

The highest As-induced increases in metabolite levels were observed for ribulose-5-phosphate (5.4 – 5.7 fold higher) and malonate (4.8 – 5.5). Additionally, significant increases (1.2 – 2.6 fold higher) were also observed in the level of phenols and phenol-related compounds such as 4-hydroxycinnamate acid, 4-hydroxyphenyl-pyruvate, caffeate, hydroquinone, piceatannol and 4-caffeoyl quinate. Among the less representative chemical classes, polyamines and sugar alcohols, spermine and sorbitol were the most As-responsive metabolites. The spermine was 1.6 fold higher in the 1 mM As treatment, whereas sorbitol was about 1.5 fold higher in 10 and 30 mM As-treated plants. The TBARs analysis and Folin-Ciocalteu's reagent assay showed that As-treated ferns presented higher concentrations of thiobarbituric acid-reactive substances (TBARs) at 30 mM As, and higher concentrations of total soluble phenols (TSP) at 10 and 30 mM As (Fig. 4).

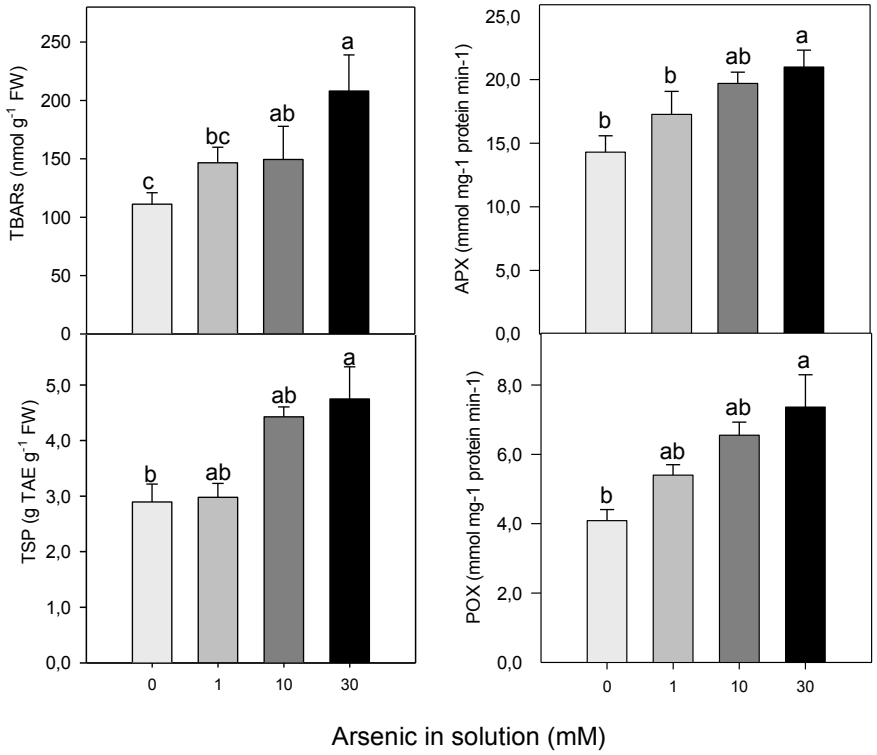

Fig. 4. Arsenic (As) effects on thiobarbituric acid-reactive substances (TBARs), total soluble phenols (TSP), activity of ascorbate peroxidase (APX) and activity of peroxidase (POX) in Pityrogramma calomelanos plants grown in Hoagland's nutrient solution cointaining 0 (control), 1, 10 and 30 mM As for three weeks. Means ± SE followed by the same letters did not differ by the Tukey's test ($p < 0.05$).

The six fatty acids reported showed no difference among control and As-treated plants. Of particular interest, dehydroascorbic acid showed a gradual reduction in response to the increase of As concentration in the solution, ranging from 43 to 64 %. Ascorbate was not altered by As treatment, however the ascorbate peroxidase (APX) showed increased activity in response to As (Fig. 4). Similarly, the activity of POX was almost two fold higher in plants exposed to 30 mM As than those of the control.

4. Discussion

Arsenic hyperacccumulation in Pityrogramma calomelanos was first reported in anthropic As-polluted soils, where ferns accumulated up to 8350 mg As kg^{-1} dry weight in their fronds (Visoottiviseth et al., 2002). In the present study, high As concentrations were found in ferns grown in hydroponic solution supplemented with arsenate for three

weeks, ranging from 3530-12100 mg kg^{-1} DW in fronds (pinnae plus stipes). This finding shows that this species has an inherent high capacity to As uptake and detoxification. The absence of As-negative effects on F_v/F_m and photosynthetic pigments suggests that As did not damaged the photosystems. The capacity of P. calomelanos to maintain P status in roots and fronds, despite the expected competition of arsenate and phosphate for P transporters (Meharg and Macnair, 1990), contributed to maintenance of energy flows in cells, especially in terms of ATP synthesis. However, in a previous study, it was observed a decrease of pinnae dry weight in plants exposed to 30 mM As (see Chapter 3).

Pinnae have been reported as the main site of As accumulation and sequestration in hyperaccumulators (Lombi et al., 2002). This study aimed to characterize the As-induced adaptive metabolic responses in pinnae of P. calomelanos that allow this species to survive under very high As concentrations. The results showed a marked trend in the reduction of glycolysis and TCA cycle metabolites-related and increase in secondary compound production, indicating that several metabolic rearrangements occurred to allow a balance between carbon metabolism and oxidative stress. Chakrabarty et al. (2009) investigated the effect of As exposure on genome-wide expression in rice seedlings (Oryza sativa) and found that As^{5+} affects mainly the cell wall, and the primary and secondary metabolisms. According to these authors, the genes coding for metallothioneins and sulfate- and GSH-conjugate-transporters are more responsive to As^{5+} than to As^{3+}.

Bona et al. (2010) reported that As predominantly affects proteins belonging to the photosynthesis and carbon fixation groups, with glycolytic enzymes playing a central role in As metabolism in Pteris vittata, a well-known As-hyperaccumulator. In P. calomelanos, the decreased glucose and glucose-6-phosphate pools, as well the increase in the oxaloacetate level, were associated with the up-regulation of a range of amino acids. The increase in total amino acid and protein contents showed that nitrogen assimilation was overall up regulated by As. In plants, nitrogen assimilation involves a sequential reduction of nitrate by nitrate reductase and nitrite reductase, and the incorporation of ammonium into glutamine and glutamate primarily by the glutamine synthase-glutamate synthase cycle (GS/GOGAT cycle) (Miflin and Habash, 2002; Masclaux-Daubresse et al., 2006).The increase in glutamine, at 10 and 30 mM As, and glutamate, at all As doses, clearly demonstrate that GS/GOGAT cycle is As-responsive. Singh et al. (2006) found a reduction of nitrate and protein concentrations in fronds of

Pteris vittata under 0.3 mM As exposure. The authors suggested the association of decreased nitrate with a possible enhancement of organic acids related to As-complexation. More evidences are necessary to clarify the differences in As metabolic responses between these two hyperaccumulators.

Cysteine, glutamate and glycine are directly related to oxidative defense, since these amino acids are the building blocks of glutathione (GSH) (Noctor and Foyer, 1998). The simultaneous increase in these three amino acids indicates the importance of GSH and its derivate compounds to As detoxification. Plants detoxify As by reducing arsenate to arsenite, which is subsequently detoxified via thiol reactive peptides, such as γ-glutamylcysteine (γ-EC), glutathione (GSH) and phytochelatins (PCs), which have been related to chelation with As^{3+} in non-hyperaccumulators (Raab et al., 2005; Dwivedi et al., 2010). According to Dave et al. (2012b), thiol metabolism is the primary detoxification strategy by which rice plants tolerate As stress. In hyperaccumulators, sulfur-rich compounds were not proved to act in As chelation (Zhang et al., 2004, Pickering et al., 2006). However, the GSH-ascorbate cycle seems to play a significant role in antioxidant defense in these species. Increase of non-protein thiols was previously reported in P. calomelanos exposed to 1 mM As for three weeks (see Chapter 1). Ascorbate and GSH are key cellular redox buffers that are used for both detoxification of ROS and transmission of redox signals (Fotopoulos et al., 2010). The enhanced APX activity and reduced ascorbate/ oxidized ascorbate (AsA/DHA) ratio also indicate the protective role of AsA in plants under higher As doses.

Higher O-acetylserine and serine levels were related to S metabolism, once the former represents the amino acid skeleton used by cysteine synthase to form cysteine, and it is synthesized through serine (Bona et al., 2010). The up-regulation of amino acids can be also associated with photorespiration. Photorespiration is considered an energy sink process that prevents the photoinhibition and provides a strong carbon drain through generation of metabolites, such as glycine, which is involved in stress protection (Wingler et al., 2000; Bauwe et al., 2010). Plants exposed to 30 mM As showed a three-fold increase in the glycine/serine ratio, suggesting an increase in the replacement of CO_2 by O_2 in the CO_2 fixation reaction catalysed by ribulose-1,5-bisphosphate (Rubisco) (Bauwe et al., 2010). Related to CO_2 fixation, Rubisco subunits were the most As-responsive proteins in P. vittata under As stress (Bona et al., 2010). Increase in GABA, a non-protein amino acid synthesized from glutamate, has been also

associated with carbon-nitrogen balance and ROS scavenging (Song et al., 2010; Liu et al., 2011).

There was a significant increase of proline and asparagine in the 10 mM As treatment. These amino acids, especially proline, have been considered essential for stress tolerance because of their role as osmolytes, free radical scavengers, electron sinks, stabilizers of macromolecules and cell wall components (Matysik et al., 2002; Szabados and Savouré, 2010). Contrasting with P. calomelanos, decrease in essential amino acid concentrations was observed in high As accumulating rice genotypes (Dave et al., 2013a). Additionally, sorbitol (sugar alcohol) and spermine (polyamine) were other osmoprotectants up-regulated by As in P. calomelanos. Polyamines are involved in membrane protection, reduction of oxidative stress and maintenance of the plant water status (Groppa and Benavides, 2008; Quinet et al., 2010). Arsenate was reported to increase the proportion of spermidine and spermine and decrease the proportion of putrescine in the polyamine pool of Atriplex atacamensis (Vromman et al., 2011).

The aromatic amino acids phenylalanine, tyrosine, and tryptophan are not only essential components of protein synthesis, but also serve as precursors for a wide range of secondary metabolites, including hydroxycinnamic acids, alkaloids, lignins and flavonoids (Tzin and Galili, 2010). In this study, the increase in tyrosine was associated with the enhanced level of 4-hydroxyphenyl-pyruvate, which is an intermediary of plastoquinone and tocopherol synthesis, and with the increase in 4-hydroxycinnamate (Maeda and Dudareva, 2012). The increase in caffeate and 4-caffeoyl-quinate is probably a consequence of phenylalanine pool consumption toward phenol synthesis. Phenolic compounds play a beneficial role in protecting plants from the harmful effects of reactive oxygen species generated from exposure to toxic metal(loid)s (Michalak, 2006). Increase of phenols in leaf cells of P. calomelanos plants exposed to As was proved by histochemical test in a previous study (Chapter 3). Polyphenol piceatannol, which was shown highly As-responsive in P. calomelanos, is a derivative of ferulic acid and has been proved to interact with polar headgroups of the lipid bilayer contributing to membrane integrity (Wesołowska et al., 2009).

In general, the metabolism of amino acids plays an important role in plant stress resistance through osmotic adjustment, accumulation of compatible osmolytes, intracellular pH regulation and detoxification of reactive oxygen species (ROS) (Sharma and Dietz, 2006). The five-fold increase of ribulose-5P pool in P. calomelanos suggests the importance of the oxidative pentose phosphate pathway (OPPP) to supply the

energetic expenses of amino acids biosynthesis, once this pathway is a major source of reducing power (NADPH) (Kruger and von Schaewen, 2003). Enzymes of glycolysis and OPPP have been reported to be up-regulated under heavy metal exposure in order to sustain the cells in terms of energy demand, reducing molecules, and carbon skeletons required for the synthesis of amino acids and metabolites involved in metal chelation (Sarry et al., 2006; Kieffer et al., 2009).

Furthermore, the high increase in the malonate pool suggests that the acetate-malonate pathway may play a significant role in secondary product biosynthesis, as reported by Shetty et al. (2014). Malonate is also a major product of lipid peroxidation and the increased TBAR content in the 30 mM As treatment indicates the occurrence of cellular damages in pinnae of P. calomelanos under very high As exposure. In a previous study, higher activities of APX and POX and the increase in non-protein thiols were pointed as mechanisms to prevent lipid peroxidation in pinnae of P. calomelanos exposed to 1 mM As (see Chapter 1). Higher As exposure can extrapolate the tolerance limit of this species, promoting morphophisiological damages and growth restriction (see Chapter 3). Similarly, it has been demonstrate that Pteris vittata has a higher capacity to increase enzymatic and non-enzymatic antioxidants that limit As toxic effects when its internal concentration is around 700-3000 mg kg^{-1} DW (Cao et al., 2004; Singh et al., 2006), while higher As accumulation can induce decrease in biomass (Cao et al., 2004; Singh et al., 2006).

Taken together, these results indicate that P. calomelanos has a great scavenging ability due to its capacity to up-regulate biosynthesis of amino acids and, consequently, antioxidant metabolites, without significant damages to central carbon metabolism. At extremely high As concentrations, its protective mechanisms may become inefficient to block the increasing ROS production leading to oxidative damages.

5. Acknowledgments

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Supplemental Table SI. Arsenic (As) effects on maximum quantum yield of PSII (F_v/F_m) in Pityrogramma calomelanos ferns grown in Hoagland's nutrient solution (pH 5.5) amended with As (1, 10 and 30 mM) for three weeks.

Treatment	**F_v/F_m**
Control	0.838 ± 0.003 a[1]
1 mM As	0.835 ± 0.005 a
10 mM As	0.836 ± 0.003 a
30 mM As	0.827 ± 0.008 a

[1] Values presented are means of four biological replicates ± SE. Means followed by the same letters did not differ by Tukey's test ($p < 0.05$).

Supplemental Table SII. Relative metabolite content in pinnae of arsenic(As)-treated (1, 10 and 30 mM) and control (without As) plants of Pityrogramma calomelanos at 5-7 frond age. Ferns were grown in Hoagland's nutrient solution (pH 5.5.), and exposed to As for three weeks. Fresh pinna samples were collected at midday in stored at -80 ºC. Metabolites were determined as described in the "Materials and Methods". Data are normalized with respect to the mean response calculated for the control plants. Values presented are the mean ± SER of six biological replicates; values set in bold and underline type were judged to be significantly different from the control ($P < 0.05$), following the performance of Students t-tests.

Amino acids	**Control**	**1 mM As**	**10 mM As**	**30 mM As**
Alanine	1.00 ± 0.34	1.17 ± 0.29	1.10 ± 0.17	1.08 ± 0.22
Arginine	1.00 ± 0.10	**1.97 ± 0.16**	**2.39 ± 0.24**	**2.76 ± 0.30**
Asparagine	1.00 ± 0.14	1.54 ± 0.25	**1.96 ± 0.28**	1.57 ± 0.32
Aspartate	1.00 ± 0.07	**1.37 ± 0.12**	**1.29 ± 0.07**	0.98 ± 0.12
beta-Alanine	1.00 ± 0.08	1.32 ± 0.14	1.27 ± 0.13	1.09 ± 0.05
Cysteine	1.00 ± 0.13	**2.60 ± 0.40**	**2.99 ± 0.53**	**3.56 ± 0.41**
GABA	1.00 ± 0.02	**1.32 ± 0.10**	**1.27 ± 0.10**	**1.84 ± 0.36**
Glutamate	1.00 ± 0.07	**1.32 ± 0.05**	**1.28 ± 0.06**	**1.21 ± 0.05**
Glutamine	1.00 ± 0.20	1.37 ± 0.36	**1.87 ± 0.24**	**2.29 ± 0.50**
Glycine	1.00 ± 0.10	1.30 ± 0.15	**1.60 ± 0.22**	**4.20 ± 0.84**
Histidine	1.00 ± 0.21	1.32 ± 0.21	1.10 ± 0.16	1.55 ± 0.22
Hydroxyproline	1.00 ± 0.15	1.00 ± 0.09	1.03 ± 0.11	1.11 ± 0.13
Isoleucine	1.00 ± 0.08	1.43 ± 0.17	1.14 ± 0.16	1.87 ± 0.53
Leucine	1.00 ± 0.07	**1.44 ± 0.11**	1.25 ± 0.14	1.97 ± 0.49
Lysine	1.00 ± 0.07	**1.41 ± 0.12**	1.12 ± 0.11	1.51 ± 0.36
Methionine	1.00 ± 0.12	1.13 ± 0.10	0.90 ± 0.07	0.86 ± 0.14
O-acetyl-serine	1.00 ± 0.05	**1.40 ± 0.11**	**1.45 ± 0.08**	**2.98 ± 0.35**
Phenylalanine	1.00 ± 0.07	0.96 ± 0.05	0.94 ± 0.06	1.00 ± 0.07
Proline	1.00 ± 0.15	1.37 ± 0.19	**1.58 ± 0.11**	0.96 ± 0.14
Pyroglutamate	1.03 ± 0.14	1.29 ± 0.27	**1.64 ± 0.17**	**1.94 ± 0.32**
Serine	1.00 ± 0.07	**1.24 ± 0.06**	**1.38 ± 0.03**	**1.49 ± 0.15**
Threonine	1.00 ± 0.07	1.26 ± 0.11	1.24 ± 0.09	**1.39 ± 0.12**
Tryptophan	1.00 ± 0.14	1.27 ± 0.19	1.12 ± 0.13	2.21 ± 0.49
Tyrosine	1.00 ± 0.12	1.32 ± 0.09	**1.35 ± 0.10**	1.79 ± 0.39
Valine	1.00 ± 0.06	**1.39 ± 0.12**	**1.32 ± 0.09**	**1.66 ± 0.28**
Organic acids				
2-oxoglutarate	1.00 ± 0.15	1.39 ± 0.25	1.21 ± 0.08	1.23 ± 0.16
Ascorbate	1.00 ± 0.12	0.80 ± 0.28	1.11 ± 0.12	0.99 ± 0.15
Citramalate	1.00 ± 0.17	1.04 ± 0.15	0.90 ± 0.06	0.81 ± 0.09
Citrate	1.00 ± 0.17	1.11 ± 0.16	0.85 ± 0.08	0.78 ± 0.13
Dehydroascorbate	1.00 ± 0.09	**0.57 ± 0.09**	**0.44 ± 0.07**	**0.34 ± 0.06**
Fumarate	1.00 ± 0.13	0.94 ± 0.16	0.68 ± 0.06	0.58 ± 0.10
Glutarate	1.00 ± 0.06	1.02 ± 0.09	1.03 ± 0.05	1.84 ± 0.04

Glycerate	1.00 ± 0.13	0.90 ± 0.19	0.87 ± 0.16	0.77 ± 0.15
Malate	1.01 ± 0.09	1.22 ± 0.09	1.05 ± 0.08	0.98 ± 0.15
Maleate	1.00 ± 0.26	0.74 ± 0.07	0.83 ± 0.22	0.68 ± 0.06
Malonate	1.00 ± 0.28	**4.84 ± 1.00**	**5.52 ± 0.83**	**5.23 ± 1.14**
Oxaloacetate	1.01 ± 0.03	**1.18 ± 0.05**	1.03 ± 0.06	**1.13 ± 0.03**
Phosphoenolpyruvate	1.02 ± 0.27	1.45 ± 0.22	1.33 ± 0.21	1.03 ± 0.35
Pyruvate	1.00 ± 0.30	1.14 ± 0.27	1.07 ± 0.16	1.05 ± 0.21
Succinate	1.00 ± 0.17	1.04 ± 0.15	0.90 ± 0.06	0.81 ± 0.09
Sugars and trioses phosphate				
Fucose	1.00 ± 0.09	0.93 ± 0.07	0.85 ± 0.05	0.84 ± 0.09
Fructose	1.00 ± 0.11	0.75 ± 0.09	0.69 ± 0.15	1.01 ± 0.09
Fructose-6-phosphate	1.00 ± 0.17	1.16 ± 0.09	0.93 ± 0.10	0.81 ± 0.09
Galactose	1.00 ± 0.21	1.28 ± 0.07	1.32 ± 0.06	1.33 ± 0.06
Glyceraldehyde-3-phosphate	1.01 ± 0.38	1.43 ± 0.49	1.15 ± 0.39	1.23 ± 0.52
Glucose	1.00 ± 0.06	**0.44 ± 0.06**	**0.51 ± 0.20**	**0.52 ± 0.07**
Glucose-6-phosphate	1.02 ± 0.63	0.90 ± 0.79	0.87 ± 0.62	**0.77 ± 0.89**
Isomaltose	1.00 ± 0.21	0.91 ± 0.10	0.60 ± 0.13	0.88 ± 0.11
Lactose	1.00 ± 0.07	1.28 ± 0.12	**1.27 ± 0.08**	1.04 ± 0.23
Lactulose	1.00 ± 0.13	1.50 ± 0.18	1.32 ± 0.11	1.30 ± 0.14
Maltose	1.00 ± 0.08	0.90 ± 0.11	0.85 ± 0.11	**0.64 ± 0.05**
Mannose	1.00 ± 0.07	0.81 ± 0.15	**0.59 ± 0.04**	**0.66 ± 0.11**
Melibiose	1.00 ± 0.15	1.33 ± 0.20	1.27 ± 0.15	0.90 ± 0.18
Sucrose	1.00 ± 0.06	1.06 ± 0.05	1.00 ± 0.04	0.98 ± 0.03
Trehalose	1.00 ± 0.22	1.08 ± 0.05	1.06 ± 0.24	1.15 ± 0.12
Xylose	1.00 ± 0.41	1.45 ± 0.78	1.04 ± 0.46	0.70 ± 0.19
Sugar alcohols				
Galactinol	1.00 ± 0.08	0.86 ± 0.23	0.63 ± 0.21	0.94 ± 0.05
Mannitol	1.00 ± 0.03	1.09 ± 0.03	1.01 ± 0.04	0.99 ± 0.04
myo-Inositol	1.00 ± 0.08	0.95 ± 0.15	0.84 ± 0.06	1.01 ± 0.11
Sorbitol	1.00 ± 0.07	1.01 ± 0.12	**1.51 ± 0.16**	**1.50 ± 0.17**
Fatty acids				
2-ethyl-hexanoic acid	1.00 ± 0.22	1.08 ± 0.05	1.06 ± 0.24	1.15 ± 0.12
Docosanoic acid	1.00 ± 0.13	1.23 ± 0.03	0.98 ± 0.08	0.86 ± 0.05
Hexacosanoic acid	1.00 ± 0.09	0.91 ± 0.09	0.92 ± 0.09	0.75 ± 0.21
Nonoic acid	1.00 ± 0.02	1.09 ± 0.03	1.00 ± 0.04	0.98 ± 0.04
Octadecanoic acid	1.00 ± 0.09	1.00 ± 0.05	1.00 ± 0.07	0.97 ± 0.03
Palmitic acid	1.00 ± 0.09	1.00 ± 0.05	1.00 ± 0.07	0.97 ± 0.03

Polyamines				
Citrulline	1.00 ± 0.32	1.03 ± 0.46	0.90 ± 0.30	1.44 ± 0.18
Spermidine	1.00 ± 0.13	**1.58 ± 0.15**	1.35 ± 0.15	1.24 ± 0.30
Putrescine	1.00 ± 0.15	1.27 ± 0.08	1.45 ± 0.52	1.58 ± 0.64
Others metabolites				
2-Amino-phenol	1.00 ± 0.09	0.96 ± 0.22	0.89 ± 0.11	0.82 ± 0.22
2,7-Anhydro-beta-sedoheptulose	1.00 ± 0.08	**0.52 ± 0.06**	0.58 ± 0.21	**0.53 ± 0.07**
4-Caffeoyl-quinate	1.00 ± 0.16	1.52 ± 0.45	2.63 ± 1.70	**2.63 ± 0.48**
1,3-Dihydroxyaceton	1.00 ± 0.09	0.91 ± 0.09	0.92 ± 0.09	0.75 ± 0.21
4-Hydroxy-benzoate	1.00 ± 0.05	1.06 ± 0.15	0.98 ± 0.05	0.97 ± 0.06
4-Hydroxycinnamate	1.00 ± 0.10	**1.45 ± 0.10**	1.25 ± 0.09	1.36 ± 0.15
3-Hydroxy-flavone	1.00 ± 0.10	0.93 ± 0.07	1.11 ± 0.09	0.93 ± 0.10
4-Hydroxyphenyl-pyruvate	1.00 ± 0.11	**1.99 ± 0.21**	**1.89 ± 0.33**	**1.98 ± 0.19**
2-Methyl-1,4-phthoquinone	1.00 ± 0.04	1.01 ± 0.14	**0.63 ± 0.13**	**0.27 ± 0.13**
1-Pyrroline-2-carboxylate	1.00 ± 0.03	**1.12 ± 0.03**	1.08 ± 0.06	0.98 ± 0.04
Benzoate	1.00 ± 0.06	1.05 ± 0.03	1.00 ± 0.07	0.96 ± 0.06
Caffeate	1.00 ± 0.07	**1.23 ± 0.15**	1.11 ± 0.07	1.19 ± 0.06
Ferulate	1.00 ± 0.14	1.26 ± 0.10	1.07 ± 0.12	1.19 ± 0.13
Galacturonate	1.00 ± 0.14	1.01 ± 0.17	0.74 ± 0.06	0.82 ± 0.17
Gluconate	1.00 ± 0.07	0.83 ± 0.11	**0.64 ± 0.04**	**0.67 ± 0.13**
Glucuronate	1.00 ± 0.18	0.77 ± 0.16	0.56 ± 0.08	0.61 ± 0.05
Glycerol-2-phosphate	1.00 ± 0.05	1.16 ± 0.08	1.01 ± 0.05	0.92 ± 0.06
Glycerol-3-phosphate	1.00 ± 0.14	0.87 ± 0.23	0.96 ± 0.06	0.86 ± 0.20
Hydroquinone	1.00 ± 0.07	**1.26 ± 0.08**	1.12 ± 0.07	1.15 ± 0.10
Nicotinic acid	1.00 ± 0.22	1.26 ± 0.10	0.99 ± 0.22	1.23 ± 0.22
Phosphorate	1.00 ± 0.07	1.01 ± 0.09	0.94 ± 0.05	0.90 ± 0.02
Piceatannol	1.00 ± 0.21	**1.65 ± 0.12**	**1.54 ± 0.07**	1.41 ± 0.10
Ribonate	1.00 ± 0.23	0.92 ± 0.07	0.78 ± 0.05	0.57 ± 0.13
Ribulose-5-phosphate	1.00 ± 0.14	**5.73 ± 0.60**	**5.69 ± 1.21**	**5.35 ± 0.76**
Uracil	1.00 ± 0.32	0.81 ± 0.07	0.81 ± 0.10	0.78 ± 0.09

FINAL CONSIDERATIONS

Pityrogramma calomelanos showed a high potential to arsenic (As) accumulation reaching As concentrations of 3000-12000 mg kg^{-1} DW in fronds after relative short exposure times (from two to three weeks). Pinnae, as expected, was the main site of As accumulation, however, high As concentrations (up to 6500 mg Kg^{-1} DW) were observed in stipes and roots of ferns exposed to higher As doses (10-30 mM As). It shows that under acute exposure (extremely high As dose in short time-exposure) these fern parts accumulate, temporary or not, the excess of As.

Arsenic tolerance in P. calomelanos was related to its higher capacity to As translocation and sequestration in pinnae, mainly as free arsenite; adjustments in mineral uptake and translocation; and its efficient antioxidant system compound by enzymatic (SOD, CAT, APX and POX) and non-enzymatic (non-protein thiols, phenols, amino acids, sugar alcohols and polyamines) components. The increased antioxidant capacity, under As exposure, was explained by the up-regulation of amino acid biosynthesis in pinnae that in turn increased the protein and secondary metabolites production.

At extremely high As concentrations (e.g. 30 mM As) these protective mechanisms may become inefficient culminating in the increase of oxygen reactive species. The increase of free radicals promote cellular and tissues damages such as lipid peroxidation, morphological injuries in pinnae that culminate in marginal and apical necrosis, and decrease of maximum chlorophyll fluorescence and biomass. Slight morphological changes were observed in roots, highlighting the protective role of the border-like cells, especially to the root tip, and probably the role of cortical root cells in As detoxification. However, the increase of lipid peroxidation and decrease of fresh weight observed to this organ under lower As dose (1 mM As) suggest that roots of P. calomelanos can be arsenate-sensitive, fact that reinforce the importance of As reduction and root-to-shoot translocation.

Altogether, these findings suggest that P. calomelanos can be successfully used in the remediation of moderate As-contaminated sites. Adult plants with higher biomass are preferable because their higher potential to extract As from the medium. Experiments with low-dose and long-time exposures (chronic exposure) are needed to clarify the dose-response-mediated mechanisms of As-tolerance in P. calomelanos, in

order to improve its performance in field. The harvesting of plants as soon as the appearing of visual symptoms (as necrosis and shriveling of young fronds) can also upgrade the phytoremediation efficiency.

Additionally, the micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF) can be used for As determination and localization in biological samples, by a fast and non-destructive way, configuring a valuable tool for phytoremediation studies and environmental monitoring programs.