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Free amino acid regulation in fronds and roots of two *Pteris cretica* L. ferns under arsenic stress

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Abstract: In the present study, free amino acid (AA) regulation in the arsenic (As) hyperaccumulating ferns was evaluated in a pot experiment to determine the relationship between As stress and the characteristic change in metabolism of AAs. The ferns *Pteris cretica* cv. Albo-lineata (Pc-Al) and cv. Parkerii (Pc-Pa) were exposed to As treatments at 0, 20, 100, and 250 mg As/kg for 90 days. Greater As content, as well as higher biomass production, were identified in Pc-Al compared with Pc-Pa. Ferns showed changes in the stress metabolism of free AA homeostasis. These results indicate a disturbance in nitrogen metabolism and depletion of pool assimilated carbon metabolism. In the fronds and roots, Pc-Pa accumulated higher amounts of free AAs than Pc-Al. The total free AA content, as well as the ratio of the main AA family pathway (glutamate family), were increased by the accumulation of toxic As in the ferns. Results suggest that Pc-Al tolerates higher As doses better due to changes in AA biosynthesis; however, at higher As doses, Pc-Pa upregulated AA biosynthesis due to As toxicity. The most abundant free AAs of ferns was glutamine, which was enhanced by As. Furthermore, the ratios of selected individual free AAs revealed a characteristic phenotype difference between ferns.

Keywords: amide; metalloid; pyruvate family; serine family; shikimate family; toxicity

Arsenic (As) is a non-essential metalloid that is toxic to plants (Begum et al. 2016). Although As affects plant growth, development, and productivity, it is usually associated with oxidative stress, which affects the regulation of a diverse range of metabolic pathways (Rodríguez-Ruiz et al. 2019).

Plants often synthesize amino acids (AAs) that accumulate predominantly in tissues under As stress (Kumar et al. 2014). Amino acids play critical roles in plants, including providing the building blocks for membrane proteins and enzymes and acting as substrates for the biosynthesis of essential primary metabolites and phytohormones interacting with many branches of metabolism (Pratelli and Pilot 2014, Ros et al. 2014). Several AAs play key roles as precursors for the synthesis of various classes of biologically active toxic or antioxidant secondary metabolites in plants (Less et al. 2010). Furthermore, AAs are used as carriers of assimilated nitrogen (N) between various organs through the phloem and xylem (Pratelli and Pilot 2014).

Studies have indicated that As changed the AA content in tissues of different plants (Pavlík et al. 2010, Tripathi et al. 2013, Okunev 2019, Rodríguez-Ruiz et al. 2019). However, few have investigated the content of AAs in As-hyperaccumulator ferns (Ashraf et al. 2011, Campos et al. 2016, Pavlíková et al. 2017). This study aimed to investigate changes in the free AA regulation of two ferns, *P. cretica* cv. Albo-lineata and cv. Parkerii under different As

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treatments. We compared free AA contents in fronds and roots of both ferns in relation to the level of toxic As accumulation in the plant parts. Our objective was to evaluate the effect of As on the regulation of free AA homeostasis, including AA families related to N metabolism, and to verify the possibility to characterise typical phenotypic differences between the studied ferns.

MATERIAL AND METHODS

Plant material and chemical analysis. A pot experiment was carried out under greenhouse conditions (natural photoperiod; temperature 22–24 °C; relative humidity ~ 60%) using As-hyperaccumulating ferns, *P. cretica* L. cv. Albo-lineata (Pc-Al) and cv. Parkerii (Pc-Pa). Ferns at the 10 fronds stage were purchased from a garden centre Tulipa Praha, in the Czech Republic. Five kg Haplic Chernozem from a non-polluted area in Prague-Suchdol, Czech Republic (total organic carbon 1.83%, cation exchange capacity 258 $mmol_+$/kg, pH_{KCl} 7.1, total As 16 ± 1.7 mg/kg, water-soluble As 0.15 ± 0.02 mg/kg and As extraction efficiency 20%) was used per pot. Each kg of soil was mixed with 0.5 g N, 0.16 g P, and 0.4 g K (applied as NH_4NO_3 and K_2HPO_4). Control pots were not supplemented with As (As0). The As treatment pots were spiked with a solution of Na_2HAsO_4, which corresponds to contents of 20 (As1), 100 (As2), and 250 (As3) mg As/kg, and the final As content in the soil equalled the As spiked dose with a 20% As extraction efficiency. In the experiment, there were three replicates per treatment and one plant per replicate. Ferns were grown in these pots for 90 days. Details of harvesting and analysis of As and free AAs were described previously by Pavlíková et al. (2020).

Statistical analysis. Statistical processing of the results was carried out using Statistica 12.0 software (StatSoft, Tulsa, USA). All data were checked for homogeneity of variance and normality (Levene and Shapiro-Wilk tests). The data are presented as the mean ± standard deviation (n = 3). A one-way analysis of variance (ANOVA) followed by post-hoc comparison with Fisher's *LSD* (least significant difference) test ($P < 0.05$) was used to identify significant differences between treatments for individual ferns. Correlation analysis was performed using Pearson's linear correlation (r; $P < 0.05$).

RESULTS AND DISCUSSION

Arsenic accumulation and biomass production of ferns. Arsenic content in fronds and roots of Pc-Al and Pc-Pa was treatment-dependent (Table 1). The As content in fronds of Pc-Al and Pc-Pa increased with increasing As dose in the soil as shown by Pearson's linear correlation (r = 0.92 and r = 0.99, respectively; $P < 0.001$). The same trend was observed in roots of both ferns (Pc-Al: r = 0.87, $P < 0.001$; Pc-Pa: r = 0.96, $P < 0.001$). All treatments of Pc-Al fronds and roots had markedly higher As accumulation than Pc-Pa.

Plants exhibit a wide variation in their response to As (Campos et al. 2016). There are a number of fern species, especially belonging to the family Pteridaceae, identified as As-hyperaccumulators that are able to accumulate at least 1 000 mg As/kg

Table 1. Arsenic (As) accumulation and frond and root biomass production of *Pteris cretica* cv. Albo-lineata (Pc-Al) and cv. Parkerii (Pc-Pa)

		As content (mg/kg DW)		Biomass production (g FW/plant)	
		fronds	roots	fronds	roots
Pc-Al	As0	14.6 ± 0.4^a	13.8 ± 4.2^a	54.0 ± 0.9^a	21.1 ± 3.6^a
	As1	568.2 ± 17.5^b	409.7 ± 68.3^b	61.1 ± 6.4^c	21.5 ± 1.3^a
	As2	2 764.6 ± 163.0^c	753.9 ± 39.4^c	54.1 ± 4.5^a	22.9 ± 1.5^a
	As3	3 668.1 ± 645.6^d	995.0 ± 212.5^d	46.4 ± 0.4^b	14.6 ± 0.4^b
Pc-Pa	As0	nd	4.1 ± 0.02^a	21.7 ± 1.1^a	6.6 ± 0.8^c
	As1	8.4 ± 1.6^a	6.5 ± 2.9^a	23.3 ± 2.9^a	10.1 ± 0.9^d
	As2	85.4 ± 7.6^b	21.6 ± 2.5^b	14.1 ± 1.1^c	5.6 ± 0.1^b
	As3	193.8 ± 11.7^c	123.9 ± 10.4^c	5.9 ± 1.4^b	4.1 ± 0.4^a

nd – value was not detected (limit of detection: < 0.3 mg/kg DW (dry weight)). Lower-case letters indicate significant differences by Fisher's *LSD* (least significant difference) test ($P < 0.05$). Data with the same letter were not statistically different. As0 – control; As1 – 20, As2 – 100, As3 – 250 mg As/kg; FW – fresh weight

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of dry mass in shoots (Claveria et al. 2019). In the present study, As content in Pc-Al was > 1 000 mg/kg, whereas As content in Pc-Pa was under the limit of hyperaccumulation (Table 1). Similar results for Pc-Pa were shown in our previous study (Pavlíková et al. 2020), as well as for Pc-Al (Zemanová et al. 2020). In contrast to our results, Luongo and Ma (2005) determined As content in Pc-Pa > 1 000 mg/kg and identified this fern as an As-hyperaccumulator.

Furthermore, As accumulation affected the growth of ferns (Table 1). The fresh biomass production of fronds and roots of Pc-Al were on average, 4.2- and 3.4-fold higher, respectively, compared to Pc-Pa (Table 1). Despite that Pc-Al grew without visible symptoms of toxicity in all As treatments, frond and root biomass decreased in the highest As3 treatment (by 14.3% and 32.2%, respectively). In Pc-Pa at the same As a treatment, necrosis on frond blades was visible, and frond and root biomass decreased (by 58.2% and 26.1%, respectively). Our results showed the toxicity of the highest As treatment for frond and root biomass production of ferns (Table 1). Results of the correlation indicated a stronger As effect on frond and root biomass production of Pc-Pa than Pc-Al. In Pc-Pa, negative correlations were calculated between fresh weight and As content in the soil, as well as As contents in fronds and roots (r = –0.68 to –0.97, P < 0.001). Conversely, negative correlations were confirmed only between fresh weight and As contents in soil and fronds (r = –0.51 to –0.72) for Pc-Al. Similarly, the changes in total and frond biomass production were shown for *P. cretica* from different As contaminated soils (Fayiga and Ma 2005). In a study with Pc-Al, As stress in roots after 122 days caused lignification and led to deformation of root cell walls; hence, frond biomass of Pc-Al was affected by As stress only in young fronds (Zemanová et al. 2020). Conversely, As toxicity was not observed for Pc-Al and Pc-Pa exposed to 10 mg/L for 14 days in a hydroponic experiment, suggesting that *P. cretica* has the ability to detoxify As (Luongo and Ma 2005). However, the capacity for As detoxification can be compromised in ferns under high As doses (Campos et al. 2016).

Free amino acid regulation in ferns under As. The synthesis of AAs is affected by the content of metals/metalloids in the plants and depends largely on the processes and products of photosynthesis and respiration (Ashraf et al. 2011). Stress tolerance of plants is related to energy availability, as most of the stresses affect the overall plant energy status (Planchet and Limami 2015). To avoid a large reduction in the cellular energy pool, AA metabolism is linked to the tricarboxylic acid (TCA) cycle, which is the source of carbon (C) skeletons for biosynthesis (Miflin and Lea 1976, Pavlík et al. 2012). The changes in free AAs appear as a response to different stresses and are important for plant metabolism when AA homeostasis is essential for plant growth, development, and defence against environmental stress (Zhu et al. 2018). Chaffei et al. (2004) suggested an increase in the proportion of high N:C AAs as a protective strategy in plants. Campos et al. (2016) showed that the As-hyperaccumulator *Pityrogramma calomelanos* tolerates high As concentrations due to its ability to upregulate the biosynthesis of AAs and antioxidants, without greatly disturbing central C metabolism. It has been reported that As exposure leads to changes in AA pools in non-As-hyperaccumulators (Dwivedi et al. 2010, Pavlík et al. 2010, Okunev 2019).

In the present study, free AA regulation in fronds and roots of Pc-Al and Pc-Pa were investigated to detect differences between ferns under As treatment. Our results revealed the qualitative and quantitative differences, as well as some similarities in AA regulation between ferns (Figures 1 and 2, Table 2). Arsenic treatments led to an increase in the total free AA content (tAAs) in fronds and roots of Pc-Al compared to the control (Figure 1A). Except in the As1 treatment in fronds, the same trend, an increase with increasing As treatments, was confirmed for Pc-Pa (Figure 1A). The highest As3 treatment increased tAAs in the fronds of Pc-Al and Pc-Pa, and tAAs content was 3.1- and 6.0-fold higher, respectively, compared to control. A similar effect of As3 treatment was shown in the roots of these ferns. This upregulation of tAA content confirmed the toxic effect of this As level. The tAAs were on average 1.7- and 2.1-fold higher in fronds of Pc-Al and Pc-Pa, respectively, than in roots. The same trend of free AA accumulation was observed in tomato plants under As exposure (Okunev 2019). Compared to Pc-Al, greater tAAs content was determined in fronds and roots of Pc-Pa, on average 1.9- and 1.5-fold higher, respectively (Figure 1A). These results showed a higher sensitivity of Pc-Pa to As when compared to Pc-Al. The results of Pearson's linear correlation confirmed a relationship between tAAs content and As content in both ferns (Pc-Al: r = 0.86–0.89, P < 0.001; Pc-Pa: r = 0.56–0.95, P < 0.05).

In addition to the tAAs content, the ratios of major AA families were also affected by As treat-

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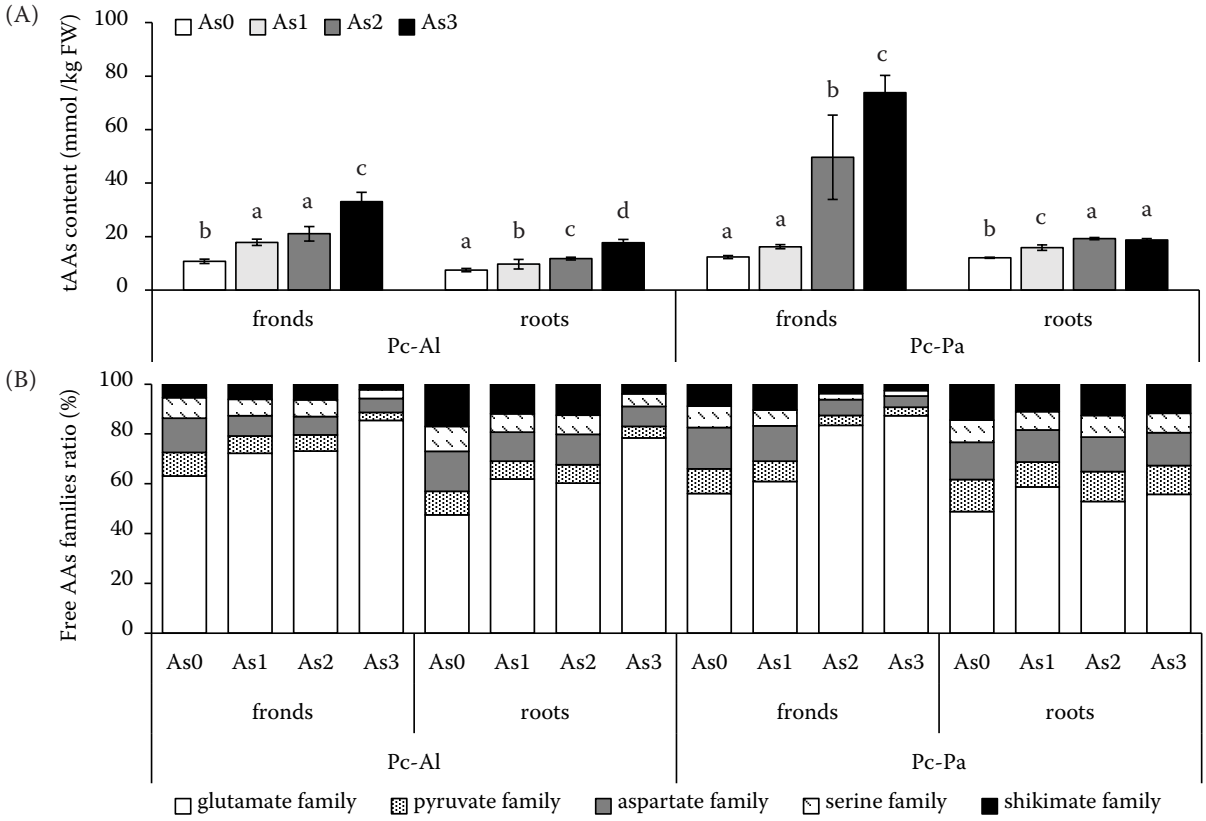

Figure 1. Effect of arsenic (As) treatments on (A) total content of free amino acids (tAAs) and (B) free amino acid (AA) family ratios in fronds and roots of *Pteris cretica* cv. Albo-lineata (Pc-Al) and cv. Parkerii (Pc-Pa). Lower-case letters indicate significant differences by Fisher's *LSD* (least significant difference) test ($P < 0.05$). Data with the same letter were not statistically different. As0 – control; As1 – 20; As2 – 100, As3 – 250 mg As/kg; FW – fresh weight

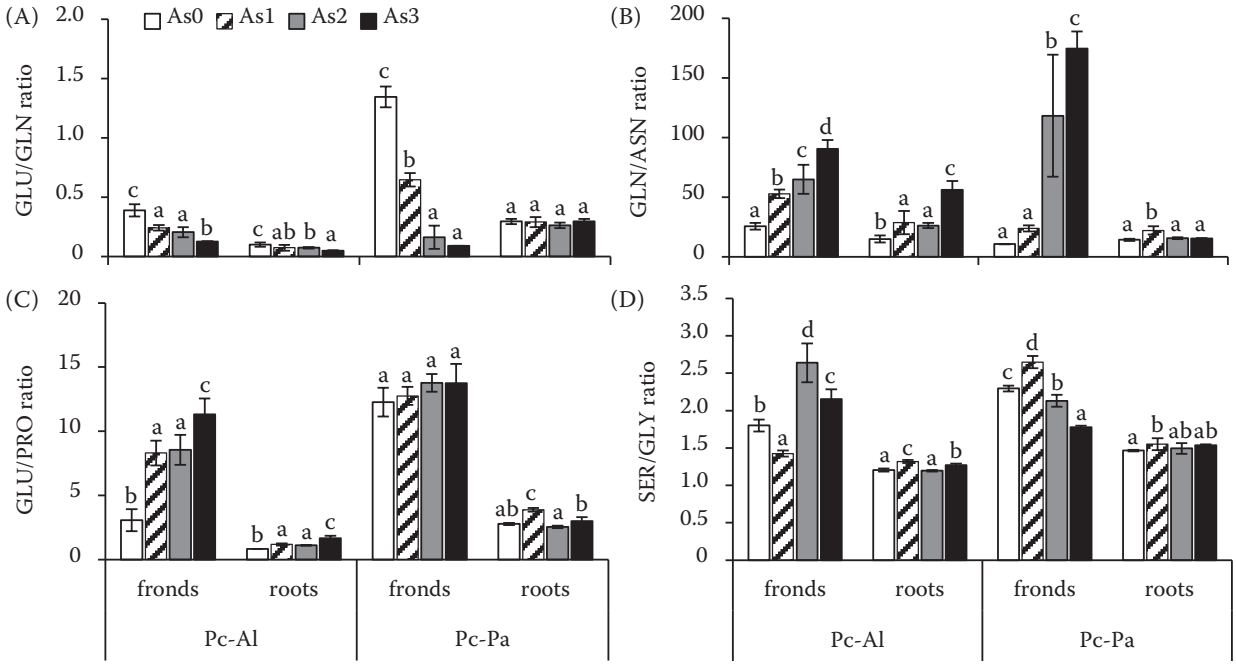

Figure 2. Effect of arsenic (As) treatments on ratios of (A) glutamic acid (GLU)/glutamine (GLN); (B) GLN/asparagine (ASN); (C) GLU/proline (PRO) and (D) serine (SER)/glycine (GLY) in fronds and roots of *Pteris cretica* cv. Albo-lineata (Pc-Al) and cv. Parkerii (Pc-Pa). As0 – control; As1 – 20, As2 – 100, As3 – 250 mg As/kg

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Table 2. Effect of arsenic (As) treatments on the content of free amino acids (AAs, mmol/kg FW (fresh weight)) in fronds and roots of *Pteris cretica* cv. Albo-lineata (Pc-Al) and cv. Parkerii (Pc-Pa)

AAs	Pc-Al fronds				Pc-Pa fronds			
	As0	As1	As2	As3	As0	As1	As2	As3
ALA	0.32 ± 0.01^{a}	0.60 ± 0.03^{c}	0.68 ± 0.04^{d}	0.39 ± 0.004^{b}	0.52 ± 0.05^{a}	0.57 ± 0.02^{b}	0.98 ± 0.01^{c}	1.52 ± 0.04^{d}
ASN	0.17 ± 0.001^{b}	0.18 ± 0.01^{a}	0.19 ± 0.01^{a}	0.26 ± 0.02^{c}	0.26 ± 0.01^{b}	0.24 ± 0.005^{a}	0.30 ± 0.01^{c}	0.34 ± 0.01^{d}
ASP	0.69 ± 0.03^{a}	0.68 ± 0.04^{a}	0.69 ± 0.07^{a}	0.92 ± 0.05^{b}	1.13 ± 0.04^{b}	1.41 ± 0.12^{c}	1.96 ± 0.11^{a}	2.09 ± 0.16^{a}
GABA	0.25 ± 0.0003^{d}	0.22 ± 0.001^{a}	0.24 ± 0.001^{c}	0.24 ± 0.0001^{b}	nd	0.24 ± 0.0002^{a}	0.28 ± 0.0001^{c}	0.28 ± 0.0004^{b}
GLN	4.31 ± 0.46^{b}	9.70 ± 0.82^{a}	12.08 ± 2.26^{a}	23.91 ± 3.10^{c}	2.82 ± 0.12^{a}	5.69 ± 0.69^{a}	36.02 ± 15.96^{b}	58.50 ± 5.79^{c}
GLU	1.70 ± 0.39^{b}	2.35 ± 0.30^{a}	2.43 ± 0.34^{a}	3.03 ± 0.32^{c}	3.80 ± 0.38^{a}	3.66 ± 0.19^{a}	4.77 ± 0.25^{b}	5.25 ± 0.57^{b}
GLY	0.32 ± 0.01^{b}	0.48 ± 0.01^{c}	0.39 ± 0.02^{a}	0.37 ± 0.01^{a}	0.32 ± 0.01^{b}	0.28 ± 0.001^{a}	0.39 ± 0.005^{c}	0.58 ± 0.01^{d}
HIS	nd	nd	nd	0.41 ± 0.002	nd	nd	nd	nd
ILE	0.26 ± 0.0002^{c}	0.23 ± 0.003^{b}	0.25 ± 0.002^{a}	0.25 ± 0.001^{a}	0.26 ± 0.003^{a}	0.28 ± 0.001^{b}	0.38 ± 0.004^{d}	0.35 ± 0.01^{c}
LEU	0.33 ± 0.003^{ab}	0.30 ± 0.001^{c}	0.33 ± 0.01^{b}	0.33 ± 0.002^{a}	0.34 ± 0.003^{a}	0.35 ± 0.002^{b}	0.45 ± 0.004^{d}	0.43 ± 0.001^{c}
ORN	nd	0.34 ± 0.0002^{a}	0.37 ± 0.0004^{c}	0.36 ± 0.0003^{b}	nd	nd	nd	nd
PHE	0.58 ± 0.02^{a}	0.63 ± 0.02^{b}	0.84 ± 0.03^{d}	0.74 ± 0.03^{c}	0.56 ± 0.005^{a}	0.74 ± 0.02^{b}	0.77 ± 0.02^{c}	0.81 ± 0.01^{d}
PRO	0.55 ± 0.03^{b}	0.28 ± 0.003^{a}	0.28 ± 0.004^{a}	0.27 ± 0.002^{a}	0.31 ± 0.005^{b}	0.29 ± 0.002^{a}	0.35 ± 0.001^{c}	0.38 ± 0.002^{d}
SER	0.57 ± 0.03^{a}	0.69 ± 0.03^{ab}	1.02 ± 0.14^{c}	0.80 ± 0.07^{b}	0.74 ± 0.003^{a}	0.75 ± 0.02^{a}	0.84 ± 0.04^{b}	1.04 ± 0.01^{c}
THR	0.37 ± 0.004^{a}	0.37 ± 0.01^{a}	0.41 ± 0.02^{b}	0.40 ± 0.01^{b}	0.39 ± 0.01^{a}	0.38 ± 0.005^{a}	0.50 ± 0.02^{b}	0.57 ± 0.01^{c}
TRP	nd	nd	nd	nd	nd	0.45 ± 0.001^{b}	0.51 ± 0.001^{a}	0.51 ± 0.002^{a}
TYR	nd	0.46 ± 0.0003^{a}	0.50 ± 0.001^{b}	nd	0.53 ± 0.0002^{c}	0.50 ± 0.001^{b}	0.58 ± 0.001^{a}	0.58 ± 0.001^{a}
VAL	0.36 ± 0.003^{a}	0.32 ± 0.003^{b}	0.36 ± 0.01^{a}	0.35 ± 0.003^{c}	0.37 ± 0.01^{b}	0.39 ± 0.004^{c}	0.55 ± 0.002^{a}	0.56 ± 0.01^{a}
	Pc-Al roots				Pc-Pa roots			
	As0	As1	As2	As3	As0	As1	As2	As3
ALA	0.30 ± 0.001^{a}	0.32 ± 0.002^{b}	0.38 ± 0.0001^{d}	0.36 ± 0.0005^{c}	0.48 ± 0.01^{b}	0.46 ± 0.01^{a}	0.67 ± 0.004^{d}	0.60 ± 0.01^{c}
ASN	0.20 ± 0.001^{b}	0.19 ± 0.002^{a}	0.24 ± 0.001^{d}	0.23 ± 0.002^{c}	0.29 ± 0.01^{a}	0.29 ± 0.001^{a}	0.42 ± 0.004^{c}	0.40 ± 0.01^{b}
ASP	0.35 ± 0.003^{b}	0.34 ± 0.001^{a}	0.42 ± 0.002^{c}	0.45 ± 0.004^{d}	0.62 ± 0.01^{b}	0.84 ± 0.02^{a}	0.93 ± 0.01^{c}	0.84 ± 0.02^{a}
GABA	nd	nd	nd	nd	nd	nd	nd	nd
GLN	2.93 ± 0.59^{b}	5.32 ± 1.77^{a}	6.20 ± 0.50^{a}	12.84 ± 1.19^{c}	4.22 ± 0.25^{b}	6.36 ± 0.99^{a}	6.61 ± 0.42^{a}	6.11 ± 0.22^{a}
GLU	0.29 ± 0.01^{a}	0.37 ± 0.02^{b}	0.46 ± 0.01^{c}	0.65 ± 0.07^{d}	1.24 ± 0.04^{b}	1.82 ± 0.06^{a}	1.73 ± 0.05^{a}	1.82 ± 0.19^{a}
GLY	0.34 ± 0.0002^{b}	0.31 ± 0.0003^{a}	0.41 ± 0.001^{d}	0.39 ± 0.001^{c}	0.44 ± 0.002^{a}	0.46 ± 0.002^{b}	0.67 ± 0.004^{d}	0.58 ± 0.002^{c}
HIS	nd	nd	nd	nd	nd	nd	nd	0.91 ± 0.002
ILE	0.31 ± 0.0002^{b}	0.28 ± 0.0003^{a}	0.37 ± 0.0002^{d}	0.34 ± 0.0004^{c}	0.40 ± 0.001^{a}	0.42 ± 0.001^{b}	0.59 ± 0.002^{d}	0.56 ± 0.01^{c}
LEU	0.40 ± 0.0003^{b}	0.37 ± 0.0003^{a}	0.48 ± 0.0002^{d}	0.46 ± 0.0004^{c}	0.53 ± 0.003^{a}	0.56 ± 0.002^{b}	0.82 ± 0.01^{d}	0.80 ± 0.01^{c}
ORN	nd	nd	nd	nd	nd	0.68 ± 0.001^{a}	1.16 ± 0.04^{c}	1.01 ± 0.02^{b}
PHE	0.60 ± 0.002^{b}	0.56 ± 0.003^{a}	0.71 ± 0.001^{d}	0.69 ± 0.003^{c}	0.90 ± 0.02^{b}	0.87 ± 0.01^{a}	1.19 ± 0.01^{d}	1.09 ± 0.01^{c}
PRO	0.35 ± 0.0002^{b}	0.31 ± 0.0001^{a}	0.42 ± 0.0002^{d}	0.39 ± 0.0002^{c}	0.45 ± 0.001^{a}	0.47 ± 0.001^{b}	0.68 ± 0.01^{c}	0.61 ± 0.003^{d}
SER	0.41 ± 0.01^{a}	0.41 ± 0.005^{a}	0.49 ± 0.01^{b}	0.49 ± 0.01^{b}	0.64 ± 0.004^{a}	0.71 ± 0.03^{b}	1.00 ± 0.05^{d}	0.89 ± 0.01^{c}
THR	0.35 ± 0.002^{b}	0.33 ± 0.001^{a}	0.42 ± 0.001^{d}	0.40 ± 0.002^{c}	0.48 ± 0.01^{a}	0.49 ± 0.002^{b}	0.71 ± 0.004^{d}	0.66 ± 0.001^{c}
TRP	nd	nd	nd	nd	nd	nd	nd	nd
TYR	0.67 ± 0.001^{b}	0.60 ± 0.0003^{a}	0.75 ± 0.001^{c}	nd	0.85 ± 0.0004^{a}	0.89 ± 0.0004^{b}	1.23 ± 0.0002^{d}	1.11 ± 0.003^{c}
VAL	nd	nd	nd	nd	0.55 ± 0.001^{a}	0.56 ± 0.01^{b}	0.81 ± 0.004^{d}	0.78 ± 0.01^{c}

Lower-case letters indicate significant differences by Fisher's *LSD* (least significant difference) test ($P < 0.05$). Data with the same letter were not statistically different.
nd – value was not detected. As0 – control; As1 – 20, As2 – 100, As3 – 250 mg As/kg

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ments (Figure 1B). The changes in tAA contents and family ratios were caused by As as a result of affected N metabolism in ferns. Arsenic impacted N metabolism in non-As-hyperaccumulators (Finnegan and Chen 2012, Pathare et al. 2013), as well as in As-hyperaccumulators (Pavlíková et al. 2020). Disturbances to AA metabolism can be lethal for plants because AAs are major N carriers in the plants' long-distance transport system and N storage molecules (Okumoto et al. 2016).

In our experiment, 18 free AAs and amides were found in fronds and roots of both ferns in detectable quantities in at least one treatment (Table 2). The determined free AAs and amides belonged to the five major AA family pathways in plants: (*i*) the glutamate family (GlF) – glutamic acid (GLU), glutamine (GLN), histidine (HIS), proline (PRO), γ-aminobutyric acid (GABA), and ornithine (ORN); (*ii*) pyruvate family (PyF) – alanine (ALA), leucine (LEU), and valine (VAL); (*iii*) aspartate family (AsF) – asparagine (ASN), aspartic acid (ASP), threonine (THR), and isoleucine (ILE); (*iv*) serine family (SeF) – glycine (GLY) and serine (SER); and (*v*) shikimate family (ShF) – phenylalanine (PHE), tyrosine (TYR), and tryptophan (TRP). The AA regulation showed clear differences between fronds and roots and phenotypic differences between ferns (Figures 1 and 2, Table 2). The main difference in AA regulation between ferns and their plant parts was in HIS, ORN, GABA, TRP, TYR, and VAL. In the fronds of ferns, unlike other free AAs, HIS, ORN, and TYR content was only observed in As treatment of Pc-Al. Their contents were not determined in control (Table 2). The same phenomenon was observed for GABA and TRP in fronds of Pc-Pa. Furthermore, HIS, ORN, and VAL were not determined in roots of Pc-Al, as well as GABA and TRP in roots of both ferns (Table 2).

The results of free AA analysis revealed that important changes in the AA metabolism of ferns under As treatments were not only in contents of individual AA but also in the ratios of certain free AAs of specific AA families (Figure 2). The influence of As treatment and differences between ferns were shown in ratios of GLU/GLN, GLN/ASN, GLU/PRO, and SER/GLY. Significant responses and clear trends of As treatments were shown in Pc-Al compared to Pc-Pa, especially in roots (Figure 2).

Among the AA families, the GlF ratio was major in fronds and roots of ferns and was increased by As treatments compared to the control (Figure 1B). An increase of 22% and 38% on average in As treatments were shown in fronds of Pc-Al and Pc-Pa, respectively. Additionally, in the roots of Pc-Al and Pc-Pa, the ratio increased by 41% and 14%, respectively, on average in As treatments. According to González-Orenga et al. (2019), the GlF pathway is more strongly regulated under stress. This family ratio, together with the second most abundant family (AsF), made up more than 60% of the total free AA contents in ferns. A similar abundance of these two families was observed by Kovács et al. (2011) and Gulyás et al. (2017) in control variants of wheat and *Arabidopsis thaliana*.

Biochemically, the GlF is close to the "entry point" of inorganic N into organic N metabolism. Furthermore, the C skeleton of GLU and GLN is directly connected to primary energy metabolism, the TCA cycle (Okumoto et al. 2016). In ferns, GLU and GLN were identified in the fronds and roots of all treatments (Table 2). The main free AA in ferns was GLN, whose content of tAAs ranged from 40% to 72% in Pc-Al and from 23% to 79% in Pc-Pa (Table 2). GLN is not only the major AA used for N transport but also is a key metabolite that acts as an amino donor and pool for other free AAs (Zemanová et al. 2016). The contents of GLN and GLU were increased by As treatment and indicate that the GS/GOGAT cycle is As-responsive in the fronds and roots of ferns (Table 2). Similar results were shown by Campos et al. (2016) for the As-hyperaccumulator *P. calomelanos*.

Furthermore, our results showed a decrease in the GLU/GLN ratio by As treatment in fronds of Pc-Al and Pc-Pa (Figure 2). Similarly, in our previous study, GLU/GLN ratio decreased with As levels in fronds of Pc-Al and Pc-Pa, which indicated a negative As effect by depletion of the C pool as a source for the biosynthesis of other free AAs (Pavlíková et al. 2020).

Another free AA from the GlF, PRO, plays a role in the growth and stress response of plants (Okumoto et al. 2016). Regulation of PRO biosynthesis from GLU is important in relation to the primary function of these AAs; i.e., GLU is an essential substrate for chlorophyll biosynthesis, adenosine triphosphate, cytokinins, glutathione, and phytochelatins (Sharma and Dietz 2006, Pavlík et al. 2012). In the present study, differences in the GLU/PRO ratio were observed between ferns, which is an important factor of PRO biosynthesis regulation from GLU determined by genotype-environment interaction (García-Ríos et al. 1997). Our previous results showed that plants with a higher GLU/PRO ratio are better adapted to Cd stress (Zemanová et al. 2016). The results pre-

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sented in this study suggested that it is the same for As stress, due to an increase of the GLU/PRO ratio in the fronds and roots of Pc-Al (Figure 2). High storage of GLU accumulation and PRO biosynthesis allows ferns to gain PRO from storage and use GLU for the ascorbate-glutathione cycle.

In the fronds and roots of ferns, the GlF was followed by the AsF, with ratios ranging from 6–16% in Pc-Al and 4.5–16.5% in Pc-Pa. Furthermore, the AsF ratios decreased with As treatments in fronds and roots of ferns (Figure 1B). A significant decrease was observed in fronds of Pc-Pa (73%) and roots of Pc-Al (50%) with the highest As3 treatment. The relationship between the AsF ratio and As treatments was confirmed by correlation analysis in fronds and roots of Pc-Al (fronds: $r = 0.91$, $P < 0.001$; roots: $r = 0.78$, $P < 0.001$) and Pc-Pa (fronds: $r = 0.88$, $P < 0.001$; roots: $r = 0.71$, $P < 0.01$). The AsF pathway is connected to cellular energy metabolism, and this connection might have a major role in the physiological plant response to various abiotic stresses that cause energy deprivation (Kirma et al. 2012). This family pathway leads to four key essential AAs, including lysine (LYS), methionine (MET), THR, and ILE, *via* several metabolic branches (Wang et al. 2018). Of the free AAs in the AsF, only ASP, ASN, ILE, and THR were found in the fronds and roots of ferns of all treatments (Table 2). In our study, LYS and MET were not detected in Pc-Al and Pc-Pa, whereas their contents were determined in another As-hyperaccumulating fern, *P. calomelanos* (Campos et al. 2016).

In ferns, ASP is the main free AA of the AsF, and it is synthetised from oxaloacetate and feeds into the synthesis of other free AAs of the AsF (Zhu et al. 2018). ASN is used to store and transport N from sources to sinks (Chaffei et al. 2004, Zemanová et al. 2016). Similarly, Pavlík et al. (2010) detected an ASP increase under As stress in spinach; however, compared to our results, ASN decreased under As stress. In the As-hyperaccumulator *P. calomelanos*, ASP and ASN increased only under low As exposure (Campos et al. 2016). In contrast, Ashraf et al. (2011) showed a lack of ASP content in *Pteris vittata*. Although in plants ASN and ASP are pools of amino groups under stress (Lea et al. 2007), in ferns, this role was filled by GLN and GLU, as was suggested by our results. The efficacy of N assimilation and C availability is reflected by changes in ASN and GLN (Nikiforova et al. 2006); therefore, the GLN/ASN ratio was calculated in ferns. This ratio increased in all As treatments in Pc-Al fronds and roots, whereas in Pc-Pa fronds, this was only significant in higher As treatments (Figure 2). In the fronds of ferns, the GLN/ASN ratio confirmed negative effects of As3 treatment on N and C metabolism, especially for Pc-Pa; when compared to the control, there was a 16-fold increase in the GLN/ASN ratio in Pc-Pa, whereas, in Pc-Al, the increase was only 3.5-fold. According to Nikiforova et al. (2006), in plants, ASN and GLN are usually in much higher molar amounts than those of all other AAs, and their increase provides a huge N and C sink. However, in studied ferns, a negative N:C ratio was regulated only by free GLN due that ASN was the least abundant free AA (Table 2).

Among five main free AAs families, the PyF pathway is linked to primary C metabolism, including glycolysis and the TCA cycle (Planchet and Limami 2015). In ferns, PyF ratios decreased by As treatment in frond and roots (Figure 1B). The largest decreases (66% compared to control) were found in fronds of ferns with the highest As3 treatment, which suggests a disturbance in energy metabolism by this treatment. These results were supported by an increase in ALA of fern fronds, where ALA was the predominant AA from the PyF (Table 2). A strong increase (192%) compared to the control was shown in Pc-Pa fronds. According to Avezedo Neto et al. (2009), ALA increases under stress, suggesting that glycolysis and thus, pyruvate content and respiration increased to sustain the higher energy demand of stress conditions or to provide C skeletons for the photorespiratory cycle.

The SeF pathway is linked to the plant defence system under different stresses, especially due to the phosphorylated pathway of SER biosynthesis (Ros et al. 2014). Serine family ratios decreased by As treatments in fronds and roots of ferns (Figure 1B), and a significant effect of the highest As3 treatment showed a decrease of 57% and 61% compared to the control in Pc-Al and Pc-Pa, respectively. Two AAs of the SeF, GLY, and cystein, are involved in the plant defence system to As exposure due to biosynthesis of phytochelatins and antioxidant metabolites (Emamverdian et al. 2015). Phytochelatins play the main role in the defence system to As toxicity in non-As-hyperaccumulators (Caille et al. 2005), but in As-hyperaccumulators of the *Pteris* species, they have a limited role in As tolerance (Zhao et al. 2003, Raab et al. 2004). Among the free AAs of the SeF, we found only GLY and SER in the fronds and roots of ferns (Table 2). In Pc-Al fronds and Pc-Pa roots, GLY

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and SER increased with all As treatments, while in Pc-Pa fronds and Pc-Al roots, an increase was shown only in the As2 and As3 treatments. An increase of these AAs can suggest an increase in the photorespiration pathway for the reduction of negative As effects in the photosystem. Similarly, an increase of GLY and SER under As exposure occurred in the As-hyperaccumulator *P. calomelanos* (Campos et al. 2016), while a decrease of these free AAs was shown in non-As-hyperaccumulators under As stress (Begum et al. 2016). In plants, GLY and SER are produced through photorespiration in leaves (Fritz et al. 2006) as precursors for phospholipids and purine synthesis and the main sources of one-carbon units (Morot-Gaudry et al. 2001). According to Bai et al. (2006), the pool of free GLY is normally much lower than that of SER, with the ratio dependent on the degree of irradiance and photorespiration. Similarly, our results showed a higher abundance of SER than GLY in ferns (Table 2). Proteogenic SER is important as a building block of proteins and enzymes and as a precursor of many biomolecules with diverse biological roles, i.e., lipids and AAs, such as CYS and TRP (Tzin and Galili 2010, Ros et al. 2014). In fern fronds, the SER/GLY ratio increased with higher As treatments in Pc-Al, while the opposite effect of As treatments was observed in Pc-Pa (Figure 2).

The ShF pathway is involved in plant responses to abiotic and biotic stresses as components of protein synthesis or precursors of secondary metabolites, i.e., auxins (Tzin and Galili 2010, Liu et al. 2011). It has been reported that about 20% of total fixed C flows through the shikimate pathway under normal conditions (Ni et al. 1996), whereas under stress, C flow through the shikimate pathway increases (Corea et al. 2012). The ShF ratio was more abundant in roots of ferns than in fronds (Figure 1B). A decrease in the ShF ratio was shown in the roots of ferns. In fronds, the ratio decreased in the highest As3 treatment, by 60% and 70% compared to the control in Pc-Al and Pc-Pa, respectively. All AAs of the ShF, including PHE, TRP, and TYR, play a major role in the regulation of plant development and defence responses (Zhu et al. 2018). Studies have shown variable changes in aromatic AAs under As stress in non-As-hyperaccumulators, i.e., Begum et al. (2016) detected a decrease of PHE, TYR, and TRP in rice, whereas Tripathi et al. (2013) and Okunev (2019) showed an increase of TYR and PHE by As exposure in rice and tomato plants, respectively. In our study, only PHE was found in the fronds and roots of ferns in all treatments and increased with higher As treatments (Table 2). A higher accumulation of PHE was found in Pc-Pa roots than Pc-Al roots by As treatment. Thus, the toxicity of As treatments seems to be higher in Pc-Pa, as PHE is a substrate for the phenylpropanoid pathway, which produces a wide range of antioxidative metabolites and phenolic compounds (Fritz et al. 2006). According to Tripathi et al. (2013), induction of PHE in As-tolerant rice cultivars suggests the activation of secondary metabolism for As tolerance; however, a reduction in As-sensitive rice cultivars indicates higher levels of As toxicity. Other aromatic AAs, TRP, and TYR showed variations in abundance, i.e., TYR was detected in all treatments of Pc-Pa fronds and roots, but only in some treatments of Pc-Al (Table 2). In Pc-Pa, the results indicated that TRP and TYR may be involved in defence under As treatments by reducing oxidative damage. In contrast to our results, in another As-hyperaccumulator, *P. calomelanos*, only TYR significantly increased under As exposure (Campos et al. 2016).

The present study revealed that both ferns had different levels of sensitivity to As treatment. Higher As accumulation was shown in Pc-Al ferns, where frond and root biomass production was also higher compared to Pc-Pa. In ferns, a disturbance in energy metabolism due to changes in N and C metabolism by As treatment was shown in the total free AA content and regulation of individual free AAs. The results showed that Pc-Al shared several aspects of AA metabolism with Pc-Pa under As treatment. When considering As accumulation and free AA metabolism, Pc-Pa showed higher sensitivity to As doses than Pc-Al.

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