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Evaluation of Multiple Responses Associated with Arsenic Tolerance and Accumulation in *Pteris vittata* L. Plants Exposed to High As Concentrations under Hydroponics

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Received: 7 October 2020; Accepted: 5 November 2020; Published: 7 November 2020



Abstract: Chinese brake fern (*Pteris vittata* L.) is recognized as an arsenic hyperaccumulating plant. Mechanisms underlying this capability and the associated hypertolerance have been described even if not completely elucidated. In this study, with the aim to expand the knowledge on the matter, an experimental trial was developed to investigate an array of responses, at the morphological, physiological, and biochemical level, in *P. vittata* plants exposed to high As concentrations in a long-term experiment under hydroponics. Results confirmed the ability of fern plants to both tolerate and accumulate a remarkable amount of As, especially in fronds. Notably, in As-treated plants, a far higher As content was detected in young fronds compared to old fronds, with bioaccumulation (BCF) and translocation (Tf) factors in accordance. At the biochemical level, As treatment affected macro and micronutrient, thiol, and phytochelatin concentrations in fronds of treated plants differently than that of the control. Physiological measurements accounted for a reduction in the photosynthetic activity of As-treated plants in the absence of visual symptoms of damage. Overall, the observed As tolerance and accumulation processes were discussed, evidencing how young fronds developed during As treatment maintain their physiological status while accumulating a high As content. Such indications could be very useful to improve the effective utilization of this plant species for phytoremediation of As-polluted water.

Keywords: phytoremediation; photosynthesis; thiols; heavy metals; water pollution; phytoremediation

1. Introduction

Arsenic (As) is a natural element, classified as a toxic and carcinogenic metalloid, that is widely distributed throughout terrestrial ecosystems and living organisms. A particular concern is posed by the excessive presence of this metalloid in groundwater, threatening millions of people throughout the world due to consumption of contaminated water and its use in agriculture [1]. In this regard, the contribution of anthropogenic activities has been clearly highlighted [2,3]. Among them, agricultural and industrial effluent discharges have been recognized for their adverse effects on the aquatic environment due to their high As concentration [4]. The urgent request to reduce pollutant contamination in environmental compartments has forced the advancement of innovative and sustainable technologies, among which NBS (nature-based solutions) has attracted increasing interest in the last few years [5]. In line with the statement

reported in the EU document (<https://ec.europa.eu/research/environment/index.cfm?pg=nbs>), NBS can be defined “as solutions that are inspired and supported by nature, which are cost-effective, simultaneously provide environmental, social and economic benefits and help build resilience ...”. In this context, it is worth mentioning that the possible utilization of plants “to remove polluting compounds from the environment or make them less harmful” was postulated more than 25 years ago by Cunningham and Berti [6], so defining phytoremediation.

Plant metal tolerance can be considered one of the best examples of an evolutionary process driven by anthropogenic activities [7]. Metal tolerance in plants can be achieved by different mechanisms [8], such as enhanced metal accumulation, due to metal sequestration or compartmentalization. Plants that accumulate metals are, therefore, considered metal accumulators.

Plants can also display an extreme form of tolerance called hyper-tolerance, allowing the accumulation of exceptionally high amount of heavy metals in their tissues, so as to reach a metal concentration in the aboveground biomass higher than that in the soil. These plants are called hyperaccumulators [9]. To evaluate the capability of a plant to hyperaccumulate metals, some descriptive indexes, such as the bioconcentration factor (BCF: ratio of metal concentration within plant tissues to that in soils) and the translocation factor (TF: ratio of metal concentration in the aboveground plant tissues to that in the roots) have been proposed [10]. For example, it is generally accepted to define a plant as an arsenic hyperaccumulator if it shows a BCF > 1 and a TF > 1 as well as it accumulates > 1000 µg arsenic per g of plant biomass [9].

Arsenic is a nonessential element for plants, and it is taken up via the phosphate transport system, being phosphorous its chemical analogue [11]. At high concentrations, if not safely sequestered, this metalloid interferes with basic plant metabolic processes, by reacting with sulfhydryl groups of enzymes and tissue proteins, leading to severe damages for plants [12,13].

In 2001, Ma et al. [14] reported that a fern, *Pteris vittata* L., could accumulate extremely large concentrations (up to 23,000 mg kg⁻¹) of this toxic element in its above ground biomass. Several other fern species have been reported by Zhao et al. [15] and Srivastava et al. [16] to hyperaccumulate As to concentrations with significant differences in their accumulation ability linked to the growth conditions [17]. *P. vittata* shows the common traits associated with metal/metalloid hyperaccumulation, including: enhanced root uptake, efficient root-to-shoot translocation, and a far elevated tolerance through internal detoxification [15,18].

Detoxification of arsenate by plants may occur through reduction to arsenite and its sequestration in vacuoles by thiol-containing compounds, such as glutathione (GSH) and phytochelatins (PCs), considered to be the main ligands of many metals and metalloids [19].

PCs are a family of sulfur-rich peptides characterized by a general structure as (γ-GluCys)_n-Gly (*n* = 2–11) and they are synthesized from glutathione (GSH), a tripeptide containing cysteine (Cys) [20]. These compounds play a key role in constitutive and adaptive tolerance to As in several nonhyperaccumulating plant species [15,21], while in hyperaccumulating plants, even if induced and accumulated in *P.vittata* leaflets [22], PC induction did not appear as the main process responsible for As hypertolerance [23]. In this regard, Cao et al. [24] proposed that in *P. vittata*, an important role in detoxification was exerted by non-enzymatic antioxidants, such as GSH and other -SH compounds, especially at high As concentrations (50–200 mg kg⁻¹).

At the physiological level, the tolerance response of plants to excess metals and metalloids relies on the plasticity of the photosynthetic process, involving structural and functional changes in the photosynthetic machinery [19,25–27]. As photosynthesis is one of the physiological processes most susceptible to environmental stress, photosynthetic efficiency can be considered a bioindicator of environmental pressure in plants under stress conditions [28]. In this context, analysis of chlorophyll fluorescence represents a useful tool for evaluating plant tolerance to pollutants, since it provides an estimation of the photosynthetic activity in a non-destructive way [29]. Moreover, the use of chlorophyll fluorescence imaging allows the evaluation of the heterogeneity in photosynthetic functions throughout a leaf by image analysis of the quantum efficiency of photosystem II (PSII) in plants subjected to several

stresses, including heavy metals [30–33], salinity stress [34], pharmaceuticals [35,36], and emerging contaminants [37].

Hydroponics is a very suitable growth system to study plant metal tolerance and accumulation, and more in general for the evaluation of its phytoremediation ability, especially in plants belonging to the aquatic or riparian compartment [35,38]. Several studies aimed at highlighting the ability of *Pteris* plants to tolerate and accumulate As under hydroponics or semi-hydroponics are reported in the literature [39–41] but to the best of our knowledge no studies dealing with morphological, physiological, and biochemical processes in the same experimental trial are present in the literature.

This study was aimed to investigate the tolerance and accumulation capability of *P. vittata* exposed to high concentrations of As in a long-term hydroponic experiment, evaluating responses at a physiological and biochemical level. The final goal of this work was to shed light on the adaptive mechanisms occurring in this As-hyperaccumulating plant species, which could be exploited for its profitable utilization of in the mitigation of As pollution in different environmental matrices.

2. Materials and Methods

2.1. Experimental Set-Up

Three-month-old *Pteris vittata* L. plants were obtained from a nursery established several years ago in an open field genotype collection at our institute (formerly IBAF-CNR) with proper shaded light and soil humidity conditions. Young plants were transferred to pots filled with peat and sand for 2 weeks in greenhouse. At the 3–4 frond stage, plants were transferred from pots to a semi-hydroponic growth system in the greenhouse, washing the roots carefully with water to remove soil residues. A semi-hydroponic growth system was chosen to assure both the metalloid availability in the water medium and the anchoring role of the sand substrate. Silica sand for agricultural and horticultural purposes, with controlled granulometry (0.1–1 mm), was used after washing and sterilization steps. Silica sand was chosen because in previous experiments this substrate showed the best performance for growing ferns, while pure hydroponic plant growth was unsatisfactory (data not shown). The growth system module consisted of two polyethylene tanks (0.81 m²). Tank 1 contained sand to anchor plants, and tank 2 was used as a water drainage collector. A pumping system (tank 3) forced the drained water to move continuously (water flow 20 L h⁻¹) from tank 2 to 1 to assure oxygenation of the solution and to avoid the formation of pollutant and nutrient gradients (Figure 1). A nutritive solution was prepared with 0.7 g L⁻¹ of soluble fertilizer with NPK 20-20-20 (Nutri-Leaf, Miller, Hanover, PA, USA) especially formulated for hydroponics, was added every ten days to tank 2. The circulating solution volume was 130 L, and pH was maintained in the range 6.5–7.0. Two similar modules were created to establish a control (module 1) and treatment (module 2). Fifteen plants, homogeneous in size and root development, were assigned to each module.

These modules were placed in a greenhouse and plants were exposed to controlled air temperature (25 °C) and photon flux density of 300 μmol m⁻² s⁻¹. Initial As concentration in the irrigation water used and sand was 14.1 ± 0.98 μg L⁻¹ and 1.7 ± 0.15 mg kg⁻¹, respectively.

After 17 weeks of pre-treatment conditions, in the treatment module, Na₂HAsO₄·7H₂O was added to tank 2 twice to reach a concentration of 7 mg L⁻¹ in the acclimatizing phase (13 days) and then to 21 mg L⁻¹ in the treatment phase of nine weeks. Every ten days, nutrient solution (as described above) was added to maintain the initial level. At the end of the experimental period, control and treated plants were collected, washed, separated into fronds, rhizomes, and roots and weighed. In treated plants, fronds were also divided into old (OF) and young (YF) fronds, depending on their completed or uncompleted unrolling at the beginning of the treatment, respectively. In particular, the fronds that were already unrolled were marked and indicated as old. Control plants were collected without separation in young and old fronds because similar As concentrations were found between them in preliminary experiments (data not shown). Immediately after physiological measurements, plant parts were harvested, and a stock was rapidly frozen in liquid N₂ and stored at -80 °C for biochemical

analyses. Another stock of samples was oven-dried for 7 days at 60 °C, weighed, and ground to fine powder for As, metals, macro and micronutrients concentration analysis.

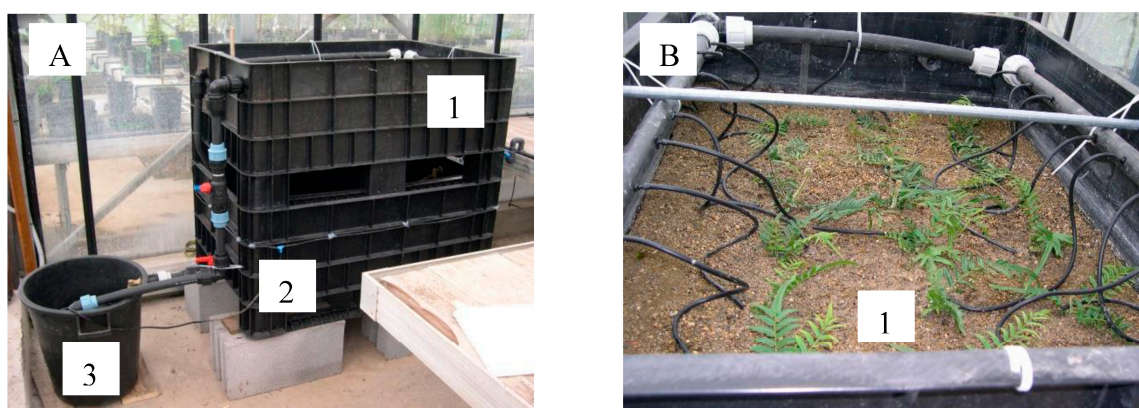


Figure 1. Semi-hydroponic growth system module (A) used in the experimental trial (1, tank with silica sand hosting plants; 2, drainage tank; 3, tank with pumping system). Fern plants growing in the tank 1 filled with silica sand (B).

2.2. Arsenic, Macro and Micro Nutrient Analysis

For chemical analysis, samples mineralization was performed by microwave assisted acid digestion (U.S. EPA. 2007). Specifically, the methods EPA 3051A/2007 was used. Plant samples referred to root, rhizome, and fronds. Briefly, samples were dissolved in concentrated nitric acid and concentrated hydrochloric acid using microwave heating. The samples and acids were placed in fluorocarbon polymer (PFA) microwave vessels. The vessels were sealed and heated in the microwave unit (175 °C for 15 min). After cooling, the vessel contents were filtered, centrifuged, and then diluted to volume and analyzed. The total metal (Al, Cd, Cr, Na, Ni, Pb) and metalloid (As) concentrations were measured by ICP-MS (Thermo Jarrell, Ash Iris Advantage, Thermoelectron, Waltham, MA, USA) following the methods UNI EN ISO 17294-2:2016 and EPA 6010 D2014 for biomass and soil, respectively. The analyses of micro (B, Cu, Fe, Mn, Zn) and macro nutrients (Ca, Mg, K, P) in the extracts were carried out by ICP-MS (Thermo Jarrell, Ash Iris Advantage, Thermo Electron, Corp., Milford, MA, USA). A QC procedure was performed by a daily five-point calibration with multi elemental standard followed by a secondary standard calibration, analyses of a certified reference material (CRM) added to samples, and a matrix spike and matrix spike duplicate (MS/MSD) were performed. All reagents were of ultrapure grade (Sigma-Aldrich, St. Louis, MO, USA).

For highlighting the accumulation potential towards As of fern plants used in this study, the bioconcentration factor (BCF) was calculated as the ratio of As concentrations in fronds to concentration in the substrate [10]. To describe the ability of the plants to translocate As in the different plant organs, the translocation factor (TF) was calculated. This index is defined as the ratio of As concentrations in fronds to those in roots [10]. In addition to frond/root, TF was calculated also for rhizome/root and frond/rhizome to better understand As distribution within the plants [42].

2.3. Thiol Analysis

Thiol (Cys, GSH) and polythiol (PCs) analyses were performed in fronds and roots. Samples were homogenized in liquid N₂ with three volumes of extraction buffer (0.1 N HCl, 1 mM EDTA, 400 mg mL⁻¹ PVPP) and centrifuged (15,000 × g) for 25 min at 4 °C. Supernatant aliquots (100 µL) were mixed with 150 µL 0.2 M HEPES and 25 µL 3 mM DTT, then some drops of 2 N NaOH were added to correct pH to 8.0. After 1 h of dark incubation at room temperature, 15 µL 30 mM monobromobimane were added to the assay buffer that was successively incubated in darkness for 15 min at room temperature. To stop the reaction, 75 µL 10% acetic acid was mixed into the solution, centrifuged

(15,000 × g) for 5 min at 4 °C and filtered (0.2 µm), to be ready for the injection into an HPLC system (Beckman-Coulter, Brea, CA, USA). Phytochelatins (PCs) were separated on a C18 reverse-phase column (250 × 4.6 mm, 5 µm pore size; Grace Davison, Deerfield, IL, USA). Elution was performed with a linear gradient (A = MeOH with 0.25% acetic acid, pH corrected to 4.3 with NaOH; B = H₂O with 0.25% acetic acid, pH corrected to 4.3 with NaOH) as follows: 90–60% B in 15 min, 60–20% in 5 min, 20–90% in 1 min, re-equilibration in 90% B for 5 min, at a flow rate of 1 mlmin⁻¹). Chromatograms were recorded and integrated using the 32 KARATTM Software 5.0 (Beckman-Coulter, Brea, CA, USA). PCs were identified by comparison with standard PC samples purified from *Silene vulgaris* (Moench). Quantification was performed with a Jasco fluorescence detector (model FP 2020 Plus; Tokyo, Japan; excitation 380 nm, emission 480 nm).

2.4. Imaging of Chlorophyll Fluorescence and Leaf Chlorophyll Content

Chlorophyll fluorescence image of fern leaves was performed using a MINI-Imaging PAM (Walz, Effeltrich, Germany) in order to investigate the heterogeneity of chlorophyll fluorescence parameters under metalloid stress conditions. The MINI-Imaging-PAM employs blue LEDs, with a peak wavelength at 450 nm, for pulse modulated measuring light, continuous actinic illumination, and saturation pulses. The charge-coupled device (CCD) camera has a resolution of 640 × 480 pixels. Pixel value images of the fluorescence parameters were displayed using a false color code ranging from black (0.000) through to red, yellow, green, blue, and pink (1.000). Leaves were dark adapted for at least 30 min before determining F_o and F_m (minimum and maximum fluorescence, respectively). The maximum quantum yield of PSII photochemistry (F_v/F_m) was determined as (F_m – F_o)/F_m. Subsequently, leaves were adapted to a photosynthetic photon flux density (PPFD) of 150 µmol m⁻² s⁻¹ for at least 10 min to reach a steady-state condition. A saturating light pulse was then applied to determine the maximum fluorescence (F'_m) and steady-state fluorescence (F_s) during the actinic illumination. Saturation pulse images and values of the chlorophyll fluorescence parameters were captured. The quantum efficiency of PSII photochemistry, ΦPSII, was calculated according to [43] using the formula (F'_m – F_s)/F'_m. Calculation of quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined at each saturating pulse, following the equation NPQ = (F_m – F'_m)/F'_m [44]. The measured values of NPQ were divided by four to display values less than 1.000. The apparent photosynthetic electron transport rate (ETR) was calculated as follows: ETR = ΦPSII × PPFD × 0.5 × Abs, where Abs is the apparent absorptivity of the leaf surface and 0.5 is the fraction of light absorbed by PSII antennae [29]. The Abs value was automatically calculated pixel by pixel from the R (red) and NI (near infrared) images using the formula: Abs = 1 – (R/NI). Chlorophyll fluorescence determinations were obtained from n = 5 leaves. Furthermore, measurements of total chlorophyll content were performed by the chlorophyll meter readings (SPAD-502, Minolta, Osaka, Japan) on the same leaves previously used for chlorophyll fluorescence determinations. SPAD values were converted to chlorophyll content (µg cm⁻²) using the following equation [45]:

$$\text{Chlorophyll content} = (99 \times \text{SPAD}) / (144 - \text{SPAD}). \quad (1)$$

2.5. Statistics

All results were expressed as an average of 5 replicates, at least. On normally distributed data, one or two-way ANOVA was applied to statistically analyze data as affected by one factor (plant organs) or two factors of variability (plant organs and treatment) using the SPSS software tool (Chicago, IL, USA). *T*-test was applied to analyze mean data about chemical element content in root and rhizome of control/treated plants. Separation of means was performed by Tukey's multiple test at a significance level of *p* ≤ 0.05.

3. Results

On average, the total biomass at the beginning of the experiment was 2.07 ± 0.33 g D.W. per plant, while at the end of the experiment, it reached 15.14 ± 2.43 g D.W. in control and 14.16 ± 1.76 g D.W. in As-treated plants (Figure 2A). At the frond level, the old leaves (see Materials and Methods for details) accounted for the largest part of the biomass, with young (unrolled) leaves showing around one-sixth of the total biomass. In this regard, no statistical difference between control leaves and old leaves of treated plants was observed. Data revealed a significant biomass reduction in the roots of As-treated plants compared to the control, while no statistical difference was observed between the rhizome of As-treated and control plants.

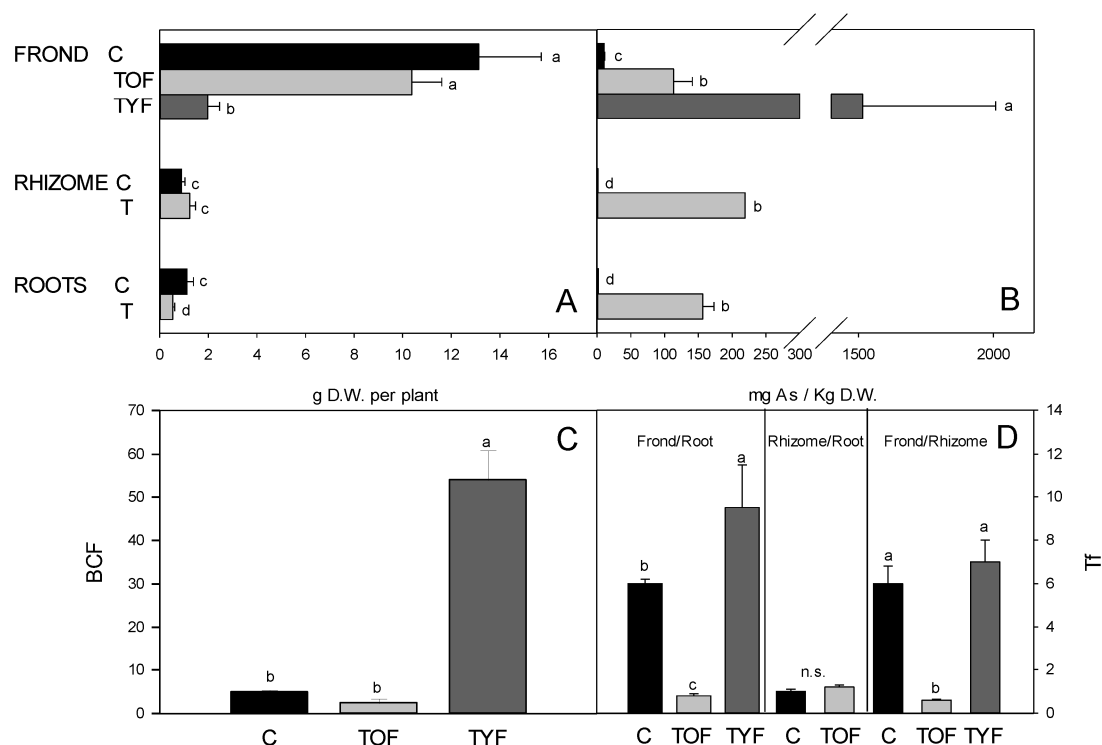


Figure 2. Biomass allocation (A), arsenic concentration (B), bioconcentration factor (BCF, (C)), translocation factor (Tf, (D)) in *Pteris vittata* L. plants treated (T) or not (C) for 9 weeks with 28 mg L^{-1} $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ under hydroponic conditions in greenhouse (TOF, treated old fronds; TYF, treated young fronds). Horizontal (A,B) and vertical bars (C,D) represent means ($n = 5$) \pm S.D. Two-way ANOVA (A,B) or one-way ANOVA (C,D) were applied; different letters indicate significant difference according to Tukey's test ($p \leq 0.05$).

After 9 weeks of growth in control or As-spiked solution, fern plants showed different As concentrations in the sampled organs (Figure 2B). In control plants, the metalloid concentration followed a trend with root = rhizome < frond. Notably, in As-treated plants, a highly significant difference in As accumulation between the young and the old fronds was found, with the concentration of the metalloid in the former more than 13 times higher compared to the latter, which had already completed unrolling after the adaptation step.

To better evaluate the ability to accumulate As in plant organs, the bioconcentration factor (BCF) was calculated. The BCF value in *P. vittata* fronds was higher than 1 (Figure 2C), as commonly found in hyperaccumulating plants. Interestingly, no difference was observed in leaves of control plants and old fronds of As-treated plants while young fronds exhibited the highest BCF value (above 50). The translocation factor (Tf, Figure 2D) was calculated to evaluate the capability of the plants to transfer the metalloid along the plant axis within the three sampled organs of fern plants (roots, rhizomes,

and fronds). Tf values were always higher than 1 except for As-treated plants where the ratio old frond/root and old frond/rhizome was 0.7 ± 0.1 and 0.5 ± 0.1 , respectively.

In As-treated plants, Tf calculation revealed that, both frond/root and in frond/rhizome, Tf values of young fronds were notably higher compared to old fronds. In frond/root, Tf values in young fronds were markedly higher than the control, while no statistical differences were observed between Tf values related to frond/rhizome in young fronds of treated plants and total fronds of control plants. Tf values related to rhizome/root were not significant.

In Table 1, macronutrient (Ca, Mg, K, P), micronutrient (B, Cu, Fe, Mn, Zn), and metal (Al, Cd, Cr, Na, Ni, Pb) concentrations within plant organs are shown and as well as a statistical comparison between control and As-treated plants. A significant increase in B, Cd, Cr, Cu, Fe, Ca, K, and Pb concentrations and a decrease in Na concentration in the roots of treated plants with respect to control plants was observed. In the rhizome, an enhancement of Cu concentration and a decrease in Ni and P content were highlighted in treated plants in comparison with control plants. In As-treated plants, only the concentration of K was higher in young fronds compared to old fronds with Al, B, Fe, Ca, Na, Mn, and Mg concentrations higher in old fronds than in young ones. Overall, in control fronds, the concentrations of most analyzed elements were similar to treated old fronds except for Cd, Cu, Ca, and Na concentrations, which were significantly lower in control plants.

Table 1. Chemical element concentration in *Pteris vittata* L. plants treated (T) or not (C) for 9 weeks with $28 \text{ mgL}^{-1} \text{ Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ under hydroponic conditions in greenhouse (TOF, treated old fronds; TYF, treated young fronds). In each column, mean values ($n = 5 \pm \text{SE}$) are reported. One-way ANOVA was applied to analyze mean data in root, rhizome, and frond, respectively. Separation of means was performed by Tukey's test (different letters indicate significant difference at $p \leq 0.05$; In each column and within each organ, letters of significance were assigned as follows: small letter, frond; superscript letters, rhizome; capital letters, root).

| | mg/kg | Al | B | Cd | Cr | Cu | Fe | Ca | Na | Mn | Mg | K | Ni | P | Pb | Zn |
|---------|-------|-------------|------------|------------|-------------|-----------------------|-------------|----------------|--------------|------------|-------------|-------------|-------------------------|---------------------------|-------------------------|------------|
| Frond | C | 500 ± 62a | 81 ± 3.7ab | 0.8 ± 0.1b | 4.6 ± 0.7 | 8.3 ± 0.4b | 318 ± 72a | 10972 ± 465b | 5108 ± 708b | 33 ± 3.9a | 4358 ± 253a | 302 ± 6.7b | 1.9 ± 0.6 | 1643 ± 127b | 3.9 ± 0.2 | 43 ± 8.4a |
| | TOF | 527 ± 42a | 107 ± 6.9a | 1.4 ± 0.1a | 6.9 ± 1.2 | 12.6 ± 0.9a | 332 ± 61a | 18211 ± 824a | 12496 ± 824a | 31 ± 3.4a | 5660 ± 382a | 300 ± 7.6b | 2.1 ± 0.3 | 2114 ± 134ab | 6.0 ± 1.3 | 33 ± 3.1ab |
| | TYF | 276 ± 30b | 70 ± 8.5b | 1.3 ± 0.2a | 5.7 ± 0.2 | 11.6 ± 0.6a | 50 ± 2.6b | 4559 ± 450c | 2512 ± 232c | 6.1 ± 0.3b | 2055 ± 145b | 353 ± 11.4a | 2.2 ± 1.2 | 2356 ± 117a | 6.2 ± 1.0 | 18 ± 2.9b |
| Rhizome | C | 2101 ± 211 | 170 ± 35 | 1.8 ± 0.07 | 5.6 ± 0.1 | 99 ± 13 ^b | 2257 ± 25 | 38322 ± 4643 | 5569 ± 467 | 264 ± 36 | 3859 ± 380 | 300 ± 26 | 10.8 ± 1.5 ^a | 10956 ± 1969 ^a | 9.9 ± 1.1 ^b | 308 ± 59 |
| | T | 2262 ± 557 | 189 ± 29 | 2.3 ± 0.4 | 8.5 ± 1.6 | 257 ± 43 ^a | 2037 ± 197 | 45222 ± 5867 | 4556 ± 492 | 264 ± 52 | 3223 ± 573 | 276 ± 14 | 6.2 ± 1.2 ^b | 4962 ± 830 ^b | 13.1 ± 2.2 ^a | 237 ± 78 |
| Root | C | 2271 ± 1277 | 74 ± 19A | 1.6 ± 0.4B | 10.5 ± 1.1B | 290 ± 77B | 4279 ± 996B | 35518 ± 10375B | 1623 ± 241A | 354 ± 93 | 5887 ± 1054 | 151 ± 39B | 24 ± 5.5 | 6890 ± 1893 | 9.9 ± 1.7B | 344 ± 100 |
| | T | 4485 ± 470 | 173 ± 9B | 2.6 ± 0.1A | 17.9 ± 0.8A | 801 ± 22A | 6365 ± 412A | 56723 ± 3196A | 776 ± 214B | 454 ± 28 | 5210 ± 323 | 231 ± 5.3A | 17 ± 0.8 | 7086 ± 813 | 16.0 ± 1.3A | 204 ± 37 |

To evaluate the plant biochemical response for defence and metal transport, sulfur compounds such as phytochelatin (PCs) and two thiols, cysteine (Cys) and glutathione (GSH), were investigated in roots and fronds of fern plants (Figure 3).

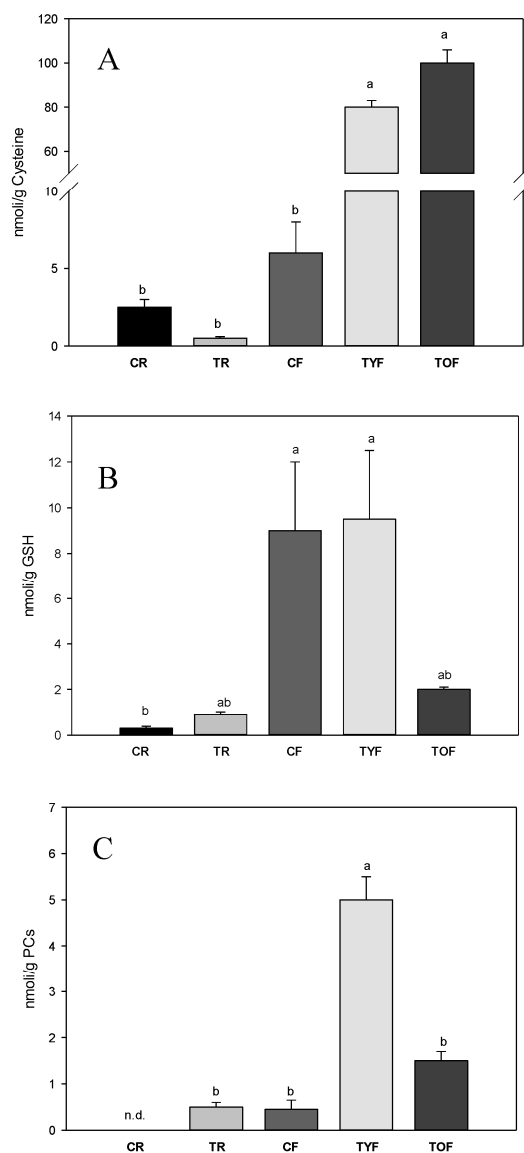


Figure 3. Thiol ((A), cysteine; (B), glutathione) and total phytochelatin ((C), PCs) content in *Pteris vittata* L. plants treated (T) or not (C) for 9 weeks with $28 \text{ mg L}^{-1} \text{ Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ under hydroponic conditions in greenhouse (CR, control root; CF, control fronds; TR, treated root; TYF, treated young fronds; TOF, treated old fronds). Vertical bars represent means ($n = 5$) \pm S.D. Two-way ANOVA was applied; different letters indicate significant difference according to Tukey's test ($p \leq 0.05$; nd = not detected).

In both control and treated plants, thiol and PC content showed lower concentrations in roots than in fronds. Cys content under As treatment increased significantly in fronds compared to control plants, with the old fronds showing a Cys content higher than young fronds. No significant differences in roots were observed between control and As-treated plants. The GSH content in treated plants decreased in old fronds compared to young fronds. This decrease was significant in comparison with control fronds. Similar to Cys, GSH content was not significantly different in roots of the control and As-treated plants.

PC concentration was higher in treated young fronds in comparison with old treated and control fronds, with the content of old fronds unaffected by As treatment. PCs were not detected in control roots, while an induction of these polythiols was observed as a consequence of As treatment.

On the photosynthetic level, chlorophyll fluorescence parameters in the control and As-treated leaves were measured and shown in Table 2. A representative image of chlorophyll fluorescence in two control and As-treated leaves measured at the end of As treatment is reported in Figure 4. All parameters were affected by As treatment. In As-treated plants, compared to the control, F_v/F_m and Φ_{PSII} showed a decrease by around 13% and 45%, respectively, while NPQ showed an increase of 3.5-fold (Table 2; Figure 4). Moreover, the electron transport rate (ETR) was around 50% lower in As-treated plants compared to the control, showing a detrimental effect of metalloid on the overall photosynthetic capacity (Table 2). In addition, leaf chlorophyll content exhibited a significantly lower value in treated plants compared to the control, which was also highlighted by the analysis of PAR-absorptivity of the leaf surface (Abs) (Table 2), showing a trend similar to chlorophyll content.

Table 2. Total chlorophyll content and chlorophyll fluorescence parameters measured in *Pteris vittata* L. plants treated (As-treated) or not (control) for 9 weeks with $28 \text{ mg L}^{-1} \text{ Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ under hydroponic conditions in greenhouse at the end of the experiment. Maximum quantum yield of PSII photochemistry (F_v/F_m) measured in dark adapted leaves and quantum efficiency of PSII photochemistry (Φ_{PSII}), non-photochemical quenching (NPQ), electron transport rate (ETR), and PAR-absorptivity (Abs) measured at steady state with actinic light illumination of $150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Mean data \pm SE; $n = 5$). Data followed by different letters in the same column are significantly different (Tukey's test, $p \leq 0.05$).

| Treatment | F_v/F_m (r.u.) | Φ_{PSII} (r.u.) | NPQ (r.u.) | ETR ($\mu\text{mol Electrons}$ $\text{m}^{-2}\text{s}^{-1}$) | Abs (r.u.) | Tot Chl Content ($\mu\text{g cm}^{-2}$) |
|------------|---------------------|-------------------------|---------------------|--|---------------------|---|
| Control | 0.750 ± 0.001 a | 0.458 ± 0.004 a | 0.731 ± 0.033 b | 29.5 ± 0.3 a | 0.867 ± 0.003 a | 43.4 ± 0.4 a |
| As-treated | 0.655 ± 0.004 b | 0.248 ± 0.007 b | 2.584 ± 0.031 a | 15.6 ± 0.4 b | 0.839 ± 0.004 b | 34.8 ± 0.5 b |

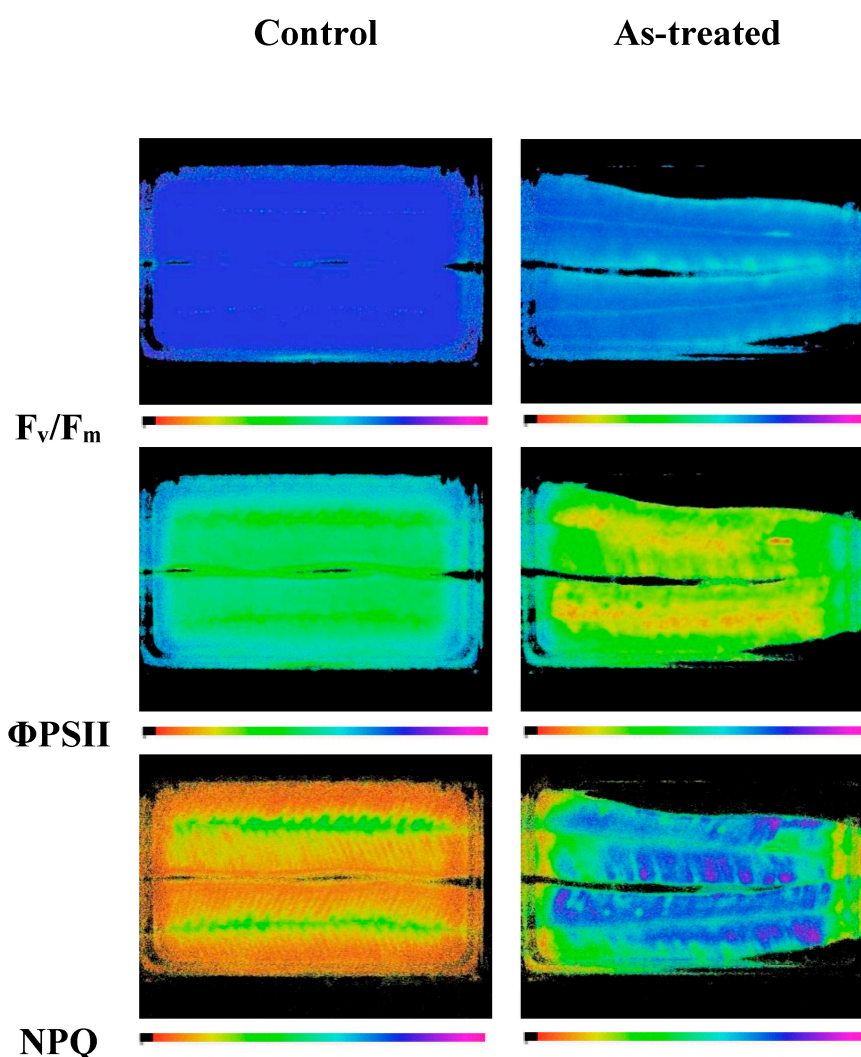


Figure 4. Chlorophyll fluorescence images of photochemistry (F_v/F_m) in a dark-adapted leaf and PSII photochemistry (Φ_{PSII}) and non-photochemical quenching (NPQ) at steady-state with actinic illumination of $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in control and arsenic-treated leaves of *Pteris vittata* measured at the end of the experiment. The false color code depicted at the bottom of the images ranges from 0.00 (black) to 1.00 (pink).

4. Discussion

P. vittata can tolerate and accumulate high levels of As in its organs, even against a concentration gradient. For this reason, this plant is considered an As-hyperaccumulator able to efficiently remove this metalloid from soil [14]. Tolerance mechanisms to As exposure were largely described in fern plants [15,18], but few works are reported in the literature investigating physiological and biochemical responses, associated with arsenic accumulation and tolerance, in a long-term and high As concentration experiment in hydroponics [46–48].

Toxicity responses in fern plants treated with different As concentrations have been reported [18,46]. Toxic symptoms such as plant growth reduction, chlorosis and necrosis of leaf tips and leaf margins, and impairment of the photosynthetic process, have been reported [17,49,50].

In this study, *Pteris vittata* plants exposed to 28 mg L^{-1} of As for 9 weeks exhibited some visible toxic symptoms (not shown). In particular, at the end of the treatment period, old fronds that had already developed before As exposure had necrosis of the tips and margins in some leaflets and leaf withering, whereas young fronds, emerging during As treatment, showed no symptoms of toxicity.

This feature was consistent to those reported by Li et al. [49], who described how As did not affect the chloroplast ultrastructure of young fronds but damaged most of the membrane systems of chloroplasts in mature pinna, resulting in dark-brown leaf margins in *P. vittata*. Biomass production is another important parameter that can be used to evaluate the capability of plants to tolerate the presence of heavy metals in growing solutions. In this study, a significant reduction in root biomass in treated plants occurred when compared with the control, while biomass of other plant parts was not affected (Figure 2A). The reduction of root biomass following As treatment represents a typical toxic symptom induced in plants by this metalloid [51,52].

Arsenic absorbed by the plants was accumulated prevalently in the fronds, followed by the rhizome and roots (Figure 2B). The same behavior was also observed in control plants. There was a significant difference in As concentration in the fronds between old and young fronds (113.20 ± 28.02 and 1516.20 ± 492.31 , respectively). These results were in agreement with Tu and Ma [46] who reported the highest As accumulation in young fronds, followed by mature and old fronds, in plants exposed to $<30 \text{ mg kg}^{-1}$ As, but in As-treated plants at $>200 \text{ mg kg}^{-1}$, the opposite trend was described.

Rhizomes showed a notable concentration of arsenic without a decrease in biomass under our treatment conditions (Figure 2A,B). This plant part could be very important in limiting the translocation of the toxic element from roots to fronds, accumulating a large amount of As, especially when plants grow in heavily contaminated soils. It was suggested by Liao et al. [53] that rhizomes in *P. vittata* can act as “buffer-storage” for As restricting As translocation to aerial parts to avoid the toxic effects on physiological processes occurring in the fronds.

BCF is a useful index to evaluate the potential of a plant to phytoextract metals from a substrate and concentrate them into its organs. It is commonly utilized to identify hyperaccumulating plants that can be so defined if the BCF value is higher than 1. In literature, the BCF value reported for *P. vittata* fronds was significantly higher than 1 [54], also reaching a value of 184 [55]. In the present work, BCF was 13.4 times higher in young than in old fronds of As-treated plants. This finding underlines the ability of this plant to bioconcentrate this toxic element, especially in fronds that were completely developed during the treatment (Figure 2C). An accumulation of As in the above ground biomass of non-polluted plants was also observed. In fact, due to the residue levels of As in the sand, in control plants, the total plant BCF was 6.23 ± 0.5 , proving the capability of this fern to accumulate the toxic element also in very low arsenic presence.

Beyond the extraction of arsenic from the soil, *P. vittata* efficiently translocates this element from roots to fronds. In this work, the Tf calculation (Figure 2D) showed a higher capability of young fronds to translocate As from roots compared to old fronds, evidencing the capability of organs developed under treatment to activate metabolic processes to act as a sink for the absorbed arsenic [42,55].

It has been reported that toxic metals may interfere with the uptake of other elements by plants causing a notable detrimental effect on plant growth. In fact, among these chemical elements, there are some essential macro- and micro-nutrients beyond other non-essentials metals [56,57]. In this study, the chemical element concentrations within plant organs were modified differently by As treatment (Table 1). In fact, in the roots of treated plants, an increase for all elements, except for Na, was observed, particularly for B, Cr, and Cu, even if only eight out of 15 elements evidenced statistically significant differences with respect to the control.

In rhizomes, the concentration of elements was substantially unaffected by As treatment since a difference between the control and treated plants was only ascertained in three out of 15 elements. In contrast, in fronds, the concentrations of nutrients were notably altered by exposure of the plants to the metalloid. Only four out of 15 elements did not have a change in titer between the control and young or old, treated fronds. There was an increase of macronutrient P and K concentrations in young treated fronds with respect to the control. Conversely, young treated fronds showed a remarkable reduction compared to the control for Ca and Mg, as well as in micronutrients, such as Al, Na, Mn, Fe, and Zn. Under As treatment, the old fronds did not reveal a remarkable change in element composition compared with the control, except for an enhancement in Na, Ca, Cu, and Cd. An enhancement of Ca

concentration in mature fronds of *P. vittata* following As treatment was shown by Li et al. [49] and Kumar et al. [57] supporting the hypothesis of a role in As detoxification.

Considering the whole plant, a general trend of increase in nutrient absorption in response to arsenate could be evidenced in accordance with previous findings [24,56].

In the present study, in fern plants exposed to arsenate in a long-term hydroponic experiment an induction of thiols and PCs occurred (Figure 3). This finding agrees with previously reported data [22,24,58] obtained in different experimental conditions. In particular, PCs were induced in roots by As treatment and their content was enhanced markedly in young treated fronds in comparison with old treated fronds and control fronds. These observations are in agreement with the As content detected in different plant parts and with the lack of visible toxic effects in young fronds, confirming the role of PCs as bio-ligands for As, even in plants exposed for several weeks at a notable arsenate concentration. Beyond many other important functions in plant cells, Cys and GSH are involved in PC formation, and changes of their concentration in plants exposed to metals have been extensively reported [59,60]. In old fronds, a higher Cys content and a lower GSH content compared with young fronds occurred (Figure 3). Moreover, old fronds showed a far lower PC content as previously discussed. In this regard, preferential accumulation of a low molecular thiol, such as Cys, could be an adaptive mechanism to reduce As transport and storage, explaining the lower As accumulation in old fronds in comparison to young fronds.

GSH content did not vary in roots or young fronds of arsenic-treated plants compared with the control as previously reported [61].

In this study, analyses of chlorophyll fluorescence parameters and images were used to investigate the effects of As exposure on photosynthetic activity in fronds of *P. vittata*. To highlight these effects, two representative frond types characterized by no visual symptoms of damage (chlorosis and necrosis) but with low (control) and high (young treated) As content were chosen. No study about the utilization of this technique on *P. vittata* was found in the literature. As previously described, data of chlorophyll fluorescence images indicated a reduction of the photosynthetic activity in As-treated leaves of *P. vittata* in comparison to the control (Table 2 and Figure 4). Arsenic treatment significantly reduced the F_v/F_m values in comparison to the control (Table 2 and Figure 4). According to Maxwell and Johnson [29], an F_v/F_m ratio in the range of 0.75 to 0.83 is the approximate optimal value for many plant species, confirming that control plants were not affected by As treatment. Lower values of this parameter indicate that a proportion of the PSII reaction centers is damaged or inactivated, a phenomenon commonly observed in plants under stress [62]. To study the possible changes in PSII photochemistry, chlorophyll fluorescence characteristics under the steady state of photosynthesis were investigated. Results showed that As treatment caused a notable reduction of Φ_{PSII} and ETR values with respect to the control, highlighting a negative effect of this metalloid on photosynthetic capacity (Table 2). Furthermore, Φ_{PSII} and ETR are two widely used chlorophyll fluorescence parameters that are employed to measure photochemistry and the overall photosynthetic capacity of plants [29,43]. Consistently, significant decreases in F_v/F_m (19.9%) and Φ_{PSII} (36.1%) were reported by [17] in *P. vittata* plants exposed to 100 mg As kg⁻¹ in comparison to the control. The NPQ values showed, if compared to the control, a significant increase in As-treated plants. High values of NPQ in plants exposed to As indicate the presence of a non-radiative energy dissipation mechanism [63], in which a higher proportion of absorbed photons is lost as thermal energy, instead of being used to drive photosynthesis [64]. Such thermal deactivation of excess energy represents a protective mechanism that maintains the high oxidative state of the primary electron acceptors of PSII and reduces the probability of photodamage [65]. As shown by images of chlorophyll fluorescence, the parameters measured in control leaves revealed a more homogeneous pattern of distribution of chlorophyll fluorescence, whereas they showed an appreciable heterogeneous pattern of light utilization and photosynthetic activity in As-treated leaves (Figure 4).

In this context, the inhibition effect of As on chlorophyll synthesis was reviewed [19]. Accordingly, our study showed a significant reduction of chlorophyll content in As-treated plants in comparison to

control plants (Table 2). The analysis of apparent absorptivity of the leaf surface (Abs) showed a trend similar to chlorophyll content. In fact, a strong correlation between leaf absorbance and total chlorophyll content has been described in the literature [66], confirming a negative effect of this metalloid on the photosynthetic apparatus. Similar findings were also observed by Zemanová et al. [50], highlighting a reduction of the chlorophyll content, F_v/F_m , and photosynthetic rate in *P. cretica* plants exposed to high As concentration.

5. Conclusions

This study showed that *P. vittata* can activate physiological and biochemical processes targeted to As hyperaccumulation and tolerance even in extremely toxic growth conditions such as exposure to elevated concentrations of arsenate in hydroponics for a long period. The relationship between bioaccumulation of the metalloid and the capability to tolerate its toxic effects varied remarkably among plant organs. Particularly, organs developed during As treatment, such as young fronds, can preserve their physiological status, even with an appreciable reduction of photosynthetic efficiency, despite the elevated As concentration in their tissues. A role for PCs in storing As in young fronds, limiting toxic effects at the physiological level, was hypothesized. Taken together, the results can provide useful indications to further substantiate the suitability of this plant species for phytoremediation of As-polluted waters.

Author Contributions: A.M. conceived, designed and supervised the experiments; M.Z. and F.P. supervised the experiments; M.Z. supervised the biochemical analyses, V.I. and L.P. performed the experimental work on plant growth; F.P. performed chlorophyll content and chlorophyll fluorescence parameters analyses; M.Z. and F.P. performed the data elaboration; A.M. and M.Z. wrote the paper with the contribution of F.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Acknowledgments: Authors wish to thank Aromolo (CREA, Italy) for the assistance in chemical element analysis.

Conflicts of Interest: The authors declare no conflict of interest.

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