ARSENIC HYPERACCUMULATION BY *PTERIS VITTATA* L.- ARSENIC TRANSFORMATION, UPTAKE AND ENVIRONMENTAL IMPACT

By

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To my daughter, Lisa Annie Johnson

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The arsenic (As) hyperaccumulating fern, *Pteris vittata* (Chinese brake fern), is capable of taking up arsenate (AsV) and arsenite (AsIII). The physiological aspects pertaining to the transformation of As in the media of the fern, its uptake mechanisms, speciation in the biomass and the impact of the hyperaccumulated arsenic on insects were studied.

The role of the fern and microbes in AsIII oxidation in the growth media and the location of AsIII oxidation and AsV reduction in *P. vittata* biomass were studied. Arsenic speciation was performed in the growth media, roots, rhizomes, rachis, pinnae, fronds, and sap of *P. vittata*. Arsenite was rapidly oxidized in the growth media by microbes and was then further oxidized in the roots of *P. vittata*. Arsenate reduction mostly occurred in the rhizomes and pinnae of *P. vittata*. Arsenite translocation from the roots to the fronds was more rapid than arsenate.

The mechanism of AsIII uptake was hypothesized to be via aquaporin transporters and was studied using competitors of AsIII uptake, glycerol and antimonite (SbIII), and also an inhibitor of aquaporin transporter, silver nitrate ($AgNO_3$). The presence of glycerol or SbIII had no impact on AsIII or AsV uptake by *P. vittata*. However, the

presence of 0.01 mM $AgNO_3$ reduced the AsIII concentrations in the fronds and roots respectively, indicating that AsIII uptake might be via an aquaporin transporter different from the glycerol and SbIII transporters.

Arsenic hyperaccumulation by *P. vittata* may serve as a defense mechanism against herbivore attack. A study was conducted to examine the effects of arsenic concentrations on scale insect (*Saissetia neglecta*) infestation of *P. vittata*. Scale insects were counted as percentage fallen from the plant to the total number of insects after 1 week of As-treatment. The higher arsenic concentrations in the fronds resulted in higher percentage of dead and fallen scale insects indicating that arsenic may help *P. vittata* defend against herbivore attack.

CHAPTER 1
INTRODUCTION

The ubiquitous presence of arsenic (As) in the environment through natural or anthropogenic sources has lead to a wide range of research areas in its occurrence, toxicity and remediation. Arsenic is found in geological formations and is released to the atmosphere, water and soil either naturally through volcanoes (Signorelli, 1999) or by As mining for commercial purposes (Smedley and Kinniburgh, 2002). These pathways result in the transformation of stable non-bioavailable arsenic to more bioavailable and toxic forms.

Arsenic in water is of major environmental concern in terms of human, animal and plant health due to its solubility and bioavailability. Once As enters a cell, it acts by binding onto thiol groups or replaces phosphates in the biochemical pathway and behaves as a carcinogen by creating chromosomal abberations. Due to the health effects caused by As, strict regulations have been imposed by the WHO setting the standards for As in drinking water as 10 $\mu g\ L^{-1}$. The introduction of strict regulations requires efficient and cost-effective remediation methods for As removal from water. The existing remediation methods include oxidation, coagulation, lime softening, ion exchange, nano filtration, reverse osmosis, electro dialysis and phytoremediation (Mohan and Pitmann, 2007)

Plants sensitive to As are either killed or have stunted growth when exposed to high concentrations. However, a certain group of plants called hyperaccumulators are able to tolerate and accumulate high concentrations of arsenic in its tissue. The use of these plants to accumulate high concentrations of As from the soil or water in the biomass, which can be harvested, is termed phytoremediation.

The first hyperaccumulator of arsenic, *Pteris vittata* L (Chinese brake fern) was discovered by Ma et al. (2001). This fern produces a large plant biomass compared *Thlaspi sp.* and *Brassica* sp., to and is efficient in arsenic uptake. The fern can accumulate as much as 2.3% arsenic in its biomass and tolerate arsenic concentrations as high as 1,500 mg kg^{-1} in soil. *P. vittata* is unique in its As hyperaccumulating capacity and hence may have a different mechanism of uptake and tolerance to As compared to other plants. There are several hypotheses set forth as to why certain plants are able to hyperaccumulate As (Boyd, 2004). These include metal tolerance, competition with neighboring plants, drought tolerance and defense against herbivores.

Pteris vittata can be used in phytoremediation of arsenic-contaminated sites and also as a model plant in the study of several physiological mechanisms in arsenic uptake and metabolism that can be related to other living organisms. One of the major pathways of As movement in an ecosystem is plant uptake. Arsenic may undergo changes in the rhizosphere in the presence of bacterial communities and root exudates before being taken up by the fern. This transformation can be of commercial value if the arsenic is converted to less toxic forms. The uptake and translocation of As in the fern depends on the species of As in the media. Since *P. vittata* is unique in its ability to accumulate high concentrations of arsenic in its biomass it may have developed a unique uptake mechanism specific for As uptake. This may be different from other plants where As is mainly taken up by phosphate or water channels. Once accumulated the As is transformed to AsIII and sequestered in the vacuoles in the frond of *P. vittata*. The accumulation of As in the biomass of *P. vittata* may have a negative impact on the herbivores that feed on the fern. This is based on the hypothesis of defense mechanism

of plant hyperaccumulation where the metal in the tissue itself or an organic chemical produced as a result of hyperaccumulation or a combined effect might result in repulsion or death of insects feeding on it. Hence, it is important to understand the threshold level of hyperaccumulated arsenic beyond which herbivores are impacted. This can also imply that plant hyperaccumulation can be a natural pest control measure if the concentrations of arsenic accumulated in the insects are not high enough to affect the food chain.

It is important to understand the fate of arsenic in the growth media and rhizosphere of *P. vittata*, its uptake, biomass speciation and insect defense mechanism and hence the major objectives in this research were to understand the 1) effects of the rhizosphere of *P. vittata* on As speciation in the media, 2) localization of oxidation or reduction of arsenic in the tissue, 3) uptake and translocation of arsenic, and 4) impact of arsenic hyperaccumulation on insect infestation in *P. vittata.*

CHAPTER 2
REVIEW OF LITERATURE

Arsenic: Origin and Occurrence

Arsenic is a carcinogenic metalloid of major environmental concern. It ranks the 20th in elemental abundance in the earth's crust with an average concentration of 2-3 mg kg^{-1} (Tanaka, 1988), 14th in seawater and 12th in the human body (Mandal and Suzuki, 2002). Arsenic is extracted for commercial purposes from lead and copper ores, which have 2-3% arsenic and from gold ores (11% As). China is the world's largest producer of As at 30,000 tons per year and the United States is a major consumer (U.S. GS, 2008). The background concentration of As in the U.S. does not exceed 15 mg kg^{-1} but concentrations from 0.2 to 40 mg kg^{-1} have been reported by Walsh et al. (1977). The known natural oxidation states of As include -3, 0, +3 and +5, of which the most abundant and of environmental concern are +3 [arsenite (AsIII)] and +5 [arsenate (AsV)] (Cullen et al., 1989; Johnson and Hiltbold, 1969; Chatterjee et al., 1999).

Arsenic is present in over 245 sulfide (chalcophilic) minerals associated with ultramafic early forming rocks (Abzalov et al., 1997). These minerals include arsenopyrite (FeAsS), arsenolite (As_2O_3), realgar (AsS), olivinite (Cu_2OHAsO_4), cobaltite (CoAsS), proustite (Ag_3AsS_3) and orpiment (As_2S_3) and are found in high temperature hydrothermal veins and in pegmatites or coarse igneous or sedimentary rocks (Allard, 1995; Reimann and deCaritat, 1998). The presence of these minerals in soils is determined by the geological history of a particular soil (Kabata-Pendias and Adriano, 1995). Volcanoes are a major source of arsenic on the earth's surface through magma or fumaroles (Signorelli, 1997).

Arsenate and Arsenite in the Environment

Arsenate with a negative charge is strongly adsorbed to the surface of minerals, such as ferrihydrite and alumina, which reduces arsenate mobility. Its adsorption decreases with increasing pH, due to the increasing negative surface potential, leading to repulsion (Mahimairaja et al., 2005). Arsenite, on the other hand, is neutral at pH of 4-9 and hence has a weaker pH dependence on adsorption. It adsorbs less strongly and to fewer minerals, which makes it more mobile (Smedley and Kinniburgh, 2002). Both AsV and AsIII form similar surface complexes with goethite by bidendate binuclear complexes with 2 adjacent iron octahedral corner sites (Manning, 1998). The activity of AsV and AsIII is controlled by surface complexation reactions on metal hydro-oxide and clay minerals of Al, Mn and Fe (Goldberg, 1986).

The species of arsenic in water depends on the pH and redox potential existing in that system as indicated in the pE/pH diagram (Figure 1-1). In aerobic conditions As exists predominantly as arsenate (H_3AsO_4, $H_2AsO_4^-$, $HAsO_4^{2-}$ and AsO_4^{3-}) and under submerged and reduced environments as arsenite (H_3AsO_3, $H_2AsO_3^-$ and $HAsO_3^{2-}$) (Onken and Hossner, 1996; Yan et al., 2000; Abedin et al., 2002).

The species of As that predominates in a system depends on the capacity of the environment to reduce or oxidize arsenic in the presence of electron donors or acceptors based on abiotic or biotic factors. The abiotic factors include the presence of chemical oxidants or reductants and biotic processes requires living organisms particularly microbes.

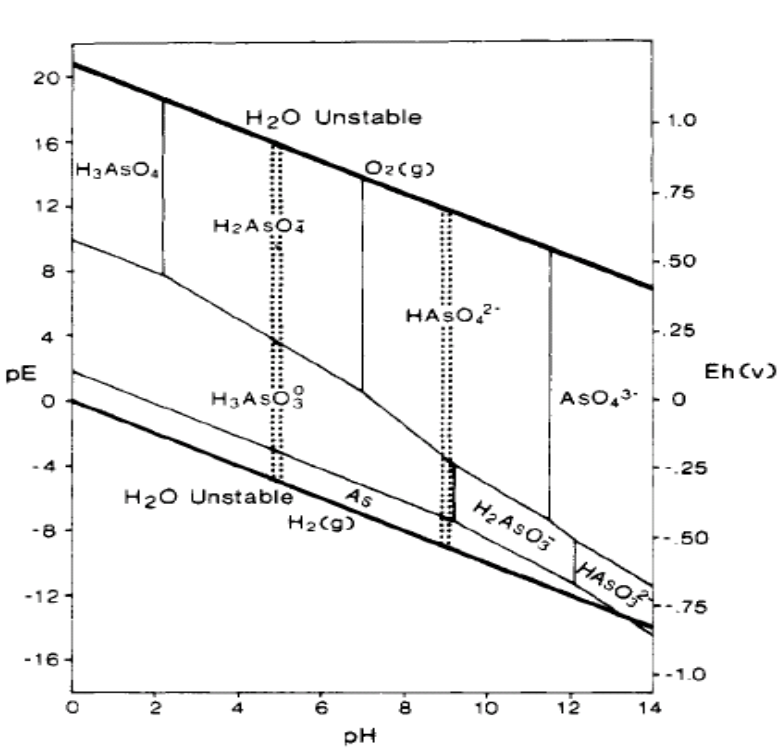

Figure 1-1. pE/pH diagram of As in water system at 25°C (Smedley and Kinniburgh, 2002)

Arsenate Reduction

Arsenic adsorbs on both iron and aluminum oxides or hydroxides but iron oxide is the most important sink of both AsIII and AsV in aquatic and terrestrial environments. When arsenate is present in submerged soils, it may get directly reduced to AsIII abiotically in the presence of sulfide as an electron donor at pH <5 (Rochette et al., 2000). Once AsIII is formed, the total soluble As decreases as a result of the formation of AsIII sulfide complexes.

There are 2 biotic pathways by which AsV adsorbed on metal oxide minerals are converted to AsIII. In the first pathway FeIII oxides undergo a reductive dissolution allowing the release of AsV into the aqueous phase as indicated in Figure 1-2 (Inskeep et al., 2002). Here microbes utilize FeIII as a terminal electron acceptor (Jones et al.,

2000). For example, the FeIII reducing *Shewanella alga* can release AsV from scorodite at 35 µM h^{-1} with 10 mM lactate as carbon source (Cummings et al., 1999). These bacteria however, cannot reduce AsV to AsIII.

The released AsV gets reduced to AsIII by biotic or abiotic pathways. Arsenate reduction occurs primarily by the action of dissimilatory arsenic respiring prokaryotes (DARPs) such as *Sulphospirillum* that respire AsV and release AsIII (Oremland and Stolz, 2005). A specific anaerobic bacteria *Sulphospirillum barnessi* is capable of both reductive dissolution of FeIII and reduction of AsV by using FeIII and AsV as terminal electron acceptors (Zobrist et al., 2000). This reductive dissolution of FeIII and subsequent release of AsV depends on crystallinity and surface area of the substrate. The dissolution is faster for amorphous ferrihydrate than crystalline goethite (Jones et al., 2000).

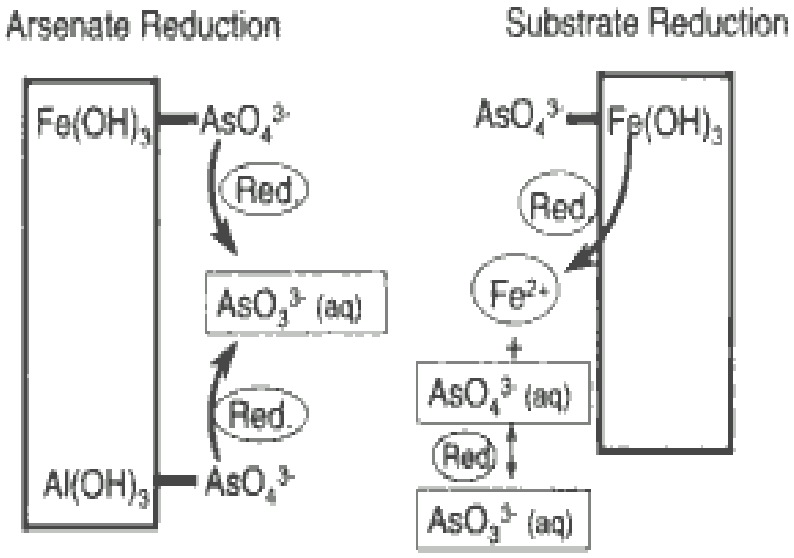

Figure 1-2. Pathways for the reductive dissolution of sorbed arsenic through reduction to arsenite (left) or degradation of substrate (right) (Inskeep, 2002).

In the second pathway of AsV reduction from iron oxides, the AsV first gets reduced to AsIII and then is released upon reductive dissolution of FeIII oxides (Inskeep et al., 2002) (Figure 1-2).

Certain microbes can reduce AsV to AsIII under oxic and anoxic conditions. These microbes do not use AsV as an electron source. Instead they follow an arsenic resistance mechanism where AsV enters into the cell, undergoes reduction to AsIII and is finally effluxed from the cell. These arsenic resistant bacteria are known to have an *ars* operon, which contains genes that encode for arsenate reductase (ArsC) that reduces AsV to AsIII. This *ars* operon detoxifies arsenic by the efflux of produced AsIII (Macur et al., 2001; Kaur et al., 2009).

Arsenite Oxidation

The rate of oxidation of AsIII to AsV with O_2 is very slow at neutral pH but is faster at highly alkaline or acidic solutions (Kolthoff, 1921). It is stable in water at 25°C (Tallman and Shaik, 1980) with a ½-life of 1 year at pH levels less than 9 (Eary and Schramke, 1990). The predominant abiotic oxidation mechanism of AsIII is by manganese minerals (MnIII and MnIV) (Oscarson et al., 1983; Brannon and Patrick, 1987). The MnIII oxide has been shown to oxidize AsIII and adsorb both AsIII and AsV species (Chiu and Hering, 2000) whereas MnIV oxides adsorb AsV (Tani et al., 2004).

There are two types of AsIII oxidizers, the chemoautotrophic arsenic oxidizers (CAO) and the heterotrophic arsenic oxidizers (HAO). The chemoautotrophs obtain energy by the oxidation of electron donating groups, using CO_2 as C source whereas heterotrophs require organic carbon as C source (Kulp et al., 2004). Several bacterial strains are known to oxidize AsIII to AsV using respiratory and non-respiratory enzymatic systems (Oremland and Stolz, 2003). An AsIII oxidase enzyme on the outer

surface of the cytoplasmic membrane in these bacteria is responsible for this oxidation (Ilyaletdinov and Abdrashitova, 1981). For example, the AsIII oxidase enzyme of the bacteria *Alcaligenes faecalis* is a Fe-molybdenum complex with azurin and cytochrome c, which acts as an electron acceptor from AsIII for AsIII detoxification (Figure 1-3). The molybdenum centre contains an oxygen atom, which results in the oxidation of AsIII to AsV. The electrons released then pass through an electron transport chain to the Fe-S complex in the enzyme and finally to the electron acceptor azurin or cytochrome c (Ellis et al., 2001). Microbially mediated AsIII oxidation substantially reduces the half life of AsIII to 1.8 hr. (Philips and Taylor, 1976).

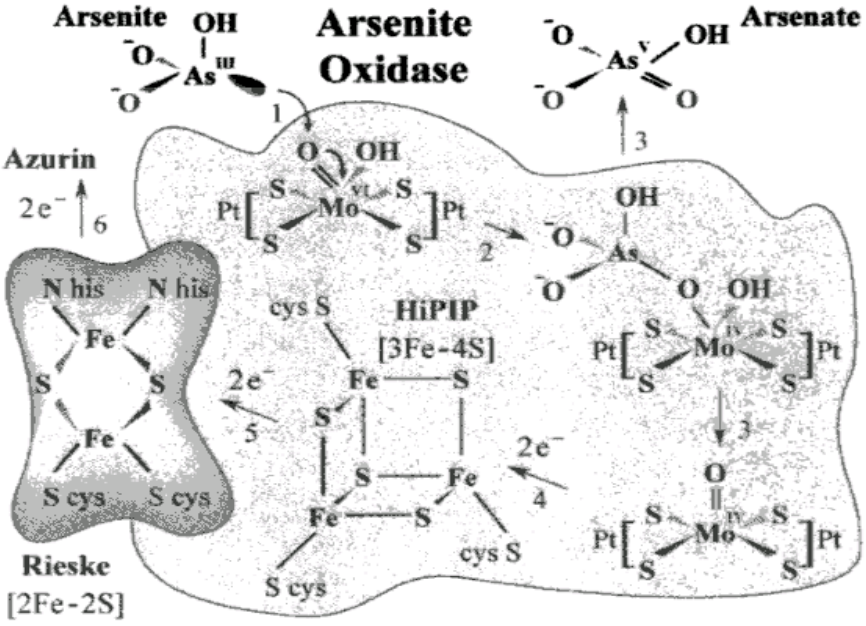

Figure 1-3. Hypothetical model of an arsenite oxidase enzyme (Anderson et al., 1992; Ellis et al., 2001)

Considering a real scenario, As that is present in the reduced form in minerals as AsS, As_2S_3 and FeAsS are attacked by CAO's, resulting in the oxidation of AsIII and iron and sulfide. The construction of wells accelerates this process in the presence of oxygen or fertilizers like nitrates. The AsV is then adsorbed on oxidized mineral

surfaces like ferrihydrite or alumina. DARP's respire the adsorbed AsV, resulting in the release of AsIII into aquifers (Oremland and Stolz, 2003) This leads to high arsenic concentrations in drinking water in areas like West Bengal Ganges river delta regions where millions of people are exposed to ground water As contamination (Madhavan and Subramanian, 2006). Research during the last 19 years has shown that nearly 569,749 square km in Bangladesh and eastern India with a population of about 500 million are at risk of arsenic poisoning (Chakraborti et al., 2001). Other countries affected include China, Mongolia, Nepal, Afghanistan, Pakistan, Argentina, Chile and several parts of the United States and Europe.

Applications of Arsenic

The wide uses of arsenic are mainly based on its toxicity. Calcium and lead arsenates are used as herbicides and insecticides (Abernathy, 1983). Application of P fertilizers to soils previously contaminated with lead arsenate has resulted in the release of arsenic to shallow groundwater (Peryea, 1991). Arsenic is also used as a feed additive for poultry as roxarsone for increased growth rates due to its action against intestinal parasites. It is also of medicinal value. Salvarsan or "salvation by arsenic" was used for the treatment of syphilis, phenylarsenic acid for trypanosomal infections, and As_2O_3 for treatment of leukemia (Zhu et al., 1999; Jones, 2007). Other applications include its use in circuits and semiconductors, transistors, bullets, fireworks, paper, pesticides, pigments and metal adhesives (Ishiguro, 1992).

A major portion of arsenic currently used in the United States (~96%) is imported from China as As_2O_3, and the remaining as As metal. Arsenic was used in the United States mainly for agriculture and further for pressure treating lumber with chromate copper arsenate. Regulations by the EPA on the use of arsenic in agriculture and in

residential areas have, however, significantly reduced the use of arsenic for land application or wood treatment (Jones, 2007).

Toxicity and Health Effects

Inorganic Arsenic

The natural abundance of this metalloid and anthropogenic sources has made it ubiquitous and hence a threat to the biosphere. In a 1984 health assessment, the U.S. EPA classified arsenic as a class A human carcinogen, and Smith et al. (1992) showed arsenic as a prominent source of cancer mortality in the world. Due to the devastating impact of arsenic contamination in the environment, the U.S. EPA and the WHO in 2006, adopted a new permissible limit for arsenic in drinking water at 10 $\mu g\ L^{-1}$, replacing the old standard of 50 $\mu g\ L^{-1}$. Arsenite is more mobile and more toxic to biota and plants than arsenate (Korte and Fernando, 1991) and the more toxic arsine gas is produced under highly reduced conditions (Buchet and Lauwerys, 1981).

The major route of arsenic poisoning is by ingestion via drinking water and food or inhalation. Inhalation exposure can be from the smelters or mining activities. Arsenic forms stable bonds with S and C in organic compounds and can react with sulfhydryl groups of cysteine in proteins and result in enzyme inactivation. Arsenite retained in the body has the capacity to inactivate sulfhydryl groups (thiols), consequently increasing the reactive oxygen species (ROS). Glutathione reductase is an enzyme that protects the cells from these oxidants that can affect DNA replication and repair. The presence of ROS results in cell damage due to the inhibition of glutathione reductase and can result in cancer (Cuzick et al., 1992). Most of the symptoms are related to the skin due to its high keratin content, which contains several sulfhydryl groups to which AsIII binds

(Styblo et al., 1996). Arsenate does not react with sulfhydryl groups, but since it is an analog to phosphate it substitutes phosphates in ATP synthesis and cell function. Other health hazards related to arsenic include the organs that directly function with arsenic excretion such as the gastrointestinal tract, the circulatory system, liver, kidney and skin (Hughes, 2002). Symptoms include hyperpigmentation, hyperkeratosis, loss of hair and skin cells, neuropathy and skin cancer.

Organic Arsenic

Methylation acts as a detoxifying mechanism where the inorganic arsenic is converted to mono methylarsonic acid (MMAA) and dimethylarsinic acid (DMAA) as the methylated forms are less reactive with tissues and are excreted from the body (Braman and Foreback, 1973). Here a methyl group from S-adenosylmethionine is added to trivalent As for methylation (Stolz et al., 2006). Certain fungi, yeast and bacteria can methylate arsenic to arsine gas, which is a deadly form of arsenic that affects the nervous system. Marine organisms have higher arsenic content in their tissue compared to terrestrial organisms. Shrimps and lobsters are observed to have methyl arsine concentrations as high as 200 mg kg^{-1} (Chapman, 1926). The methylated form of As in mammals (Aposhian, 1997), fungi, and algae (Edmonds and Francesconi, 1993; Cullen and Reimer, 1989), undergoes further transformation by its incorporation into organic molecules and form arsenocholine, arsenobetaine or arsenosugars (Edmonds and Francesconi, 1993).

Arsenic Remediation

There are several methods to remediate arsenic from contaminated soils and water, which include physical, chemical and biological techniques. Soil physical remediation methods include excavation, capping, soil solidification, soil flushing and

vitrification whereas chemical methods include electrokinetics. Compared to soils, As is more problematic in surface and ground waters as it is easily ingested via drinking water. Arsenic remediation in water employs granular metal oxide adsorbent method, ion exchange or coagulation-microfiltration methods. The granular metal oxide adsorbent method uses activated alumina, iron-oxide coated sand and granular ferric hydroxide and works on the principle of adsorption. However, all the above methods are only effective if AsIII is first converted to AsV resulting in a negative charge for better adsorption (Clifford and Ghurye, 2002). This can be achieved by using oxidants such as chlorine, ozone or permanganate (Frank and Clifford, 1986). Ion exchange technology is the best available technology recommended by U.S. EPA (Ghurye et al., 1999). Here strong base anion exchange resins like polystyrene divinylbenzene polymers are used. In the remediation process of coagulation and microfiltration, As is adsorbed on hydrolyzing metal salts such as ferric chloride or alum (Brandhuber and Amy, 1998). Biological treatments in soil and water include microbial remediation and phytoremediation.

The microbial remediation of As involves the reduction and oxidation of arsenic and hence is not an effective detoxification mechanism. Here certain bacteria such as *Pseudomonas arsenitoxidans* can gain energy in the presence of AsIII by oxidizing AsIII and *Sulfurospirillum arsenophilum* and *Sulfurospirillum barnessii* can reduce AsV (Inskeep et al., 2002).

Phytoremediation is the use of hyperaccumulator plants that can tolerate high concentration of contaminants in their tissue to remediate soil ad water. Conventional methods of arsenic remediation would cost billions of dollars in the U.S. (Salt et al.,

1995) and hence less expensive methods of remediation are required for a long term remediation strategy. Phytoextraction of one acre of sandy loam soil to a depth of 50 cm will cost $60,000-100,000 compared to the cost of excavation and storage at $400,000 (Salt et al., 1995). Another advantage is that the contaminants can be remediated in situ. There are different kinds of phytoremediation techniques based on the media that is to be remediated and on the required end result. Phytoextraction extracts metals from the soil into the harvestable parts of the plants. This depends on the rate of uptake and the biomass of the plant. Rhizofiltration requires plants that have rapidly growing roots to absorb and precipitate heavy metals from solution. Here surface sorption occurs by chelation, ion exchange and adsorption (Salt et al., 1995). A soil contaminated with metals results in erosion and further spread of the contamination. To minimize this effect plants can be used to stabilize the soil and hence prevent further movement of the contaminants through a process called phytostabilization. The plants may also be able to convert the contaminants from a soluble oxidation state to an insoluble oxidation state.

Heavy metal contaminants in soil exist in several forms such as free soluble metal ions, ions occupying exchange complexes, organically bound metals, precipitated or insoluble complexes and in silicate minerals. Phytoremediation can be efficiently used only when the metals are present in available form (Salt et al., 1995). Chelating agents, pH, root exudates and microbes can influence the bioavailability of metals in soils (Harter, 1983; Uren, 1981; Blaylock and James, 1994).

Hyperaccumulators can take up metals by using metal chelating molecules like siderophores with mugeneic and avenic acid (Kinnersley, 1993). Plant roots may have

metal reductases that increase the metal availability or may have the capacity to acidify the soil environment (Salt et al., 1995). The metals once taken up by the roots are either stored in the roots or translocated symplastically into the xylem vessels. Hence the symplastic transport is the rate limiting step in translocation of metals. Once the metals enter the plant system detoxification can occur either by chelation, compartmentalization or precipitation (Mathys, 1977; Krotz et al., 1989; van Steveninck et al., 1994).

With the rising remediation expenses using traditional methods, phytoremediation is a promising method of remediating heavy metal contaminated soil and water. Improvements can be made in phytoremediation techniques by introducing genes that can enhance hyperaccumulation. Efforts should also be taken in following appropriate agronomic practices with addition of amendments, which can improve phytoextraction. A total of 450 angiosperms have been identified so far as heavy metal hyperaccumulators of As, Cd, Co, Cu, Ni, Pb, Sb, Se, and Zn (Rascio and Navari-Izzo, 2010).The hyperaccumulators should be capable of accumulating >10 mg g^{-1} of Zn, >1 mg g^{-1} As, Co, Cr, Cu,Ni, Pb, Sb, or Se and >0.1 mg g^{-1} Cd in the aerial parts without phytotoxic damage (Verbruggen et al., 2009). Twenty five % of the hyperaccumulators belong to the family Brassicaceae and genera *Thlaspi* and *Alyssum* (Macnair et al., 1998). The major accumulators of arsenic are *Pteris vittata, P. cretica, P. multifida, P. oshimensis, P. bhaspericaulis* and *P. fauriei* (Wang et al., 2007) and *Pityrogramma calomelanosis* (Francesconi et al., 2002).

Pteris vittata in Phytoremediation

The discovery of the first arsenic hyperaccumulator fern *Pteris vittata* L. (Chinese brake fern) by Ma et al., (2001) has made it possible for arsenic remediation. In a study

by Natarajan et al. (2008), the fern was capable of reducing the arsenic concentration levels in water from 130 to 10 μg L^{-1} in 8 h.

Life Cycle

P. vittata belongs to the phylum Pteridophyta. They are tetraploid vascular plants with haploid chromosome number n = 58 (Beri and Bir, 1993; Srivastava et al., 2007). Similar to other ferns, it follows an alternation of generation between a tetraploid sporophyte generation and a diploid gametophyte generation (Xie et al., 2009). The sporophyte stage of the fern is well adapted to moist and shady environments. Fern leaves are called fronds and if the stems are underground they are referred to as rhizomes. Emerging new fronds are called croziers or fiddleheads. When fertile fronds mature, reproductive structures called sori are formed on the undersurface of the pinnae(divisions of the compound frond) (Bondada et al., 2006). A sorus contains clusters of sporangia, which contain tetraploid spore mother cells that divide by meiosis to produce diploid cells. These diploid cells are released from the pinnae of the sporophyte and germinate to form a prothallus. The prothallus houses both the antheridia, the male reproductive structures and the archaegonia, the female reproductive structures (Gumaelius et al., 2004). Upon fertilization, the zygote divides rapidly to form an embryo and further division results in a sporophyte. Both the sporophyte and the gametophyte are capable of As hyperaccumulation (Ma et al., 2001 and Gumaelius et al., 2004).

Physiology

In a normal plant, heavy metal phytotoxicity may result in changes in the physiological processes at cellular or molecular levels as a result of inactivation of enzymes, blocking functional groups and disrupting membrane integrity (Rascio and

Navari-Izzo, 2010). A hyperaccumulating plant may be able to overcome these effects and accumulate large concentrations of metals in its tissue. The arsenic hyperaccumulating fern *P. vittata* is capable of accumulating up to 2.3% As in the fronds (Ma et al., 2001). Studies indicate that once As is taken up by *P. vittata*, As is translocated to the shoots (Cao et al., 2004), sequestered in the pinnae (96% of total As) and are stored in the vacuoles of the upper and lower epidermis, as revealed by EDXA analyses (Lombi, et al. 2002). Studies also show that As may be sent to tiny hairs that guard the reproductive cells near the edge of the fronds (Pickering et al., 2006).

The hyperaccumulator *P. vittata* can take up 4.8 to 5.6 times higher arsenic than the non hyperaccumulator *Pteris ensiformis*. Once taken up, *P. vittata* is highly efficient in translocating As from the roots, rhizomes and the fronds compared to *P. ensiformis*, which was ineffective in reducing AsV to AsIII (Singh and Ma, 2006). In a study where excised *P. vittata* tissue was exposed to As, AsIII predominated in the fronds and AsV in the roots (Tu et al., 2004b). These ferns were also found to accumulate As in the tissue when sprayed with AsV and AsIII on the pinnae surface (Bondada et al., 2004). This indicates that the fern can readily take up As through specified transporters, which may be different for AsV and AsIII. Compared to the fronds, lower concentrations of As are stored in the roots. Root exudates released by *P. vittata* may act as chelators, which enhance metal uptake, translocation and resistance. The dissolved organic carbon released *P. vittata* roots may change the rhizosphere pH, which might result in an increase in As uptake (Gonzaga et al., 2006)

Arsenic Uptake

Arsenate is an analogue of inorganic P and hence its transport into cells is assumed to be via a P transporter. Bacteria with a defective P uptake system exhibited

increased tolerance to AsV (Willisky and Malamy, 1980). AsV uptake in carcinoma cells inhibited P uptake, indicating a common transport system (Huang and Lee, 1996). AsIII, on the other hand, is neutral and hence its uptake is mediated by aquaporins, which allow the passage of neutral solutes. The main function of aquaporins is in osmoregulation by transport of water molecules and neutral solutes. The pores of the channels are believed to be narrow so that water molecules move through a single file (Maurel and Chrispeels, 2001). A glycerol transporter or aquaglyceroporin, GlpF, mediates SbIII uptake in *E. coli* as a mutation in the gene resulted in increased tolerance (Sanders et al., 1997). *Saccharomyces cerevisae* Fps1 gene mediates the uptake and efflux of glycerol from yeast cells (Sutherland et al., 1997) and it is homologous to the *E. coli* GlpF gene. Disruption of this Fps1 gene confers AsIII and SbIII tolerance. Aquaporins in *E. coli* (AQP-Z) are water selective whereas aquaglyceroporins in *E. coli* (Glpf) can transport neutral solutes such as glycerol or urea (Gomes et al., 2009). Mercury and silver block the water channels by binding to a cysteine residue in the pore (Kuwahara et al.,1997).

Microflora Associations

P. vittata has unique characteristics of arsenic hyperaccumulation and it may also house a number of microorganisms on its phyllosphere and rhizosphere, that may aid in the transformation or hyperaccumulation of arsenic. Microbes have a highly resistant system against AsIII and AsV and both AsV reducing and AsIII oxidizing system exists in microbes for the detoxification of As. An arsenic resistant proteobacterium was isolated from the fronds of *P. vittata* grown in an As-contaminated site (Rathinasabapathi et al., 2006). The bacterium was resistant to AsIII, AsV and antimony. The plant roots and microbes result in a combined release of carbon in the

form of sugars, organic acids and amino acids. Several AsV resistant bacteria were identified from the rhizosphere of *P. vittata* by Huang et al. (2010). These include *Naxibacter sp*., *Mesorhizobium sp*., *Methylobacterium sp*., *Enterobacter sp*. and etc.

Insect Deterrence

There may be several hypotheses why a plant would hyperaccumulate high concentrations of arsenic. The phenomenon of hyperaccumulation could have developed over a period of time during evolution in order to evade certain herbivores prevalent in the environment. There are studies which do and do not indicate a positive correlation between metal accumulation and herbivore deterrence. Laboratory trials indicating insect deterrence may not be seen in a field situation (Noret et al., 2007). Deterrence also depends on the mode of feeding in herbivores (Jhee et al., 2005). It is hence important to understand the herbivore defense mechanism in *P. vittata* using different test herbivores and pathogens in lab and field conditions and also monitor the synthesis of organic constituents in the presence of As.

CHAPTER 3
ARSENIC TRANSFORMATION IN THE GROWTH MEDIA OF *P. VITTATA*- THE ROLE OF MICROBES OR ROOT ENZYMES

Arsenic Transformations

Of the two predominant inorganic forms of arsenic, AsV and AsIII, AsIII is more toxic (Smith et al., 1992). This makes AsIII a greater environmental concern than AsV in terms of its environmental occurrence and transformation pathways. Both AsV and AsIII can complex with iron oxides like goethite by forming bidendate, binuclear complex by complexing with two adjacent iron octahedral corner sites (Manning et al., 1998; Parfitt et al.,1975). However, AsV has a strong affinity for other metal hydroxides like aluminum and clay minerals (Goldberg, 1986). Since AsIII exists as a neutral species at pH < 9.2, it is less strongly adsorbed onto minerals in aquifers and soils making it more mobile, bioavailable and hence more toxic than AsV (Hering, 1996; Bhattacharya et al., 2004). This difference in their environmental behavior and toxicity makes it important to understand arsenic speciation and transformation in the environment.

The presence of plants in a soil system with different forms of As may have an effect on its speciation. *P. vittata* has an extensive fibrous root system. The roots release exudates, which can form a carbon source and house a number of microbial communities (Al Agely et al., 2005). In nature, several AsIII oxidizing and AsV reducing bacteria have been identified from different sources, which control arsenic transformation in the rhizosphere (Leblanc et al., 1995; Blum et al., 1998; Stolz et al., 1999).

Arsenate can be remediated by adsorption, ion exchange or coagulation based on the fact that it is charged. However, AsIII is a neutral species at normal waste water pH levels (H_3AsO_3) and is more difficult to remediate than AsV because of its low affinity for

adsorbents. The AsIII has to be first converted to AsV by pretreatment before conventional remediation methods such as precipitation, ion exchange, lime softening or coagulation are carried out. This oxidation of AsIII requires oxidants such as ozone, H_2O_2, manganese oxides (Hug and Leupin, 2003; Driehaus et al., 1995) or TiO_2 (Bissen et al., 2001). Due to the presence of a pretreatment process in remediation, these techniques are often costly, which requires further processing. This intermediate step of AsIII oxidation by chemical means can be avoided if existing phytoremediation methods can be used to oxidize AsIII in the media. The rhizosphere of *P. vittata* exude organic acids (Tu et al., 2004a) and enzymes nourishing a number of microbial communities, which may alter AsIII stability and hence oxidize AsIII.

Arsenate is taken up via the P transporters in *P. vittata* (Wang et al., 2002). The transporters involved in AsIII uptake are unknown in *P. vittata*, but research in a number of plants and microbes shows that AsIII is taken up via aquaglyceroporins (Meng et al., 2004; Isayenkov and Maathuis, 2008). Knowledge of arsenic speciation in the media is hence important as it controls the arsenic uptake mechanism by *P. vittata* (Mathews et al., 2010). The objective of this study was to understand the dynamics of arsenic transformation in the growth media and *P. vittata* tissues with specific objectives (1) to determine arsenic transformation in the growth media in the presence or absence of *P. vittata* and (2) to examine the role of microbes and plants in AsIII oxidation in the growth media.

Materials and Methods

Experimental Setup

Mature *P. vittata* with spores on the underside of the fronds were collected and placed in plastic containers for a week. The spores that settled at the bottom of the

containers were then spread on germination soil mix (Jungle growth mix) or sand: vermiculite mix (1:1) and sprayed with 0.2x Hoagland's solution (HS) daily to maintain the moisture of the germination mix. After 4-7 days green algae like growth was observed followed by the gametophytic stage. In about 2-3 weeks, the sporophytes develop by the fertilization of the male and the female gametes in the gametophytic stage (Figure 3-1). The sporophytes were then transplanted to potting mix in separate pots and watered with 0.2x HS for 3-4 months until ready for use.

Figure 3-1. One month old *P. vittata* sporophytes

Arsenic Speciation under Natural Conditions

Ferns 4 months of age with 15-18 cm frond length were acclimatized in HS at 0.2-strength with pH adjusted to 5.7 with 1 mM KOH-MES buffer for 2 weeks. They were maintained under constant aeration with a 12 h photoperiod and a photon flux of 350 µmol m^{-2} s^{-1} using cool and warm white fluorescent lamps. The temperature was maintained at 23–28°C and relative humidity at 70%. After two weeks of acclimation, the ferns were transferred to opaque containers containing 1 liter of test solution spiked with AsV (Na_2HAsO_4 .$7H_2O$) or AsIII (Na_2AsO_3) (Sigma, St. Louis, MO). The arsenic was

provided in de-ionized (DI) water in all experiments to minimize P competition for AsV unless otherwise indicated. All experiments were performed in triplicates.

In the first experiment, AsIII oxidation in the growth media was determined in solution containing 0.10 mM AsIII with and without *P. vittata*. Both plant and solution samples were taken after 24 h. In the second experiment, AsIII oxidation and AsV reduction were determined in the growth media containing 0.27 mM AsIII or AsV with or without *P. vittata*. Aqueous solution samples were taken at intervals of 1 h, and 1, 2, 4 and 8 d and plant samples were taken after 8 d.

Arsenic Speciation under Sterile Conditions

Efforts were made to sterilize 4 month old *P. vittata* from microbes. These ferns were previously raised in potting mix (Jungle growth mix,). The intact *P. vittata* fronds and roots were washed thoroughly in tap water and then rinsed in autoclaved water three times. These were then dipped in 10% bleach for 7 minutes and then immersed in autoclaved cooled water. The ferns were then placed in ½ Murashige and Skoog media (Macro+ Micronutrients) with 2% (w/v) sucrose and 0.8% agar. In another trial the ferns were washed in tap water and rinsed in autoclaved water five times. Here, the ferns were dipped in 10% bleach for 10 minutes. The ferns survived in both intact plant sterilization trials. The first set with 7 minutes of 10% bleach treatment indicated immediate microbial contamination in the media. In the second trial with 10% bleach treatment for 10 minutes, white cloudy liquid was observed in the agar medium after 2 d indicating microbial contamination.

As a result of the failed sterilization of the intact ferns, the ferns were raised under sterile conditions from the spore stage. Surface sterilization of spores was done using 10% bleach and 0.5% Tween 20 for 4 min and followed by four washes with sterile

water. The spores were then germinated in autoclaved magenta boxes with sterile ½ MS media and 2% (w/v) sucrose and 0.8% (w/v) agar at pH 5.7 in sterile G7 boxes.

The gametophytes were formed in 2 weeks and they were subsequently sub-cultured into fresh media every 2 weeks. After two months, the ferns of 5–7 cm in size were placed in autoclaved containers with the roots inside 5 mL of 0.10 mM sterile AsIII solution. They were then placed in sealed autoclaved G7 boxes for 1 d and 14 d under aseptic conditions. A separate set of containers containing 5 mL of 0.10 mM AsIII without *P. vittata* was placed in the G7 box as a control to understand the speciation of AsIII after 14 d in the absence of the fern. The growth media and *P. vittata* tissue were sampled for arsenic speciation.

A B

Figure 3-2. Propagation of *P. vittata* under sterile conditions (A) germinated spores and (B) *P. vittata* sporophyte

Arsenic Speciation in Sonicate Extract

Arsenic transformation in the media can be due the presence of specific enzymes in the roots or due to the effect of microbial activity or both. For this, a study was done where the root extracts were tested with As to understand the speciation of AsIII. Here

0.1 g of root samples of *P. vittata* were cut to 1 cm and sonicated in 10 mL DI water for 2 h. The sonicate was either used as such or boiled at 100ºC for 10 minutes to inhibit enzyme activity or filtered using 0.2 µm filter to remove microbes. Following this 5 mL of the sonicate was used to make a final concentration of 1.33 µM AsIII and 1.33 µM AsV mixture of 100 mL. The media was analyzed after 1h, 1d, 4d and 8d for arsenic speciation.

Arsenic Analysis

The growth media samples were analyzed immediately for arsenic speciation. The media was diluted and speciated for AsV and AsIII directly using an arsenic speciation cartridge (Waters SPE cartridge), which retains arsenate (Meng et al., 2001). For fern arsenic analysis, the ferns were harvested and separated into fronds and roots. *P. vittata* roots were placed under running distilled water, rinsed with ice cold phosphate buffer (1 mM Na_2HPO_4, 10 mM MES and 0.5 mM Ca $(NO_3)_2$, pH 5.7) and washed once again with distilled water to remove arsenic adsorbed on the root surface. The ferns were then flash-frozen in liquid nitrogen and stored at -80°C. For arsenic speciation, the samples were ground using liquid nitrogen. 0.1 g of this fern sample was extracted with methanol:water (1:1 v/v) under sonification for 2 h (Zhang et al., 2002). By this method both total As and AsIII will be obtained and the difference between them gives AsV. Since AsV and AsIII are predominant in the growth media (100%) and in *P. vittata* biomass (>95%), other arsenic species were not considered in this study.

For total arsenic analyses a part of the sample was oven-dried (65°C for 2 d). The air-dried plant tissue was ground (20-mesh), digested with concentrated HNO_3 (1:1, v/v), and followed by 30% H_2O_2 for arsenic determination (U.S. EPA, 1983; method 3050). Arsenic in the growth media and digested plant tissues were determined by a

graphite furnace atomic absorption spectrophotometer (GFAAS; Varian 240Z, Walnut Creek, CA). In addition, standard reference materials from the National Institute of Science and Technology (Gaithersburg, MD) and appropriate reagent blanks, internal standards and spikes were used to ensure method accuracy and precision, which was within 100 ± 20% of the expected quality control checks.

Data Analysis

The treatment effects were determined by analysis of variance according to the linear model procedure of the Statistical Analysis System (Freund et al., 1986). Treatment means were separated by Duncan's multiple range tests using a level of significance of $p < 0.05$.

Results and Discussion

Arsenic Speciation under Natural Conditions

Media arsenic speciation

The impact of fern roots and associated microbes on As transformation in the growth media were examined. The tissue As concentrations were also analyzed to understand the difference in uptake in the presence of AsV or AsIII in the media. Arsenite was stable in the solution at 1 d (Figure 3-3 A) in the absence of *P. vittata*. It has been shown that abiotic AsIII oxidation in water in the presence of atmospheric oxygen is slow at 25°C (Frank and Dennis, 1986; Scott and Morgan, 1995), and this is supported by its long half life of 1 year in water (Eary and Schramke, 1990). However, in the presence of *P. vittata* roots in the solution, >67% AsIII in the media was oxidized to AsV after 1 d (Figure 3-3 A).

Arsenic transformation in the growth media, with and without *P. vittata*, was monitored for 8 d where *P. vittata* grew in solutions containing 0.27 mM AsIII or AsV.

After 1 d of the experiment 18% of AsIII was oxidized to AsV (Figure 3-4A). Compared to the experiment using 0.10 mM AsIII (Figure 3-3A), which had 67% oxidized, the oxidation rate in the experiment using 0.27 mM AsIII was slower i.e., 18% (Figure 3-4A). Hence, it is indicative that there is a capacity of AsIII to be oxidized in the growth media, and this oxidation is concentration dependent. The data also suggests that for most hydroponic experiments where AsIII is supplied to *P. vittata*, AsIII oxidation occurs within 1 d. In the case of AsV, no reduction to AsIII was observed with or without *P. vittata* even after 8 d (Figure 3-5A). This indicates that AsV is stable under natural oxidized conditions and also due to the constant aeration used in the experiment. From this experiment it is clear that *P. vittata* is critical for AsIII oxidation in the growth media since no oxidation occurred in its absence.

Biomass arsenic speciation

The speciation of As in the media may or may not have an effect on As speciation in the biomass. It is known that AsV dominates in the roots of *P. vittata* whereas AsIII dominates in the fronds (Wang et al., 2002; Singh and Ma, 2006). When *P. vittata* was treated with 0.1 mM AsIII, after 1 d, 60% of As in the roots were AsV whereas up to 90% in the frond was AsIII (Figure 3-3B). Here 70% of AsIII was oxidized to AsV in the media (Figure 3-3A).

To better understand the dynamics of arsenic transformation in the biomass of *P. vittata*, root and frond samples were collected for arsenic speciation over an 8-d period where *P. vittata* grew in 0.27 mM AsIII or AsV solution. Arsenic concentrations in the roots and fronds increased with increasing exposure time from 1 h to 8 d (Figures 3-4 and 3-5). Since no P was supplied in the growth media, one can compare the uptake rate of AsIII and AsV by *P. vittata* without the competitive effect of P on AsV. However,

there were no significant differences in the uptake rates between AsIII and AsV by *P. vittata* from 1h to 8d ($p < 0.05$). Since AsIII and AsV were stable during 1 h of exposure in the growth media (Figures 3-4A and 3-5A), those data are more compelling. After 1 h of exposure, the arsenic concentrations in the roots and fronds were 7 and 12 mg kg^{-1} for the AsIII treatment (Figures 3-4B and 3-4C) compared to 5 and 17 mg kg^{-1} for the AsV treatment (Figures 3-5B and 3-5C).

Similar data were obtained after 8 d of exposure, where the arsenic concentrations in the roots and fronds were 23 and 73 mg kg^{-1} respectively for AsIII treatment (Figures 3-4B and 3-4C) compared to 37 and 58 mg kg^{-1} for AsV treatment (Figures 3-5B and 3-5C). Though arsenic species in the growth media did not impact plant uptake rate by *P. vittata*, they directly impacted arsenic species in the roots. For example, in the AsV treatment, all arsenic was present as AsV in the growth media (Figure 3-5A), and also in the roots (Figure 3-5B). On the other hand, in the AsIII treatment, after 1 and 2 d exposure, 100% and 82% of arsenic was present as AsIII in the growth media (Figure 3-4A), which resulted in 50% and 21% AsIII in the roots (Figure 3-4B). After 4 d and 8 d of exposure, there was no AsIII detected in the growth media or in the roots (Figures 3-4A and 3-4B). Unlike the roots, AsIII dominated in the fronds (Figures 3-4C and 3-5C). Though there was no detectable AsIII in the roots from either AsIII or AsV treatment after 1 h exposure (Figures 3-4B and 3-5B), 75% and 47% AsIII were detected in the fronds (Figures 3-4C and 3-5C). This is consistent with the literature that most arsenic is present as AsV in the roots and AsIII in the fronds in *P. vittata* (Singh and Ma, 2006). Based on the data, the following inferences were made (1) both AsIII

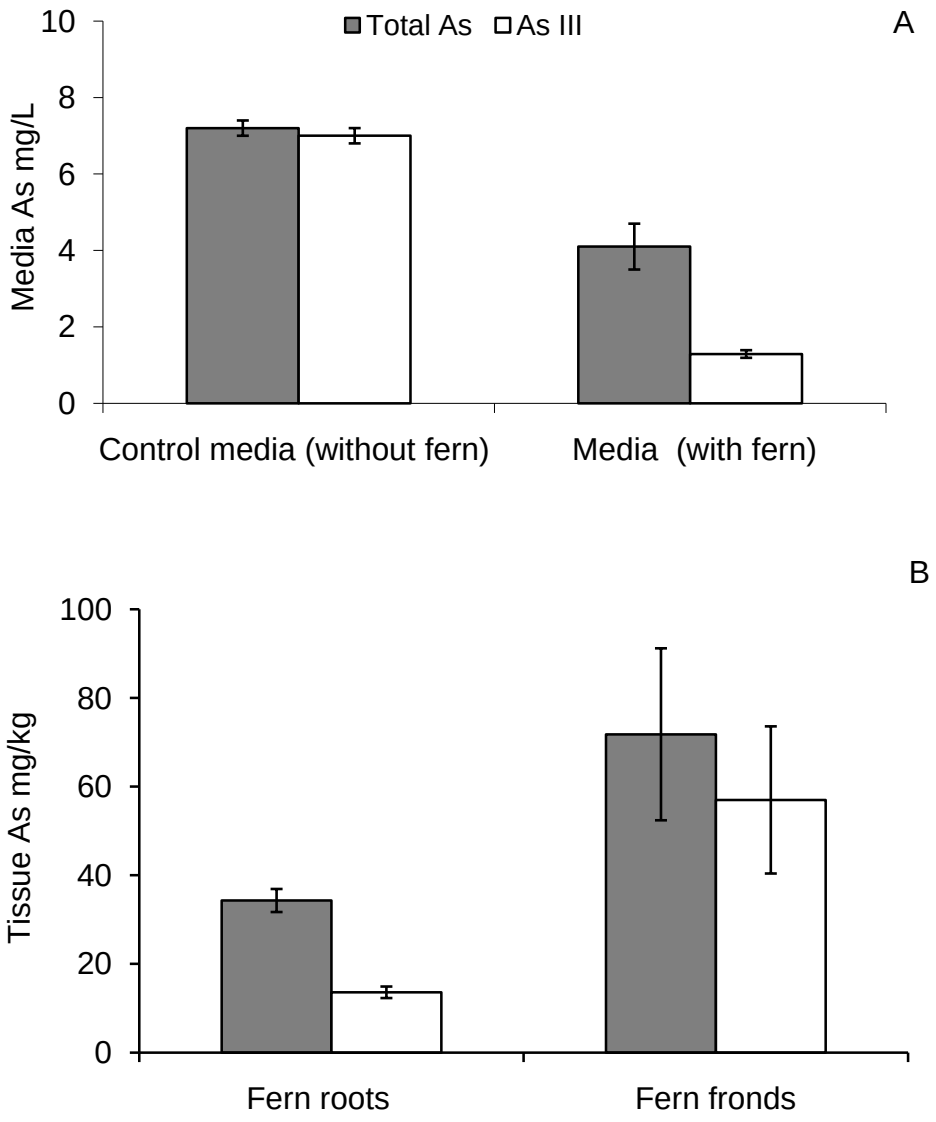

Figure 3-3. As speciation when treated with 0.10 mM AsIII for 24 h A) the growth media and B) P. vittata tissue.

and AsV were translocated from the roots to the fronds as both AsIII and AsV were detected in the fronds; (2) AsIII was translocated more rapidly than AsV as relatively more AsIII was detected in the fronds treated with AsIII than AsV; and (3) the AsIII in the fronds came from two sources: translocation of AsIII from the roots and reduction of AsV in the fronds.

The amounts of AsIII in the fronds were 60–89% for the AsIII treatment compared to 47–98% for the AsV treatment. Hence, the data may argue against the hypothesis that AsV is reduced to AsIII in the roots (Su et al., 2008). First of all, though no AsIII was detected in the roots in all AsV treatments (Figure 3-5B), 47–98% of AsIII was detected in the fronds (Figure 3-5C). If AsV were reduced in the roots, then at least some AsIII should be detected. Secondly, after 1 d and 2 d of exposure, 50% and 21% AsIII was detected in the roots of the AsIII treatments (Figure 3-4B) compared to no AsIII in the AsV treatments (Figure 3-5B). Yet the increased AsIII concentrations in the roots in the AsIII treatment didn't translate to greater arsenic concentrations in the fronds (Figure 3-4C).

After 1 d and 2 d of exposure, arsenic concentrations in the fronds were 26 and 54 mg kg^{-1} for the AsIII treatment compared to 25 and 33 mg kg^{-1} for the AsV treatments (Figures 3-4C and 3-5C). The data clearly showed that (1) *P. vittata* was able to take up both AsIII and AsV and translocated them from the roots to the fronds, and (2) regardless of arsenic species supplied, AsV dominated in the roots while AsIII dominated in the fronds. This would indicate the presence of an arsenite oxidizing enzyme in the roots and arsenate reducing enzyme in the fronds of the fern. The activity of microbes in the oxidation of As III is another possibility.

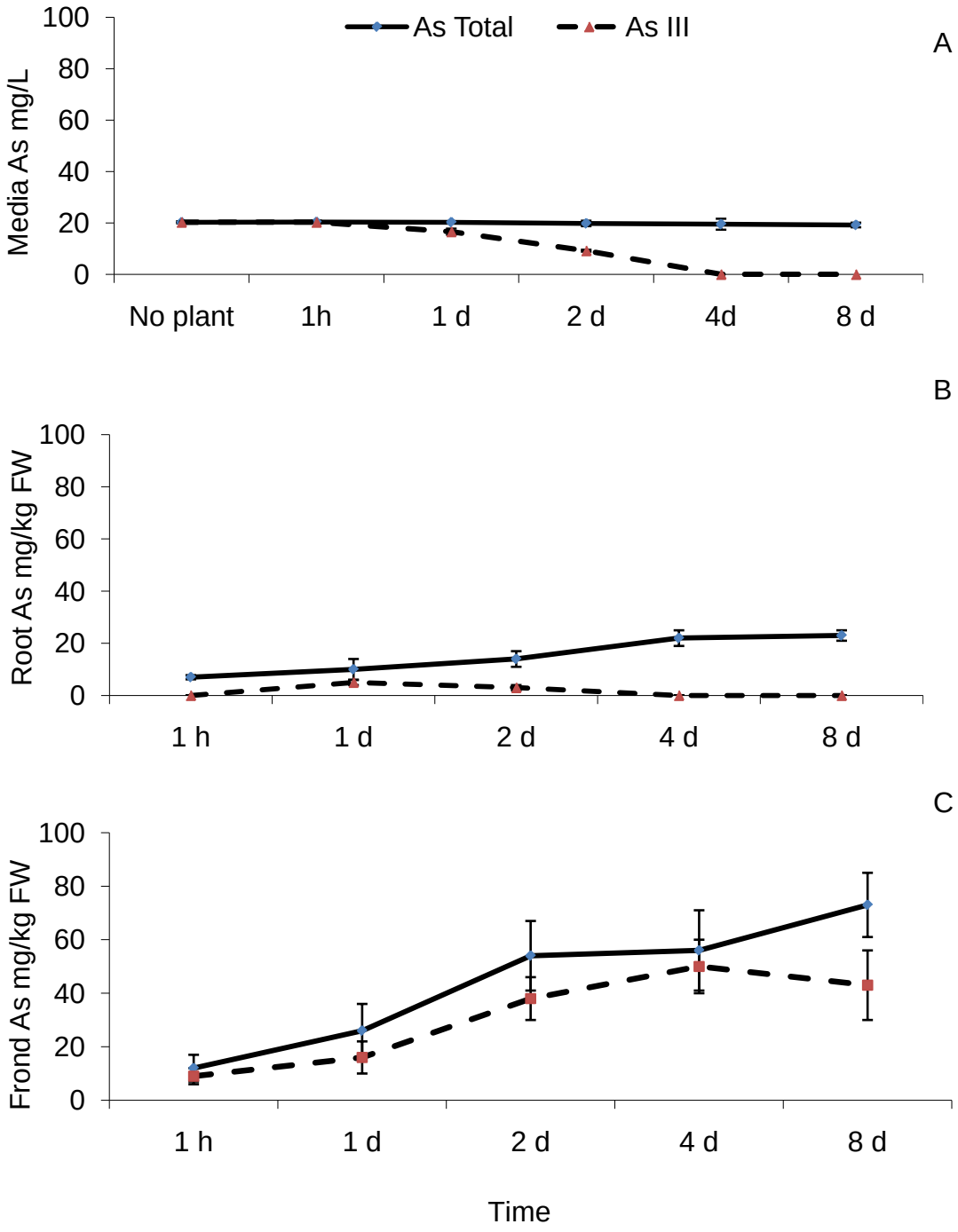

Figure 3-4. Arsenic speciation when treated with 0.27 mM AsIII for 8 d. A) growth media, B) roots and C) fronds of *P. vittata*.

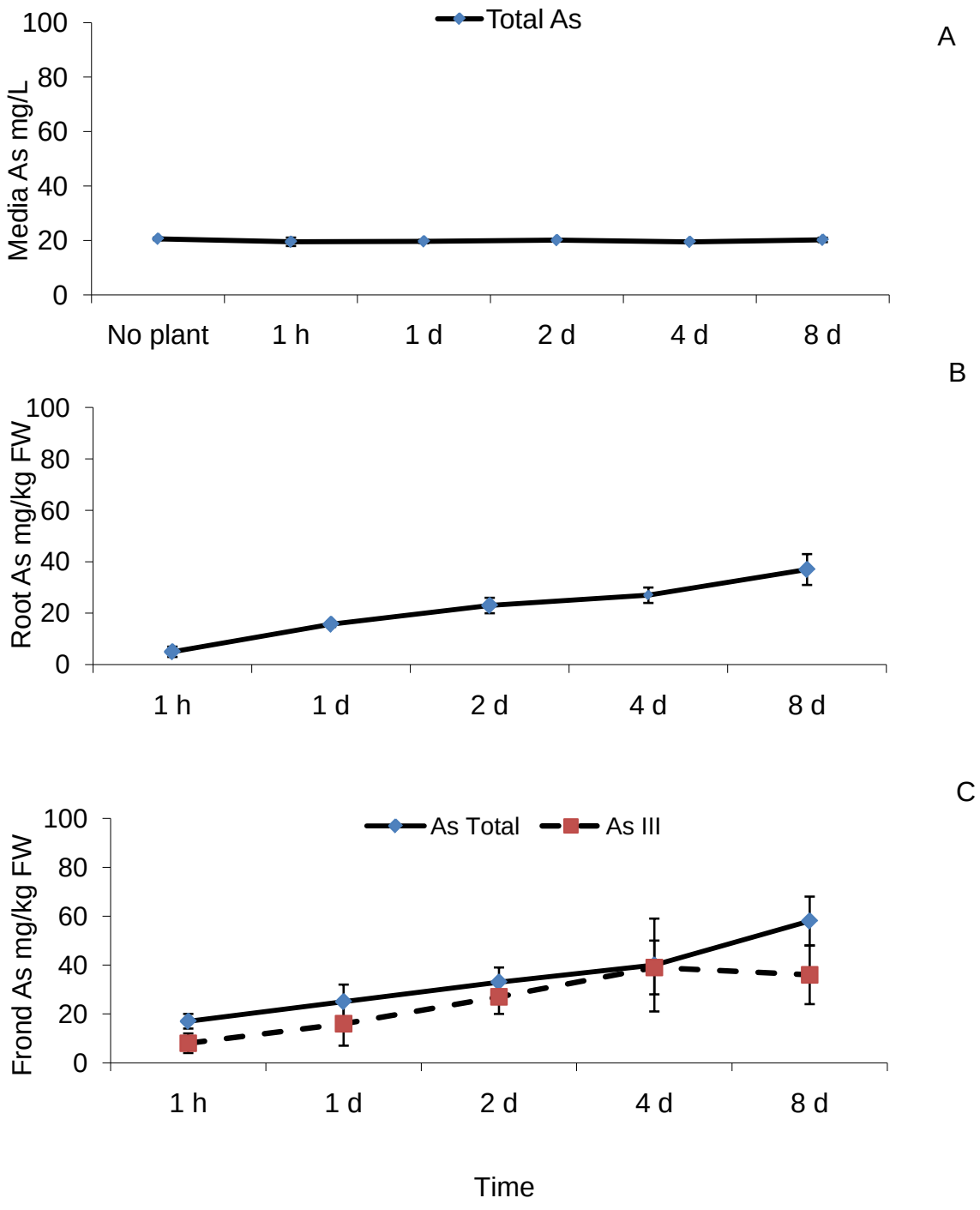

Figure 3-5. Arsenic speciation when treated with0.27 mM AsV for 8 d. A) growth media, B) roots and C) fronds of *P. vittata*

Though the data did not support the hypothesis that AsV reduction occurs primarily in the roots, they supported the hypothesis of faster translocation of AsIII than AsV from the roots to fronds. Except for 1 h data, arsenic translocation was more rapid with AsIII than AsV treatment. For example, the translocation factor, which is defined as the ratio of arsenic concentrations in the fronds to the roots, for AsIII treatment was 2.5–3.9 compared to 1.4–1.6 for the AsV treatment (Figures 3-4B-C and 3-5B-C).

To help determine the location of arsenic reduction in *P. vittata*, arsenic speciation was conducted in the roots and fronds of *P. vittata* after growing in 0.10 mM AsIII for 1 d (Figure 3-1B). Though only ~33% AsIII was present in the growth media (Figure 3-3A), ~42% and 86% of AsIII were detected in the roots and fronds (Figure 3-3B), respectively. Since no difference was observed in the uptake rate of AsIII and AsV by *P. vittata* in the absence of P, the data were consistent with the hypothesis that AsIII reduction occurred in the roots. This was possible since rhizomes were not separated from the roots in this experiment.

Arsenic Speciation under Sterile Conditions

It is unclear whether *P. vittata* or microbes are responsible for AsIII oxidation. Arsenite oxidizing bacteria may require specific carbon sources to oxidize AsIII. For example, a strain of AsIII oxidizing bacteria N-26 can grow heterotrophically in the presence of carbon sources like acetate, succinate, fumarate, pyruvate, malate, mannitol, sucrose, glucose, arabinose, fructose, trehalose, raffinose, maltose, xylose, galactose, lactate, salicin, glycerol, lactose and inositol but not on citrate, sorbitol or rhamnose (Ehlrich, 2002). Hence, *P. vittata* may have played three roles: (1) it provides the microbes with the C sources via root exudates; (2) it exudes enzymes to oxidize AsIII; and (3) its roots provide the source of microbes for AsIII oxidation. Though

microbial oxidation of AsIII in both oxic and anoxic environment has been reported, no report is available on AsIII oxidation by plant roots (Oremland and Stolz, 2003; Kulp et al., 2008).

To separate the effect of plant from microbes, arsenic speciation in the growth media was determined under sterile conditions. It was observed that no AsIII oxidation occurred with or without *P. vittata* after 14 d (Figure 3-6A). It should be noted that the ferns were grown under aseptic conditions from the spore stage indicating no impact of microbes on its rhizosphere. Hence, microbes and not *P. vittata* were directly involved in AsIII oxidation in the growth media. However, under natural conditions, *P. vittata* may have facilitated microbially mediated AsIII oxidation by providing the microbes associated with the roots (Al Agely et al., 2005) and the required carbon sources via root exudates (Tu et al., 2004a). Here, the original source of microbes would be from the potting mixture or the soil where *P. vittata* grew before transferring to hydroponic system.

Since AsIII was unstable in water and possibly in the soil solution in the presence of *P. vittata*, efforts should be made to monitor arsenic species in the growth media while comparing AsIII and AsV uptake by *P. vittata*. However, the data from the sterile experiment indicates that AsIII was stable and it clearly supports the hypothesis that AsIII was predominantly oxidized in the roots including rhizomes. After growing *P. vittata* in the sterile media containing 0.10 mM AsIII for 1 d, no AsV was present in the media (Figure 3-6A). However, 35% AsV was present in the roots (Figure 3-6B). The results suggested that once taken inside the roots, AsIII was oxidized to AsV in the roots. Similarly, 48% AsV was present in the roots after 14 d exposure though AsV was

absent in the growth media (Figure 3-6B). There was a higher concentration of AsIII in the roots in the sterile experiments as the rhizomes were too small to be separated from the roots. But the presence of 48% AsV clearly supports the hypothesis that AsIII was predominantly oxidized in the roots including rhizomes.

Arsenic reduction predominantly occurs in the pinnae based on studies using excised *P. vittata* (Tu et al., 2004b). After exposing excised *P. vittata* to 0.67 mM AsV for 2 d, 86% and 24% AsIII was detected in the excised pinnae and roots including rhizomes. Hence, AsV reduction occurs in both the roots and pinnae and the pinnae have much more reducing power than the roots since the arsenic concentration in the excised pinnae is 15-fold greater than that in the roots. The fact that 92% and 61% AsIII were detected in the pinnae and roots after exposing excised *P. vittata* to 0.67 mM AsIII for 2 d was consistent with the hypothesis that AsIII oxidation occurred in the roots (Tu et al., 2004b). This indicates more oxidation in the roots and more reduction in the pinnae regardless of whether AsIII or AsV was supplied to *P. vittata*.

Arsenite Oxidation in Sonicated Extract

To further study the effect of microbial or root enzymatic activity on arsenic transformation, the sonicated extract of the roots were tested in a mixture of AsV and AsIII solution. The *P. vittata* root sonicated extract maintained at room temperature (RS) resulted in 32% oxidation of AsIII in the mixture of AsV (1.33 µM) and AsIII (1.33 µM) in 1 h, 43% in 1d and 100% after 4d indicating that either the root extract or microbes have resulted in AsIII oxidation (Figure 3-7). Boiling the sonicate (RSB) to 100ºC for 10 minutes may have inhibited enzymatic activity in the AsIII and AsV mixture, which showed a decrease in oxidation by 16%-29% compared to the sonicated extract at room temperature for 1 h.

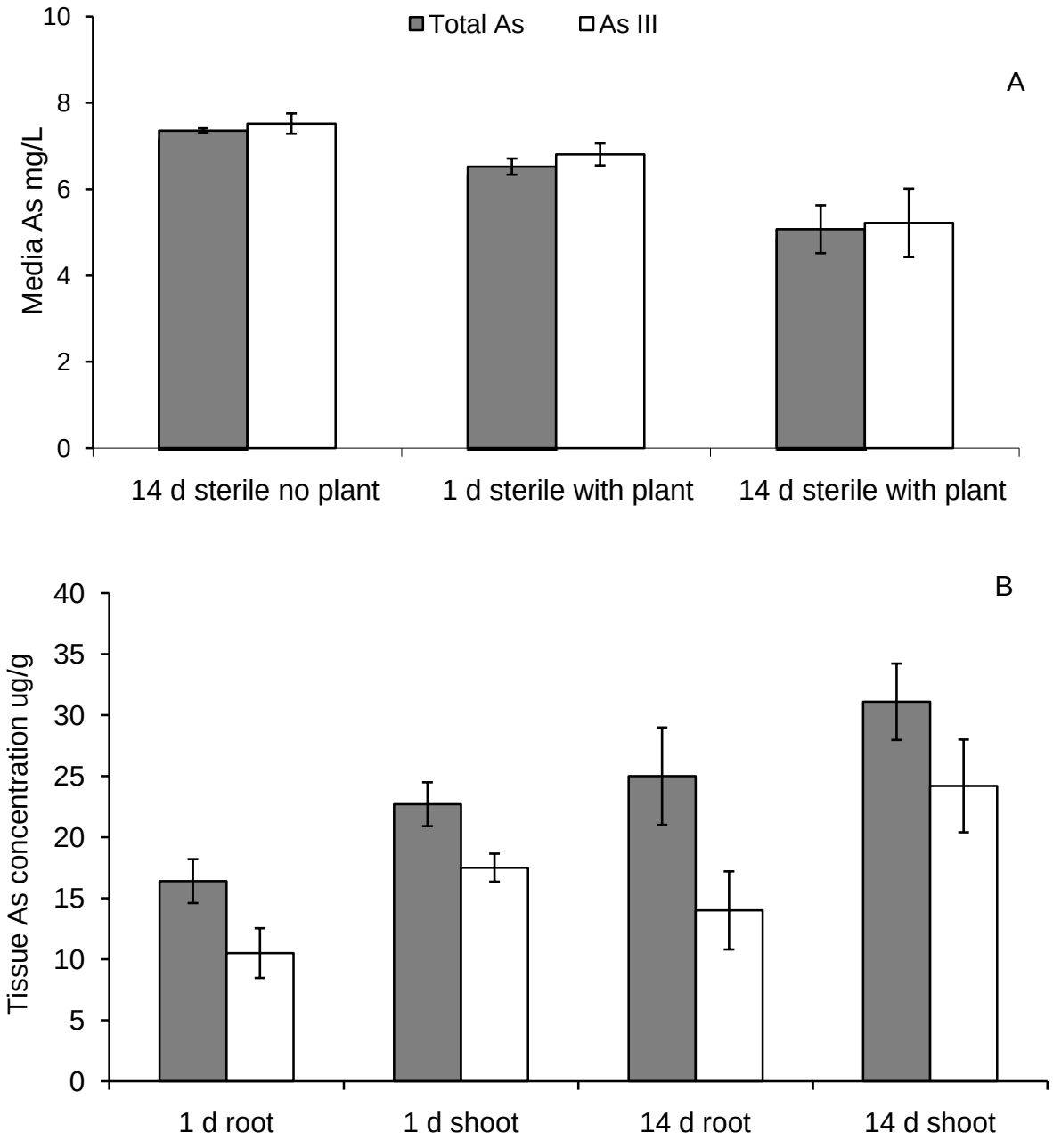

Figure 3-6. As speciation when treated with 0.10 mM AsIII for 1 and 14 d under sterile conditions. A) growth media with and without *P. vittata* and B) *P. vittata* roots and fronds.

However, filtration of the sonicated extract with a 0.2 µm filter indicated an oxidation similar to the sonicated extract that was boiled.

At 8 d, the sonicated extract when boiled or filtered resulted in an oxidation of 5-33% of the AsIII after 8 d compared to 100% oxidation after 8 d when untreated root sonicated extract was used. Hence a decrease in microbial population by filter sterilization or boiling resulted in a decrease in oxidation of AsIII as enzymatic activity is not affected by filtration. This reduction in AsIII oxidation indicates a predominant microbial role in oxidizing AsIII.

Future Research

The results shed new insights into the dynamics of arsenic transformation in the growth media and *P. vittata* tissues. It was clearly demonstrated that AsIII oxidation occurred in the media and the roots of *P. vittata*. This research has made use of the hydroponic conditions rich with microbes, sterile conditions and root extracts. The results of the study indicate the arsenite oxidation to be microbially mediated. This study will open the door to a number of research ideas such as the presence of a novel arsenite oxidizing bacteria in the rhizosphere of *P. vittata*, the characterization of root exudates in *P. vittata* and identify the carbon source required for the growth of these bacteria and also to investigate the use of this fern in arsenite oxidation and remediation in water treatment plants.

While arsenate reductase and cytosolic triosephosphate isomerase from the fronds were previously implicated in arsenate reduction (Ellis et al., 2006; Rathinasabapathi et al., 2006), others have reported arsenate reductase activities in protein extracts from the roots with rhizomes (Duan et al., 2005; Liu et al., 2009).

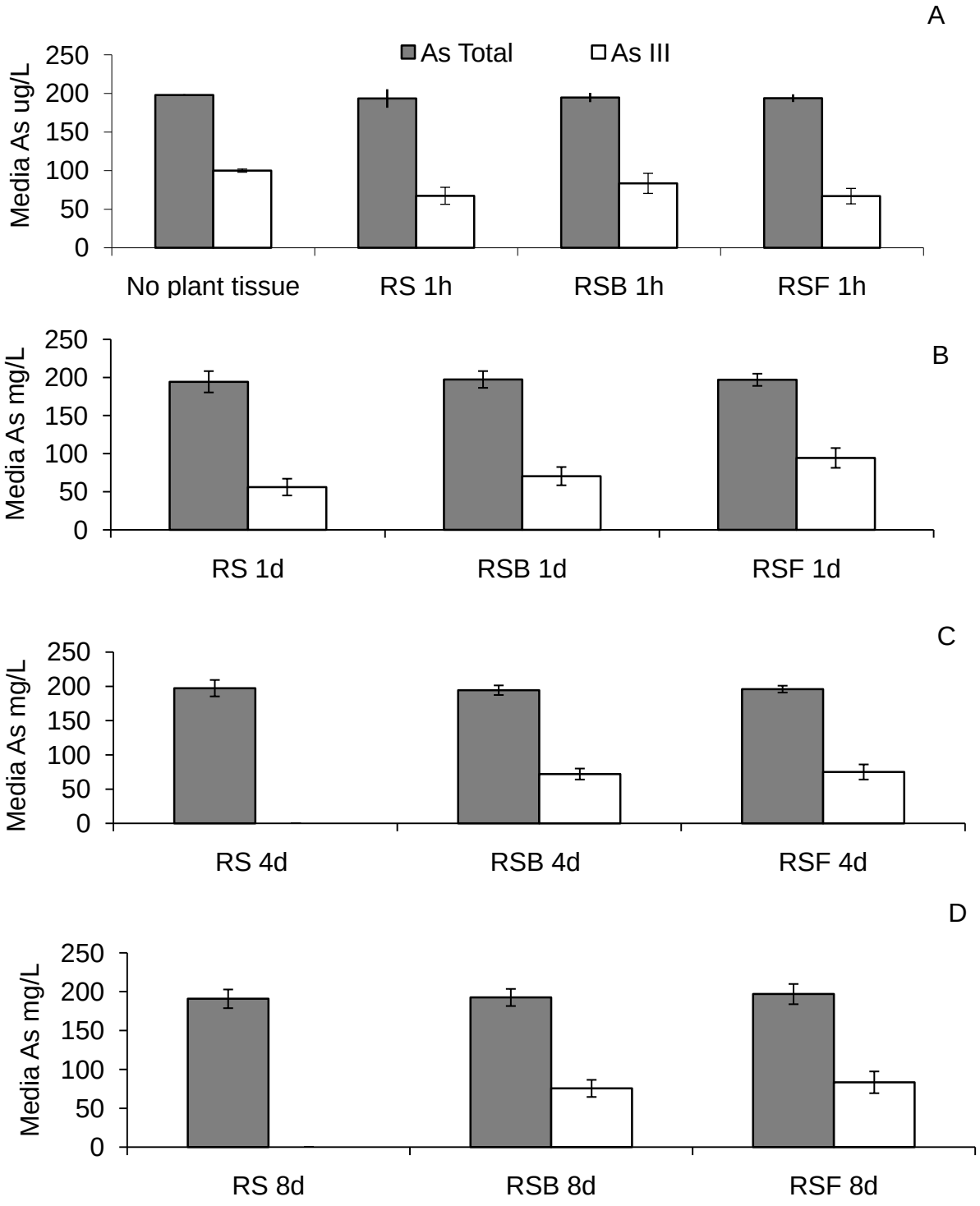

Figure 3-7. As speciation in sonicated extract of *P. vittata* roots incubated for 8 d with 1.33 µM AsV and 1.33 µM AsIII (untreated (RS), boiled (RSB) and filtered (RSF)) A) 1 h treatment B) 1 d treatment C) 4 d treatment D) 8 d treatment

CHAPTER 4
ARSENIC TRANSFORMATION AND SPECIATION IN *PTERIS VITTATA* BIOMASS AND XYLEM SAP

Arsenic Speciation

Under natural conditions plants growing in soils uncontaminated with arsenic have a low translocation factor of As, less than 0.1 (Cullen and Reimer, 1998). Unlike hyperaccumulators, these plants, which are called excluders, have restricted uptake and restricted translocation of As (Zhao et al., 2010). It is important to understand the speciation of arsenic in plants to understand its toxicity to herbivores at higher trophic levels (Zhao et al., 2010). Plants growing in As contaminated environments take up As and the species of As depends on plant species. Arsenic is predominantly present as AsIII in many plants (Dhankher et al., 2002). Unlike other plants, hyperaccumulators of As contain AsIII as uncomplexed species due to low phytochelatin content in tissue (Raab et al., 2004). It is believed that *P. vittata* takes up AsIII and AsV by the roots (Wang et al., 2002; Fayiga et al., 2005), translocates AsIII and AsV from the roots to fronds (Kertulis-Tartar et al., 2005; Singh and Ma, 2006), reduces AsV to AsIII in the fronds (Bondada et al., 2004; Tu et al., 2004b), and transports AsIII into vacuoles for storage (Lombi et al., 2002).

In the biomass of *P. vittata*, inorganic arsenic species AsV and AsIII account for more than 95% of the total arsenic with minimal organic species (Zhang et al., 2002), therefore, these two species are considered in most arsenic speciation studies. To better understand the different parts in *P. vittata* and the localization of As in the fern tissue a schematic of the fern has been provided (Figure 4-1). The below ground biomass of this fern includes rhizomes (horizontal stems) and roots whereas their aboveground biomass consists of the fronds (leaves).Fronds are composed of a stipe

(leaf petiole), rachis (continuation of central stalk) and pinnae (leaflets) that are attached to the rachis. Based on the fact that arsenic is predominantly present as AsIII in excised pinnae exposed to 0.67 mM AsV (86% AsIII) (Tu et al., 2004), and in pinnae supplied with foliar AsV at 1.3 mM (65–86% AsIII) (Bondada et al., 2004), it is hypothesized that AsV reduction occurred primarily in the pinnae. Unlike pinnae, both AsIII oxidation and AsV reduction occur in the roots including rhizomes. After 1 d exposure, 61% of the arsenic is present as AsIII in excised roots exposed to 0.67 mM AsIII (71% AsIII in the growth media) and 76% as AsV in excised roots exposed to 0.67 mM AsV (97% AsV in the growth media) (Tu et al., 2004). Hence, it is hypothesized that AsIII oxidation occurs in the roots exposed to AsIII (71% AsIII in the growth media compared to 61% AsIII in the roots), and AsV reduction occurs in the roots exposed to AsV (97% AsV in the growth media compared to 76% AsV in the roots) (Kertulis-Tartar et al., 2005). On the other hand, regardless of arsenic species supplied (0.5 µM AsIII or AsV), AsIII dominates the xylem sap of *P. vittata*. Here, Su et al. (2008) hypothesized that AsV reduction mainly occurs in the roots, and the reduced AsIII is then rapidly translocated to the fronds. The data are consistent with Duan et al. (2005) who showed arsenic reductase activity in the roots but not in the fronds. However, in both experiments, they did not separate the roots from the rhizomes. Therefore, it is unclear whether AsV reduction occurs in the roots and/or rhizomes. The overall objective of this study was to understand the dynamics of arsenic transformation in different parts of the fern and in the sap of *P. vittata.* The specific objectives were to investigate the location of arsenic oxidation and reduction in the sap and biomass of *P. vittata*. The results from this study should shed light on the mechanisms of arsenic hyperaccumulation by *P. vittata*.

Materials and Methods

Six-month old *P. vittata* with 8–12 fronds (50–60 cm in height) were used. Larger ferns were chosen to ensure easier sap collection. After acclimation, the ferns grew in 0.2-strength HS containing 0.10 mM AsV for 8 d. Sap was collected by 2 different methods. Young succulent fronds (cut 1 cm above the rhizomes) were used to collect xylem sap via a portable Scholander pressure chamber (PMS 1000, MPS Instrument Co., Corvallis, OR). Also, sap oozing out of the cut where the frond was separated 1 cm above the rhizomes was collected using a pipette and considered sap from the rhizomes. A total of 50 µL sap was obtained from each of the cut fronds. Separately, fresh fronds, rhizomes and roots from the same ferns, which were used for sap collection, were used for arsenic speciation. The pinnae, rachis ± stipe and the roots (without rhizomes) were divided into three sections, i.e., upper, middle and lower 1/3 based on their length (Figure 4-1) to understand the location of arsenic transformation. The rhizomes as a whole were used for As speciation.

Results and Discussion

Arsenic was supplied as AsV in the hydroponic media and there was a predominance of AsV in the roots. Based on the fact that AsV dominates the roots and AsIII dominates the fronds of *P. vittata*, it is hypothesized that AsV reduction occurs primarily in the fronds whereas AsIII oxidation occurs mainly in the roots. Su et al. (2008) conducted sap arsenic analysis and proposed that arsenic reduction occurs in the roots, and the reduced AsIII is then rapidly translocated to the fronds. However, in their experiment, they did not separate the rhizomes from the roots; therefore the sap they collected was actually from the rhizomes.

Xylem Sap Arsenic Speciation

To better understand the location of arsenic transformation in *P. vittata*, arsenic speciation was conducted in the sap collected from the fronds and rhizomes after exposure to 0.10 mM AsV for 8 d (Figure 4-3A). Sap from the fern rhizome and the frond were used to understand the speciation of arsenic in the fern. The efforts to collect sap from the roots of large fern plants were unsuccessful. The arsenic concentration in the rhizomes sap was 8-fold greater than that in the growth medium, i.e., 61 compared to 7.5 mg L^{-1} (Figure 4-3A).

This is consistent with the observation of Su et al. (2008) who reported 18–51 times greater arsenic concentration in the sap than the initial concentration of 5 µM in the growth media. Though arsenic concentration in the rhizomes sap was much lower than that in the frond sap, i.e., 61 versus 650 mg L^{-1}, AsIII dominated both saps (Figure 4-3A).This is consistent with the fact that when treated with AsIII or AsV most plants have a predominance of AsIII in its xylem sap (Zhao et al., 2009). But this characteristic varies from plant to plant as rice loads AsIII into xylem more efficiently than wheat or barley (Su et al., 2008). This may indicate a highly developed AsIII reducing system in the roots. The roots and shoots of rice indicate AsV reduction but since AsIII is present in the xylem sap, the root may be an important location for AsV reduction (Duan et al., 2007). However, in the case of the fern sap analysis performed just above the rhizome indicates a predominant AsIII, which puts forth the question whether the rhizomes are key centers of AsV reduction and not the roots.

Biomass Arsenic Speciation

The roots were separated into lower, middle and upper third after separating from the rhizomes (Figure 4-1).Total arsenic concentrations decreased from 82, to 70, and to

50 mg kg^{-1} as it traveled from the lower to middle and to upper roots (Figure 4-3B). However, regardless of the root location, AsV dominated the roots with 92–93% being AsV.

In most experiments reported in the literature, the roots and rhizomes are not separated (Singh and Ma, 2006). This is partially because many people are unaware the presence of rhizomes and partially because, for young ferns, rhizomes are not easily separable from the roots. Rhizomes are actually underground stems from which the roots and fronds arise (Figure 4-1). They also transport water and nutrients up and down its length (Foster, 1984). However, more AsIII was present in the frond sap (86% AsIII) than the rhizome sap (71% AsIII), again suggesting further AsV reduction in the fronds. Arsenic speciation data in the rhizome sap (71% AsIII; Figure 4-3A) was consistent with that in the rhizome tissue (68% AsIII; Figure 4-3B). The data suggests that when arsenic was translocated from the roots (7% as AsIII) into the rhizomes (71% as AsIII), some of the AsV was reduced to AsIII in the rhizomes (Figure 4-3B). After the mixture of arsenic (71% AsIII) was translocated from the rhizomes into the pinnae, some of the AsV was reduced to AsIII in the pinnae (>90% as AsIII) (Figure 4-3B). Since rhizomes are underground stems for ferns, they may have similar function as the fronds (Figure 4-1).

The hypothesis that arsenic reduction occurred in the pinnae is also supported by the arsenic speciation data in the rachis (main leaf stalk with attached leaflets), which was separated into lower, middle and upper third (Figure 4-1). The lower portion of the rachis included the stipe (petiole without leaflets), and fewer pinnaes compared to the middle and upper rachis. As arsenic moved from the lower to upper rachis, arsenic

concentrations increased from 47 to 160 and to 244 mg kg^{-1} with proportionally more being AsIII (23%, 71% and 72%). The data are consistent with those of Pickering et al. (2006) who reported 76% AsIII in the rachis based on Xray absorption spectroscopy analysis. The fact that much higher AsIII was observed in the upper rachis (71–72% AsIII where more pinnae are located) than the lower rachis (23% AsIII with much fewer pinnae) supports the hypothesis that arsenic reduction occurred in the pinnae.

If arsenic were mainly reduced in the rhizomes and then translocated from the rachis to the pinnae, then one would not expect substantial changes in arsenic speciation along the rachis. Though pinnae were separated from the rachis, the arsenic concentrations and speciation in the rachis was probably affected by pinnae, which had much greater arsenic concentration (463–588 mg kg^{-1}) and was dominated by AsIII (90–100%) (Figure 4-3). The fact that substantially more AsIII was present in pinnae (90–100%) than that in rachis (23–72%) is consistent with the hypothesis that arsenic reduction occurred in the pinnae. AsV reduction predominately happened in the rhizomes and the pinnae of *P. vittata* though limited AsV reduction also occurred in the roots. The results also open new questions into the tissue distribution of arsenic reducing and oxidizing enzymes in *P. vittata*.

Research Findings

The results reported here are consistent with the fact of the presence of arsenate reducing enzymes in the fronds and rhizomes but not in the roots. The presence of an arsenite oxidizing enzyme in the fern roots have to be studied using molecular techniques.

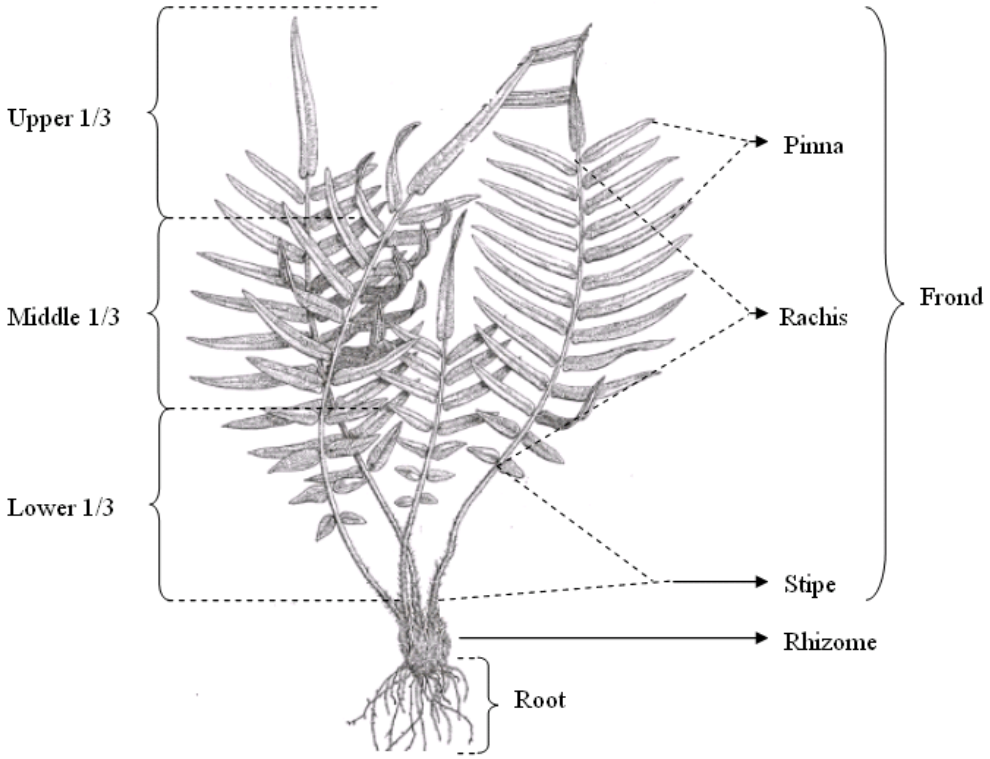

Figure 4-1. Schematic of different parts of *P. vittata* including root, rhizome, stipe, pinna, rachis, and frond. Modified from http://www.bioscripts.net/flora/plantas/FI/Pteris%20vittata.jpg.

Figure 4-2. Scholander pressure chamber

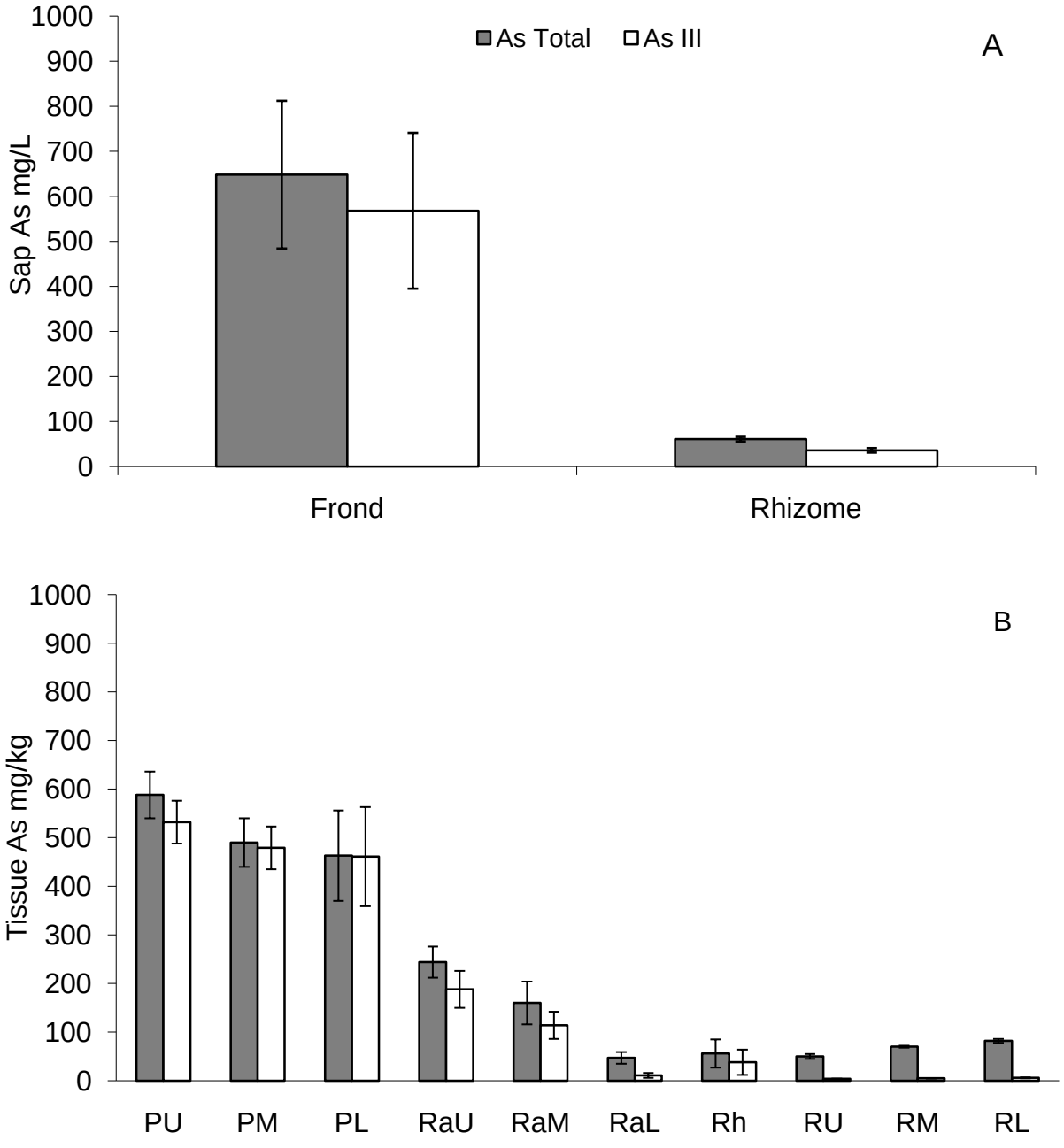

Figure 4-3. Arsenic speciation in the sap and tissue of *P. vittata* treated with 0.1mM AsV for 8 days. A) Sap from fronds and rhizomes, and B) in the pinnae (P), rachis (Ra), rhizomes (Rh), and roots (R). U, M and L = upper, middle and lower and the lower rachis includes the stipe with few pinnae (see Figure 4-1).

CHAPTER 5
UPTAKE AND TRANSLOCATION OF ARSENITE AND ARSENATE BY *PTERIS VITTATA* L.: EFFECTS OF GLYCEROL, ANTIMONITE AND SILVER

Arsenic Uptake and Translocation

The speciation of As depends on the environmental conditions a plant is in and this greatly affects the uptake rate and mechanism of As into the plant. The mechanisms of plant arsenic uptake depend on arsenic species as they are structurally and chemically different. Most plants take up AsV via phosphate transporters whereas they take up AsIII via aquaglyceroporin transporters.

Arsenate, with dissociation constants 2.2, 6.97 and 11.5 behave as oxyanions in solution, i.e., $HAsO_4^{2-}$ and $H_2AsO_4^-$ at pH 5-7 (Jeon et al., 2009), which are similar to phosphate, HPO_4^{2-} and $H_2PO_4^-$ (Teo et al., 2009). As chemical analogs, they compete for entry through the membrane phosphate transport system. Moreover, AsV can repress genes involved in the phosphate starvation response suggesting that AsV may interfere with the phosphate signaling mechanism (Catarecha et al., 2007) and can be taken up by plants instead of phosphate. This competitive effect has been observed in *E. coli* (Willsky et al., 1980), yeast (Yompakdee et al., 1996), and plants including barley (Lee, 1988), wheat (Zhu, 2006), rice (Meharg, 2004), *H. lanatus* (Meharg and Macnair, 1992), and *Brassica napus* (Quaghebeur and Rengel, 2005). *Arabidopsis thaliana* mutants defective in P transporters are more tolerant to AsV (Catarecha et al., 2007). Studies also indicate that AsV uptake into *P. vittata* is by the phosphate transporter (Wang et al., 2002).

Arsenite (H_3AsO_3), on the other hand, is present as neutral species at pH <9.2 (O'Day et al., 2006) and are transported via aquaglyceroporins in *E.coli* (Meng et al., 2004), yeast (Robert et al., 2001) and human cells (Liu et al., 2002). In plants a nodulin

26-like intrinsic proteins (NIPs) are the structural and functional equivalents of bacterial and mammalian aquaglyceroporin (Wallace et al., 2005). Hence a similar mechanism of AsIII uptake is expected in plants as well. In rice roots, Lsi1 (OsNIP 2;1) is a major route of entry for silicic acid (Ma et al., 2006) and AsIII (Ma et al., 2008) and mutation in this protein resulted in a 60% loss of AsIII uptake. NIP channels in rice, Lsi2, allow bidirectional transport of AsIII as well as the efflux of As from the exodermis and endodermis cells towards the stele (Ma et al., 2008) and mutation to this gene resulted in a dramatic effect on AsIII loading into the xylem (Ma et al., 2007). This may be a prominent step in *P. vittata* as well, where efficient AsIII efflux systems pump As into the xylem.

Several analogues of AsIII compete with it during uptake into living cells. Antimonite (SbIII) is one such analogue, which is chemically and structurally similar to AsIII. The pKa values of SbIII and AsIII are 11.8 and 9.2, respectively, and hence both exist as neutral solutes [$Sb(OH)_3$ and $As(OH)_3$] in the environment (Meng et al., 2004) and may compete for plant uptake. Several proteins have been identified that transport both AsIII and SbIII and this includes AtNIP 5;1, AtNIP 6;1, AtNIP 7;1, OsNIP 3;2, OsNIP 2;1 and OsNIP 2;2 (Bienert et al., 2008). A similarity also exists among AsIII, SbIII and a specific conformation of glycerol, $C_3H_5(OH)_3$, where the 3 hydroxyl groups occupy nearly the same positions as $As(OH)_3$ and $Sb(OH)_3$ with similar charge distribution and volume.

The Sb content in the earth's crust is low and is elevated in Sb mining areas (Flynn et al., 2003). Though SbIII is not essential for organisms, it is taken up by living cells through the same channel as glycerol and AsIII. The glycerol facilitator of *E. coli*

(GlpF) takes up SbIII and a lack of this transporter in mutants makes them resistant to both SbIII and AsIII (Sanders et al., 1997). Similarly, the glycerol facilitator in yeast (fsp1) can take up both SbIII and AsIII (Wysocki et al., 2001). Mammalian aquaglyceroporins AQP7 and AQP9 also allow SbIII and AsIII to permeate (Liu et al., 2002). The proteins that have been studied to transport both AsIII and glycerol in plants include AtNIP 1;1, AtNIP 1;2 and AtNIP 6;1 (Kamiya et al., 2009 and Tanaka et al., 2008). The smaller diameter of $As(OH)_3$ and $Sb(OH)_3$ is an additional advantage for transit though the narrowest region of the glycerol transporter channel (Bhattacharjee, 2008). Hence it can be predicted that a glycerol facilitator that transports glycerol may also be responsible for AsIII and SbIII uptake in *P. vittata* and the presence of SbIII or glycerol in the substrate may inhibit AsIII uptake by the cells.

The function of these aquaporins can be inhibited by chemicals that interfere with the permeation of water and neutral solutes. Some of the known aquaporin inhibitors include mercury, silver, gold, copper, phloretin, tetraethyl ammonium salts and acetazolamide compounds (Haddoub, 2009). Silver is a powerful inhibitor of aquaporins due to their interaction with sulfhydryl groups of cysteine near the conserved NPA motif, which blocks the constriction regions of the channel. Silver inhibition is rapid and not reversible by mercaptoenthanol (Niemietz and Tyerman, 2002). In human red blood cells, the inhibition of aquaporins was 200 times more potent than mercury compounds (Sha'afi, 1977). Silver nitrate has been tested on peribacteriod membranes of soybean nodules, plant plasma membrane vesicles and human red blood cells. Compared to the widely used mercurial compounds for aquaporin inhibition, which is toxic to plants (Patra and Sharma, 2000), silver nitrate is less phytotoxic and more specific.

In the presence of transition metals like silver, the pKa of the thiol moiety is lowered from 8.5 to 6 which helps in its co-ordination with transition metal ions (Scozzfava et al., 2001). Another amino acid that can interact at silver concentrations of greater than 200 µM is histidine (Wells et al., 1995).

Using AsIII competitors and aquaporin inhibitors serves as an important preliminary tool to understand the uptake mechanism of AsIII in *P. vittata*. The objective of this paper was to understand if the aquaglyceroporin channels are responsible for AsIII uptake in *P. vittata* by investigating 1) the competitive effects of glycerol and SbIII, and 2) the inhibiting effect of aquaporin $AgNO_3$ on AsIII and AsV uptake by *P. vittata.*

Materials and Methods

Experimental Setup

P. vittata ferns, 4 months of age and 15-18 cm in height, purchased from Milestone Agriculture Inc. (Apopka FL, U.S.A.) were used for this study. Efforts were taken to ensure uniform plants were used for the study. The ferns were acclimatized in an aerated hydroponic system with 0.2 strength HS and pH adjusted to 5.7 with 1 mM KOH-MES [(2-(N-morpholino) ethanesulphonic acid] buffer for 2 weeks. During the experiments photon flux of 350 $\mu molm^{-2}s^{-1}$ was used using cool and warm white fluorescent lamps with temperature maintained at 23-28°C and relative humidity 65-70%.

After acclimatization in HS, the ferns were acclimatized in a solution of 0.5 mM MES (pH 5.7) and 0.5 mM $CaCl_2$ for 1 day. Following this they were transferred to opaque containers containing 1 L of solutions spiked with 0.1 mM AsV ($Na_2HAsO_4.7H_2O$) or AsIII (Na_2AsO_3) (Sigma, St. Louis, MO). The arsenic was provided in deionized water in all experiments to minimize P competition for AsV.

Time Dependent Uptake Study

An experiment was conducted to study the time dependent uptake of AsIII and AsV into the fern. *P. vittata* were grown in 1L solution of 0.1 mM AsIII or AsV for 1, 2, 4, 6 and 24 h. The root samples were analyzed to study the short term influx of AsIII into *P. vittata*. The water samples were analyzed to understand AsIII stability in the media in the presence of the fern. The results of both experiments helped to decide the time required for the uptake competition and inhibitor experiments.

Competition and Inhibition Study

Glycerol [$C_3H_5(OH)_3$] and SbIII (potassium antimonyl tartarate) at concentrations 0, 0.1, 1, 10, and 100 mM were used with 0.1 mM AsIII or AsV. The inhibition studies used silver nitrate ($AgNO_3$) at concentrations 0, 0.001, 0.01 and 0.1 mM against 0.1 mM AsIII or AsV. For the inhibition study the ferns were first treated with $AgNO_3$ for 1 h before addition of AsIII or AsV to ensure prior inhibition of the aquaporin. The concentrations of As, glycerol, SbIII and Ag used showed no phytotoxic effects on *P. vittata*. All experiments were performed in triplicate and the duration of the study was 1h. This was to minimize conversion of AsIII to AsV by oxidation. Following the completion of the experiment and to further clarify the effect of silver nitrate on aquaporin inhibition and AsIII uptake, this experiment was repeated for 2 h using the most effective concentration of $AgNO_3$.

After the 1 h or 2 h treatment, the water samples were collected for arsenic speciation. The fern was washed with distilled water followed by rinsing in ice-cold phosphate buffer (1 mM Na_2HPO_4, 10 mM MES and 0.5 mM $Ca(NO_3)_2$, pH 5.7) to ensure As desorption from the root surface. Following this, the plants were again washed with distilled water. The plant parts were blotted dry, weighed and stored in

-80°C for As speciation analysis. For arsenic speciation in the fern, the samples were ground using liquid nitrogen and extracted with methanol: water (1:1 v/v) under sonication for 2 h (Zhang et al., 2002). AsV and AsIII were separated using an arsenic speciation cartridge (Waters SPE cartridge), which retains arsenate (Meng et al., 2001). For total arsenic, air-dried fern tissue was ground (20-mesh), digested with concentrated HNO_3 (1:1, v/v), and followed by 30% H_2O_2 (U.S. EPA method 3050).

Total As and Sb in the growth media and fern tissues were determined by a graphite furnace atomic absorption spectrophotometer (GFAAS; Varian 240Z, Walnut Creek, CA). In addition, standard reference materials from the National Institute of Science and Technology (Gaithersburg, MD) and appropriate reagent blanks, internal standards and spikes were used as quality checks and were within 100±20% of the expected values.

Data Analysis

The treatment effects were examined by analysis of variance based on the linear model procedure of the Statistical Analysis System (SAS Institute Inc., 1986).

Results and Discussion

A preliminary experiment on arsenic stability in the growth media indicates that In the presence of *P. vittata*, AsIII was unstable in the growth media whereas AsV was stable (Figure 5-1A). AsIII was stable for 1 h, after which it was gradually transformed to AsV. In comparison, AsV was stable beyond 24 h since it was maintained under an aerated oxidized system (Figure 5-1A). The influx of both AsIII and AsV into *P. vittata* roots was linear for up to 8 h (Figure 5-1B). To minimize AsIII oxidation in the media, 1 h was chosen for all the uptake experiments. During this period, the difference in AsIII and AsV uptake by *P. vittata* was minimal (Figure 5-1B).

Effects of Glycerol and SbIII on AsIII Uptake

AsIII uptake in rice (Meharg and Jardine, 2003) and Arabidopsis (Kamiya et al., 2009) is via aquaporins, which can transport water or neutral solutes. Aquaporins can either be water channel aquaporins or glycerol transporters (aquaglyceroporin) and are known to allow the passage of water, glycerol, carbon dioxide, nitric oxide, ammonia, hydrogen peroxide and metalloids such as AsIII, SbIII, boric acid, and silicic acid. The major difference between the two is in the constriction regions of the channel. The narrow constriction in water specific aquaporins have a diameter of water molecule at 2.8 Å and aquaglyceroporins have a diameter of glycerol molecule at 3.4 Å (Beitz, 2004).

The speciation analysis of the growth media samples in the glycerol and SbIII treatments indicated limited AsIII oxidation (2-10%) (Figures 5- 2A and 5- 4A), indicating that most of the As taken up by the plant was AsIII. This is if there was no oxidation of AsIII at the surface of the roots due the impact of microbial activity. Separate control experiments with 0.1 mM AsV treatment in the presence of different concentrations of glycerol (Figures 5-3B and 5-3C) and SbIII (Figures 5-5B and 5-5C) showed a similar trend as AsIII with no significant difference in plant As uptake. This was expected as AsV is taken up by the ferns through a phosphate transporter.

Studies by Nagarajan and Ebbs (2007) where 0.1 mM of SbIII had no impact on accumulation of As by *P. vittata* when treated with 0.1mM AsIII for 8 h indicates that the uptake of AsIII by the fern may be by a different mechanism compared to that of an SbIII transporter. The total arsenic concentrations in the fronds with the glycerol treatment (4.6 to 6.3 mg/kg As) were slightly greater than those in the SbIII treatment (4.4 to 5.7 mg/kg As).

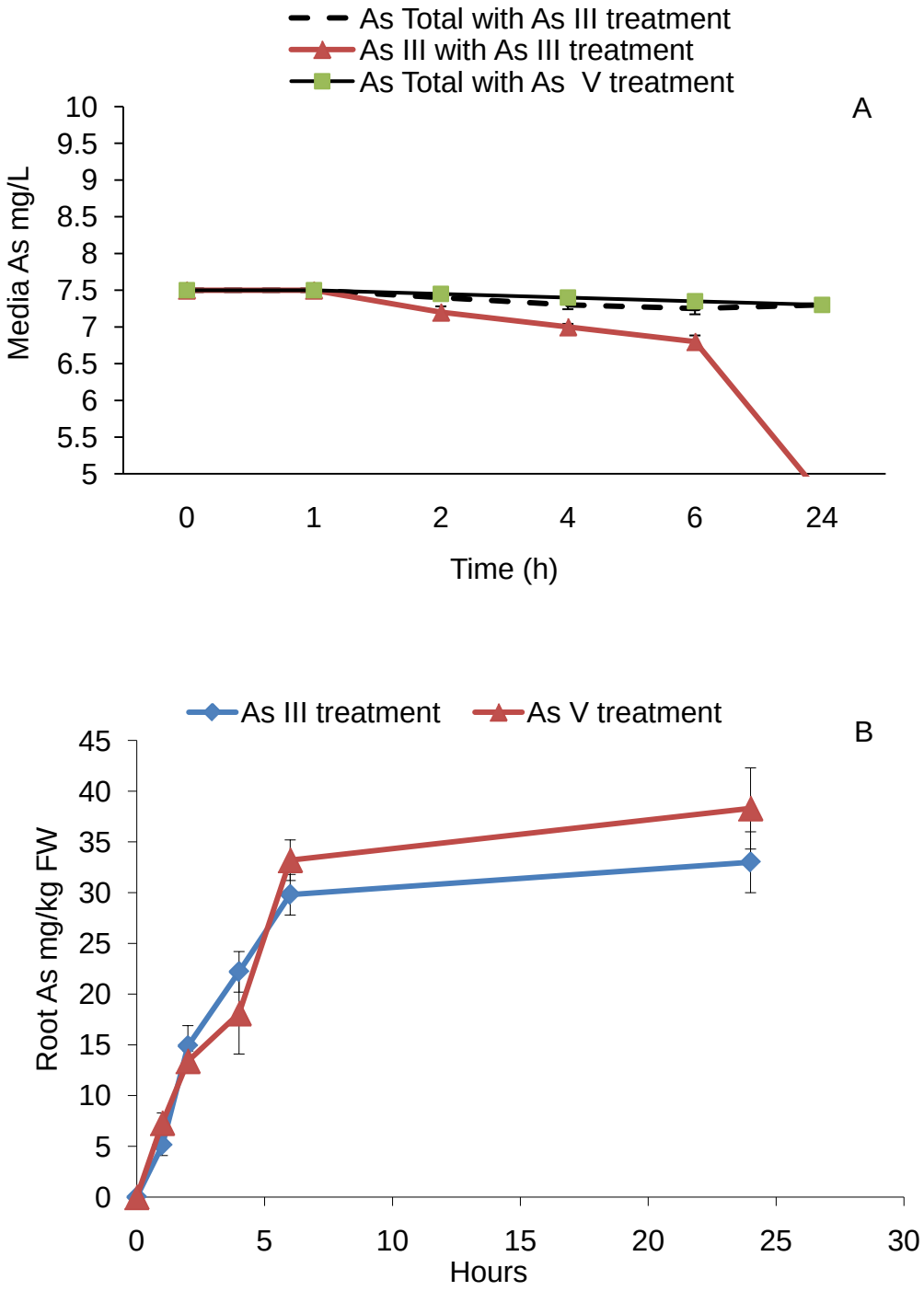

Figure 5-1. Arsenic speciation in the media and root of *P. vittata* exposed to 0.1 mM AsIII or AsV for 1, 2, 4, 6 and 24 h. A) Media i) As total and AsIII with AsIII treatment ii) As total with AsV treatment, and B) total As concentrations in the roots of *P. vittata*.

The root arsenic concentrations were 3.8-6.2 mg kg^{-1} and 3.9-4.2 mg kg^{-1} with the glycerol and SbIII treatments, respectively. The increased As uptake in the presence of glycerol may be due to the positive impact it has on microbes. Plant growth promoting rhizobacteria are known to enhance metal accumulation by enhancing plant growth or sequestering metal ions inside the cell walls (Khan, 2009).

Glycerol in the media can act as a carbon source for the microbes promoting As accumulation by *P. vittata*. On the other hand, SbIII is known to inhibit microbial activity in soil (An et al., 2009), causing reduced As uptake with SbIII compared to glycerol. The arsenic in the fronds was predominantly AsIII ranging from 78 to 96% in both glycerol and SbIII treatments whereas the roots contained almost all AsV (Figures 5-2B and 5-3B).

In addition to As, SbIII concentrations in *P. vittata* were analyzed. There was an increase in SbIII concentrations in the fronds and roots with an increase in SbIII concentration in the media (Figure 5-6).The fact that Sb concentrations in the roots (45 – 5,742 mg kg^{-1}) were much higher than those in the fronds (3.2 - 13 mg kg^{-1}) was observed by Muller et al., 2009. This indicates that *P. vittata* was an efficient accumulator of SbIII in the roots, but was ineffective in translocating SbIII from the roots to fronds during the 1 h experiment. For example, the highest Sb translocation factor (TF; ratio of Sb in the fronds to roots) in *P. vittata* was 0.07 (Figure 5-6). In comparison, the highest As TF in *P. vittata* treated with AsIII and SbIII was 2.4 (Figure 5-4), which indicates that, upon uptake, *P. vittata* translocated 71% As to the fronds and 7% Sb to the fronds. This shows that though AsIII and SbIII are analogs there were differences in

their uptake and translocation in *P. vittata*. It is possible that they were taken up through different aquaporin channels or transporters.

Wang et al. (2010) had similar studies on AsIII uptake with competition experiments using analogs of AsIII, silicic acid and boric acid. The treatments had no impact on the uptake of AsIII, which agrees with this study. However, silicic acid [$Si(OH)_4$] has a molecular diameter of 4.38 Å, which is larger than that of $As(OH)_3$ (4.11 Å; Ma et al., 2008) and of the ar/R region of the aquaglyceroporin and hence silicic acid may not be an effective competitor of AsIII in uptake studies. Also, the permeability for boric and silicic acid is not wide spread in all members of the NIPs (Maurel et al., 2008) whereas AsIII permeability is observed in several different subclasses of NIPs (Zhao et al., 2009)

In the rice study a dose dependent reduction in AsIII uptake was observed when similar treatments using glycerol and SbIII were performed on excised roots indicating that AsIII was taken up by glycerol transporters (Meharg and Jardine, 2003). The fact that glycerol and SbIII had no effect on AsIII uptake may indicate a separate uptake pathway of AsIII in *P. vittata*.

Effects of Silver Nitrate on AsIII Uptake

Silver, an aquaglyceroporin inhibitor, was used to understand the mechanisms of AsIII uptake by *P. vittata*. Mercurial compounds are also inhibitors of aquaporin activity, which are commonly used for uptake studies. However, at higher concentrations Hg may be phytotoxic to plants and a reduction in AsIII uptake may result from its phytoxic effect. Moreover, studies indicate that 0.01 mM Hg had no impact on AsIII uptake by *P. vittata* when treated with 0.015 mM AsIII for 2 d (Wang et al., 2010).

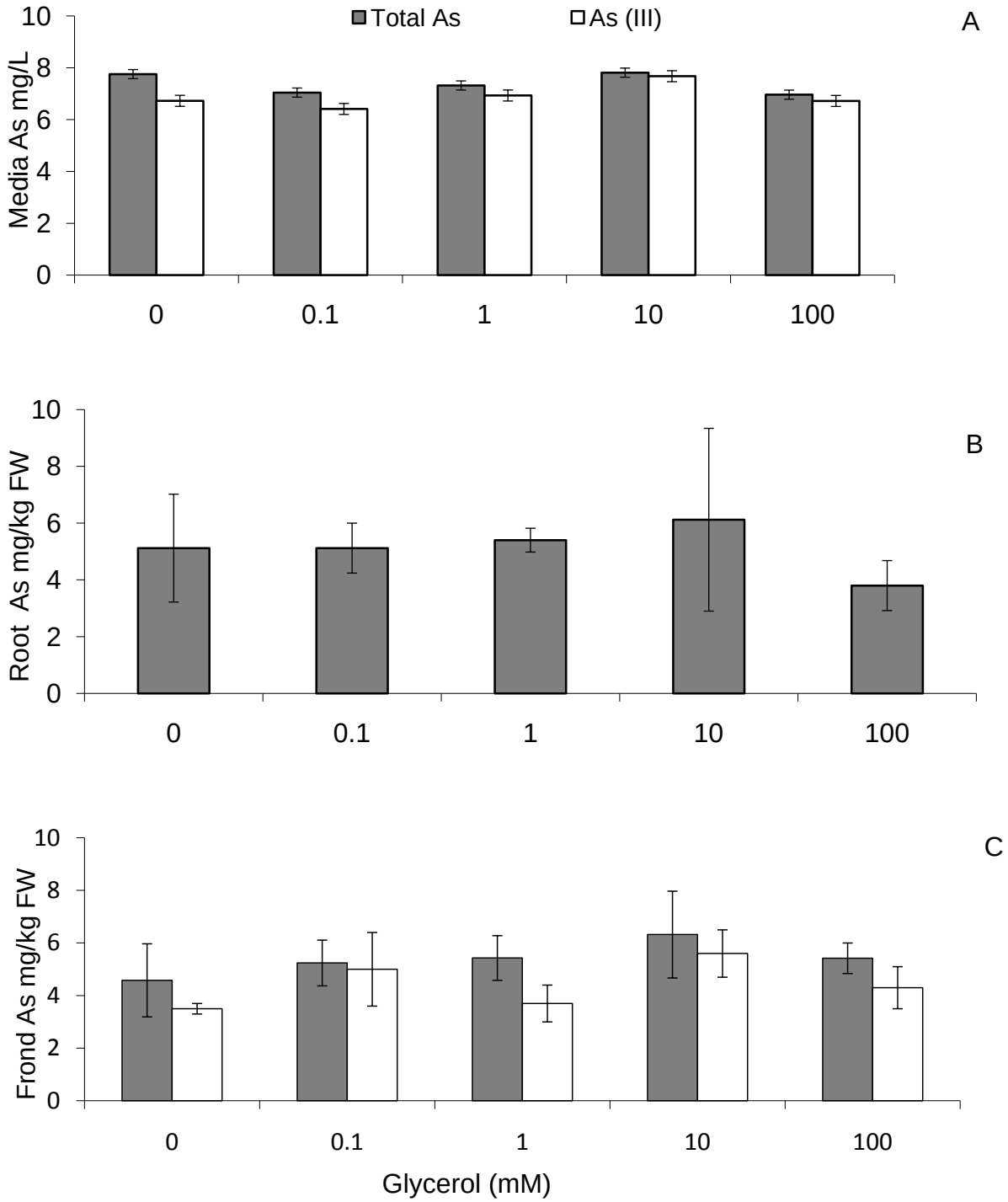

Figure 5-2. Effect of glycerol on arsenic speciation in *P. vittata* when treated with 0.1 mM AsIII for 1 h in the A) media, B) roots and C) fronds

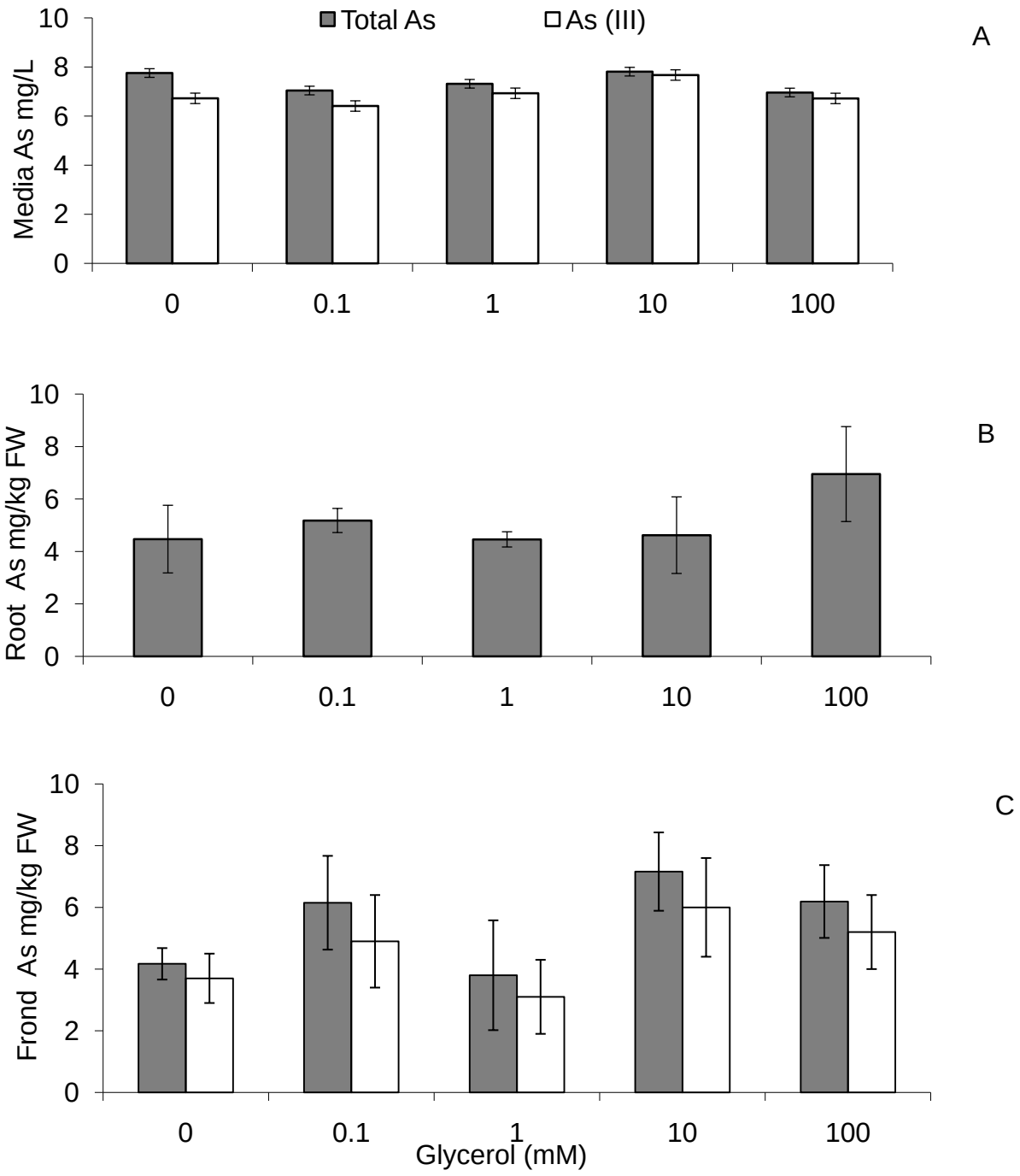

Figure 5-3. Effect of glycerol on arsenic speciation in *P. vittata* when treated with 0.1 mM AsV for 1 h A) media, B) roots and C) fronds

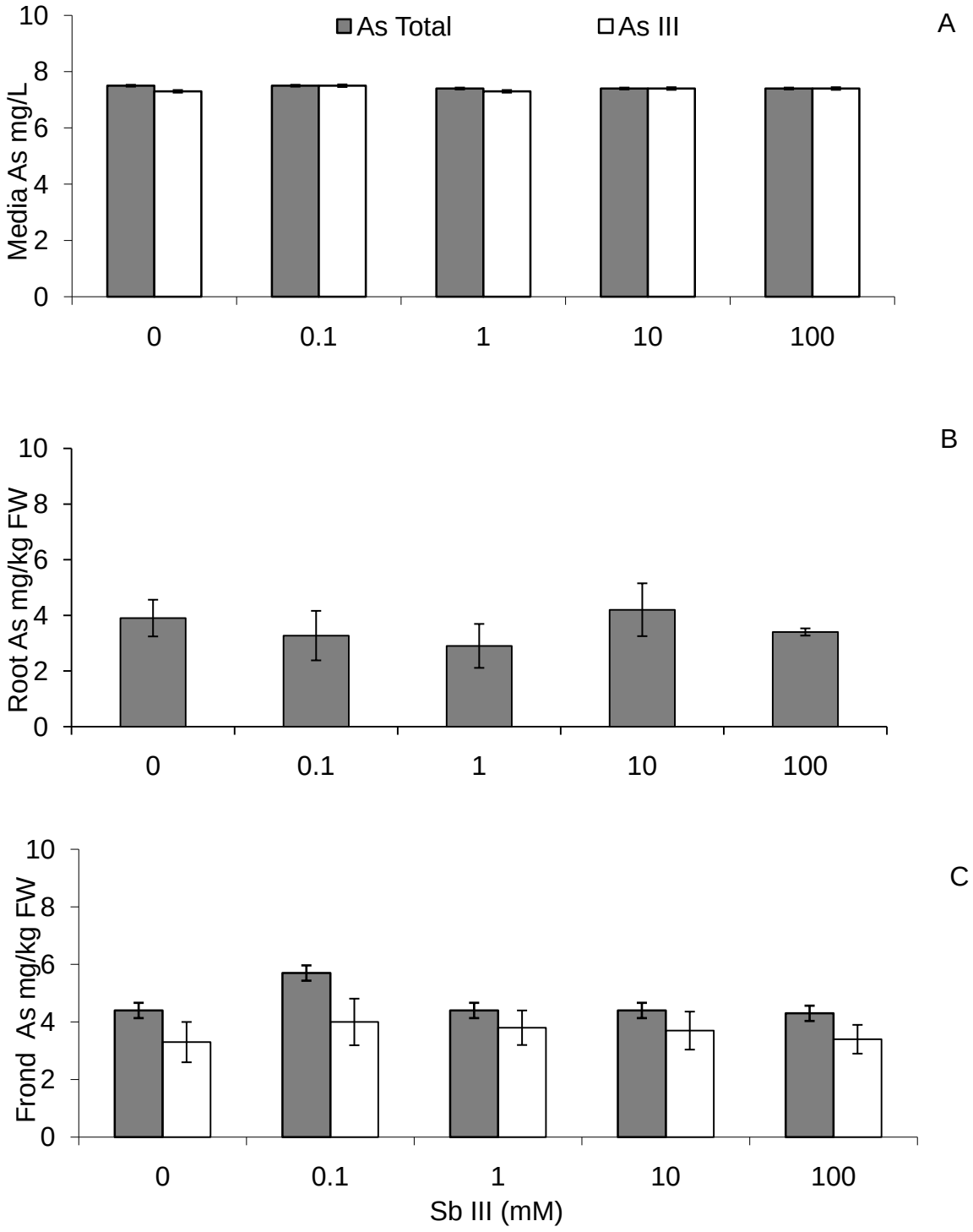

Figure 5-4. Effect of SbIII on arsenic speciation in *P. vittata* when treated with 0.1 mM AsIII for 1 h A) media, B) roots and C) fronds

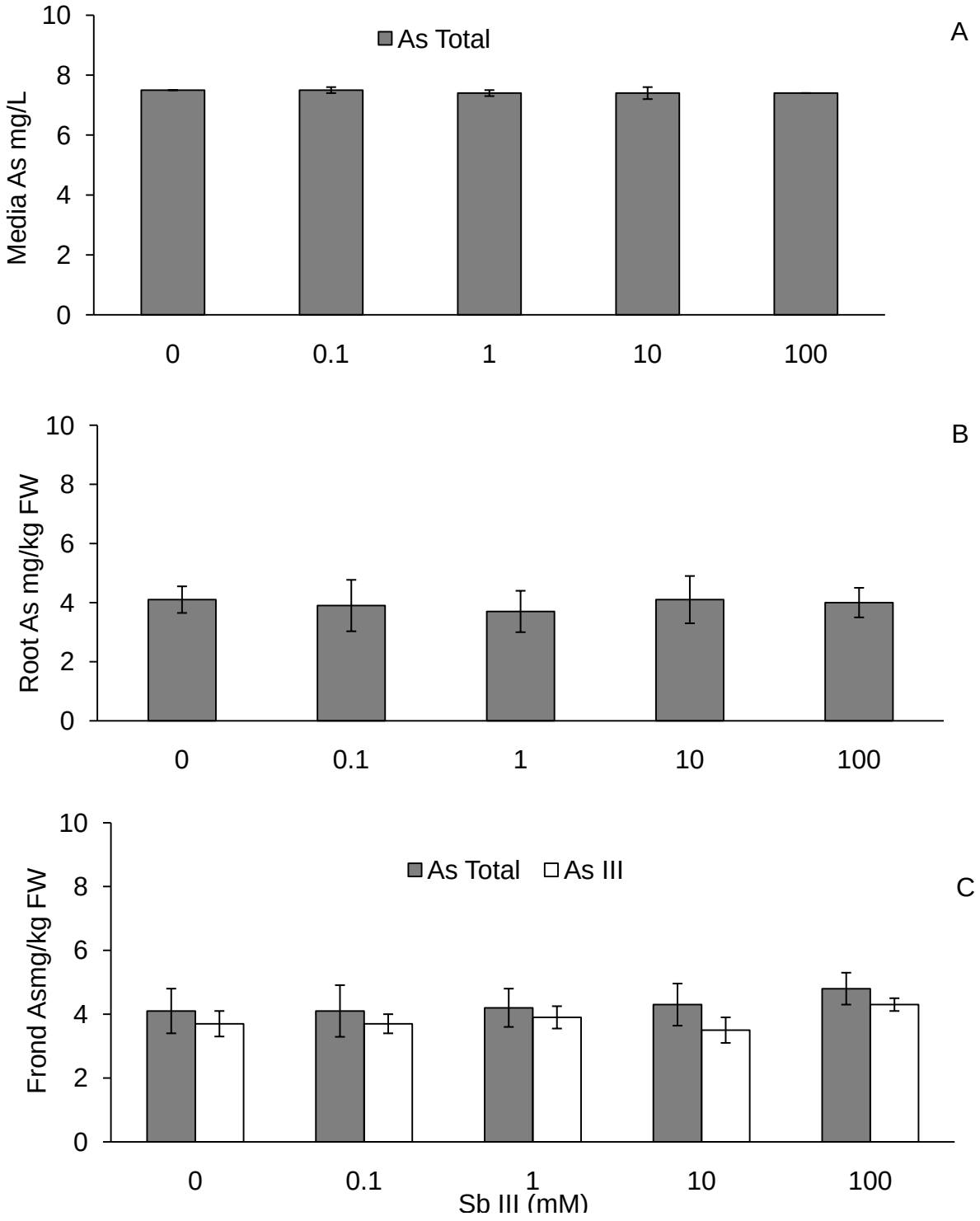

Figure 5-5. Effect of SbIII on arsenic speciation in *P. vittata* when treated with 0.1 mM AsV for 1 h A) media, B) roots and C) fronds

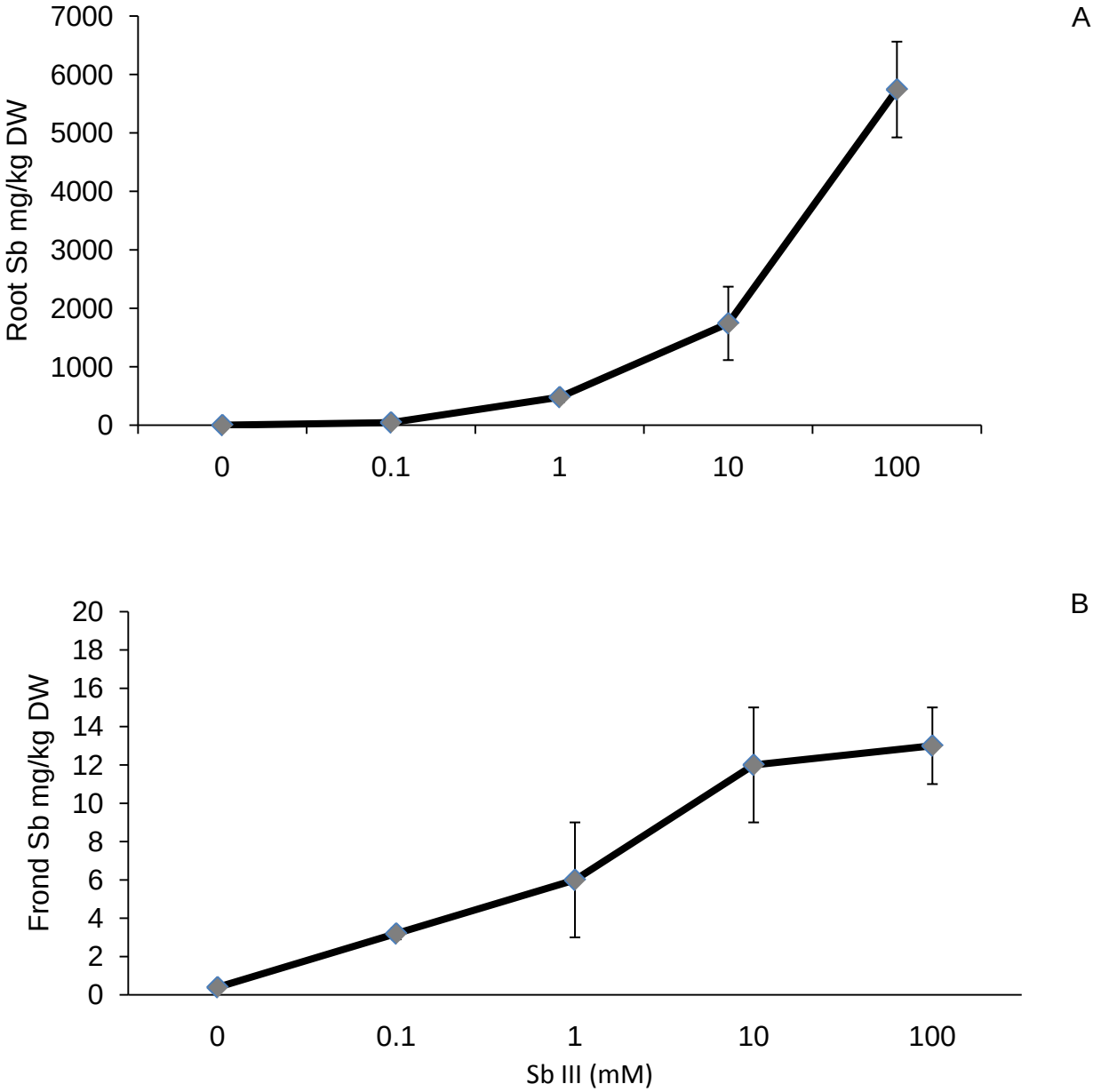

Figure 5-6. Concentration of Sb in *P. vittata* when treated with 0.1 mM AsIII and different concentrations of SbIII for 1 h. A) roots and B) fronds

$AgNO_3$ is a potential inhibitor of aquaporins with reduced phytotoxicity (Niemietz and Tyerman, 2002) and hence was used in this study. The function of the inhibitor can be further substantiated by the fact that the pore diameter of an aquaporin is 2.8 Å and the ionic radius of Ag^+ is 2.5 Å. Hence elements with ionic radii similar to silver may be potent inhibitors of aquaporins. The presence of 0.001 and 0.01 mM Ag had little effect on AsIII oxidation in the media, with 4-6% AsIII being oxidized after 1 h, which was comparable to the control at 6% (Figure 5-7A). However, at 0.1 mM Ag, the amount of oxidized AsIII increased to 13%.

Unlike glycerol or SbIII, the presence of Ag significantly reduced As uptake by *P. vittata* (Figure 5-7). As the Ag concentrations increased from 0 to 0.001 and to 0.01 mM, the arsenic concentrations in the roots decreased from 3.8 to 2.5 and to 1.4 mg kg^{-1}, (Figure 5-7B)and those in the fronds from 5.1 to 5.0 and to 3.0 mg kg^{-1} (Figure 5-7C). The impact was most pronounced at 0.01 mM Ag, with As reduction being 64% in the roots and 58% in the fronds. The fact that arsenic concentrations in the roots and fronds at 0.1 mM Ag were greater than those at 0.01 mM Ag may be attributable to the increased AsV concentration in the growth media (13% compared to 6%).

To confirm the impact of Ag at 0.01 mM, the experiment was repeated for 2 h, which showed similar results (Figure 5-9). At 0.01 mM Ag, arsenic concentrations in the fronds and roots were reduced by 63 and 48% compared to the control (Figure 5-9A). Similar to this study, a significant decrease in As accumulation was observed when *P. vittata* was treated with 10 and 100 µM Ag^+ for 15 d (Nagarajan and Ebbs, 2007).

Compared to AsIII uptake by *P. vittata*, the impact of Ag on AsV uptake was much less. Regardless of the Ag concentrations used, Ag had little impact on arsenic

concentrations in the fronds, ranging from 4.9 to 5.2 mg kg^{-1} (Figure 5-8C). However, with Ag concentrations increasing from 0 to 0.001 to 0.01 mM, arsenic concentrations in the roots decreased from 3.8 to 3.5 and 2.7 mg kg^{-1} (Figure 5-8B). It seemed that Ag had some impacts on root arsenic concentrations, with the highest reduction at 0.01 mM Ag at 29%. When the time period of the experiment was increased from 1 h to 2 h, 0.01 mM Ag reduced As concentration in the roots by 26% (Figure 5-9B). The difference in Ag impact on AsIII and AsV uptake by *P. vittata* indicates that AsIII uptake was different from AsV and it depended on a pathway, which was inhibited by $AgNO_3$.

This study confirms the fact that there was a decrease in AsIII uptake by *P. vittata* in the presence of $AgNO_3$ but no effect in the presence of glycerol or SbIII. This indicates that AsIII may be taken up by another transporter unique to the fern and the transporter is inhibited by Ag. The transporter can be another NIP protein or a protein that has a completely different function compared to that of an aquaporin. Though it is proved that aquaporins or glycerol facilitators are responsible for AsIII uptake in *E. coli*, yeast or mammals, certain other transporters have been recently tested to understand their role in AsIII uptake. A glucose transporter permease and hexose transporter in yeast has been shown to mediate AsIII uptake (Liu et al., 2004; Boles and Hollenberg, 1997). It is seen that in the presence of glucose, AsIII is taken up by the glycerol transporter Fps1p, and in the absence of glucose it is taken up by the glucose transporter. These transporters are analogs to mammalian GLUT permeases, which are also known to take up both AsIII and monomeythylarsenite, MMA (III) (Liu et al., 2006). This indicates that *P. vittata* may have a separate AsIII uptake pathway.

AsIII Oxidation in the Media and *P. vittata*

To minimize AsIII oxidation, 1 h was used in this experiment. Based on the preliminary data, little AsIII oxidation was observed in the growth media within 1 h (Figure 5-1A). However, limited AsIII oxidation occurred during the experiment. For example, in the glycerol experiment, 1.7-8.9% AsIII was oxidized. This means some of the As was taken up as AsV instead of AsIII and it had be to accounted for.

In addition to the limited oxidation of AsIII in the growth media (Figure 5-2A), AsIII can be oxidized to AsV on the root surface, which are rich in microbial population (Mathews et al., 2010). The oxidized AsV may be taken up by the plant immediately and would not contribute to AsV concentration in the media. This means though no AsV was detected in the media, some of the As was taken up as AsV by *P. vittata*, which may follow the phosphate transporter pathway and therefore would not be inhibited by glycerol or SbIII.

This hypothesis was supported by arsenic speciation in the roots. Though only 1.7-8.9% AsV was present in the growth media, almost all As was present as AsV in the roots in the glycerol (Figure 5-2B) and SbIII (Figure 5-4B) treatments. Part of the AsV may be taken up by phosphate transporters in *P. vittata*. In addition, AsIII-oxidized AsV on the root surface has also contributed to AsV concentrations in the roots. In contrast, most of the As in the fronds was present as AsIII, ranging from 69 to 96% in the presence of AsIII and glycerol (Figure 5-3C). Even in the AsV and glycerol treatment, 81 to 85% of the As in the fronds was present as AsIII (Figure 5-4C). Similar data were observed in the AsIII-SbIII and AsV-SbIII treatment, with 72 to 98% and 82 to 95% arsenic present as AsIII in the fronds (Figures 5-4C and 5-5C).

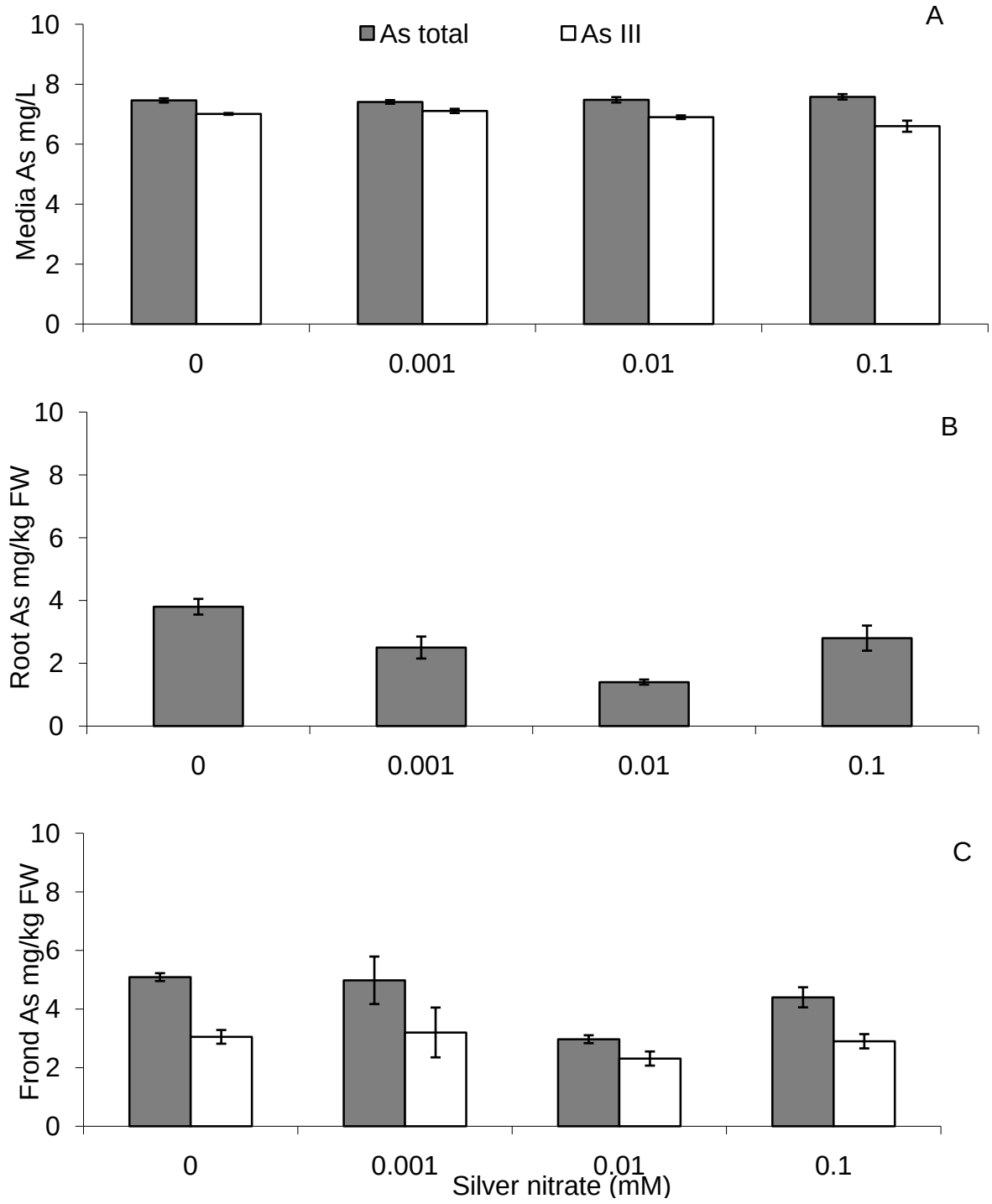

Figure 5-7. Effect of $AgNO_3$ on arsenic speciation in *P. vittata* when treated with 0.1 mM AsIII for 1 h A) media, B) roots and C) fronds.

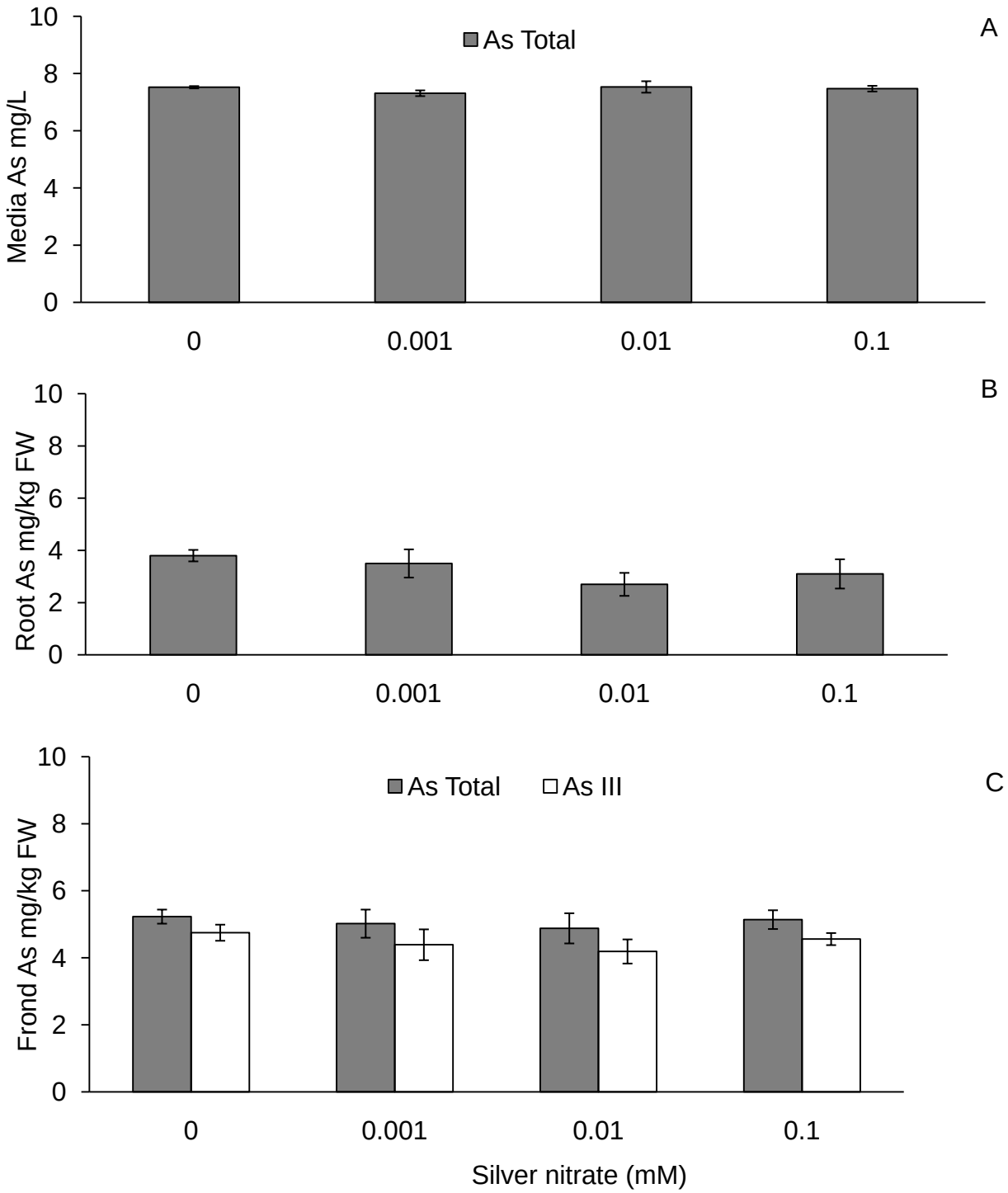

Figure 5-8. Effect of $AgNO_3$ on arsenic speciation in *P. vittata* when treated with 0.1 mM AsV for 1 h A) media, B) roots and C) fronds.

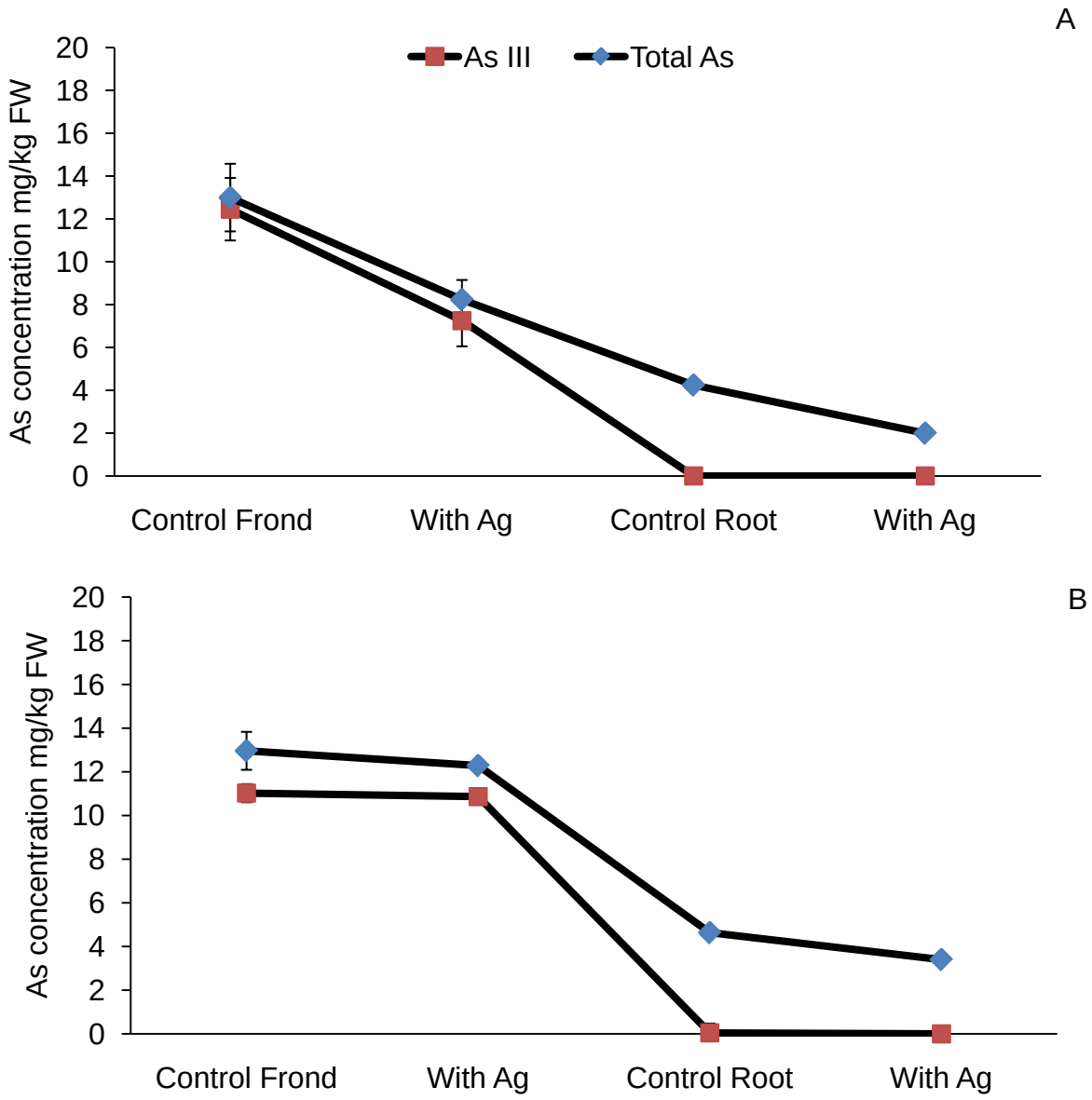

Figure 5-9. Effect of AgNO3 on arsenic speciation in the fronds and roots of *P. vittata* after exposure to 0.1 mM arsenic for 2 h A) AsIII and B) AsV

Research Findings

Aquaglyceroporin competitors such as glycerol and SbIII showed no significant impact on AsIII uptake. However, silver ions had a negative effect on As III uptake which means the transporter of AsIII is different from that of a glycerol or SbIII transporter but is inhibited by silver. This indicates that *P. vittata* has a different mechanism for AsIII uptake, which needs to be further investigated.

CHAPTER 6
ARSENIC REDUCED SCALE-INSECT INFESTATION ON ARSENIC HYPERACCUMULATOR *PTERIS VITTATA* L.

Insect Detterence

There are several ways plants protect themselves from natural infestations by herbivores. These include constitutive defenses that with physical barriers on the plant surface and the production of allelochemicals to prevent the herbivore from completing its life cycle on the plant (Paiva, 2000). Induced defense mechanisms include both direct and indirect defenses. In direct defenses, plants use secondary metabolites that behave as insect toxins, digestibility reducers or anti-nutrients (Baldwin and Preston, 1999), whereas in indirect defenses, plants release volatile compounds after herbivore attack. These volatiles or semio chemicals (Law and Regnier, 1971) can attract natural enemies of the attacking insect or even induce defense responses in neighboring plants (Pare and Tumlinson, 1999).

The above mentioned defense mechanisms demand energy and nutrient resources of a plant, which would otherwise have been used for its vegetative and reproductive development (van Dam and Baldwin, 1998). It might be to overcome this expensive defense mechanism that plant evolution has developed an unusual phenomenon of hyperaccumulation of heavy metals in certain species (Mathews et al., 2009). Hyperaccumulators refer to plants that can accumulate large amounts of elements or compounds such as metals, in the aboveground biomass. Heavy metals accumulated in the biomass may influence the plant disease/infestation triangle, which includes pathogens/herbivores, plant susceptibility and a favorable environment (Poschenrieder et al., 2006). Boyd (2007) indicates that hyper accumulators may have

an advantage against pathogens and herbivores in comparison to non-hyperaccumulators.

Research has been on-going worldwide to understand the ecological benefits and evolutionary basis of hyperaccumulation in relation to biotic stress management. Various hyperaccumulators have been studied to determine if they are better able to defend against infestation by insects and pathogens (Poschenrieder et al., 2006). For example, nickel hyperaccumulator (*Strepthanus polygaloides*) can defend against leaf moths, hoppers, root chewers and mites (Jhee et al., 2005; Jhee et al., 2006); zinc hyperaccumulator (*Thlaspi caerulescens*) shows deterrence against hoppers (Behmer et al., 2005), and selenium hyperaccumulator (*Brassica juncea*) deters leaf chewer *Pieris rapae* (Hanson et al., 2003) and phloem feeder *Myzus persicae* (Hanson et al., 2004). Also, Galeas et al. (2008) found that, selenium hyperaccumulators have lower infestation rates by arthropod load than non-hyperaccumulators under field conditions. However, there are studies that report that certain insects may have developed tolerance to heavy metals accumulated in plants. For example, *Melanotrichus boydi* and *Plutella xylostella* prefers to feed on the Ni hyperaccumulator, *Streptanthus polygaloides* (Wall et al., 2006) and on the Se hyperaccumulator *S. pinnata* (Freeman et al., 2006), respectively.

Rathinasabapathi et al., 2007, showed that As hyperaccumulation could deter herbivore damage when the leaf chewing insects, American grasshoppers (*Schistocerca americana*), were fed arsenic treated ferns. Arsenic concentrations at 1 mM (sodium arsenate) deterred the grasshoppers from consuming the fronds but at ≤0.1 mM, arsenic had no deterrence effect. This study was the first to test arsenic-

induced defense mechanism in an arsenic hyperaccumulator. The ability of insects to avoid feeding on the plants indicates that the herbivores are able to taste the difference in plants with or without metals. The experiment was supported by choice studies where lettuce was dipped in water and arsenic solution (1.0mM) and here a similar trend of deterrence was observed. The results were consistent with the hypothesis that arsenic in the tissue was sufficient to deter grasshoppers from feeding on the fern.

Ferns are very hardy and seldom have pest problems. Though the American grass hopper is polyphagous, it is unlikely that the American grasshopper is a major natural pest of the fern. The major pests of ferns include scales, hemispherical scales and mealy bugs. Since no natural pest of this fern has been tested for elemental defense hypothesis to date, it is important to test the effect of arsenic hyperaccumulation by *P. vittata* on a natural infestation where the insects where naturally growing on the fern (Mathews et al., 2009).

A study was done focusing on the effects of arsenic on infestation of *P. vittata* by Carribean black scale (*Saissetia neglecta*). The scales are polyphagous, phloem sap sucking insects, 3–5 mm in size (De Lotto, 1969). They commonly infest Florida citrus (Fasulo and Brooks, 2004), avocado (Pena, 2003), cassava (Pena and Waddill, 1984) and many other crop plants.

Unlike the armored scales, these scales do not have a hard protective covering, and hence are called soft scales. The female scale insects move on a plant and lay eggs by parthenogenesis underneath the waxy covering. These eggs hatch over a period of 1–3 weeks. The newly hatched scales (crawlers) move around a plant until

they locate succulent new growth where they insert their piercing–sucking mouthparts into the plant to feed on the sap.

Figure 6-1.Scale Insect: *Saissetia neglecta.* (Futch et al., 2001)

The antennae and legs of adult female scales are reduced and they do not move often. The soft scale secretes a sticky fluid, and hence plants infested by these scales are seen to harbor ants as well. Unlike the armored scale, upon death these scales fall off the plant. Dead scales are normally dried up with no fluid in it (Fasulo and Brooks, 2004).

Boyd (2007) elaborated the four steps necessary to study elemental defense of a plant against herbivores. These include determining if natural enemy performance is reduced by increasing elemental concentrations in the plant, showing that high elemental concentration in the plant is sufficient to cause the defensive effect in artificial media, comparing the fitness between high and low concentration plant attacked by a natural enemy and finally comparing the effectiveness of high and low concentration plant attacked by natural enemies under natural field conditions. The study here starts

with the first approach with the objective to determine the effects of arsenic concentrations in *P. vittata* on its ability to deter infestation by scale insects.

Materials and Methods

Experiment Setup

P. vittata of similar size were grown in a hydroponic system. They were four-month old after transplanting, with 4–5 fronds. The nutrients were supplied as 0.2-strength HS with aeration. The plants were grown under a 12-h photoperiod with a photon flux of 350 µmol m^{-2} s^{-1} using cool and warm white fluorescent lamps with temperature maintained at 25 ˚C and 70% relative humidity. The experiment was performed in triplicates. The ferns were naturally infested with the Caribbean black scale 1 month after transplanting into a hydroponic system. The scales were allowed to multiply on the ferns for 3 months to allow for uniform infestation. A tray covered with a white paper was placed under each plant. The plants were spatially isolated to prevent the spread of the insects from one plant to the other. Scale counts, visible to the eyes, were taken before the experiment.

Arsenic Treatment and Scale Counting

Four months after transplanting, the scale-infested ferns were exposed to 0, 5, 15, and 30 mg kg^{-1} arsenic as sodium arsenate in the hydroponic solution. The total numbers of scales at the start of the experiment for each treatment were 170±9, 115±21, 252±77, and 195±17, respectively. Following arsenic exposure, the number of fallen-scales (presumably dead) was counted on a daily basis for 7 d until no more increase in fallen-scales was observed.

Chemical and Data Analysis

At the end of the experiment, the arsenic content in the fallen and intact-scales as well as the aboveground biomass of *P. vittata* was analyzed. The fronds as well as the insects were dried and ground to 20 mesh size and digested with concentrated HNO_3 (1:1, v/v), followed by 30% H_2O_2 for As determination (U.S. EPA, 1983, method 3050). The As concentration was determined by a graphite furnace atomic absorption spectrophotometer (Varian 240Z, Walnut Creek, CA). Standard reference materials from the National Institute of Science and Technology (Gaithersburg, MD) were used to assess method accuracy and precision (within 100±20%). The arsenic effects were determined by analysis of variance according to the linear model procedure of the Statistical Analysis System (SAS Institute Inc. 1986). Treatment means were separated by Duncan's multiple range tests using a level of significance of $p < 0.05$.

Results and Discussion

During the 3-month infestation by scales, the Chinese brake ferns could withstand the infestation. The underside and base of the leaflets and rachises were most infested by the Caribbean black scales. Ants were also seen in all plants infested with the scales, which is common in the presence of the sweet secretions of scale insects.

Arsenic Toxicity in Scales

During the 7-d arsenic exposure, the number of fallen-scales was counted on a daily basis (Figure 6-1). Arsenic accumulation in the ferns significantly impacted the scale population at high As-treatment of 15 and 30 mg L^{-1}. For the control treatment, the number of fallen-scales was limited, ranging from 2 to 4 per day. A similar trend was observed for the ferns exposed to 5 mg L^{-1} As, indicating that arsenic in the plant had

little impact on the scales. However, for ferns exposed to 15–30 mg kg^{-1} As, the impact on the scales was apparent on day 1.

Approximately 16–18 fallen-scales were observed compared to 5 for the control. There were more fallen-scales on day 2 and 3, ranging from 25 to 50 per day. The number of fallen-scales was reduced to less than 10 per day after day 5. *Pteris vittata* is able to take up arsenic within an hour of exposure and hence would have a significant concentration of As by day one (Mathews et al., 2010). After exposing to 10 and 20 mg L^{-1} As for 1 d in a hydroponic system, the arsenic concentrations in the fronds of *P. vittata* were 56 and 84 mg kg^{-1} (Singh and Ma, 2006). In a different hydroponic experiment, the arsenic in the fronds of *P. vittata* was 165 mg kg^{-1} after exposing to 7.5 mg L^{-1} As for 2 d. Hence, the As concentration in the fronds in this experiment would be expected at 56–84 mg L^{-1} and >165 mg kg^{-1} after exposing to 15 mg L^{-1} As for 1 and 2 d. If this is the case, then arsenic concentration >50 mg L^{-1} in the fronds was toxic to scales and the effect was observed after 1 d of arsenic exposure (Figure 6-2).

Arsenic Concentration and Scale Death

The total number of fallen and intact scale insects per plant at the end of one-week experiment is summarized in Figure 6-2. The control ferns with no As-treatment had approximately 29±1 fallen-scales and 141±10 intact-scales, i.e., 17%. The ferns treated with 5 mg kg^{-1} As had similar number of fallen- and intact-scale, indicating limited effect from arsenic. At 15 mg kg^{-1} As, the number of fallen scale increased significantly to 140 per plant. The number for 30 mg kg^{-1} As treatment was slightly lower at 120 per plant. Since the ferns were naturally infested with the scales, the number of scales could not be controlled for a given plant. As a result, the total number of scales for each treatment at the beginning of the experiment was different (Figure 6-2A).

To effectively assess the impact of arsenic on scale infestation of *P. vittata*, the percentage of fallen-scales to total scales per fern was used (Figure 6-3). At the end of the experiment after 7 d, the plants, and fallen and intact scales were analyzed for arsenic. Due to the short time used in this experiment, no significant change in plant biomass was observed between treatments. The fresh plant biomass for the four treatments ranged from 5.8 to 7.2 g per plant (data not shown). The total arsenic in *P. vittata* fronds increased significantly from 5.40 to 812 mg kg^{-1} as solution arsenic increased from 0 to 30 mg L^{-1} (Figure 6-3).

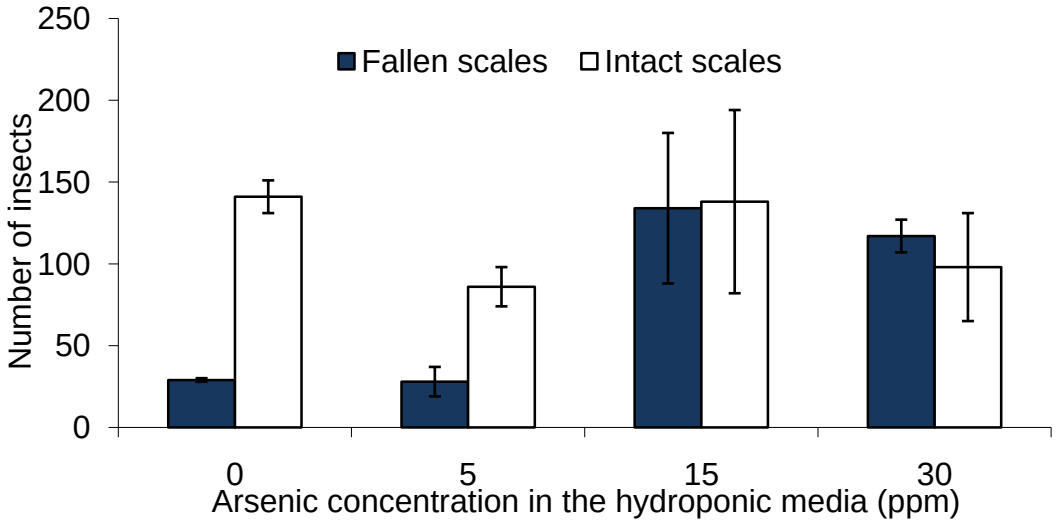

Figure 6-2**.** The total number of fallen-scale and intact-scale on the fronds of *P. vittata* after 1 week of arsenic exposure A) Graphical representation B) The number of scale insects fallen from *P. vittata* fronds exposed to different arsenic concentration for 1 week.

With increase in frond arsenic, the percentage of fallen scales from the plants increased from 17.2% in the control to 55% in the plants treated with 30 mg kg^{-1} As (Figure 6-3). There was also a decrease in the ant population associated with the scales and indicates that they might have moved to less-contaminated areas. Some ants were also observed dead along with the scale insects (data not shown). It was unclear if this resulted from the arsenic in the fronds or the scale secretions, which the ants were fed on. The majority of the fallen-scales were dry with the exception of a few scales of higher instars.

The arsenic concentrations in the fallen scales ranged from non-detectable (detection limit 2 µg kg^{-1}) in the control to 194 mg kg^{-1} in the 30 mg kg^{-1} As-treatment (Figure 6-3). Some scales fell off from the fern while others remained on the plant which may be because female crawlers of the *Saissetia* sp. are not fastened permanently on the plant until they are ready to lay eggs (Fasulo and Brooks, 2004). Another aspect is that the wax-like secretions from scale insects of later instars may harden the insect body and bind it to the plant parts. Approximately 90% of the intact-scales were darker brown in color which indicates that they were of later instars compared to the pale greenish color of the earlier instars (data not shown; Futch et al., 2001). Hence the adult scales are fastened on the plant and may remain on the plant even if they may be poisoned by arsenic. This was indicated by the arsenic concentration of the intact-scales on the plant, which ranged from non-detectable in the control to 81 mg kg^{-1} in 30 mg kg^{-1} As-treatment, which was significantly lower than those in fallen scales. Previous studies by Kertulis et al. (2005) indicate that arsenic is transported in *P. vittata* via the xylem sap. The scale insects tested here were phloem feeders and the presence of

arsenic in the scale biomass is consistent with the fact that arsenic is present in the phloem of the fern as well.

The toxic doses of arsenic on aquatic insect species are observed to be 40 mg $kg^{-1}$for midge (Holcombe et al., 1983) and water bugs (Lanzer-DeSouza and Dasilva, 1988). In this study, at the end of 7-d experiment, the frond concentration was 90 mg kg^{-1} when exposed to the lowest As at 5 mg $kg^{-1}$and no arsenic effect was observed (Figure 6-3). This corresponded to 47 and 51 mg kg^{-1} As in the fallen and intact insects, respectively. Rathinasabapathi et al. (2007) showed that arsenic accumulated at 46 mg kg^{-1} was sufficient to deter grasshoppers.

Other phloem feeders like green peach aphids on a selenium accumulator *Brassica indica* indicated a 50% reduction in population with a leaf Se concentration of 1.5 mg kg^{-1} whereas concentrations above 10 mg kg^{-1} was lethal (Hanson et al., 2004). Nickel hyperaccumulation by *Streptanthus polygaloides* had no deterrence effect on phloem feeders but deterred leaf chewing insects (Jhee et al., 2005). The present study indicates the poisoning of phloem feeder of the hyper-accumulating fern whereas the previous study by Rathinasabapathi et al. (2007) supports the deterrence against a chewing insect.

Chemical accumulation in a plant may result in the synthesis of a variety of organic compounds. These elemental and organic plant compounds may have a joint effect the on defense mechanism (Boyd, 2007). For example, Ni and certain defensive organic compound work together to repel herbivores in Nickle hyperaccumulators (Jhee et al., 2006). Hence this is an important aspect that is to be considered as it will give an overall insight on elemental defense mechanism.

Considering the research and other works mentioned, it can be inferred that metal toxicity in hyperaccumulators would vary among plant species, metal accumulated and insect species. In addition, the localization of the accumulated metal in the plant is also important.

Findings and Future Directions

Although arsenic hyperaccumulation in *P. vittata* was previously shown to be important in feeding deterrence by grasshoppers (Rathinasabapathi et al., 2007), it was unknown whether arsenic accumulation can deter natural pests of the host plant. In the current study, it was demonstrated that a scale, a natural pest of *P. vittata*, was deterred by the arsenic accumulated in the fronds. This implies that under field conditions arsenic accumulation could have a protective role and hence an evolutionary advantage for *P. vittata*. This study showed, for the first time, that scales and ferns can be employed to investigate unanswered questions on potential biological and ecological implications of arsenic hyperaccumulation in *P. vittata* and related ferns.

This study puts forth two important questions regading the threshold level of a heavy metal in the biomass and on impotance of precauitionary measures to be taken while using these plants in phytoremediation. Furture study would involve the use of different pests under lab and field conditions to study the impact of hyperaccumulated arsenic. The effect of arsenic in the tissue of the insect on organisms of higher tropic levels in the food chain should also be studied in detail.

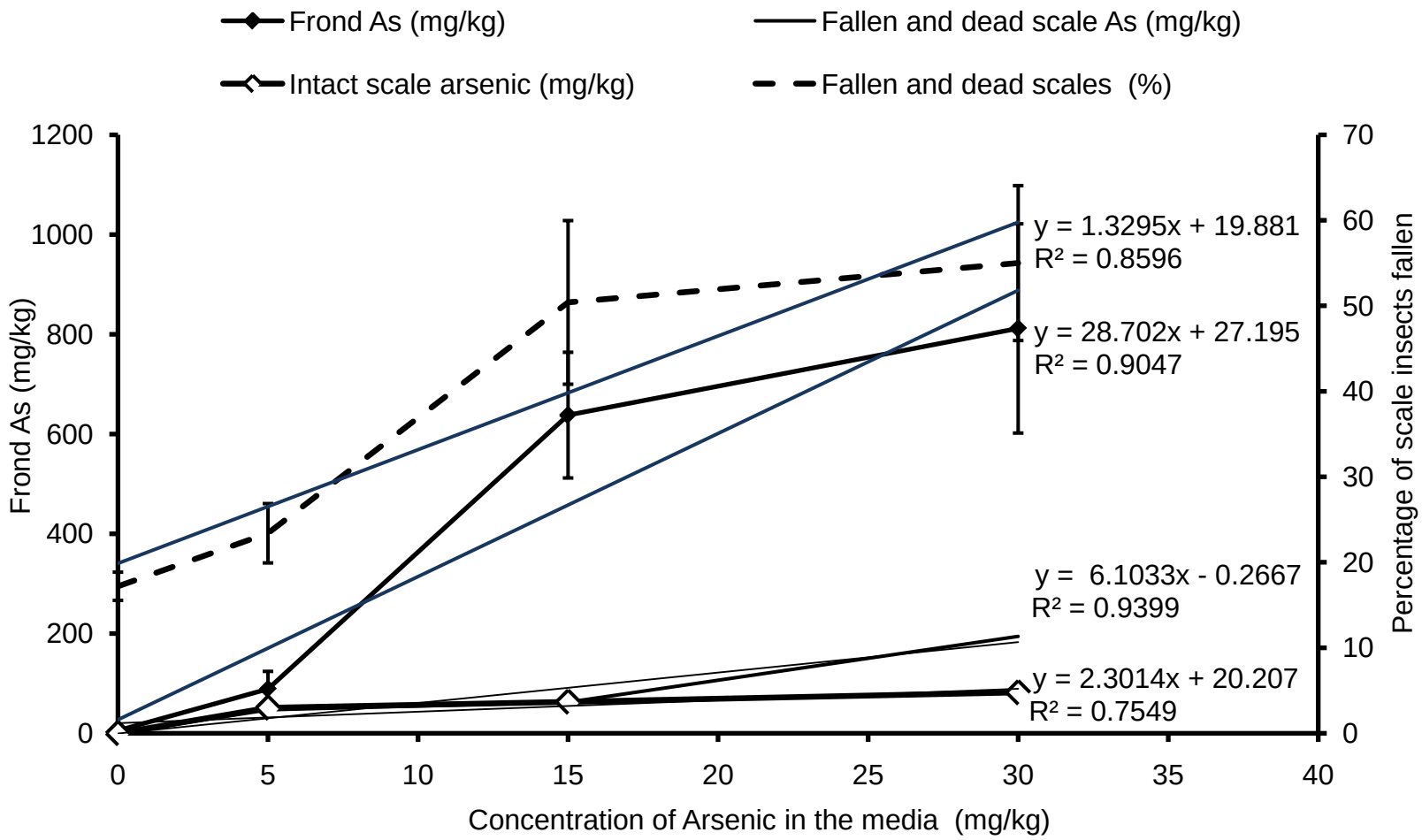

Figure 6-3. Arsenic concentrations in the fronds of *P. vittata*, fallen-scales and intact-scales (left y-axis), and % fallen-scales (right y-axis) after 1-week arsenic exposure.

CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

The unique mechanism of *P. vittata* as an As hyperaccumulator and its application in phytoremediation has been well established. However, there are many different dimensions to this fern that needs to be understood. This study was useful in determining new aspects of the fern, which can help in proposing further research objectives and also help to harness the additional capabilities of the fern in arsenic remediation and pest control.

The impact of *P. vittata* on AsIII stability in the media was substantial indicating that the presence of the fern in the media induced the AsIII oxidation with complete oxidation of 0.27 mM in 4 d. When growing the fern under sterilized conditions there was no oxidation of AsIII. The sonicated root extract which would contain both extracts from the fern root as well as from the microbes also resulted in 100% oxidation of AsIII in 4 days. This oxidation reduced substantially with boiling or filter sterilizing the sonicate. Microbes hence play a substantial role of oxidation in the media. The fern can act as a solid substrate and provide a specific carbon source for microbial activity or by itself serve as a source of microbes. Further research will be to isolate the microbes that are responsible for this oxidation and elucidate the specific role that *P. vittata* has to play in the life cycle of the microbe. For example, there may be a specific carbon source that is required by the microbe in question and hence requires *P. vittata* as a host. The effect of other As hyperaccumulating plant species on this phenomenon of oxidation is also important.

A major obstacle in traditional methods of chemical remediation of AsIII in water is the inability of AsIII to adsorb onto the chemical adsorbents used in remediation. AsV is

negatively charged and can easily be removed by adsorption or precipitation remedial measures. Hence a pre-treatment method is required to first convert the nuetral AsIII to AsV. The use of the fern can effectively avoid an expensive oxidation treatment step and also assist in phytoremediation of water.

The study on xylem sap and speciation of As in the biomass of the fern indicates the capacity of the fern to transform As into different species and sequester the As away from other physiological activities in the fern. This capacity should shed light on the As sequestration followed in other organisms including the human body. It is to be noted that the rhizomes play a major role in AsIII and this part of the fern has been given comparatively less importance in arsenic hyperaccumulation studies. The study indicates that different parts of a living organism may have different enzymes for the detoxification of As. The presence of AsIII oxidizing enzymes in the root or endogenous bacteria in the roots of the fern is a possibility that needs to be investigated in detail using molecular techniques.

Arsenite is known to be taken up by aquaglyceroporins, which can take up SbIII as well as glycerol. This indicates that both SbIII and glycerol should compete against the uptake of AsIII in the fern. Also, the aquaporin inhibitor should be able to inhibit AsIII uptake into the fern. However, in this study, there was no impact of glycerol or antimonite on AsIII uptake. This showed that AsIII may be taken up by a different mechanism unique for *P. vittata* and not via transporters that are responsible for SbIII or glycerol uptake. The inhibition of AsIII uptake by $AgNO_3$ indicates that a certain type of aquaporin may be involved in the uptake of *P. vittata* and this requires a molecular level analysis of the transporters for further understanding. Once genes responsible for AsIII

uptake are discovered, its presence in other edible plants like rice and seaweeds, which are more susceptible to AsIII uptake, can be elucidated. Once the genes responsible for AsIII uptake are known it may be possible to silence their functioning by mutations in the edible plants and, thereby, prevent AsIII uptake by those plants.

Hyperaccumulation of As may have evolved to evade insects that are a common pest of the plants. The study here indicates its impact on a sap sucking insect. Future studies would include its impact on other insect species with different feeding modes and also using laboratory trials as both the fern and the insects would behave differently in field conditions compared to the laboratory conditions. Also, since the fern has the capacity to take up high concentrations of As there are possibilities that As can be taken up by herbivores and then transferred into the food chain affecting many living organisms This requires the adoption of precautionary measures during its use in commercial level phytoremediation where very high concentrations of As are present. Under such conditions the use of screens to physically isolate the ferns from the herbivores should be considered. This phenomenon is also of interest because of the positive impacts it may have on the environment. The As hyperaccumulator that grow naturally on As contaminated sites or artificially on phytoremediation plots create an above ground biomass that have a high concentration of As. This may prevent the occurrence of pests in that area and can be beneficial for other plants as well. Alternatively, there can be a negative impact of the hyperaccumulator as well. The presence of a hyperaccumulator with high metal concentrations may result in insect attack on the non hyperaccumulators growing in the vicinity indicating an avoidance mechanism of the fern. The pests of the fern may turn to other plants as food source.

To conclude, the As hyper accumulator *P. vittata* is a major discovery in the field of phytoremediation. The physiological and molecular mechanisms of As uptake is yet to be studied in great detail to reveal more potentials of this fern as a model plant arsenic hyperaccumulator and detoxifier.

APPENDIX A
EFFECT OF ARSENIC LOADING ON ARSENIC HYPERACCUMULATION BY *PTERIS VITTATA*

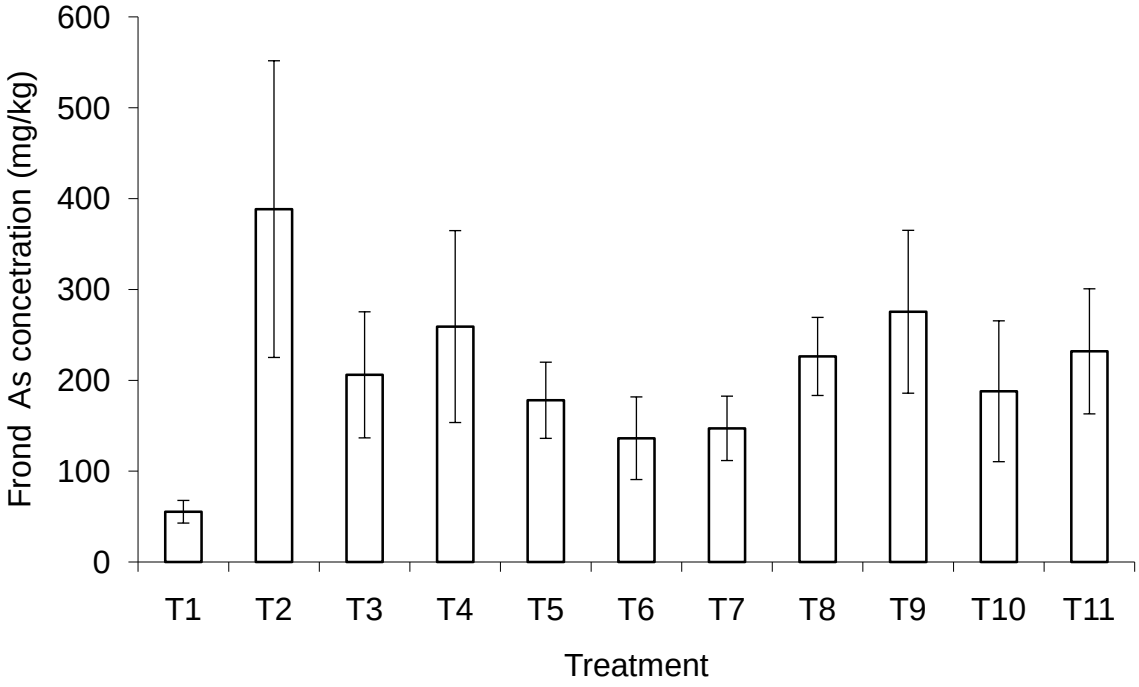

Figure A-1. Effect of arsenic loading when treated with As for 15 d on frond arsenic concentration in *P. vittata*. The treatments were as follows: T1- 0/0/0, T2- 5/5/5, T3- 0/10/5, T4- 0/5/10, T5- 5/10/0, T6-5/0/10, T7- 10/5/0, T8- 10/0/5, T9-15/0/0, T10- 0-15-0, T11- 0/0/15, where 0/5/10 can be defined as 0 mg L^{-1} arsenic for the first 5 d, 5 mg L^{-1} As on the next 5 d and 10 mg L^{-1} As in the last 5 d. The data indicates no significant difference at $p<0.05$

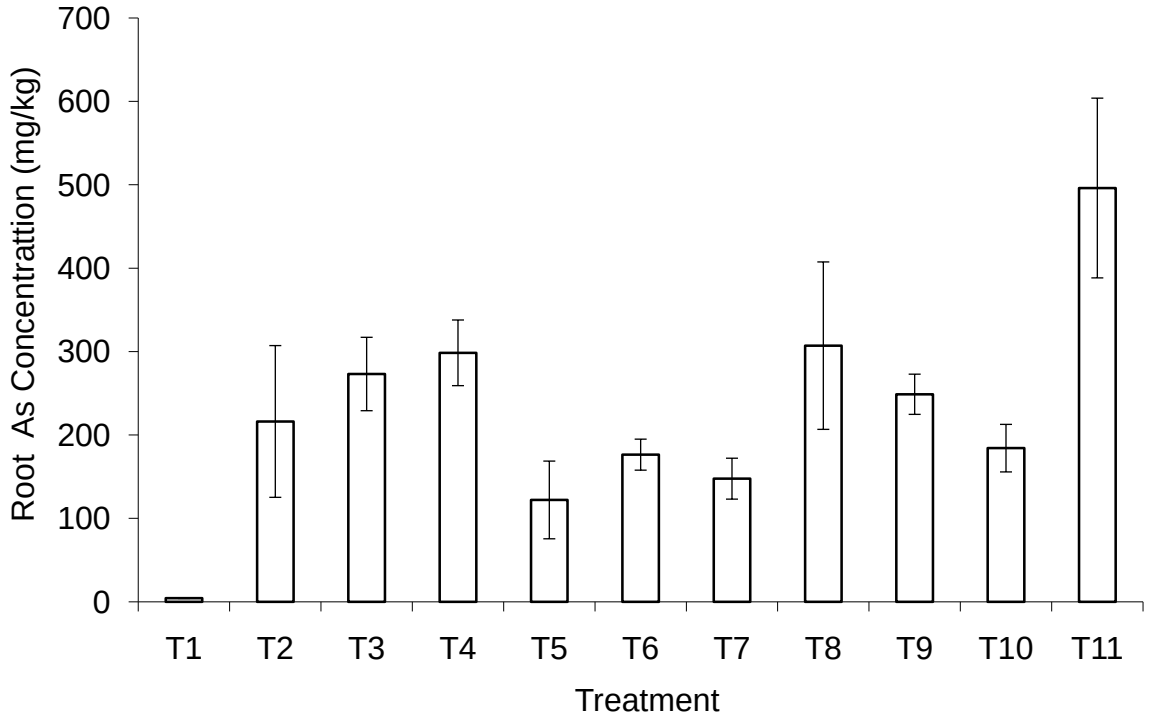

Figure A-2. Effect of arsenic loading when treated with As for 15 d on root arsenic concentration in *P. vittata*. The treatments were as follows: T1- 0/0/0, T2- 5/5/5, T3- 0/10/5, T4- 0/5/10, T5- 5/10/0, T6-5/0/10, T7- 10/5/0, T8- 10/0/5, T9-15/0/0, T10- 0-15-0, T11- 0/0/15, where 0/5/10 can be defined as 0 mg L^{-1} arsenic for the first 5 d, 5 mg L^{-1} As on the next 5 d and 10 mg L^{-1} As in the last 5 d. The data indicates no significant difference at $p<0.05$

APPENDIX B
COMPARISON OF ANTIMONY ACCUMULATION IN *PTERIS VITTATA* AND *PTERIS ENSIFORMIS*

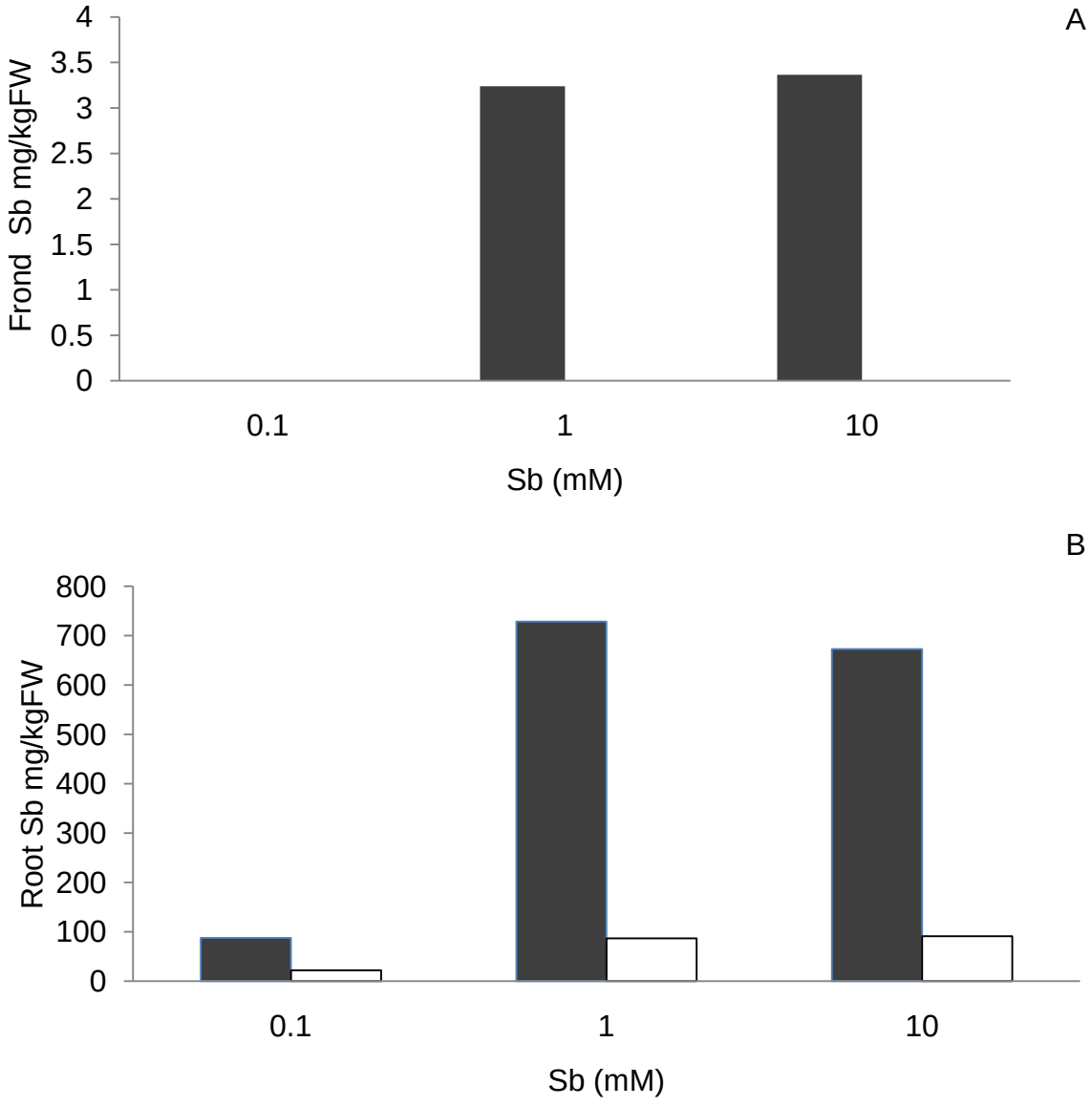

Figure B-1. Concentration of Sb in *P. vittata* when treated with different concentrations of Sb A) fronds B) roots. The solid bars indicate total Sb and the open bars indicate SbIII

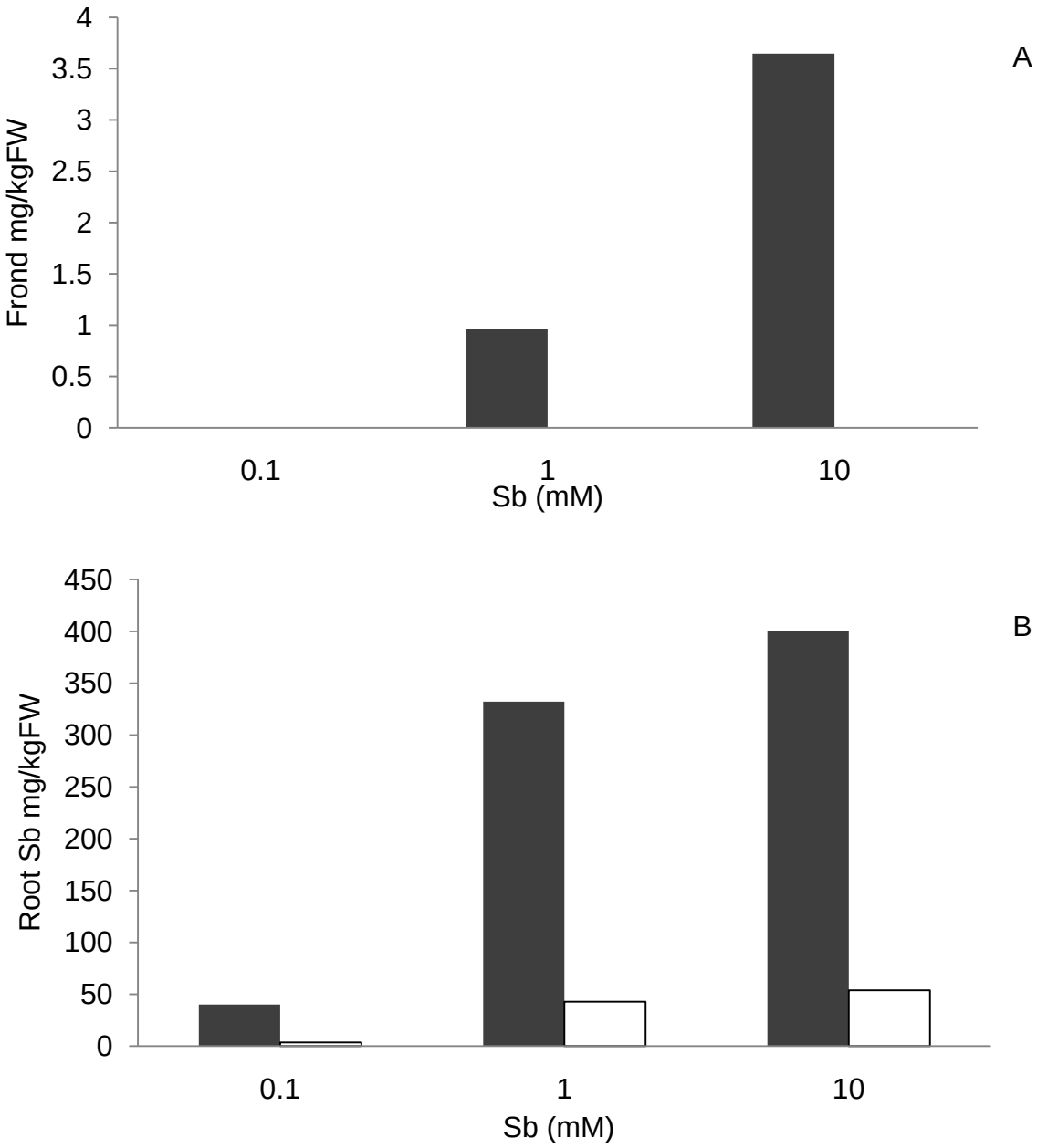

Figure B-2. Concentration of Sb in *P. ensifomis* when treated with different concentrations of Sb A) fronds B) roots. The solid bars indicate total Sb and the open bars indicate SbIII

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BIOGRAPHY

Shiny Mathews, the only child of her parents, was born and brought up in Bahrain. After her high school education she went to India to join the Kerala Agricultural University for her bachelor's degree. Following this she obtained an ICAR fellowship for pursuing a master's degree in soil science and agricultural chemistry at the University of Agricultural Sciences, Bangalore. Her master's thesis was titled "Behavior of Alachlor on Alfisols of Banglaore". She was awarded a gold medal for her academic and research performance by the university. Further, she went on to qualify for the CSIR fellowship and JN Tata scholarships, which are prestigious awards provided by the government of India. In the year 2006, she joined the University of Florida, Gainesville, Florida to pursue a PhD in soil and water science. Here she was a graduate assistant and worked on the physiological studies on the arsenic hyperaccumulator *Pteris vittata*.