PATRICIA CURY RIBEIRO

EVALUATION OF ARSENIC TOLERANCE IN *Pistia stratiotes* L. (ARACEAE): PHOTOSYNTHETIC AND RESPIRATORY METABOLISM

Dissertation presented to the Universidade Federal de Viçosa as part of the requirements of the Plant Physiology Graduate Program to obtain the tittle of *Magister Scientiae.*

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APPROVED: February 26, 2019.

Wagner Luiz Araújo

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Dedico este trabalho aos meus pais e à minha irmã, por serem os maiores incentivadores da minha carreira acadêmica. Obrigada por me ensinarem desde cedo o valor de estudar!

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BIOGRAPHY

PATRICIA CURY RIBEIRO, daughter of Décio Pinto Ribeiro and Lilian Cury Ribeiro, was born in São Paulo, São Paulo state, Brazil, on July 11th, 1992. In 2012, she started the undergraduate course in Biological Sciences in State University of São Paulo (UNESP), Jaboticabal, São Paulo state, Brazil and achieved bachelor degree in December, 2015. In February 2017, she started Magister course in Plant Physiology at Federal University of Viçosa (UFV), Viçosa, Minas Gerais state, Brazil, under the supervision of Prof. Juraci Alves de Oliveira. In February 2019, she submitted her dissertation to the defense of master´s degree.

SUMMARY

ABSTRACT

RIBEIRO, Patrícia Cury, M.Sc., Universidade Federal de Viçosa, February, 2019. **Evaluation of arsenic tolerance in *Pistia stratiotes* L. (ARACEAE): Photosynthetic and respiratory metabolism.** Adviser: Juraci Alves de Oliveira.

Arsenic (As) is a toxic element known to impair the development and growth of plants, as well as triggering various physiological changes when absorbed by plants. The plants, cultivated in nutrient solution, pH 6,5 and ½ ionic strength, were exposed in two treatments for 24, 48 and 72 hours: control (nutrient solution only) and As (1.5 mg L^{-1}). After each day, a part of the experiment was collected for later physiological analyzes. The plants exposed to As presented lower biomass gain over the days, as effect of the damages caused by the contaminant in both leaves and roots. Roots accumulated more As and lower concentrations were found in the leaves. Once As is absorbed by the roots and translocated to the leaves, As changes photosynthesis, respiration, photorespiration and in turn, changes in the synthesis and accumulation of carbohydrates were also observed. Among the effects of As on metabolism, leaves displayed decreased in proteins and increased amino acids contents, while sugars and starch were accumulated. In roots, however, decreases in the concentrations of proteins, amino acids and sugars, mainly after 24 hours were observed. Starch decreased in roots during the 72 hours of exposure to As. Although the pigments did not show differences between control and As, net assimilation rate and the efficiency of photosystem II declined in presence of As. The photorespiration and carboxylation/ oxygenation rates of Rubisco followed the photosynthetic parameters, and decreased leaf stress due to the inhibition of specific enzymes in the presence of As. In contrast, both dark and mitochondrial respiration were higher in stressed leaves, possibly due to the chemical similarity of arsenate to phosphate, which may have led to increased respiration and subsequent collapse throughout the respiratory process. As a consequence of the changes observed in photosynthesis and respiration caused by oxidative stress, the activity of TCA enzymes, TCA intermediates and the NAD, NADH, NADP and NADPH balance also showed differences between the control and As. In leaves, there was an increase in the activity of the enzymes of the TCA cycle, which is in agreement with the respiration results. In roots, the activity of enzymes decreased under stress. Fumarate concentrations were higher for contaminated roots and leaves. However, in leaves with As, an increase in malate was observed, and for roots with As was observed a decrease in the concentration of malate, which suggests that malate can be diverted

to other pathways within the cell. Among the pyridine nucleotides, all showed an increase between the contaminated leaves and in roots, the NAD^+ was not detected. Probably it was not regenerated during the respiratory process in roots with As, and as a consequence, the decrease of NADH was observed. Roots were more severely damaged, which can be explained by the greater accumulation of As in this part of the plant. Complementary analyzes related to leaf and root respiration are necessary to better understand the behavior of organic acids during As stress.

RESUMO

RIBEIRO, Patrícia Cury, M.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Avaliação da tolerância ao arsênio em *Pistia stratiotes* L. (ARACEAE): Metabolismo fotossintético e respiratório.** Orientador: Juraci Alves de Oliveira.

O arsênio (As) é um elemento tóxico conhecido por prejudicar o desenvolvimento e o crescimento das plantas, além de desencadear várias alterações fisiológicas quando absorvido. As plantas, cultivadas em solução nutritiva, pH 6,5 e ½ força iônica, foram expostas em dois tratamentos por 24, 48 e 72 horas: controle (somente solução nutritiva) e As (1,5 mg L^{-1}). Após cada dia, uma parte do experimento foi coletada para análises fisiológicas posteriores. As plantas expostas a As apresentaram menor ganho de biomassa ao longo dos dias, como efeito dos danos causados pelo contaminante nas folhas e raízes. Entre as partes analisadas das plantas, as raízes acumularam mais As e as menores concentrações foram encontradas nas folhas. Ao ser absorvido pelas raízes e translocado para as folhas, o As causou alterações na fotossíntese, respiração, fotorrespiração e, por sua vez, alterações na síntese e no acúmulo de carboidratos também foram observadas. Entre os efeitos do As sobre o metabolismo, as folhas apresentaram diminuição nos teores de proteínas e aumento nos teores de aminoácidos, enquanto os açúcares e o amido se acumularam. As raízes, no entanto, apresentaram decréscimo nas concentrações de proteínas, aminoácidos e açúcares, principalmente após 24 horas. A concentração de amido diminuiu nas raízes durante as 72 horas de exposição ao estresse. Embora os pigmentos não tenham apresentado diferenças entre o controle e o As, a taxa de assimilação líquida de CO_2 e a eficiência do fotossistema II declinaram na presença do contaminante. As taxas de fotorrespiração e carboxilação / oxigenação de Rubisco seguiram o padrão dos parâmetros fotossintéticos e diminuíram com o estresse foliar devido a inibição de enzimas específicas na presença de As. Em contraste, a respiração noturna e mitocondrial foi maior nas folhas estressadas, possivelmente devido a semelhança química entre o arsenato e o fosfato, o que pode ter levado ao aumento da respiração e subsequente colapso ao longo do processo respiratório. Como conseqüência das mudanças observadas na fotossíntese e respiração causadas pelo estresse oxidativo, a atividade das enzimas TCA, intermediários de TCA e o balanço de NAD, NADH, NADP e NADPH também mostraram diferenças entre o controle e As. Nas folhas, houve um aumento na atividade das enzimas do ciclo de TCA, o que está de acordo com os resultados obtidos anteriormente para a respiração. Nas raízes, a atividade das enzimas diminuiu sob estresse. As concentrações de fumarato foram maiores em raízes e folhas contaminadas. No entanto, nas folhas com As, foi observado um aumento de

malato, e para as raízes com As observou-se uma diminuição na concentração de malato, o que sugere que o malato pode ter sido desviado para outras vias dentro da célula. Entre os nucleotídeos de piridina, todos mostraram um aumento entre as folhas contaminadas e nas raízes, o NAD^+ não foi detectado. Provavelmente por não ter sido regenerado durante o processo respiratório para as raízes com As, não foi possível encontrar NAD^+ e, como conseqüência, foi observada a diminuição do NADH. As raízes apresentaram danos mais severos, o que pode ser explicado pelo maior acúmulo de As nessa parte da planta. Análises complementares relacionadas à respiração foliar e radicular são necessárias para melhor compreensão do comportamento dos ácidos orgânicos durante o estresse.

GENERAL INTRODUCTION

Contamination of the environment by toxic elements is becoming an increasing problem because of its high capacity for imbalance of the ecosystem. These elements, when present in high concentrations, can alter several metabolic reactions and physiological responses processes, damaging the growth and development of plants (Abbas et al., 2018). Among them, arsenic (As) has been attracting the attention of many researchers due to its high contamination capacity (Singh et al., 2015).

The weathering of rocks has been highlighted as the main natural source of As in the environment (Matschullat, 2000). Anthropogenic sources of As include the extraction of sulfite minerals, agrochemicals, fertilizers, wood preservatives, copper smelting and coal combustion (Mandal and Suzuki, 2002). Inorganic forms of As are the most common in the natural environment and include the element's most toxic chemical species, such as arsenate (AsV) and arsenite (AsIII) (O'Day, 2006). In Brazil, the most serious environmental issue involves the contamination of As in the Quadrilátero Ferrífero (QF) region of Minas Gerais (MG). The most representative natural sources of As in QF are associated with rocks containing gold sulphide complexes (Souza et al., 2018), releasing As during gold mining.

In response to As toxicity, plants may respond via increased expression of reactive oxygen species (ROS) and reactive nitrogen species (NO), as well as substitution/inhibition of transcription factors, enzymatic cofactors, antioxidative enzymes, cellular redox imbalance and unbalance in ionic transport (Cuypers et al., 2011, Gangwar and Singh, 2011, Sytar et al., 2013). The presence of As can activate a chain of events that affect growth, disrupt photosynthesis and respiration, and stimulate secondary metabolism (Pigna et al., 2010). Plants can tolerate As by many mechanisms such as phytochelation, vacuole sequestration and activation of antioxidant defense systems (Abbas et al., 2018).

The As absorbed can trigger physiological changes in roots and leaves. Biomass loss, decrease in CO_2 assimilation rate, chlorophyll and carotenoid degradation, and changes in proteins and amino acids concentrations are the most commonly reported physiological responses in research with toxic elements in plants (Chandrakar et al., 2016). Metabolic pathways are all interconnected, and when one process is affected, most of the time the reflex occurs in another dependent way. As an example, about 80% of the CO_2 assimilated during photosynthesis is used for the synthesis of sucrose (Rosa et al., 2009). If the toxic element impairs photosynthesis, directly the synthesis of other metabolites also changes. In addition to the photosynthetic apparatus, the respiratory process may suffer irreparable damage,

especially in stress with As (Farnese et al., 2017). The increase observed in respiration in stressed plants may alter the activity of several enzymes in the tricarboxylic acid cycle (TCA), which produce important intermediates that may help in the response to stress.

Among the strategies developed by plants to survive in contaminated environments, the participation of organic acids is likely fundamental. They are synthetized in the TCA cycle and the glyoxylate cycle, as well as in the photosynthetic processes of C4 and CAM (Igamberdiev and Eprintsev, 2016), they play important roles in the metabolic pathways, including energy production, precursor formation for amino acid biosynthesis, and support in modulating adaptation to the environment (Osmolovskaya et al., 2018). When the plant comes in contact with some toxic element, organic acids can act directly with the stressing agent. Thus, root exudates can bind to metals in the soil, forming stable compounds, making it difficult for metals to be absorbed by plants (Chen et al., 2017). The other function of the organic acids is to regulate the different antioxidant enzymes (Ehsan et al., 2014; Zaheer et al., 2015). In this context, a more in-depth study on the behavior of organic acids under stress by different toxic elements present in soil and water is necessary to assist the development of several species in a contaminated environment.

There are some plant species that are able to grow in contaminated areas and have adopted a number of approaches to eliminate, accumulate or hyperaccumulate toxic heavy metals (Anjum et al., 2012). Among the aquatic species, *Pistia stratiotes* has attracted attention because it has fast growth and high potential for biomass production, besides the potential for metal accumulation in their tissues (Farnese et al., 2014). *Pistia stratiotes*, also known as water lettuce, is a floating aquatic plant belonging to the Araceae family. It is considered an ornamental species, with high vegetative reproduction capacity and classified as a weed species (Lorenzi, 1982).

In this context, the aim of this study was to evaluate the changes caused in leaf and root metabolism of *Pistia stratiotes* under arsenic stress, with emphasis on the respiratory and photosynthetic process, and organic acids profile.

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CHAPTER 1: Toxicity of arsenic on photosynthesis and respiratory metabolism in *Pistia stratiotes*

ABSTRACT

The effect of arsenic (As) on the metabolic pathways and the physiological processes in leaves and roots of *Pistia stratiotes* were the main objectives of this work. Plants of *Pistia* were collected in Botanic Garden of the Federal University of Viçosa, cultivated in nutrient solution, pH 6.5 and ½ ionic strength, and submitted to two treatments: control (nutrient solution only) and As (1,5 mg L^{-1}) for up to 72 hours. After exposure to the treatments, the plants were collected and prepared for physiological analysis. The accumulation of As was higher in roots, with translocation of lower concentrations to the leaves. However, there was lower biomass gain in leaves and roots contaminated over time, when compared to controls. Although the pigments showed no difference between control and As, most of the photosynthetic parameters were affected by stress, decreasing in comparison to controls. As a consequence of oxidative stress caused by the accumulation of As in plants, changes in protein and amino acid contents were observed in leaves and roots. An imbalance between sugar and starch concentrations were also related to the decreased of the assimilation of carbon in the photosynthesis, which can lead to the accumulation of carbohydrates in the leaves. Photorespiration has declined with stress, which may be related to the effect of As on some specific enzymes, inhibiting or impairing the activity of the enzymes. In contrast, respiration increased, possibly due to the similarity between arsenate and phosphate. All of these stress-induced changes show that *Pistia stratiotes*, although able to accumulate high concentrations of As, can not respond very effectively to As stress. In general, the time factor did not show interaction during the plant responses, there was no significant difference in the majority of the results obtained for the three times analyzed.

Keywords: respiration; photosynthesis; photorespiration

RESUMO

O efeito do arsênio (As) nas vias metabólicas e nos processos fisiológicos em folhas e raízes de *Pistia stratiotes,* foram os objetivos principais deste trabalho. Plantas de Pistia foram coletadas no Horto Botânico da Universidade Federal de Viçosa, cultivadas em solução nutritiva, pH 6,5 e ½ força iônica, e submetidas a dois tratamentos: controle (apenas a solução nutritiva) e As (1,5 mg L^{-1}) por até 72 horas. Após exposição aos tratamentos, as plantas foram coletadas e preparadas para a realização de análises fisiológicas. O acúmulo de As foi maior na raiz, com translocação de menores concentrações para as folhas. No entanto, houve menor ganho de biomassa em folhas e raízes contaminadas ao longo do tempo, quando comparadas aos controles. Embora os pigmentos não tenham apresentado diferenças entre controle e As, a maioria dos parâmetros fotossintéticos foram prejudicados pelo estresse, apresentando decréscimos em comparação aos controles. Como consequência do estresse oxidativo causado pelo acúmulo de As nas plantas, alterações nos teores de proteínas e aminoácidos foram observados em folhas e raízes. Um desbalanço entre as concentrações de açucares e amido também estão relacionados com a diminuição da assimilação de carbono na fotossíntese, o que pode levar ao acúmulo de carboidratos nas folhas. A fotorrespiração decaiu com o estresse, o que pode estar relacionado com o efeito do As sobre algumas enzimas especificas, inibindo ou prejudicando a atividade das enzimas. Em contraste, a respiração aumentou, possivelmente pela similaridade entre o arsenato e o fosfato. Todas estas alterações induzidas pelo estresse demonstram que a *Pistia stratiotes*, embora consiga acumular altas concentrações de As, não consegue responder de maneira muito efetiva ao estresse por As. Em geral, o fator tempo não apresentou interação ao longo das respostas das plantas, ou seja, não houve diferença siginificativa na maioria dos resultados obtidos para os três tempos analisados.

Palavras-chave: respiração; fotossíntese; fotorrespiração

1. Introduction

Toxic elements occur naturally in the environment and some of them, even in low concentrations, play an important role in plant nutrition, while others have negative effects on various components of the biosphere (Clemens and Ma 2016). In response to human activities, the marked increase in the concentrations of these elements in the environment has caused damage to the entire ecosystem. In particular, in Brazil, the occurrence of areas degraded by the presence of certain contaminants, including arsenic (As) and others toxic metals, has become increasingly frequent and worrying (Alves et al., 2008).

The high biological toxicity of As has generated great interest in understanding the effects of this metalloid in the trophic chain. Studies have shown that the major sources of contamination are represented by water consumption or foods containing high levels of As (Sun et al., 2014). Arsenic is found in the environment in two main different forms: as arsenate (AsV) and arsenite (AsIII) (Rivas and Aguirre, 2010; Mathews et al., 2011), the second form being the most toxic due to its high affinity by sulfhydryl groups of biomolecules (Zhao et al., 2010; Flora, 2011; Farooq et al., 2016).

Although As is not a redox metal, studies show that plant exposures to this metalloid result in the formation of free radicals and the generation of reactive oxygen species (ROS), such as the hydroxyl ion (OH^-), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which at high levels generate imbalance in the system and, consequently, stress (Garg and Singla, 2011; Sharma et al., 2012). As a consequence, changes in DNA and proteins are observed, causing the decomposition of biological macromolecules and lipid peroxidation in the membranes (Souza et al., 2011).

Arsenic may impair the functioning of metabolic and biochemical pathways. In addition, As contamination can cause inactivation of key enzymes through interaction with sulfhydryl groups or indirectly by the production of ROS that causes irreversible damage to cells (Finnegan and Chen, 2012). Several metabolic pathways that operate in different cell compartments, with mitochondria, chloroplast and peroxisome can produce ROS as byproducts (Flora, 2011; Shahid et al., 2014). The effects of As on plants can be divided into physiological, morphological and biochemical damage. Among the physiological ones, it is possible to emphasize reduction of shoots and roots growth, reduction in stomatal conductance and the absorption of nutrients, chlorophyll degradation and biomass reduction. Morphological damages include reduction in leaf numbers, leaf necrosis, senescence and

defoliation. Biochemicals involve overproduction of ROS, leading to irreparable damage to carbohydrates, proteins and DNA (Karuppanapandian et al., 2011; Shahid et al., 2014; Abbas et al., 2018).

Toxic elements in high concentrations may cause plants to synthesize and accumulate clusters of metabolites, such as specific amino acids, peptides and sugars which aind in tolerance and stress response. Sucrose and glucose act as substrates for cellular respiration or as the osmolytes to maintain cellular homeostasis, whereas fructose appears to be related to the synthesis of secondary metabolites (Rosa et al., 2009). Changes in sugar concentrations also involve changes in CO_2 assimilation and other factors related to photosynthesis. Therefore, if the net assimilation rate of CO_2 decreases under certain stress, changes in sugar levels in plants can be observed (Gupta and Kaur, 2005).

Starch, considered the largest storage of carbohydrates in plants, also presents great plasticity when the plant is exposed to stressful situations (Thalmann and Santelia, 2017). In storage organs, starch is synthesized from sucrose and can be stored for months or years, aiding in the establishment of seedlings during growth. In photosynthetic cells, starch is mainly synthesized using a part of the carbon fixed in CO_2 and temporarily stored in the chloroplasts. In stressful situations where photosynthesis may be limited, plants may remove this starch as a source of energy and carbon (Thalmann and Santelia, 2017). However, it is well known that As causes significant changes and damages in the photosynthetic apparatus and pigments (Chandrakar et al., 2016), which may lead to a cascade of responses in the metabolites associated directly with photosynthesis.

Among the several methods to remove As of water, the use of aquatic plants stands out. Phytoremediation is considered an efficient, viable and low cost technology (Farnese et al., 2014). Extensive studies have been performed on the accumulation of toxic elements in aquatic plants. The main plants that function as phytoremediators in aquatic environments are part of the class of floating plants, such as *Eichhornia crassipes* (Mishra et al., 2008), *Lemna polyrrhiza* (John et al., 2008; Mishra and Tripathi, 2008) and *Pistia stratiotes* (Maine et al., 2004; Mishra et al., 2008).

Pistia stratiotes, also known as water lettuce and belonging to the Araceae family (Rahman and Hasegawa, 2011), have demonstrated great potential as accumulator of toxic compounds present in the water as Cr, Cu, Fe, Mn, Ni, Pb , and Zn (Lu et al., 2011). Although *Pistia* absorbs and accumulates high concentrations of As, Farnese et al (2014) concluded that its use in phytoremediation is still limited, as severe damages were triggered in the plant as a

result of stress, and plants subjected to As caused alterations in the photosynthetis and respiratory metabolism to tolerate the toxic effects. To complement the studies already done, new analyzes were carried out in *Pistia stratiotes* with the objective of assessing the tolerance and the integrated response system of this aquatic plant to survive under the stress caused by As.

2. Material and Methods

2.1. Plant material and growth conditions

Pistia stratiotes L. (Araceae) were collected in the Botanical garden of the Federal University of Viçosa (Viçosa, MG, Brazil) and sterilized with 1% sodium hypochlorite for 1 min and then washed in running water and distilled water. The collected plants were transferred to nutrient solution of Clark (1975), 1/2 of the ionic strength, pH 6.5, being kept in room of plants growth, with controlled light and temperature (25 ± 2 ºC, 230 μmol m^{-2} s^{-1}), and photoperiod of 16 hours. The plants remained under these conditions for three days for acclimatization. After the acclimation period, plants were transferred to containers containing 0.5 L of Clark nutrient solution (1975), 1/2 of the ionic strength, pH 6.5, and exposed in two treatments: control (nutrient solution only) and nutrient solution with As (1,5 mg L^{-1}). To observe the toxicity, absorption and effects on the metabolism of organic acids, the plants were collected at 24 , 48 and 72 hours.

2.2. Determination of absorbed arsenic

After exposure to the treatments, plants were separated into leaves and roots, washed with deionized water and stored in a conventional oven at 80ºC until constant weight was obtained. About 100 mg of the dry matter and 1.5 mL of nitroperchloric mixture was added. After digestion, the mixture was diluted to 25 mL and the determination was performed by atomic absorption spectrophotometry with hydride generation (Shimadzu AA-6701F).

2.3. Fresh mass and dry mass

Plants were collected, separated into leaves and roots and taken immediately to analytical balance to obtain the fresh weight. For each plant, 12 leaf discs with 1 cm of diameter were collected. After weighing, leaves, roots and leaf discs were taken to the stove, where they remained until the constant dry weight was obtained.

2.4. Biochemical characterization

After exposure to As for 24 h, 48 h and 72 h, the plants were collected, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis. The extraction was carried out by rapid grinding of 50 mg of material in liquid nitrogen and addition of methanol as described by Lisec et al. (2006). The methanolic extracts and precipitates were stored at -20°C for subsequent quantification of the metabolites.

2.4.1. Chlorophyll and carotenoids determination

The content of chlorophyll (a and b) and carotenoids were determined immediately after methanolic extraction using aliquots from the supernatant. It was added on the microplate 100 µL of extracts and 100 µL of methanol. The absorbance was measured at 653, 666 and 470 nm. The content of chlorophyll and carotenoids were determined following the equations suggested by Wellburn et al. (1994). Last, the total content of chlorophyll (a + b) as well as chlorophyll a/b ratio were determined.

2.4.2. Determination of protein

Protein content was determined as in Gibon et al. (2004). To the tubes containing the precipitate, corresponding to fraction insoluble in methanol, 400 µL of NaOH 0.1 M were added. After resuspending the precipitate, the samples were incubated for 1 h at 95°C. Subsequently, the tubes were centrifuged at 17 000 xg for 5 minutes. Aliquot of 5 µL of supernatant was added to a microplate containing in each well 180 µL of Bradford reagent (1/5). The absorbance was determined at the wavelength of 595 nm. The content of protein of each sample was determined using a standard curve of bovine serum albumin (BSA). The protein content was normalized by the fresh weight of the samples.

2.4.3. Determination of amino acids

Total amino acids were determined as by Gibon et al. (2004). In one microplate, a mix containing 50 µL of 1 M citrate buffer, pH 5.2 with ascorbic acid 0.2% (w / v), 50 µL of ethanol extract and 100 mL of ninhydrin solution of 1% (w / v in 70% ethanol) were added to each well. Then the plate was sealed with an aluminum tape, resistant to high temperature (3M Model 425® HD) and incubated for 20 min at 95 ° C. After incubation, the plates were centrifuged for 10 sec at 17 000 xg and subsequently the samples were transferred to a new microplate and readings were made at 570 nm. For the determination of total amino acid

content, a standard curve of leucine was performed, and the values were normalized by fresh weight of the sample.

2.4.4. Determination of sugars

The contents of glucose, fructose and sucrose were determined in the methanol soluble fraction as previously described (Fernie et al., 2001). Initially, a mix containing HEPES/KOH buffer 0,1 M pH 7, $MgCl_2$ (30 mM), ATP (60 mg mL^{-1}), NADP (36 mg mL^{-1}) and glucose-6-phosphate dehydrogenase (G6PDH) (70 U mL^{-1}) was prepared. In a microplate, 160 µL of the mix, 5 µL of the extract and 45 µL of H_20 were added to each well. The absorbance was determined at 340 nm in one minute intervals. Once the absorbance was stabilized, it was added 5 µL of hexokinase (1.5 U/reaction), 5 µL of phosphoglucose isomerase (0.7 U/reaction), and 5 µL of invertase (5 U/reaction) to determine glucose, fructose, and sucrose, respectively. To calculate the concentration of the respective sugars the following equation was used: NADPH (µmol) = ΔOD/(2,85*6,22).

2.4.5. Determination of starch

Starch content was measured as previously described (Fernie et al., 2001). To the tubes containing the precipitate resulting from methanolic extraction and 0.1 M NaOH (same extract used for quantification of proteins), 70 µL of 1 M acetic acid was added for neutralization of the extract, and vigorously homogenized. The mix for degradation of starch containing the enzymes amyloglucosidase and α-amylase diluted in sodium acetate 0.5M pH 4.0 was added to 40 µL of suspension and incubated at 55°C for 60 min. The plates were centrifuged for 10 seconds at 10 000 xg and then 40 µL of the suspension was transferred to a new plate where it was added to each well 160 µL of a mix containing HEPES / KOH buffer 1M, pH 7, MgCl2 (30 mM), ATP (60 mg mL^{-1}), NADP (36 mg mL^{-1}), and glucose-6-phosphate dehydrogenase (0.7 U μL^{-1}). The absorbances were read at 340 nm in one minute intervals. Once OD was stabilized, 5 µL of hexokinase (2 units per reaction) was added to the wells. To calculate the concentration of glucose the following equation was used: NADPH (µmol) = ΔOD/(2,85*6,22).

2.5. Gas exchange parameters

The minimum fluorescence (F_0) analysis was performed in the early morning period by excitation of foliar tissues by modulated red light of low intensity (0,03 µmol photons m^{-2} s^{-1}). Maximum fluorescence (*Fm*) was obtained by applying a pulse of 0,8 s of saturating actinic

light (8000 μmol photons m^{-2} s^{-1}). Variable fluorescence (*Fv*) was determined by the difference between F_0 and *Fm* and, from these values, the potential quantum yield of photosystem II was calculated (Van Kooten and Snel 1990).

Leaves were acclimated to actinic light (1000 μmol photons m^{-2} s^{-1}) for 60 s to obtain transient fluorescence (*Fs*), followed by a pulse of saturating light to estimate maximum fluorescence in light (*Fm*). Finally, a pulse of red-distant light was applied to obtain minimal fluorescence after acclimatization to actinic light. The photochemical efficiency of the electron transport associated with photosystem II (*FSII*), the quantum yield of CO_2 assimilation and non-photochemical quenching (NPQ) were calculated using these parameters, as proposed by Maxwell and Johnson (2000).

The net assimilation rate of carbon (*A*), stomatal conductance (*gs*) and internal CO_2 concentration (*Ci*) were determined in open system under saturated light (1000 μmol m^{-2} s^{-1}) and partial CO_2 pressure (LI-6400, Li-Cor Inc., Nebraska, USA) equipped with a blue/red light source (model LI-6400-02B, LI-COR).

2.5.1. Dark and mitochondrial respiration

Dark respiration (R_D) was evaluated pre dawn using an infrared gas analyzer (LI-6400, Li-Cor Inc., Nebraska, USA). Mitochondrial respiration during the day (R_M) was estimated from R_D, according to Bai et al. (2008). R_M was estimated at different temperatures using Q_{10} (Larcher 1983), as follows:

$$R_M = R_D Q_{10}^{(Tm - Td)/10}$$

Q_{10} is equal 2.2, Td is the foliar temperature at which R_D was measured and Tm is the foliar temperature at which the R_M was calculated.

2.5.2. Estimation of the photorespiratory rate

Photorespiratory rate (PR) of the plants subjected to As were calculated using the data obtained with the infrared gas analyzer. The flow of electrons used for carboxylation (ETRc) and oxygenation (ETRo) by Rubisco (ribulose-15-bisphosphate carboxylase/oxygenase) were also calculated, based on the following formulas (Epron et al., 1995; Valentini et al. 1995):

$$PR= 1/12[ETR_T - 4 (A + R_M)];$$

$$ETRc = 1/3[ETR_T + 8\ (A + R_M)];$$
$$ETRo = 2/3[ETR_T + 4\ (A + R_M)]$$

2.6. Statystical analysis

The experiments were conducted in a completely randomized design, with six replicates for each treatment, the data being submitted to ANOVA and the averages calculated by the Tukey test, at 5% probability. Statistical analyses were performed using the statistical GENES program (Cruz, 2008) developed at the Federal University of Viçosa. Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3. Results

3.1. Concentration of absorbed arsenic

Plants exposed to arsenic presented higher accumulation of the contaminant in the root, thus translocating smaller amounts of As to the leaves. Among the three times, plants exposed to As for only 24 hours had the lowest values for both leaves and roots. At the other times, there was no statistical difference between the amounts of arsenic absorbed by the plants (Fig 1).

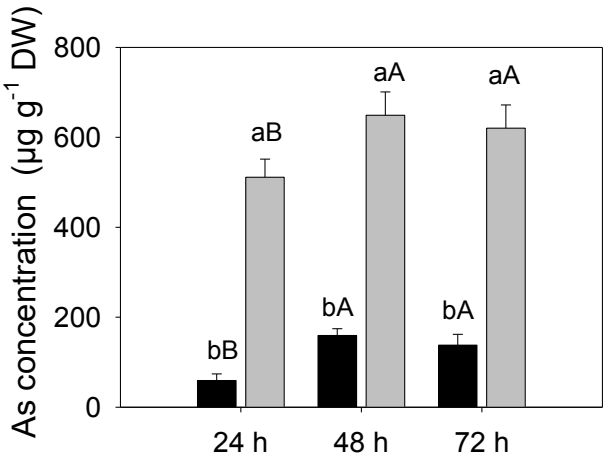

Figure 1. Arsenic concentration in leaves (■) and roots (▭) in *Pistia stratiotes* after 24, 48 and 72 h exposure to As. Averages followed by the same letters do not differ by Tukey's test. Lowercase

letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3.2. Fresh mass and dry mass

Controls and plants exposed to As did not present statistical difference in the fresh leaf weight (Fig 2A), although a greater increase in the percentage of fresh biomass in the controls over time compared to the contaminated plants was observed. From 24 h to 72 h the controls showed biomass gain around 22.5%, while in the presence of As the gain was around 6.43% (Fig 2C).

The same pattern was observed for fresh root mass. Although control and As do not present statistical difference in the three times (Fig 2B), it was observed in the controls increase of biomass of 37.13% and for the As only 18.37% (Fig 2C). These results therefore reflected in the total fresh mass, in which the averages followed a higher value pattern for the As, although a more marked increase of biomass occurred only in the controls over time.

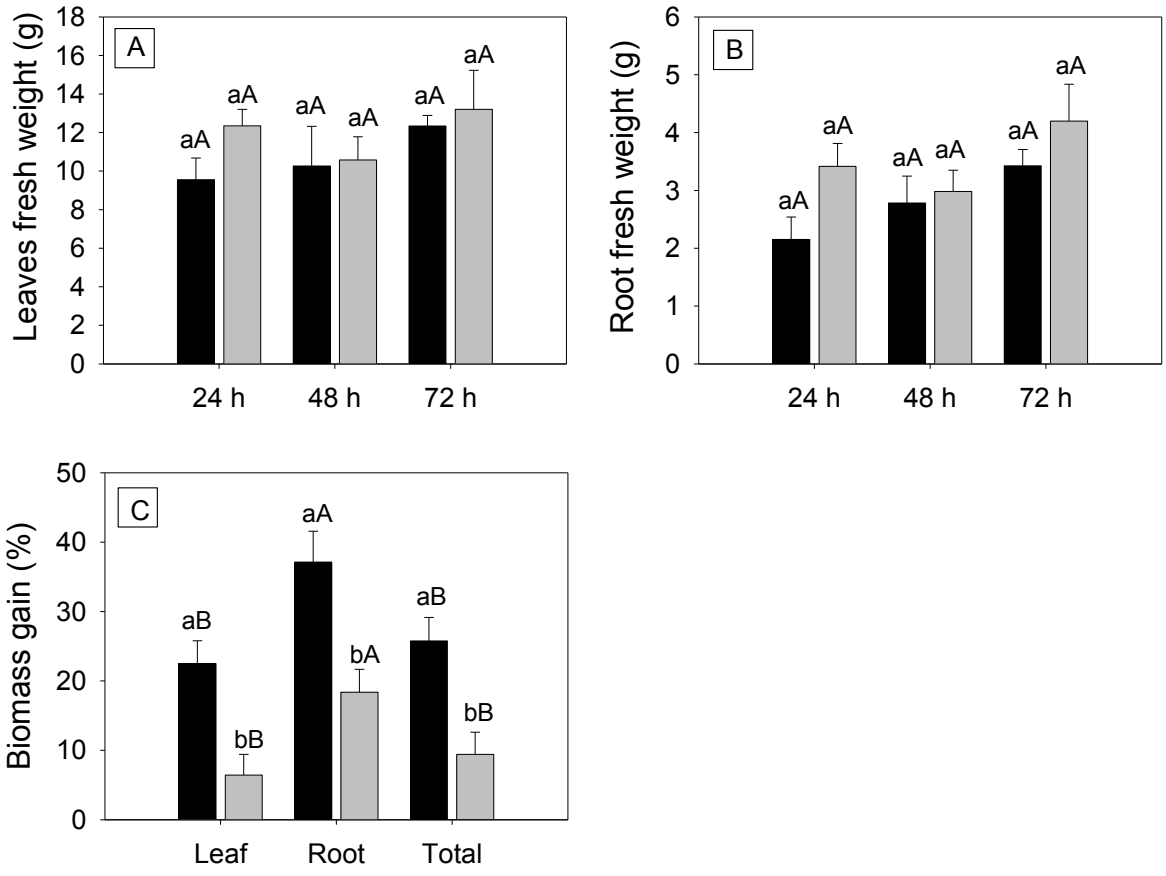

Figure 2. Leaves (A) and root (B) fresh weight in *Pistia stratiotes* exposed to As in 24, 48 and 72 hours and fresh biomass gain after 72 hours exposed to As (C) (■ Control □ As). Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

Dry weight maintained the same pattern observed for fresh weight for leaves and roots (Fig 3A and B). Although, the plants exposed to As showed higher averages over time, the gain of dry biomass was more significant for the controls. In leaves, the increase of dry biomass for the controls was 28.4% against 17.54% for the As and for roots the controls had a percentage gain of 41.1% against 10% for the As (Fig 3C).

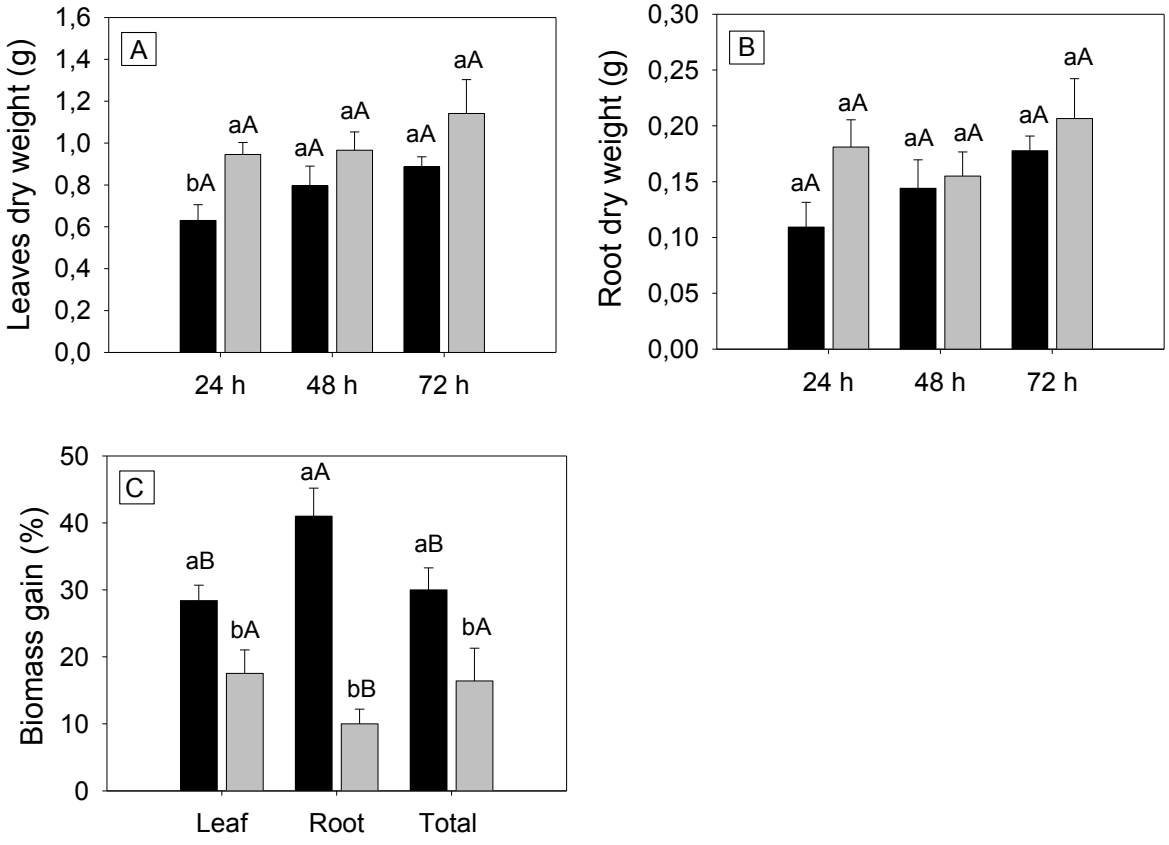

Figure 3. Leaves dry weight (A), root dry weight (B) and dry biomass gain after 72 hours (C) in *Pistia stratiotes* exposed to As after 24, 48 and 72 hours (■ Control □ As). Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between

control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3.3. Biochemical characterization

3.3.1. Chlorophyll and Carotenoids Concentrations

Chlorophyll a and chlorophyll b did not show differences between controls and treatments (Fig 4A and B). The concentration of carotenoids showed a significant difference only between the treatments in 72h, in which there was an increase in the average in leaves exposed to As (Fig 4C). The result observed in the total chlorophyll and chlorophyll a/chlorophyll b follow the patterns found in the chlorophyll concentrations mentioned above (Fig 4D and E).

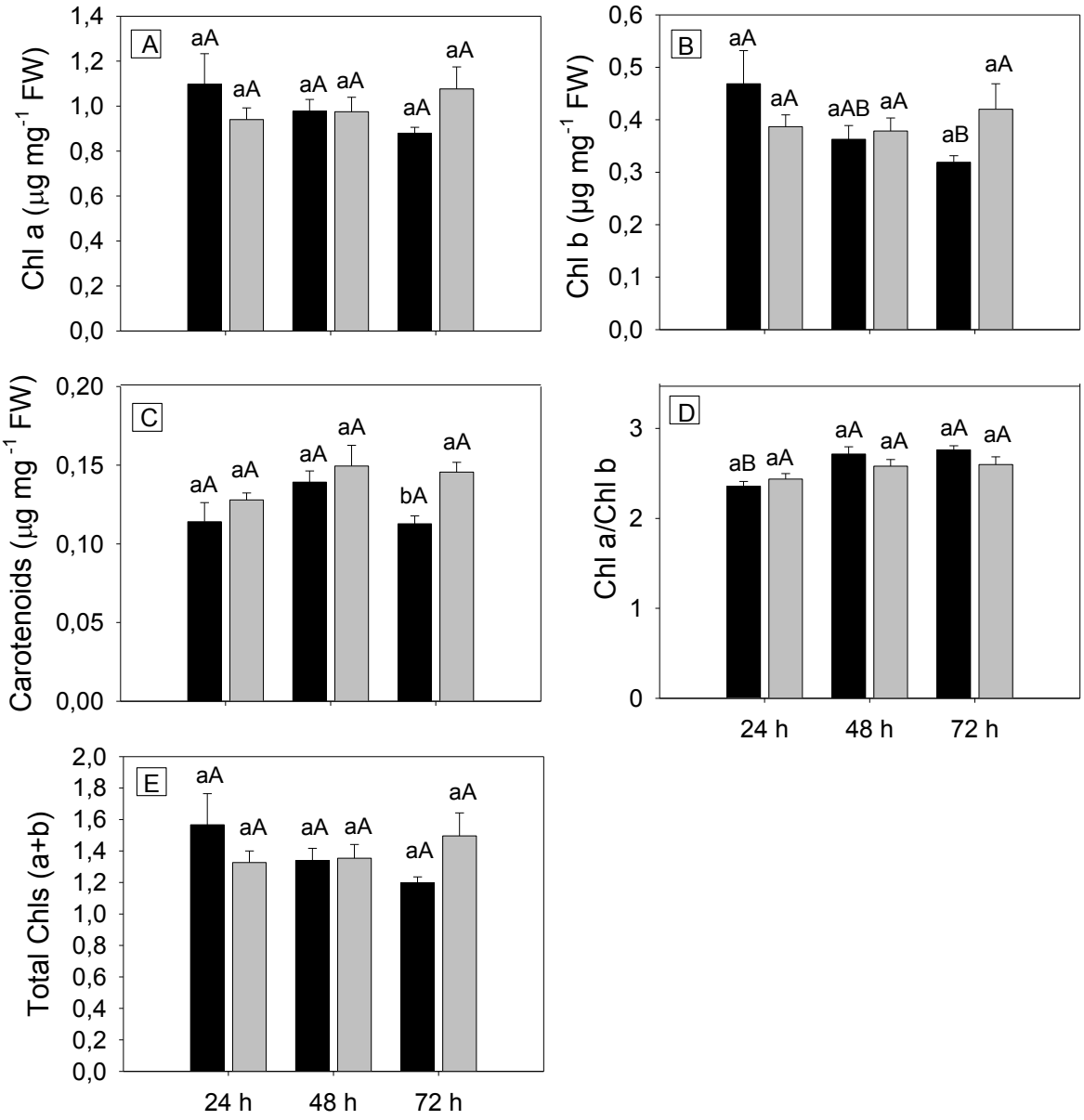

Figure 4. Concentration of chlorophyll a (A), chlorophyll b (B) and carotenoids (C), chl a/b (D) and chl a+b (E) in *Pistia stratiotes* exposed to As after 24, 48 and 72 hours (■ Control ▭ As). Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3.3.2. Concentrations of protein and aminoacids

Protein concentration in leaves and roots decreased after 24 h of exposure to As compared to controls. In contrast, after 48 h and 72 h the protein concentrations remained constant even when exposed to the pollutant (Fig.5 A and B).

Amino acids presented constricting results when comparing leaf and root. For contaminated leaves, there was an increase in the concentration of amino acids in 24h and 48h (Fig 4. C). On the other hand, in contaminated roots, there was a decrease in the concentration of amino acids in 24h. At other times, the two conditions remained constant (Fig 4.D).

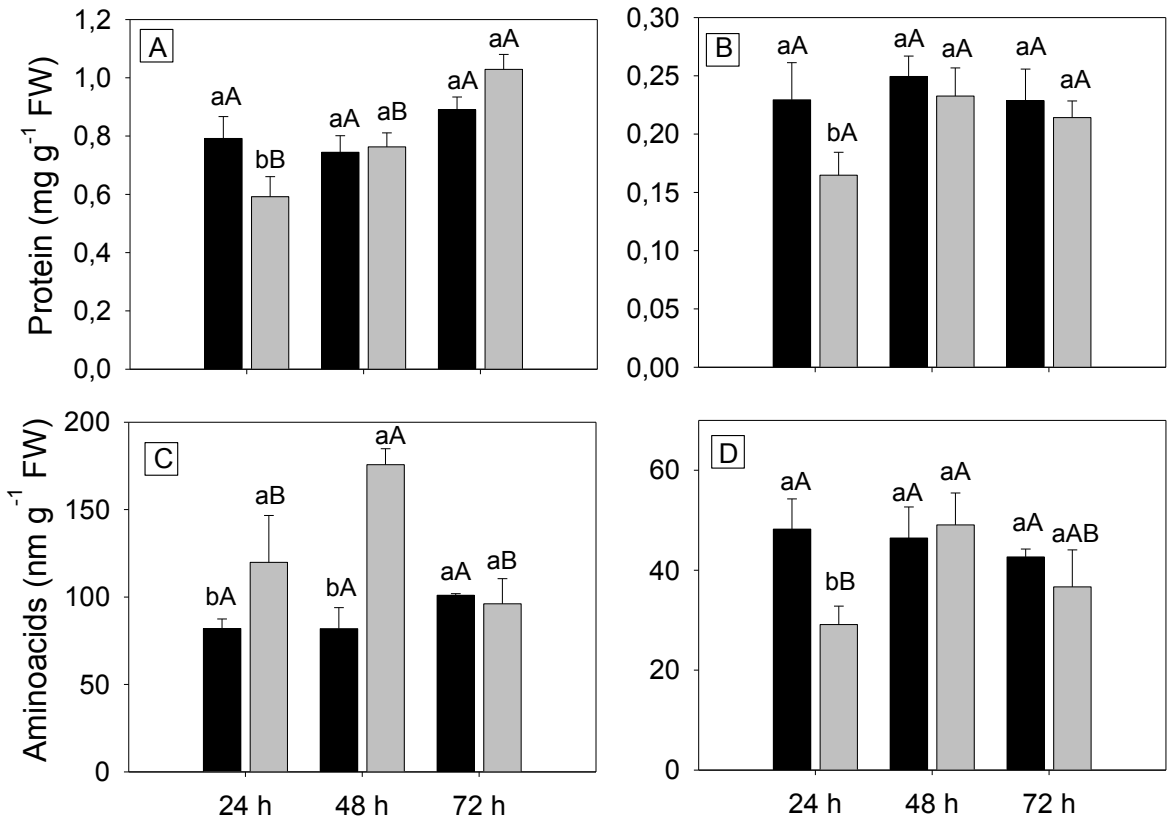

Figure 5. Protein content in leaves (A) and roots (B), amino acids content in leaves (C) and roots (D) in *Pistia stratiotes*, after exposure to As for 24, 48 and 72 h. (■ Control ▭ As). Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3.3.3. Concentration of glucose, fructose and sucrose

The glucose concentration was higher in leaves of plants exposed to As after 24 h, and for the other times, there was a significant decrease in the amount of glucose (Fig 6A). Concentrations of fructose and sucrose presented a pattern of increase in concentrations over the time for plants exposed to As (Fig 6 C and E).

In roots, the glucose concentration was lower in plants with As in 24 h and 48 h (Fig 6B). For fructose in 24 h, As treatment presented a lower concentration, unlike that observed

in other times, when fructose increased and was higher than in controls (Fig 6D). The concentration of sucrose in roots did not present significant differences between the times and the treatments (Fig 6F).

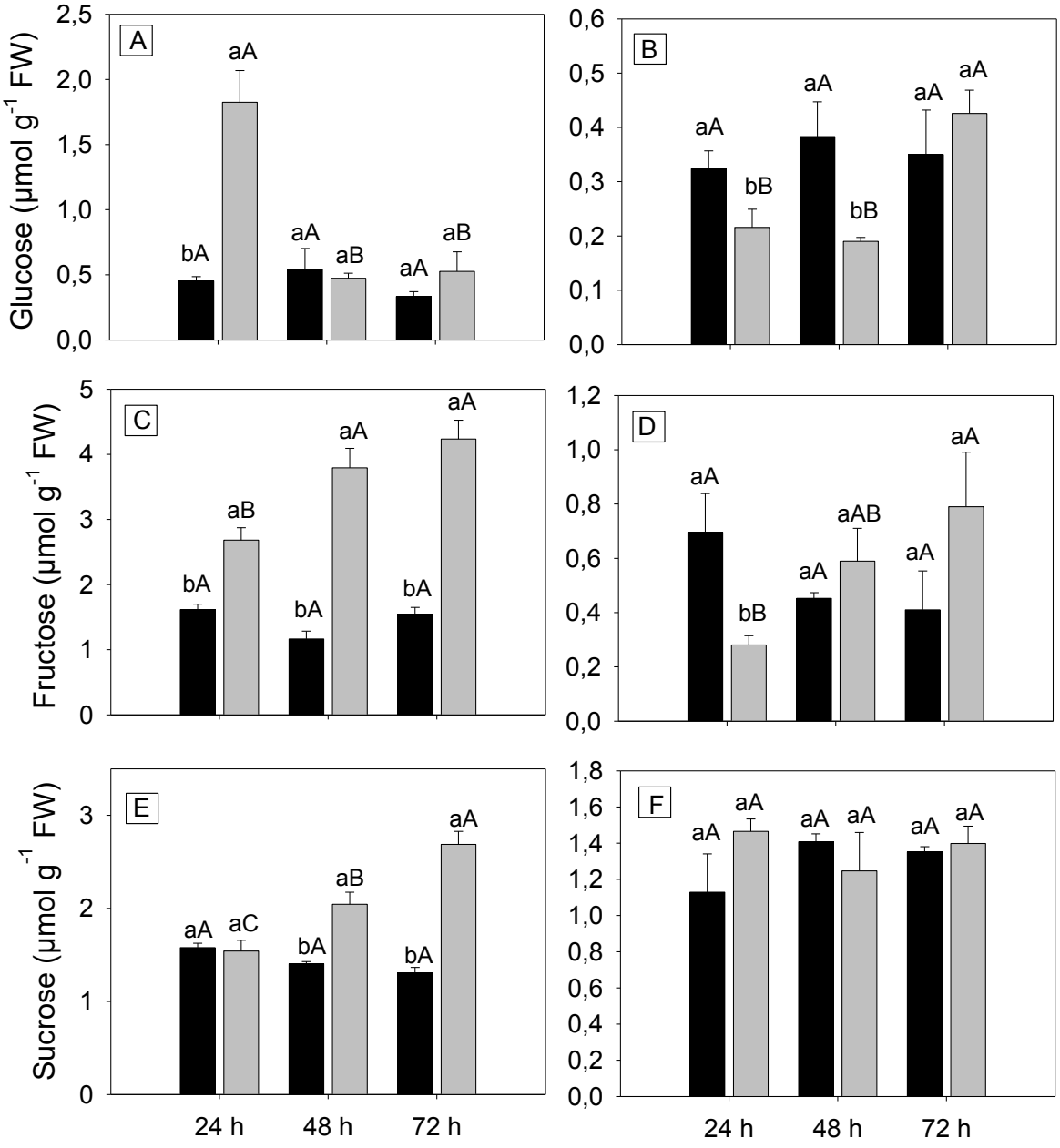

Figure 6. Concentration of glucose, fructose and sucrose in leaves (A, C and E) and roots (B, D and F) in *Pistia stratiotes* after exposure to As for 24, 48 and 72 h. (■ Control □ As). Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3.3.4. Concentration of starch

The concentration of starch in leaves with As increased between 24h and 72h, presenting higher values in comparison to controls (Fig 7A). In roots the reverse occurred, the starch concentrations decreased over time in contaminated roots (Fig 7B) and the control remained unchanged.

In general, the concentrations of all metabolites analyzed were higher in the leaves than in the roots, thus presupposing a higher activity of these metabolites in the shoot of the plants.

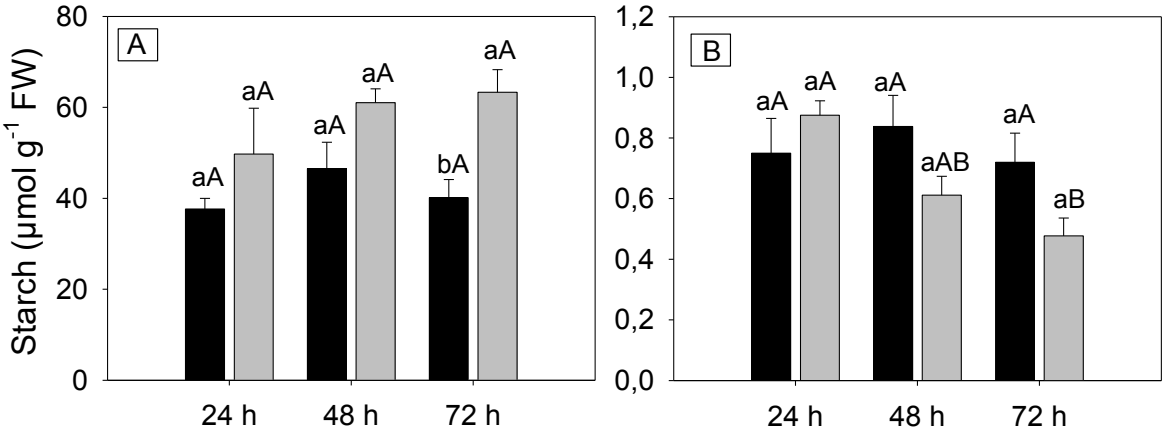

Figure 7. Starch concentration in leaves (A) and roots (B) in *Pistia stratiotes* after 24, 48 and 72 h exposure to As. (■ Control □ As). Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3.4. Analysis of gas exchange parameters

Exposure of *Pistia stratiotes* to As caused changes in photosynthesis, respiration and photorespiration. In relation to the gas exchanges, decreases in the net carbon assimilation rate (*A*) were observed in 48 and 72 h, while the stomatal conductance (gs) and internal CO_2 concentration (Ci) did not differ statistically from controls (Fig 8A, B and C).

Although the As did not trigger changes in the potential quantum yield of photosystem II (Fv/Fm), the operation efficiency of photosystem II (FSII) and the quantum yield of CO_2 assimilation were affected, presenting lower values compared to controls. The non-photochemical quenching (NPQ) of the plants exposed to As did not present a significant difference of the controls, during 72 h (Fig 8D, E, F and G).

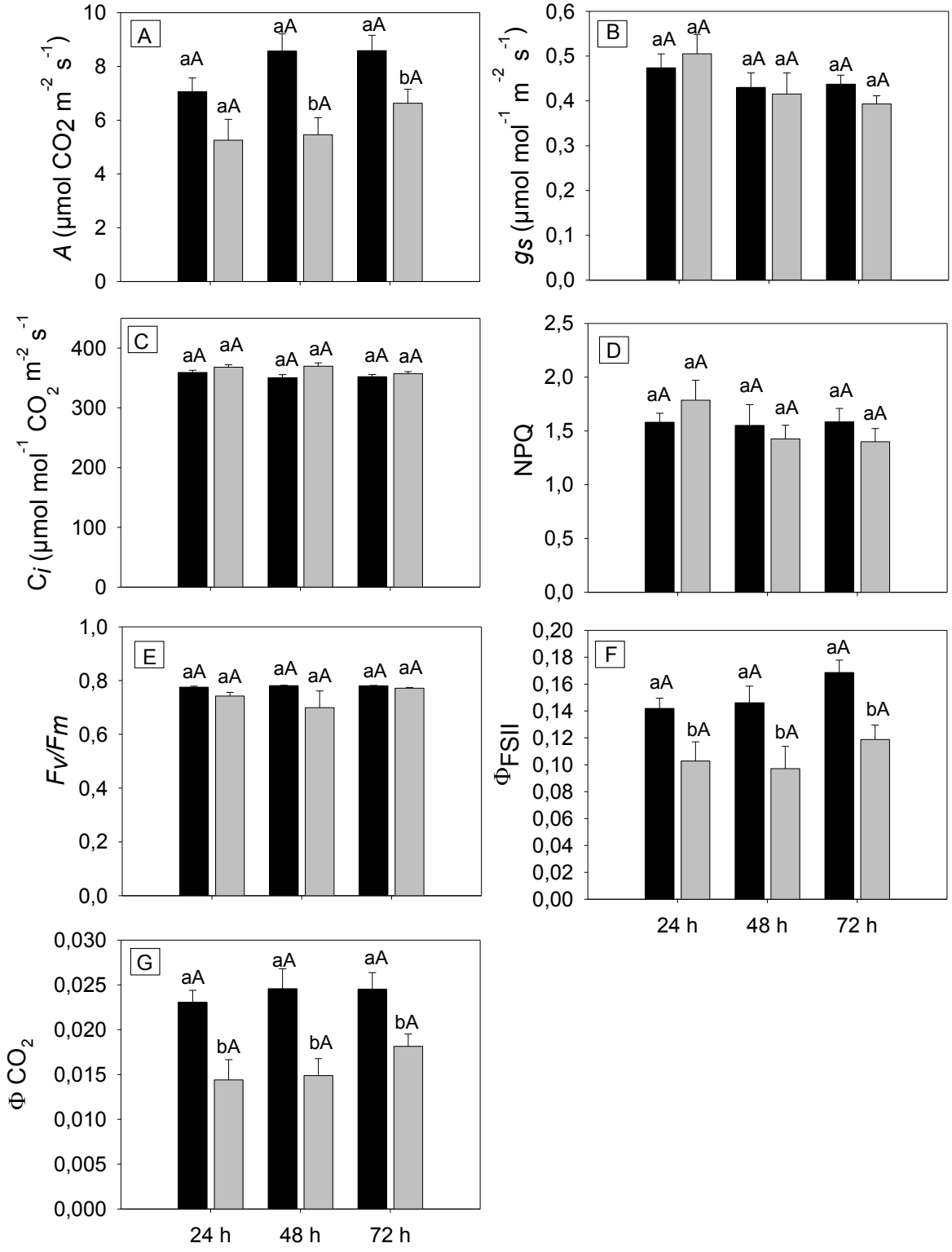

Figure 8. Rate of carbon assimilation (A), stomatal conductance (B), internal CO_2 concentration (C), non-photochemical quenching (D), potential quantum yield of photosystem II (E), operation efficiency of photosystem II (F) and quantum yield of CO_2 (G) in *Pistia stratiotes* exposed to As after 24, 48 and 72 h (■ Control ▭ As). Averages followed by the same

letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3.5. Dark respiration, mitochondrial respiration and photorespiration

Arsenic promoted increased in dark respiration (RD) and mitochondrial respiration (RM) in the leaves of *Pistia*, with the increase being more significant in 48 hours of exposure to the pollutant (Fig 9A and B).

The photorespiratory rate decreased in the presence of As in all times of exposure (Fig 9C). The same pattern was observed in the Rubisco carboxylation rate (ETRc), and in the Rubisco oxygenation rate (ETRo) and as a consequence, the ratio ETRc / ETRo remained inaltered for both treatments (Fig 9D, E and F).

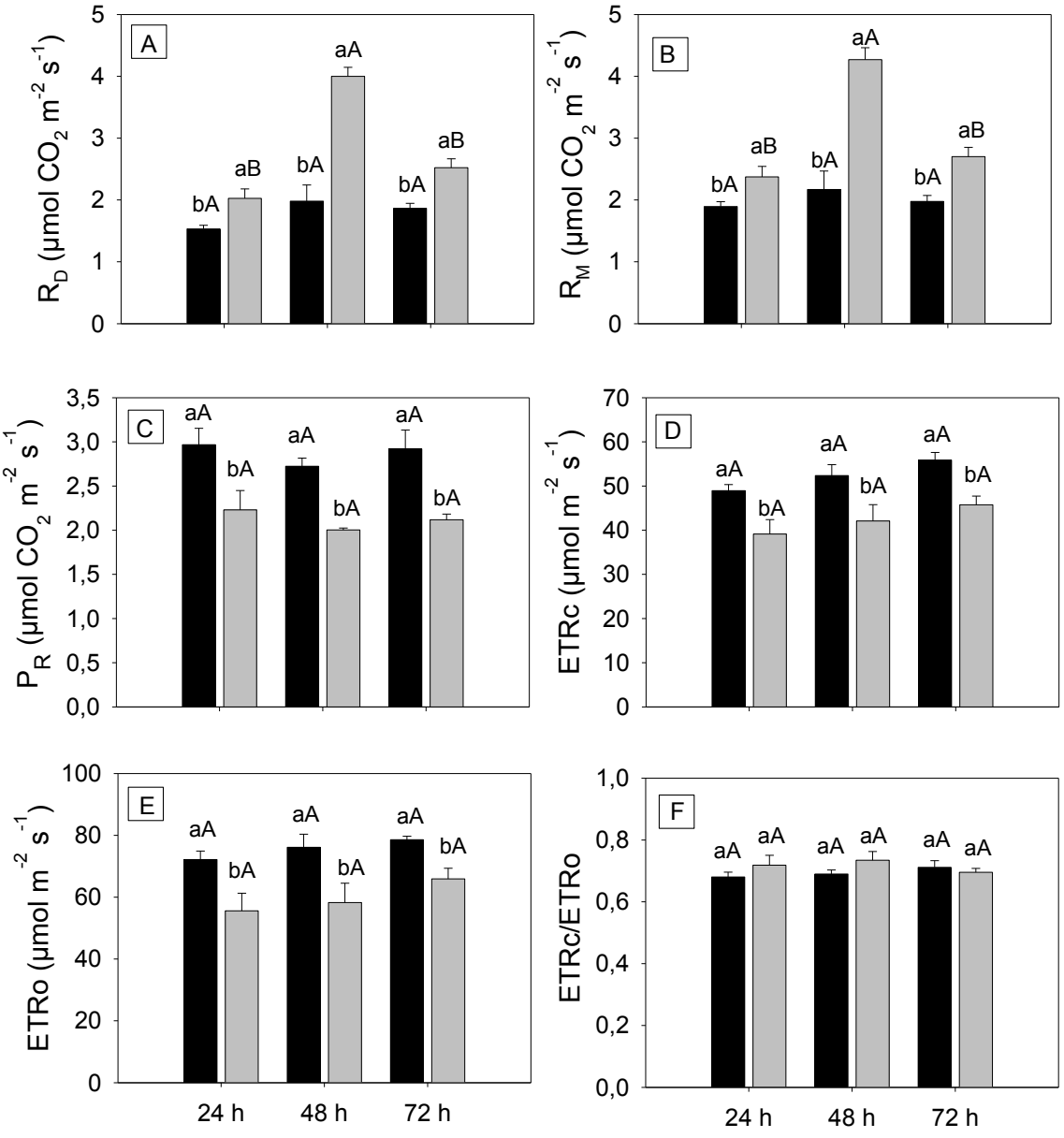

Figure 9. Dark respiration (A), mitochondrial respiration (B), photorespiration (C), Rubisco carboxylation rate (D), Rubisco oxygenation rate (E) and ETRc/ETRo (F) in *Pistia stratiotes*, comparing control with As in 24h, 48h and 72h. (■ Control ▭ As). Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

4. Discussion

Arsenic when present in water or soil in high concentration may interfere in plant growth and development, leading to reduced biomass and plant yield (Hare et al., 2019). The results obtained in this work showed lower gain of fresh and dry biomass due to the exposure of *Pistia stratiotes* to As. Similar results were obtained in other species of macrophytes submitted to As as *Azolla caroliniana*, *Salvinia minima* and *Lemna gibba* (Guimarães et al., 2012) and lettuce plants (Yañez et al., 2019).

In leaves, the decrease in biomass gain can be explained due to damage caused by As in the photochemical and biochemical part of photosynthesis (Gusman et al., 2013). These effects results in lower biomass in roots associated to toxicity by the affinity of the toxic element with sulfhydryl groups of proteins and enzymes present in the root membranes, causing imbalance of the basic functions, which can lead to cell death (Shaibur and Kawai, 2011). When it comes in contact with As, part of available energy that the plant would usually invest in biomass growth and gain is consumed by the formation of elements linked to the defense system, such as phytochelatins production and greater activity of the antioxidant system (Farooq et al., 2015).

The uptake and accumulation of As by several species is very well documented (Kumarathilaka et al, 2018; Liu et al., 2018). In this study, the highest accumulation of As was observed in the roots and lower concentrations were translocated to the shoots. This event can be explained by the ability of As to bind to the components of the root cell wall (Salt et al., 1998), by decreasing the conduction of As through water flow in the xylem (Stoeva et al., 2005) and by the complexation of the metalloid, after reduction of arsenate to arsenite, with thiol groups (Mathews et al., 2010). Similar results were observed in *Eicchornia crassipes* (Meneguelli-Souza et al., 2016) and in *Elodea canadensis* (Pico et al., 2018) in which there was greater accumulation of As in the root when in contact with contaminated solutions.

Although most plant species exposed to As presented a decrease in the concentration of photosynthetic pigments (Armendariz et al., 2016), the results obtained did not show a significant decrease in chlorophyll a, b and carotenoids. The Fv/Fm ratio, which remained constant for control and treatment with As, may explain this result, since changes in its values may be indicators of the decrease in concentration and photo-oxidation of chlorophylls (He et al., 2001). Other studies with As indicated the same response patterns for chlorophyll a and b with *Eichhornia crassipes* (Menegeelli-Souza et al., 2015), *Isatis cappadocia* (Karimi et al., 2013) and *Lactuca sativa* (Gusman et al., 2013).

Non-photochemical quenching (NPQ) has the function of minimizing the production of oxidizing molecules in the photosynthetic apparatus, dissipating excess energy as heat (Li et al., 2000). The NPQ rates did not change in function exposure to As, similar to obtained by Paiva et al. (2009) with *E. crassipes* exposed to Cr. Linearity relationships in the results between concentrations of chlorophyll a and b, carotenoids and NPQ were also discussed by Brugnoli et al. (1998) in *Zea mays* and *Herdera helix*. According to the results, it can be inferred that exposure to As for 72h was not time enough to trigger photochemical limitations in *Pistia stratiotes*.

In *Pistia stratiotes* subjected to As, no changes were observed in stomatal conductance and in internal CO_2 concentration, suggesting that there is no stomatic limitation. In this way, we can correlate the decrease in the rate of carbon assimilation with the biochemical limitations caused by the stress with As, including inhibition in the electron transport chain or inhibition in the activity of enzymes of the Calvin Cycle caused for oxidative stress (Tanaka et al., 1982; Sharma, 2012).

Increased respiration in As-exposed plants can be explained by the structural similarity between arsenate (AsV) and phosphate, both of which can compete in the mitochondria for the same active site of ATP synthase. As a result of this competition, the AsV-ADP complex is formed, leading to the reduction of the ATP concentration. This decrease in ATP is what triggers increased respiratory activity and increased formation of the AsV-ADP complex (Wickes and Wiskich, 1975; Moore et al., 1983). Due to the continuous formation of this complex, more energy is used by the cell, resulting in the accumulation of reactive oxygen species (ROS) (Finnegan and Chen, 2012). Reactive oxygen species further increase stress, which can lead to further damage to photosynthetic rates. In addition, it is likely that glycolysis and the citric acid cycle process the substrates more rapidly, in an attempt to maintain the proton gradient at a level sufficient to give continuity to the respiratory process

(Finnegan and Chen, 2012). Thus, the significant increase in respiration in 48h of exposure to As, with a subsequent decrease in respiration in 72h observed with *Pistia stratiotes*, emphasizes that this increase is not sustained for long periods, caused by high energy demand that the cell can not sustain.

Unlike respiration, the oxygenation rate of Rubisco decreased in contaminated plants. This parameter indicates a reduction in photorespiration, as a consequence of the inhibition of enzymes involved in the photorespiratory process. Glycine decarboxylase (GDC), an enzyme essential for photorespiration, contains lipoamide dehydrogenase (LPD). LPD catalyzes the transfer of electrons from the reduced dihydrolipoamide cofactor to NAD^+ as part of the enzyme reaction cycle of the complex and are inactivated in the presence of As. (Finnegan and Chen, 2012). Similar effects was previously reported for mutants of *A. thaliana* that are unable to produce the lipoamide dehydrogenase 2 enzyme component of GDC in the presence of As (Chen et al. 2014).

Exposure of plants to As may lead to excessive production of ROS, such as superoxide radical and hydrogen peroxide (Rahman et al., 2015) and cause oxidation of amino acid side chains and the formation of covalent crosslinks between them may occur, thus causing denaturation of cellular proteins (Seneviratne et al., 2017). Therefore, the decrease in protein contents in leaves and roots after 24 hours of exposure to As may be related to this mechanism. The results observed in 48 hours and 72 hours, when there was an increase in the protein content, may be related to the ability of *Pistia* to tolerate the stress or defense mechanisms developed by plants against toxic elements. Among these mechanisms, signaling molecules and proteins that express during stress comprise the complex signal transduction system.

Similar results were reported by Anjum et al. (2016) in two maize cultivars exposed to Cd and As, while one was tolerant to stress and increased in protein levels, the other was not able to combat the overproduction of ROS, thus presenting a decrease in protein. Other studies have also observed increase of proteins under stress by toxic elements (Verma and Dubey, 2003; Verbruggen et al., 2009; Kumar et al., 2015).

Precursors of proteins, amino acids also play important roles in stress tolerance in plants. Among them, the regulation of intracellular pH and ion transport, modulation of stomatal conductance and detoxification of ROS (Szabados and Savoure, 2010; Choi et al., 2011). As with proteins, the generation of ROS may lead to a decrease in amino acid levels, as observed in roots, but not in leaves, after 24 hours of exposure to As. The observed differences in

amino acid content between leaf and root can be explained by the higher concentration of As found in the roots. Xu et al. (2012) reported that plants exposed to toxic elements also accumulated specific amino acids, acting as signaling molecules, regulating ionic transport and aiding in detoxification. Histidine, proline, cysteine and glycine, along with other amino acids, increased significantly after exposure to heavy metals (Davies et al., 1987).

The metabolism of basic carbohydrates such as starch, glucose, fructose and sacarose was affected in the presence of As. Guo et al. (2017) studied the modifications caused in *Landoltia punctata* and observed induction in the accumulation of starch caused by exposure to cobalt (Co) and nickel (Ni), as was observed in *Pistia* leaves contaminated with As. Other authors, such as Appenroth et al. (2010) and Sree et al. (2015), also reported that the starch accumulation in *Spirodela polyrhiza* and *Lemna minor* was notably affected by Co and Ni. This result indicates that carbon metabolism may be disordered in the presence of toxic elements (Sree et al., 2015).

The toxic metals may restrict the enzymes involved in the synthesis/degradation of the starch (Ahmad and Ashraf, 2011), thus, the possible decrease in α-amylase activity and increase of β-amylase, it is expected in greater starch accumulation, as reported by Higuchi et al. (2015) in the stalk of *Phragmites australis* under cadmium stress. In addition to changes in enzymes, the presence of toxic elements may lead to changes in phloem translocation, thus reducing the export of photo-assimilates to other parts of the plant (Tian et al., 2010).

Starch is also becoming a key molecule in mediating plant responses to abiotic stresses. For *Arabidopsis thaliana* exposed to As, there was a decrease in the starch concentration in the leaves (Barbosa et al., 2019). These different, sometimes opposing modifications, highlight the importance of starch plasticity to respond in a variety of situations, demonstrating that it can not be considered just a storage compound. The same can be observed for sugars, which depending on the stress and the species, a type of sugar can be altered presenting accumulation or decrease in its concentration (Thalmann and Santelia, 2017).

In the presence of As, significant alterations in carbohydrate metabolism can occur, especially accumulation of sucrose and hexoses (Mishra and Dubey 2013). Several authors have reported that sugars tend to accumulate in plants exposed to different toxic elements due to changes in enzymatic activity involved in carbohydrate metabolism, a decrease in the use of carbohydrates for growth or reduction in their translocation from the source (leaves) for the root (Jha and Dubey, 2004; Devi et al., 2007; Mishra and Dubey, 2013). Vezza et al. (2018)

verified that As induced an increase in the accumulation of sugars in soybean leaves, as was observed in the leaves of *Pistia stratiotes*, in which there was an increase in soluble sugars (glucose, fructose and sucrose).

In contrast, in the root of Pistia, glucose and fructose concentration decreased and sucrose remained similar to the control levels. The same pattern was observed in the roots of soybean, in which the soluble sugars presented decrease in the roots in relation to the controls (Vezza et al., 2018). The exposure to As may reduce photosynthetic rate, and consequently decrease photoassimilate production (Myśliwa-Kurdziel et al., 2004; Stoeva et al., 2005; Vernay et al., 2007) and may also affect the transport of sugar through of the phloem even from the inhibition of photosynthesis (Ainsworth and Bush, 2011). In Pistia, the highest concentration of As found in the root can also explain all these metabolic changes reported previously.

5. Conclusions

The absorption and accumulation of As by Pistia stratiotes caused some damage to the physiological processes essential to plants. However, levels of chlorophyll and carotenoids, proteins, starch and some of the photosynthetic parameters did not suffer significant changes in response to As. These parameters contributed to the maintenance of the integrated system of stress response; allowing plants to tolerate the concentrations of As used in this experiment.

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CHAPTER 2: Alterations on organic acids metabolism in *Pistia stratiotes* subjected to arsenic

ABSTRACT

The effect of arsenic toxicity (As) on respiration and the participation of organic acids and NAD (H) and NADP (H) in the stress response were studied in *Pistia stratiotes*. The plants were collected in Botanic Garden of the Federal University of Viçosa, cultivated in nutrient solution, pH 6.5 and ½ ionic strength, and submitted in two treatments: control (nutrient solution only) and As (1,5 mg L^{-1}) for up to 72 hours. The organic acids analyzed showed an increase in leaves and roots, except malate, which showed a decrease in its concentration in the roots. This decrease may be explained by the deviation of fumarate or malate to other pathways that may act in the defense system against As. Citrate synthase, fumarase, isocitrate dehydrogenase and malate dehydrogenase, showed increased in activities on stressed leaves, which reaffirms the increase in respiration obtained previously. However, the decreased in the activity of the enzymes in root, can be explained due to the more severe stress promoted by the greater accumulation of As by this part of the plant. The same pattern was observed for the pyridine nucleotides in the leaves, mainly in the increase of NADPH, since this reduced form is important for the cycle of ascorbate and glutathione that act directly in the defense of the cells against the oxidative stress caused by the presence of ROS. In the roots, higher concentration of arsenic accumulated can lead to inhibition of respiration, which can be observed by not detecting the regeneration of NAD^+, to the point of leading the decline of NADH production. In this way, the most severe damages in the presence of As were observed in the roots. The results obtained further limit the potential of *Pistia stratiotes* for use in phytoremediation, since in high concentration of As the damages appear to be irreversible.

Key-words: pyridine nucleotides; malate; TCA cycle, fumarate

RESUMO

O efeito da toxicidade do arsênio (As) na respiração e a participação dos ácidos orgânicos e do NAD (H) e NADP (H) na resposta ao estresse foram estudadas na *Pistia stratiotes*. As plantas foram coletadas no Horto Botânico da Universidade Federal de Viçosa, cultivadas em solução nutritiva, pH 6,5 e ½ força iônica, e submetidas a dois tratamentos: controle (apenas a solução nutritiva) e As (1,5 mg L^{-1}) por até 72 horas. Os ácidos orgânicos analisados apresentaram aumento nas folhas e raízes, exceto o malato, que apresentou decréscimo em sua concentração nas raízes. Este decréscimo pode ser explicado pelo desvio de fumarato ou de malato para outras vias que podem atuar no sistema de defesa contra o As. A citrato sintase, fumarase, isocitrato desidrogenase e malato desidrogenase, enzimas do ciclo dos ácidos tricarboxílicos que foram quantificadas, apresentaram aumento nas atividades em folhas estressadas, o que reafirma o aumento na respiração obtido como resultado anteriormente. No entanto, o decréscimo na atividade das enzimas na raiz, pode ser explicada devido ao estresse mais severo pelo maior acúmulo de As por esta parte na planta. O mesmo padrão foi observado para os nucleotídeos de piridina nas folhas, principalmente no aumento de NADPH, já que esta forma reduzida é importante para o ciclo de ascorbato e glutationa que atuam diretamente na defesa das células contra o estresse oxidativo causado pela presença de ROS. Nas raízes, o acúmulo de As pode levar a inibição da respiração, o que pode ser observado pela não detecção na regeneração de NAD^+, a ponto de levar ao declínio a produção de NADH. Dessa forma, os danos mais severos na presença do As foram observados nas raízes. Os resultados obtidos limitam ainda mais o potencial da *Pistia stratiotes* para uso na fitorremediação, já que em alta concentração de As os danos parecem ser irreversíveis.

Palavras-chave: nucleotídeos de piridina; malato; ciclo do TCA, fumarato

1. Introduction

Arsenic (As) is a metalloid found in high concentrations in contaminated waters and soils and it is easily absorbed by plants. Most of the As is usually retained in the roots, and a minor part is translocated and accumulated in leaves (Kumar et al., 2015). The first response observed in plants exposed to As is an increase in the production of reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide. ROS are reactive molecules, which can oxidize various cell macromolecules, altering cellular redox balance and triggering oxidative stress (Demidchik, 2015). In general, ROS overproduction can cause changes in plant development, leading to irreversible damage to metabolic pathways leading ultimately to cell death (Abbas et al., 2018).

The tricarboxylic acids cycle (TCA) is an important connector of several metabolic pathways such as respiration, photorespiration and nitrogen assimilation (Foyer et al., 2011). The TCA cycle represents a series of chemical reactions used by organisms to generate energy. The cycle is composed by eight enzymes that bind the oxidation of pyruvate and malate (generated in the cytosol) to CO_2, with the generation of NADH for oxidation by the mitochondrial respiratory chain (Fernie et al., 2004). Among the eight enzymes, fumarase, malate dehydrogenase and 2-oxoglutarate dehydrogenase appear to be more involved in cycle regulation (Araújo et al., 2012). The TCA cycle can be considered an amphibole, since the intermediates produced along the cycle can be used in several anabolic reactions (Sweetlove et al., 2010). The enzymes that produce these important intermediates are citrate synthase (CS) that catalyzes the condensation of acetyl-CoA with oxaloacetate forming the citrate, fumarase (FUM) that catalyzes the reversible hydration of fumarate in malate and malate dehydrogenase (MDH) which oxidizes the malate to oxaloacetate, producing a molecule of NADH. The isocitrate dehydrogenase (IDH), in turn, catalyzes the conversion of isocitrate to α-ketoglutarate.

The production of organic acids (pyruvate, citrate, malate, fumarate and succinate) known to be TCA cycle intermediates, have also been correlated with tolerance to abiotic stress in plants. They help in tolerance to toxic elements and plant-microbe interactions at the root-soil interface, have important functions during nutrient deficiency and are involved in various biochemical pathways such as energy production and formation of amino acid biosynthesis precursors. They are also involved in the assimilation of ammonia, and in the production of specific compounds necessary for defense against stress (Lopez- Bucio et al., 2000; Sweetlove et al., 2010).

Organic acids, when exuded by the root, can form stable complexes with some metals in the soil, making them less available in the environment and acting as a mechanism of tolerance to stress in plants. The action of root organic acids on stress tolerance by heavy metals was characterized by Chen et al. (2017), involving the detoxification of aluminum, zinc, cadmium and lead, with the participation of malate, citrate and oxalate. In these cases, the plants use root exudation to adapt to different soil conditions, and these exudate compounds can alter the environmental condition to improve the growth and development of the species. The most well documented mechanism of detoxification mediated by organic acids is the exclusion of aluminum, which limits the absorption of Al^{3+} which may be detrimental to the roots of plants (Kochian et al., 2004; Delhaize et al., 2012).

Pyridine nucleotides, NAD(H) (nicotinamide adenine dinucleotide) and NADP(H) (nicotinamide adenine dinucleotide phosphate) are essential for the transport of electrons and are known as the central metabolites that regulate cellular redox homeostasis. The importance of NAD and NADP metabolism in plant development and stress tolerance is well known (Hashida et al., 2009; Srivastava et al., 2013). These nucleotides also play vital roles in signaling via the generation and elimination of ROS (Mittler et al., 2004). Relating the role of pyridine nucleotides in aquatic plants, Srivastava et al. (2011) demonstrated the importance of the redox state and energy balance to determine the extent of stress caused by As in *Hydrilla verticilatta.*

One of the best techniques used in phytoremediation is the use of aquatic plants (Jasrotia et al., 2017). Among them, *Pistia stratiotes* has been noted for having potential to accumulate some heavy metals (Lu et al., 2011). However, in comparison with other toxic elements, few studies have deeply analyzed the effects of exposure to As in the respiratory process of plants and the changes that this toxic element can cause in the TCA cycle and the formation of organic acids. Farnese et al. (2017) found that water lettuce leaves showed increase in respiration under stress by As. To complement previous studies, the aim of this study was to verify the changes in the respiratory process, the consequences that As brought in the formation of the intermediaries of the TCA cycle, as well as the participation of the pyridine nucleotides in the modulation of the stress response.

2. Material and Methods

2.1. Plant material and growth conditions

Pistia stratiotes L. (Araceae) were collected in the Botanical garden of the Federal University of Viçosa (Viçosa, MG, Brazil) and sterilized with 1% sodium hypochlorite for 1 min and then washed in running water and distilled water. The collected plants were transferred to nutrient solution of Clark (1975), 1/2 of the ionic strength, pH 6.5, being kept in room of plants growth, with controlled light and temperature (25 ± 2 ºC, 230 μmol m^{-2} s^{-1}), and photoperiod of 16 hours. The plants remained under these conditions for three days for acclimatization. After the acclimation period, plants were transferred to containers containing 0.5 L of Clark nutrient solution (1975), 1/2 of the ionic strength, pH 6.5, and exposed in two treatments: control (nutrient solution only) and nutrient solution with 18 mM of arsenic (Na_2HAsO_4). To observe the toxicity, absorption and effects on the metabolism of organic acids, plants were collected at 24 , 48 and 72 hours.

2.2. Obtaining material for further analysis

The plants were immediately frozen in liquid nitrogen, and stored at -80°C until further analysis. The leaves and roots were distributed in two tubes: 50 mg was used to the enzymatic activity analysis and 50 mg to determine the concentration of malate and fumarate. For quantification of pyridine nucleotides were obtained two eppendorfs of 25 mg (one for NADP and NAD, and other for NADPH and NADH). For malate and fumarate analysis, material was extracted with addition of methanol as described by Lisec et al. (2006).

2.3. Determination of malate and fumarate content

Malate and fumarate content were determined as previously described by Nunes-Nesi et al. (2007). The mix containing buffer Tricine / KOH 0.4 M, pH 9; $MgCl_2$ 10 mM, MTT (methylthiazolyldiphenyl-tetrazolium bromide) 10 mM, NAD^+ (60 mM), phenazinaetosulfato 20 mM, Triton X100 10% (v/v) and H_2O was added to 10 μL of methanol extract in a microplate reader. The absorbances were read at 570 nm in one minute intervals. Once OD was stabilized, 5 μL of malate dehydrogenase (1 U per reaction) was successively added to the wells and, after further OD stabilization, 5 μL of fumarase (0.1 U per reaction). Both enzymes were resuspended in the same buffer of the reaction medium. The determination of malate and fumarate concentrations were made from calibration curves using malic and fumaric acid as standards. The results of these organic acids were expressed in μmol g^{-1} FW.

2.4. Pyridine nucleotides

NAD(H) and NADP(H) were determined as described by Schippers et al. (2008). Around 25 mg of leaf powder were aliquoted twice into 1.5 ml tube. NAD^+ and $NADP^+$ were extracted with 250 µL of 0.1 M $HClO_4$ and NADH and NADPH were extract with 250 µL 0.1 M KOH. Reduced and oxidized forms are distinguished by preferential destruction in acid or base, respectively. The powder was immediately suspended after adding the solution by tapping and vortex. The samples were incubate 10 min on ice and centrifuged at 17 000 g for 10 min at 4ºC. 200 µL of supernatant were taken into a new tube that was boiled for 2 min, rapidly cooled on ice, and neutralized as follows. All the following steps were done on ice. The alkaline extract was neutralized by adding 200 µL of 0.1 M $HClO_4$ in 0.2 M Tris pH 8.4 and the acid extract was neutralized with 200 µL of 0.1 M KOH in 0.2 M Tris pH 8.4. The final pH of the neutralized extracts was between 8.0 and 8.5. NADP(H) was measured in the presence of 9 $U.mL^{-1}$ of glucose-6-phosphate dehydrogenase (G6PDH) grade I, 0.3M Tricine/KOH, pH 9, 12 mM Na_2-EDTA, 0.3 M of phenazine methosulfate (PMS), 1.8 mM methylthiazolyldiphenyl-tetrazolium bromide (MTT), and 9 mM glucose-6-phosphate. NAD(H) was measured in the presence of 18 $U.mL^{-1}$ alcohol dehydrogenase (ADH), 0.3M Tricine/KOH, pH 9, 12 mM Na_2-EDTA, 0.3 mM phenazine ethosulfate (PES), 1.8 mM MTT, and 1,5 M ethanol. The absorbance was followed at 570 nm at 30°C until the rates were stabilized.

2.5. Enzymatic activities

Aliquots of 50 mg of ground material were used for the protein extraction with 1 mL of the extraction buffer containing 50 mM HEPES-KOH (pH 7.5), 10 mM $MgCl_2$ (pH 7.4), 1 mM EDTA, 1 mM benzamidine, 1mM aminocaproic acid, 1 mM EGTA, 0.25% (w/v) bovine serum albumine, 1% (v/v) Triton-X100, 0.25 mM DTT, 1 mM phenylmethylsulfonylfluoride, 17.4% (v/v) glycerol. The last three components were added just prior to extraction (Gibon et al., 2004).

The total enzymatic activity of NAD dependent malate dehydrogenase, NAD dependent isocitrate dehydrogenase and citrate synthase were obtained as described by Hummel et al. (2010). The fumarase activity was assayed in the malate-forming direction as described previously (Omena-Garcia et al., 2017).

2.6. Statystical analysis

The experiments were conducted in a completely randomized design, with six replicates for each treatment, the data being submitted to ANOVA and the means calculated by the Tukey test, at 5% probability. Statistical analyses were performed using the statistical GENES program (Cruz, 2008) developed at the Federal University of Viçosa. Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3. Results

3.1. Malate and fumarate determination

In *Pistia stratiotes*, organic acids (malate and fumarate) were found in greater concentrations in leaves of plants exposed to As in comparision to controls after 48h and 72h. Malate had a higher concentration than the fumarate in both leaf and root (Fig 1A and C).

In root exposed to As, malate was smaller and fumarate was higher than control plants. In general, the time exposure to As did not significantly alter the concentration of organic acids (Fig 1B and D).

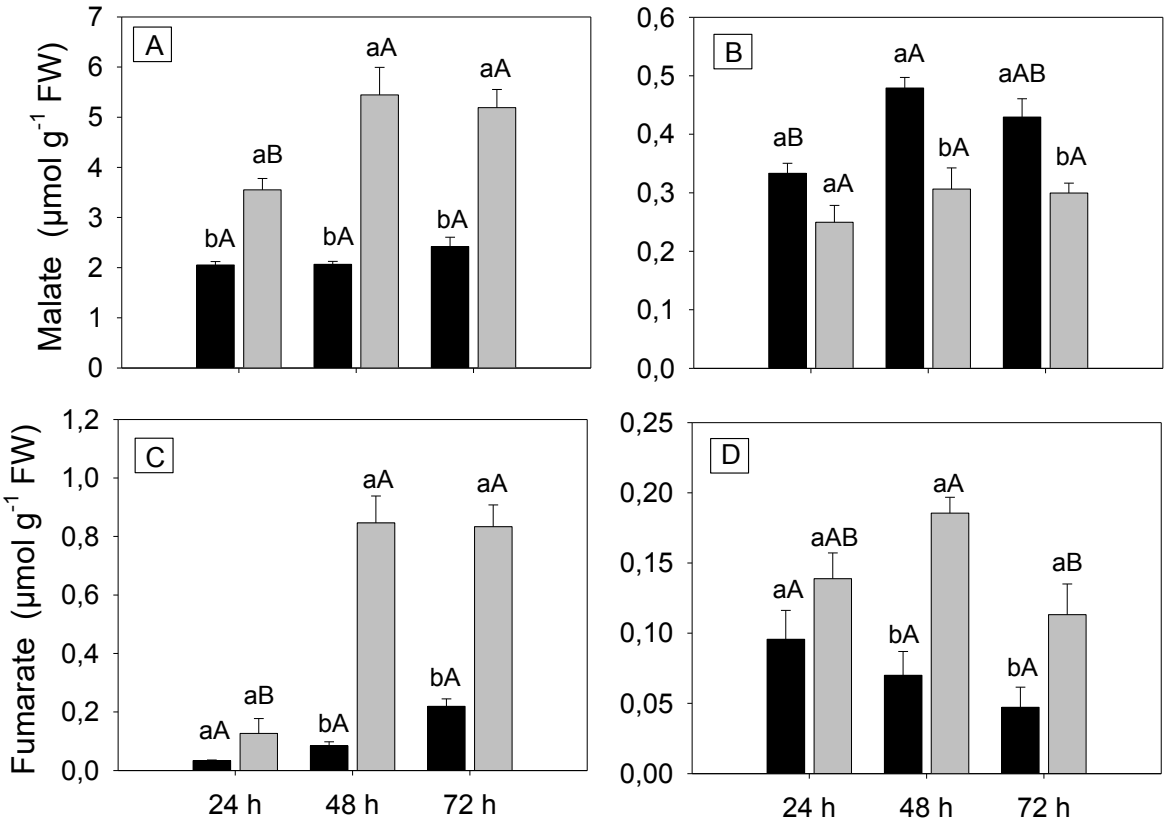

Figura 1. Malate content in leaves (A) and root (B); fumarate content in leaves (C) and root (D) in *Pistia stratiotes,* comparing control with As in 24, 48 and 72 hours. (■ Control ■ As). Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3.2. Pyridine nucleotides

NADH showed increase between 48 and 72 h in leaves of plants exposure to As. The concentration of NAD^+ was higher in 24, 48 and 72 h in stressed plants comparing to the control (Fig 2A and G). The phosphated nucleotides ($NADP^+$ and NADPH) increased in leaves exposed to As in comparision to control plants between 48 and 72 h (Fig 2C and E).

In roots, pyridine nucleotides concentrations presented no significant differences among the treatements. Although the results do not present statistical differences, the pattern of NADH decrease over time in stressed plants is evident, and conversely a gradual increase in NADPH concentration between 24h and 72h is observed (Fig 2B, D and F). It is important to note that it was not possible to detect NAD^+ in roots by the methodology used. In Fig 3 the ratio NAD/NADH (3A) in leaves and ratios NADP/NADPH in leaves and roots (3B and C).

Only after 24 hours is possible to observe statistical difference between control and As in the three graphs.

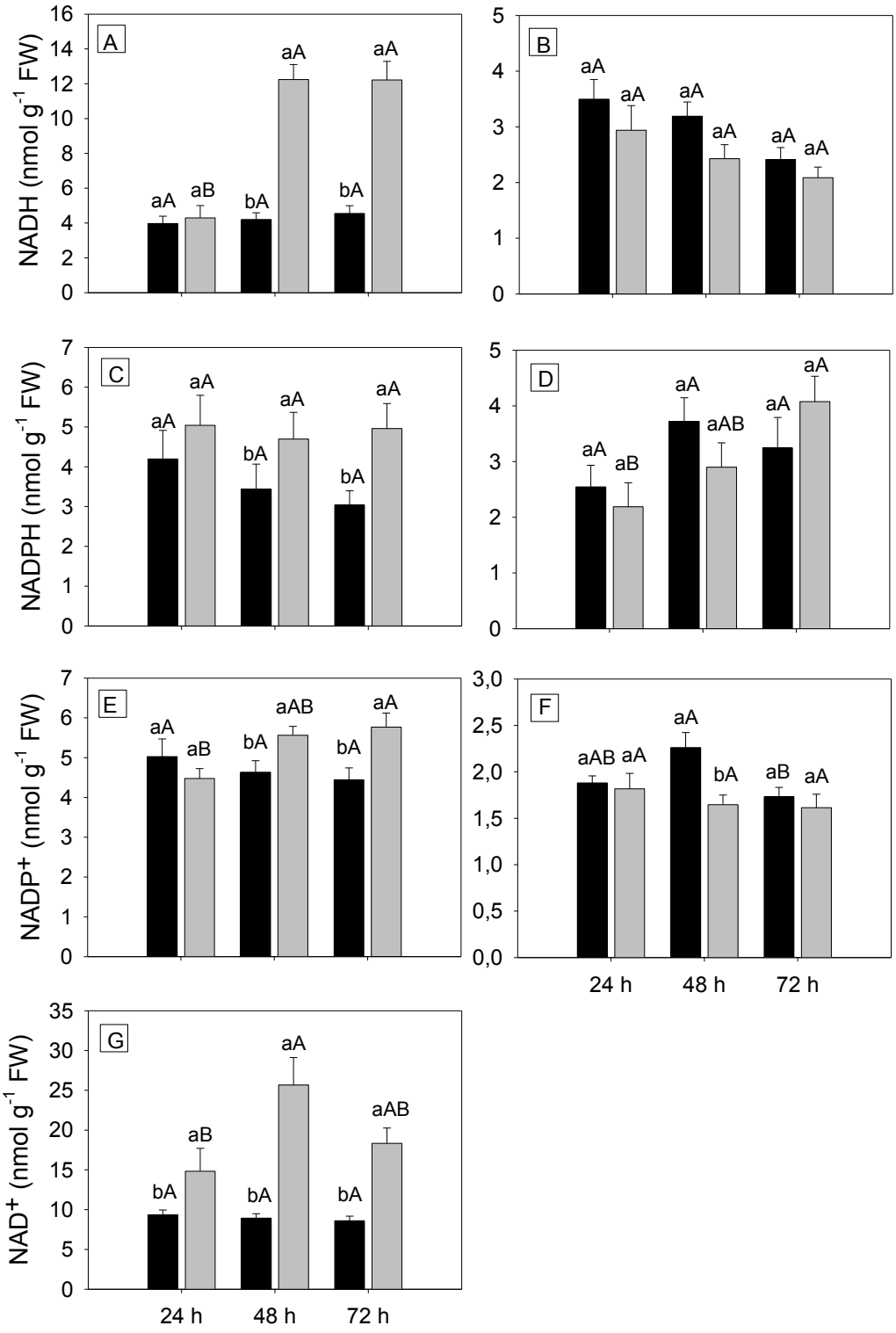

Figure 2. Concentration of NADH (A and B), NADPH (C and D), $NADP^+$ (E and F) in leaves and roots respectively, and NAD^+(G) just in leaves of *Pistia stratiotes*, comparing

control with As in 24, 48 and 72 hours. (■ Control ▭ As). Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

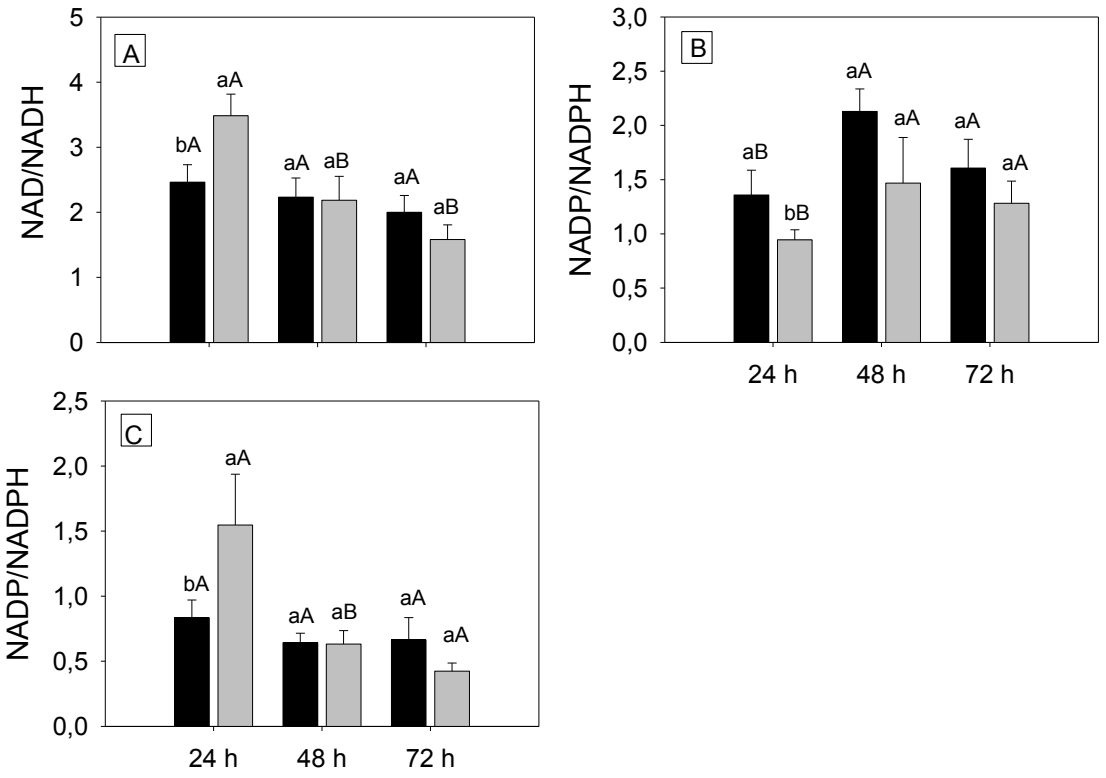

Figure 3. NAD/NADH in leaves (A) and NADP/NADPH in leaves (B) and roots (C) of *Pistia stratiotes*, comparing control with As in 24, 48 and 72 hours. (■ Control ▭ As). Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

3.3. Enzimatic activities

The activity of TCA cycle enzymes in leaves contaminated with As was modified by stress. NAD-dependent malate dehydrogenase (NAD-MDH), citrate synthase (CS) and NAD-dependent isocitrate dehydrogenase (IDH) showed higher activity in the presence of As (Fig 4A, C and G). However, fumarase (FUM) was the only enzyme that did not show differences between controls and As in leaves and roots (Fig 4E and F). In roots, for the other enzymes mentioned, presented opposite results to that observed in the leaves. With the exception of FUM and NAD-MDH that remained constant, exposure to As decreased the IDH and CS activity in comparision to the control plants (Fig 4D and H).

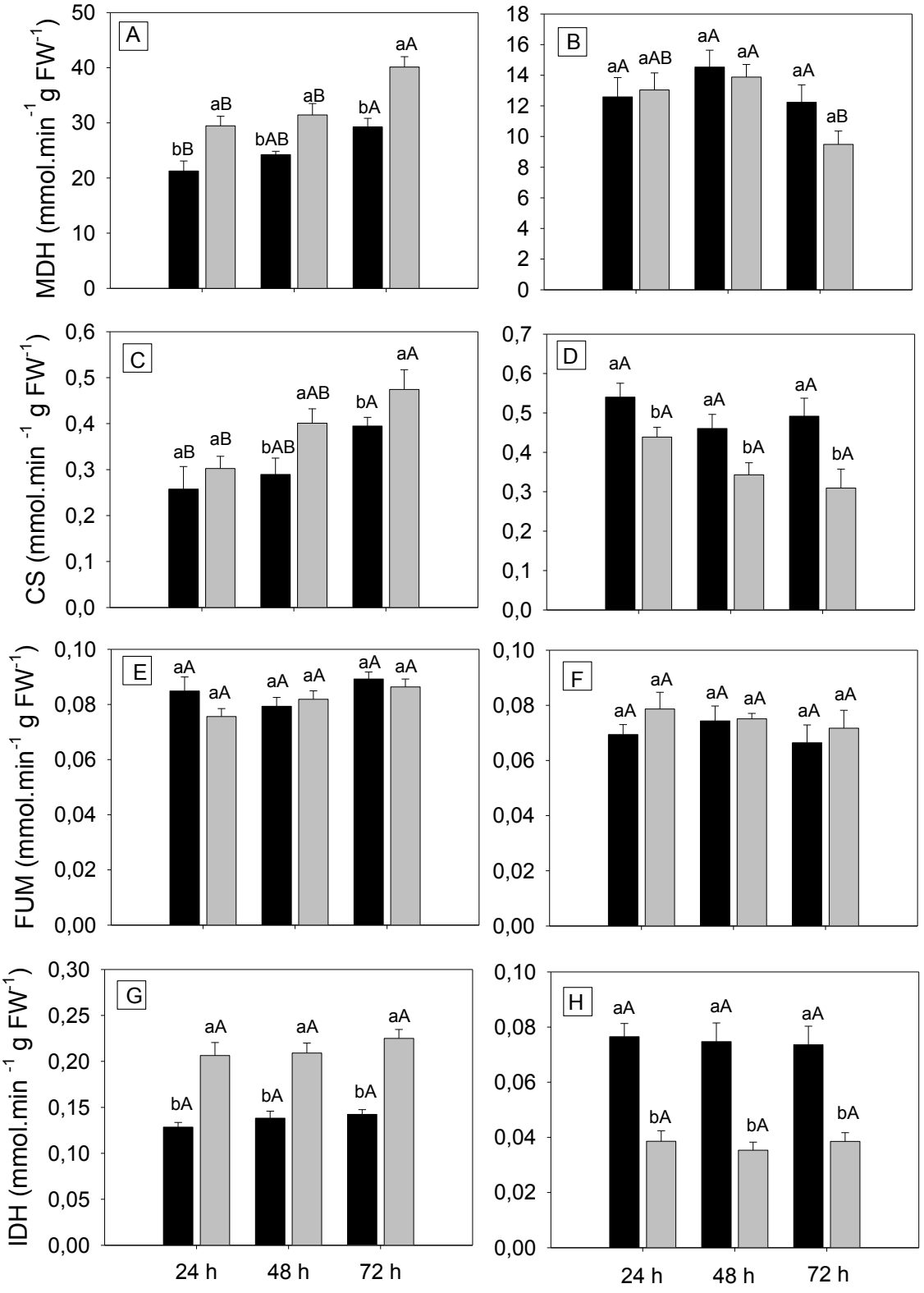

Figure 4. Activities of NAD dependent malate dehydrogenase (A and B), citrate synthase (C and D), fumarase (E and F) and NAD dependent isocitrate dehydrogenase (G and H) in leaves and roots respectively, in *Pistia stratiotes* after 24h, 48h and 72h. (■ Control □ As).

Averages followed by the same letters do not differ by Tukey's test. Lowercase letters represent the comparison between control and As within each time and uppercase letters represent the comparison between all controls and all As in the three times.

4. Discussion

Organic acids produced by TCA cycle enzymes such as citrate, pyruvate, succinate, fumarate and malate are essential because of their involvement in various biochemical pathways involving energy production, such as photosynthesis and nitrogen metabolism (Sweetlove et al., 2010). In addition, the production of these acids helps in the biosynthesis of specific compounds necessary to confer protection against stress caused by nutritional deficiencies and stress to toxic elements (Oburger et al., 2009).

The increase in the concentrations of organic acids is a physiological response that assists in the maintenance of the ionic balance, thus functioning as good indicators of stress in plants (Xu et al., 2013). Organic acids such as citrate, malate and oxaloacetate secreted by plant roots can form stable complexes with toxic elements, which prevents the binding of these elements to cell compartments (Chen et al., 2017). These acids can also protect biomolecules and cell structures from overproduction of reactive oxygen species. In this study, *Pistia* demonstrated increased malate and fumarate production as a response to stress by As. Sil et al. (2018) reported an increase in the concentration of pyruvate, citrate, succinate and malate in wheat as a response to As stress. In rice contaminated with As, an increase in the concentration of organic acids was also reported (Saha et al., 2017). The accumulator plant *Chrysopogon zizanoides* significantly increased levels of amino acids, organic acids (malate and fumarate) and coenzymes in response to exposure of lead compared to corn (Pitdala et al., 2018).

In contrast to the other results presented, the malate content in the root decreased in the treatments with As in *Pistia*. Malate can be converted to fumarate in a reversible process by enzyme fumarase and thus, one hypothesis for the decrease in malate concentration is that the fumarate or malate may have been diverted to assist other cell processes, which may be impaired due to oxidative stress. Moreover, the hydration of fumarate in malate is catalyzed by fumarase, which demonstrated a decrease in its activity in cultures of *Arabidopsis* cells subjected to oxidative stress (Sweetlove et al., 2002). To test these hypotheses, further analyzes can be made on the root of *Pistia stratiotes*.

Previous studies with several species of plants submitted to stress with cadmium, lead and arsenic indicated an increased activity of the enzymes of the respiratory process in small

concentrations of these toxic elements. In contrast, higher concentrations may lead to a decline in activity of the enzyme TCA cycle (Shao et al., 2011). As reported in the first chapter of this work, *Pistia stratiotes* respiration increased in leaves as a response to As stress. The same pattern was observed by Farnese et al. (2017), in which the low ATP production was a result of the synthesis of As-ADP, which compromised the respiratory process and caused mitochondrial membrane rupture.

Arsenate is also a de-coupling agent for glycolysis, since it can replace inorganic phosphate in the step of glycolysis which produces 1,3-bisphosphoglycerate, producing instead 1-arsenic-3-phosphoglycerate, an unstable and easily hydrolysable molecule, forming the next intermediate of the process, or 3-phosphoglycerate. Thus, glycolysis occurs, but there is no formation of the ATP molecule that would be generated from 1,3-bisphosphoglycerate (Byers et al., 1979).

The mitochondrial electron transport chain is responsible for the production of intracellular reactive oxygen species. Thus, the metabolic pathways in mitochondria become sensitive to oxidative changes in the intracellular environment, acting as sensors in stress responses (Lizhong et al., 2015). In *Pistia*, there was an increase in the activity of most enzymes in contaminated leaves, which explains the increased respiration reported in the first chapter. In contrast for the roots where there was excessive accumulation of the As, resulted in severe toxic effects on enzymes that are part of the respiratory complex. The effects caused by As can be seen more clearly in citrate synthase and isocitrate dehydrogenase over the 72 hours of exposure to As.

In root of *Pistia*, the decrease in IDH activity was also observed in peas (Bansal et al., 2002). The decrease in NAD-MDH activity may impair respiration, alter carbon metabolism (Bouthour et al., 2012) and with the decline in CS activity there will be no accumulation of citrate, which is one of the major organic acids for detoxification of cell. The results are, sometimes, conflictants, as related by Sil et al. (2018) in which As caused increase in the activities of citrate synthase and fumarase and reduction in the isocitrate dehydrogenase and malate dehydrogenase. In rice plants it was demonstrated an increase in the activities of citrate synthase and fumarase, and a decrease in isocitrate and malate dehydrogenase (Saha et al., 2017). In both studies there was greater accumulation of malate and citrate, since malate is formed by fumarate through the hydration of fumarate to malate and the citrate synthase catalyzes the combination of oxaloacetate with acetyl CoA producing the citrate. Increased

IDH and MDH activity may generate reducing power, which may play a role in redox mechanisms in plant cells (Bouthour et al., 2012).

The importance of NAD and NADP metabolism in plant development and stress tolerance is well known (Kharbech et al., 2017; Kurt-Gür et al., 2018). As observed in the leaves of *Pistia*, in general there was an increase in the activities of pyridine nucleotides in response to As. With the formation of reactive oxygen species during stress, ascorbate (ASC) and glutathione (GSH) have the function of regulating the ROS in enzymatic and non-enzymatic steps (Noctor and Foyer, 1998). In turn, NADPH is required by the ascorbate-glutathione cycle to protect against plant oxidative damage, making it an indispensable co-factor in detoxification processes. In addition to the ferrodoxin-NADP reductase present in the photosynthetic organs, the NADPH-producing enzymes are: NADP-isocitrate dehydrogenase (NADP-ICDH), NADP-malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH). Each of these enzymes contains several isoforms with different subcellular locations whose activity is modulated during the development of the plant under different stresses, such as exposure to As (Leterrier et al., 2012; Corpas et al., 2016; Ruiz-Torres et al., 2017). Therefore, these enzymes can be considered as the second line of defense against oxidative stress (Kharbech et al., 2017). These studies can explain the increase in $NADP^+$ and NADPH concentrations in both leaf and root. Due to the high demand for NADPH for protection against stress, there is an increase in the reduction of $NADP^+$ with involvement of several enzymes.

During the elimination of the reactive oxygen species, the formate is released and used as reducing power in cells with hydrogen peroxide. This defense strategy is possible through NAD^+ -degraded formate dehydrogenase (FDH) (Thomas et al., 2016). The NAD^+ -dependent FDH catalyzes the oxidation of the formate in CO_2 simultaneously with the reduction of NAD (P) + to NAD (P) H (Alekseeva et al., 2011). Thus, the level of FDH increased significantly in stressful conditions such as exposure to aluminum (Lou et al., 2016) and copper (Kurt-Gür et al., 2018) to ameliorate the tolerance of toxic metals.

Other studies have also investigated the balance of NAD/NADH and NADP/NADPH in aquatic plant species exposed to As, Cr and Zn. For *Hydrilla verticillata* exposed to As, NADPH increased along with the antioxidant system enzymes, while there was decline in NADH and increase in NAD^+ levels. This decline in NADH suggests that its consumption was required for the synthesis of ATP in the electron transport chain to allow the cells to tolerate stress (Srivastava et al., 2010). In *Pistia stratiotes* roots, the results found for NAD^+

and NADH can be explained by the possible inhibition of the respiratory process caused by As.

5. Conclusions

The As caused changes in respiratory metabolism of Pistia stratiotes. The increase in the enzymatic activities of the TCA cycle in the leaves and the changes observed in the levels of pyridine nucleotide, confirmed that *Pistia* was able to respond to stress. However, due to the greater accumulation of As in the roots, the damage to the respiratory metabolism was more pronounced than in the leaves.

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GENERAL CONCLUSIONS

The arsenic (As) absorbed by *Pistia stratiotes* triggered changes in the physiology of the plant, leading to some damages that impaired the growth and development of the species. As most metabolic pathways are interconnected, when one process is affected under stress, consequently another pathway may be impaired. In addition, As can also act on specific enzymes which can directly inhibit some metabolic pathway. Due to the greater accumulation of As in the roots, the more severe damages were reflected in the respiratory process, observed by the decrease in the activity of most of the enzymes of the TCA cycle. The leaves, which accumulated lower concentrations of As, presented more effective responses under stress. In this study, two factors were evaluated: the condition (control and presence of As) and time (24h, 48h and 72h). The condition was significantly the factor that generated the greatest differences in the results obtained. However, in a few analyzed parameters time modified the results in a standard way (increasing or decreasing).