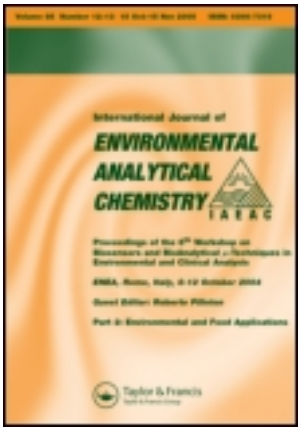

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Organic acids in two arsenic hyperaccumulators and a non-hyperaccumulator of *Pteris* exposed to elevated arsenic concentrations

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Six organic acids (oxalic, malonic, malic, citric, palmitic and linolenic acid) in two arsenic (As) hyperaccumulators (*Pteris multifida* and *Pteris vittata*) and a non-hyperaccumulator fern (*Pteris semipinnata*) exposed to different As concentrations (0, 5, 20 and 40 mg As L^{-1}) under hydroponic conditions were determined using gas chromatography-mass spectrometry (GC-MS). Three fern species were collected from an uncontaminated site, but two As-hyperaccumulators were also collected from an As-contaminated site. Furthermore, the time-course effect of organic acid production in *P. multifida* and *P. semipinnata* collected from the uncontaminated site were also studied under 0 and 20 mg As L^{-1} treatments with a sampling interval at 0, 6, 12, 24 and 36 h. After esterifying (H_2SO_4–CH_3OH), extracting (CH_2Cl_2), washing (saturated NaCl) and drying (anhydrous Na_2SO_4), these organic acids were isolated (Column: HP Ultra 2, 50 m × 0.2 mM × 0.33 μM; Carrier gas: He). There was no significant increase in the concentrations of the six organic acids in the fronds and roots of the three fern species sampled from an uncontaminated site under 5 or 40 mg As L^{-1} treatments. In addition, there were also no significant differences in the concentrations of organic acids among the three fern species under the same As concentration treatments. Concentrations of malic, oxalic and linolenic acids in the fronds and roots of two As hyperaccumulators collected from an As-contaminated or uncontaminated site and the non-hyperaccumulator collected from an uncontaminated site followed the sequence of malic acid > oxalic acid > linolenic acid. As for time-course effect, compared to *P. semipinnata*, a significant increase was observed in concentrations of oxalic and linolenic acid in *P. multifida* over time under 20 mg As L^{-1} treatment. In general, the changes of organic acid concentrations in As hyperaccumulators did not directly contribute to As accumulation.

Keywords: arsenic; fern; GC-MS; hyperaccumulator; organic acids

1. Introduction

Organic acids play an important role in transportation and sequestration of heavy metals, which in turn is related to plant tolerance to heavy metal pollution. There are two response

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mechanisms of organic acids in plants under heavy metal pollution: externally and internally. Some plants detoxify heavy metals in the rhizosphere by exuding organic acids which chelate heavy metals externally, whereas other plants detoxify heavy metals internally by forming complexes with organic acids [1].

As far as exudation of organic acids is concerned, much attention has been focused on aluminum (Al). It has been documented that the main exudation of *Cassia tora* (a plant tolerant to Al) is citrate under Al stress, with little exudation within the first 4 h but a significant increase after 6 h [2]. In soybean (*Glycine max* L.), citrate and malate efflux increased in all genotypes during the first 6 h of Al exposure, but only citrate efflux in Al-tolerant genotypes was sustained for an extended period [3]. Malic and succinic acids were increasingly excreted with Al addition in wheat (*Triticum aestivum* L.) seedlings, and Al-tolerant genotypes excreted 5- to 10-fold more malic acid than Al-sensitive genotypes [4]. The concentrations of citrate increased, but malate, succinate and α-ketoglutarate decreased in the xylem sap of *Melastoma malabathricum* with elevated Al concentration [5]. It was concluded that there were two patterns of Al-induced secretion of organic acids: having time lag and not having time lag between the addition of Al and the onset of organic acid release. The former pattern may be related to the activation of anion channels and the latter may involve genes controlling metabolism and the secretion of organic acids [6]. The composition of organic acids in the root exudates of the arsenic (As) hyperaccumulator *Pteris vittata* and non-hyperaccumulator *Nephrolepis exaltata* consisted mainly of phytic acid and oxalic acid. However, the former produced 0.46 to 1.06 times more phytic acid and 3 to 5 times more oxalic acid than the latter under As stress [7].

Accumulation of organic acids in plants under heavy metal pollution has been documented. Concentrations of malic and citric acids significantly increased in the shoots and roots of sunflower seedlings treated with Al^{3+}, Cd^{2+} or Zn^{2+}, whereas the change of fumaric acid was insignificant [8]. A high root exudation rate of Ni and the enhanced accumulation of organic acids, especially malic acid, in roots might be one of the important factors related to the tolerance of crops to toxic Ni levels [9]. In shoots of the Zn hyperaccumulator, *Thlaspi caerulescens*, malate is the most abundant organic acid, followed by citrate, succinate and oxalate. Additionally, there is a significant correlation between soluble Zn and both malate and oxalate in shoots, but not in roots [10]. It is generally accepted that the compartmentation of plant vacuole will decrease the toxicity of heavy metals, which have been combined with organic acids to form complexes [11].

Results of As species analysis in the fronds of *P. vittata* and *Pityrogramma calomelanos*, two As hyperaccumulators, showed that organoarsenic species could be detected in trace concentrations [12]. The organoarsenic species detected in a range of plants, including monomethylarsonic acid, dimethylarsinic acid, tetramethylarsonium ion, trimethylarsine oxide, arsenobetaine, arsenocholine and glycerol ribose, were summarised [13]. Dimethylarsinic acid was identified in nine green plants, trimethylarsine oxide in 12 green plants, but arsenobetaine was only found in two lichens as a major As compound from an old arsenic smelter in Austria [14]. These results show that organoarsenic compounds which may be formed via organic acids commonly exist in plants.

Up to now, limited information is available about the comparison of organic acid production in As hyperaccumulators and non-hyperaccumulators. The objectives of our present study were to: (1) determine the main organic acids in two As hyperaccumulators (*Pteris multifida* and *P. vittata*) and a non-hyperaccumulator (*Pteris semipinnata*) under external As contamination, using gas chromatography coupled with mass spectrum (GC-MS); (2) investigate whether there were any differences in organic acid concentrations

between As hyperaccumulators and the non-hyperaccumulator; (3) investigate whether there were any differences in organic acid concentrations in As hyperaccumulators between different origins: As-contaminated and uncontaminated sites; and (4) examine the concentrations of organic acid generation over time.

2. Experimental

2.1 *Tested plants*

Three fern species, including two As hyperaccumulators (*P. multifida* and *P. vittata*) and one non-hyperaccumulator (*P. semipinnata*), were used in the present study. *P. semipinnata* was collected from the Lechang lead/zinc mine (Lechang, Guangdong Province), which contained a comparatively lower As concentration in the soil (128 mg As kg^{-1}, range 63–322 mg As kg^{-1}). Two populations, one from an As-contaminated site (Chongyang As mine, Guangdong Province) and another from an uncontaminated site (campus of Sun Yat-sen University, Guangdong Province), were collected for *P. multifida* and *P. vittata*, respectively.

2.2 *Experiment I: Concentrations of organic acids in As and non-hyperaccumulators under As pollution*

Young plants (4–5 cm in height, with 3–4 fronds) were collected and cultivated in soil in a greenhouse for about 2 months. Efforts were made to ensure the uniformity in size of growing plants (with 6–7 young fronds).

The experiment was conducted under hydroponic conditions, with half-strength Hoagland's nutrition solution [15]. All tested plants, *P. vittata* and *P. multifida* (As-contaminated and uncontaminated origins) as well as *P. semipinnata* (non As-contaminated origin), were pre-cultured in As-free half-strength Hoagland's nutrition solution for 1 week under natural sunlight in a greenhouse. Arsenic was then added as $Na_2HAsO_4 \cdot 7H_2O$ to the nutrient solution to give concentrations of 0, 5 and 40 mg As L^{-1}. There were three replicates for each treatment. In other words, nine young plants of each tested fern were averagely transplanted into 3 glass bottles (250 mL each) for each treatment. The plants were allowed to grow for 3 weeks under natural sunlight in a greenhouse, with temperature varying from 20 to 30°C. In order to keep sufficient dissolved oxygen in the solution, hydrogen peroxide was added every 1 to 3 days [16]. The culture solution was changed every 3 days and the pH was adjusted to about 6.50, using 6 mol L^{-1} HCl or NaOH solution.

2.3 *Experiment II: Time-course effect of organic acid production*

In order to compare the time-course effect of organic acid production in *P. multifida* (As hyperaccumulator, uncontaminated origin) and *P. semipinnata* (non hyperaccumulator, uncontaminated origin) with and without the stress of As, 10 young plants were exposed to 0 (control) and 20 mg As L^{-1}, and then two individual plants were collected at 0, 6, 12, 24 and 36 h, respectively. There were three replicates for each treatment.

2.4 *Determination of organic acids*

The procedure followed as described by Zhang [17] with minor revisions. Standard materials (1 g) of oxalic acid, malonic acid, malic acid, citric acid, palmitic acid and linolenic acid purchased from Fluka Company (Switzerland) were dissolved in methanol and diluted to 100 mL, respectively (10 mg mL^{-1}). The stock solution (5, 2, 0.4 and 0.08 mL) was transferred to a round bottom flask (150 mL), then 1.0 mL glutaric acid-methanol (internal standard, 10 mg mL^{-1}) and 50 mL 10% H_2SO_4–CH_3OH were added in sequence. The mixture was methyl-esterised for 6 h in a water bath at 60–65°C. The esterised liquid was transferred to a separatory funnel with a volume of 150 mL containing 40 mL water, and extracted three times with CH_2Cl_2 (10 mL each time). The organic phase was combined, washed with 30 mL saturated sodium chloride (NaCl) solution, dried with sodium sulfate anhydrous (Na_2SO_4), and concentrated to 1 mL for GC-MS analysis (Figure 1). According to the ratio of peak area of organic acid to that of internal standard, the working curves and regression equations between the ratio and concentrations of organic acids were established:

Oxalic acid (OA): A (peak area of OA/peak area of internal standard) = 0.0143 + 0.724C (mg), R = 0.9992;
Malonic acid (MA): A (peak area of MA/peak area of internal standard) = 0.0186 + 0.658C (mg), R = 0.9991;
Malic acid (MAA): A (peak area of MAA/peak area of internal standard) = 0.0143 + 0.782C (mg), R = 0.9994;
Citric acid (CA): A (peak area of CA/peak area of internal standard) = 0.0192 + 1.015C (mg), R = 0.9990;

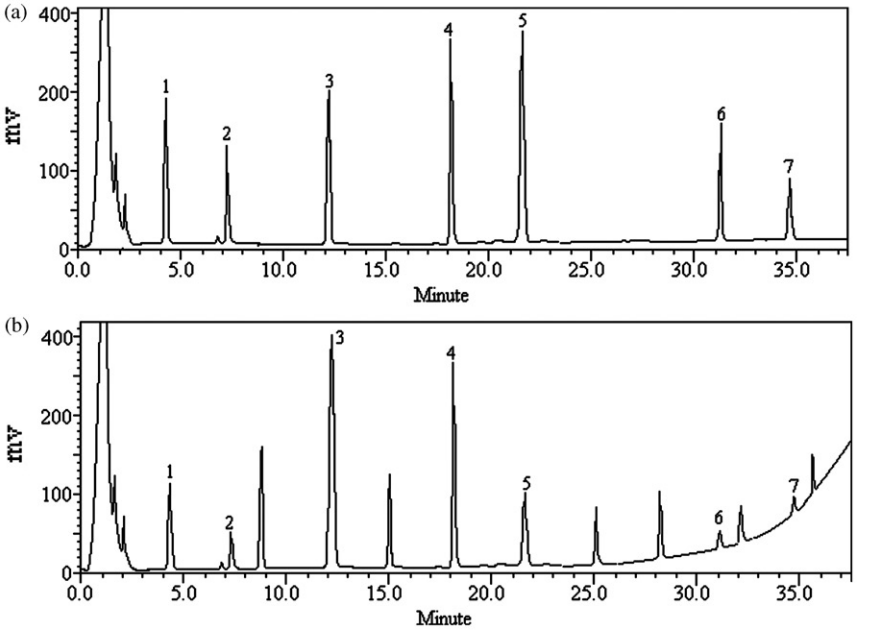

Figure 1. Chromatograms of organic acids in standard (a) and plant samples (b). (1) Oxalic acid; (2) malonic acid; (3) malic acid; (4) glutaric acid (internal standard); (5) citric acid; (6) palmitic acid; (7) linolenic acid.

Palmitic acid (PA): A (peak area of PA/peak area of internal standard) = 0.0215 + 0.684C (mg), R = 0.9989;
Linolenic acid (LA): A (peak area of LA/peak area of internal standard) = 0.0108 + 0.477C (mg), R = 0.9987.

Plants were taken out from a culture bottle, carefully washed with tap water and 0.1 mol L^{-1} HCl solution, rinsed with deionised water, separated into roots and fronds, and kept frozen at −40°C. The samples were ground to fine powder by adding 0.2 mol L^{-1} phosphate buffer solution (PBS, pH = 7.0) in liquid nitrogen, and then freeze-dried (Germany, UNICRYO FD5525S) at −55°C for 24 h. The freeze-dried fine powder was passed through a 0.149 mm sieve for further analysis.

Freeze-dried fine powder (1 g) was weighed and treated the same way as that described above, except for filtration after methyl esterification. The relative standard deviation (RSD%) varied from 2.6% to 3.4% and the recovery rate ranged from 92% to 98%.

GC-MS analyses were performed using a 6890 Plus gas spectrometer (HP Corporation, USA), equipped with a HP Ultra 2 capillary column (length: 50 m; ID: 0.2 mM; film thickness: 0.33 μM). The injection volume was 1 μL and a splitless injection method was adopted. The flow rate of helium carrier gas was 0.8 mL min^{-1}. The inlet temperature was set at 250°C while the oven temperature was initially at 40°C (held for 2 min), then increased to 250°C at 5°C min^{-1} (held for 15 min) and finally increased to 280°C at 5°C min^{-1}. Flame ionisation detector (FID) was used and its temperature was set at 300°C.

According to the ratio of peak area between sample and internal standard, the concentration of organic acid (mg) was calculated by regression equation and was reported on a percentage basis (%).

2.5 *Statistical analysis*

A statistical comparison of means was examined with one-way ANOVA for effects of As on the concentrations of organic acids in the fronds and roots, and with two-way ANOVA for time-course effects of organic acids induction in the fronds and roots using the SPSS statistical package. These two methods were followed by Tukey-HSD tests for multiple comparisons.

3. Results and discussion

3.1 *Effects of As on the concentrations of organic acids in fronds*

The concentrations of organic acids in the fronds of the three fern species under As treatment conditions were higher than those of controls, with some exceptions (Table 1). However, there were no significant differences among different As treatments within the same plant/population ($p > 0.05$). As far as the interspecific level was concerned, there were no significant differences in the concentrations of six individual and total organic acids in the fronds of three fern species collected from the uncontaminated site when treated with 5 or 40 mg As L^{-1}. In the case of *P. multifida* and *P. vittata* collected from As-contaminated site, the concentrations of malonic acid in *P. multifida* were significantly higher (46.4%) than those of the controls when treated with 5 mg As L^{-1} ($p < 0.05$). However, a similar result did not occur in *P. vittata*, suggesting that the increase of malonic acid in As hyperaccumulators collected from the As-contaminated site was not general when low

Table 1. Concentrations of organic acids in fronds of As hyperaccumulators (*P. multifida* and *P. vittata*) and non-hyperaccumulator (*P. semipinnata*) under As treatments (%, values are mean ± SE, $n = 3$).

Sources	As concentration ($mg\ L^{-1}$)	*P. multifida*	*P. vittata*	*P. semipinnata*
			Oxalic acid	
Uncontaminated site	0	2.10 ± 0.11 a-A	2.11 ± 0.05 a-A	1.91 ± 0.04 a-A
	5	2.15 ± 0.13 a-A	2.16 ± 0.13 a-A	2.05 ± 0.12 a-A
	40	2.32 ± 0.09 a-A	2.19 ± 0.17 a-A	2.18 ± 0.10 a-A
As-contaminated site	0	2.58 ± 0.19 a-A	2.56 ± 0.11 a-A	–
	5	2.79 ± 0.20 a-A	2.69 ± 0.28 a-A	–
	40	2.83 ± 0.25 a-A	2.72 ± 0.15 a-A	–
			Malonic acid	
Uncontaminated site	0	0.27 ± 0.01 a-A	0.27 ± 0.03 a-A	0.22 ± 0.06 a-A
	5	0.25 ± 0.03 a-A	0.27 ± 0.01 a-A	0.28 ± 0.01 a-A
	40	0.27 ± 0.02 a-A	0.27 ± 0.02 a-A	0.23 ± 0.02 a-A
As-contaminated site	0	0.28 ± 0.04 b-A	0.27 ± 0.01 a-A	–
	5	0.41 ± 0.02 a-A	0.29 ± 0.01 a-B	–
	40	0.48 ± 0.01 a-A	0.26 ± 0.02 a-B	–
			Malic acid	
Uncontaminated site	0	3.55 ± 0.27 a-A	3.91 ± 0.24 a-A	3.55 ± 0.17 a-A
	5	3.65 ± 0.13 a-A	3.66 ± 0.19 a-A	3.47 ± 0.21 a-A
	40	3.95 ± 0.19 a-A	3.72 ± 0.28 a-A	4.04 ± 0.29 a-A
As-contaminated site	0	4.17 ± 0.45 a-A	4.23 ± 0.26 a-A	–
	5	4.33 ± 0.38 a-A	4.34 ± 0.13 a-A	–
	40	4.41 ± 0.26 a-A	4.40 ± 0.32 a-A	–
			Citric acid	
Uncontaminated site	0	0.68 ± 0.03 a-A	0.73 ± 0.02 a-A	0.70 ± 0.02 a-A
	5	0.71 ± 0.02 a-A	0.64 ± 0.05 a-A	0.66 ± 0.05 a-A
	40	0.70 ± 0.06 a-A	0.66 ± 0.04 a-A	0.71 ± 0.01 a-A
As-contaminated site	0	0.90 ± 0.11 a-A	0.72 ± 0.04 a-A	–
	5	0.84 ± 0.04 a-A	0.69 ± 0.08 a-A	–
	40	0.98 ± 0.01 a-A	0.66 ± 0.03 a-B	–
			Palmitic acid	
Uncontaminated site	0	0.27 ± 0.02 a-A	0.27 ± 0.03 a-A	0.25 ± 0.02 a-A
	5	0.28 ± 0.03 a-A	0.28 ± 0.02 a-A	0.27 ± 0.03 a-A
	40	0.29 ± 0.01 a-A	0.28 ± 0.02 a-A	0.28 ± 0.01 a-A
As-contaminated site	0	0.31 ± 0.02 a-A	0.31 ± 0.01 a-A	–
	5	0.33 ± 0.05 a-A	0.32 ± 0.01 a-A	–
	40	0.36 ± 0.02 a-A	0.30 ± 0.02 a-A	–
			Linolenic acid	
Uncontaminated site	0	0.13 ± 0.02 a-A	0.14 ± 0.02 a-A	0.12 ± 0.01 a-A
	5	0.14 ± 0.01 a-A	0.14 ± 0.01 a-A	0.13 ± 0.01 a-A
	40	0.16 ± 0.01 a-A	0.14 ± 0.01 a-A	0.14 ± 0.01 a-A
As-contaminated site	0	0.14 ± 0.01 a-A	0.15 ± 0.02 a-A	–
	5	0.16 ± 0.02 a-A	0.16 ± 0.03 a-A	–
	40	0.16 ± 0.01 a-A	0.16 ± 0.01 a-A	–
			Total organic acids	
Uncontaminated site	0	7.01 ± 0.36 a-A	7.42 ± 0.30 a-A	6.75 ± 0.29 a-A
	5	7.18 ± 0.29 a-A	7.14 ± 0.25 a-A	6.86 ± 0.29 a-A
	40	7.69 ± 0.36 a-A	7.25 ± 0.49 a-A	7.58 ± 0.36 a-A
As-contaminated site	0	8.38 ± 0.79 a-A	8.25 ± 0.34 a-A	–
	5	8.87 ± 0.61 a-A	8.50 ± 0.38 a-A	–
	40	9.22 ± 0.50 a-A	8.50 ± 0.42 a-A	–

Note: Different small letters after data indicate significant differences in organic acid concentrations among As concentrations with the same source/population of a plant (column) ($p < 0.05$); different capital letters indicate significant differences in organic acid concentrations among or between plants treated with the same concentrations of As (row) ($p < 0.05$).

concentrations of As were added. There were no significant differences in the concentrations of oxalic acid, malic acid, palmitic aicd, linolenic acid and total organic acids in the fronds between *P. multifida* and *P. vittata* collected from the As-contaminated site when treated with the same concentrations of As. However, the concentrations of malonic acid in the fronds of *P. multifida* were significantly higher than those of *P. vittata* treated with 5 or 40 mg As L^{-1} ($p < 0.05$).

Concentrations of organic acids in the fronds of two As hyperaccumulators collected from As-contaminated or uncontaminated site and the non-hyperaccumulator collected from uncontaminated site followed the sequence of malic acid > oxalic acid > linolenic acid.

3.2 *Effects of As on the concentrations of organic acids in roots*

Similar to the fronds, the concentrations of organic acids in the roots of the three fern species under As treatment conditions were higher than those of controls, with some exceptions (Table 2). However, there were no significant differences among As treatments within the same plant or population ($p > 0.05$). As far as the interspecific level was concerned, there were no significant differences in the concentrations of six individual and total organic acids in the roots of the three plants collected from the uncontaminated site when treated with 5 or 40 mg As L^{-1}. In the case of *P. multifida* and *P. vittata* collected from As-contaminated site, the concentrations of malonic acid were much higher (50% and 39.3%) than those of the controls when treated with 5 mg As L^{-1}, respectively ($p < 0.05$). In *P. multifida* collected from the As-contaminated site, the concentrations of palmitic acid and oxalic acid were higher (42.3% and 32.1%) than those of the controls when treated with 5 and 40 mg As L^{-1}, respectively ($p < 0.05$). However, similar results were not shown in the roots of *P. vittata*. There were no significant differences in the concentrations of the six individual and total organic acids in the roots between *P. multifida* and *P. vittata* collected from the As-contaminated site when treated with the same concentrations of As ($p > 0.05$). Similar to the fronds, the concentrations of organic acids in the roots of two As hyperaccumulators collected from As-contaminated or uncontaminated site and the non-hyperaccumulator collected from uncontaminated site followed the sequence of malic acid > oxalic acid > linolenic acid.

Arsenic concentrations in the fronds of *P. multifida* and *P. vittata* (As hyperaccumulators, uncontaminated origin) were 4.4 and 6.8 times higher than those of *P. semipinnata* (non-hyperaccumulators, uncontaminated origin) (data not shown). However, as far as the interspecific level was concerned, there were no significant differences in the concentrations of organic acids in the fronds of the three fern species collected from the uncontaminated site when treated with the same concentrations of As. Based on the present results, it can be explained that the changes of organic acids in the three fern species studied were, to a great extent, a reaction to As stress, rather than a direct relation to As hyperaccumulation.

3.3 *Time-course effects of organic acid induction in fronds*

The time-course effects of oxalic acid, malonic acid, malic acid, citric acid, palmitic acid, linolenic acid and total organic acid induction in the fronds and roots of *P. semipinnata*

Table 2. Concentrations of organic acids in roots of As hyperaccumulators (*P. multifida* and *P. vittata*) and non-hyperaccumulator (*P. semipinnata*) under As treatments (%, values are mean ± SE, $n = 3$).

Sources	As concentration (mg L^{-1})	*P. multifida*	*P. vittata*	*P. semipinnata*
			Oxalic acid	
Uncontaminated site	0	1.89 ± 0.21 a-A	1.78 ± 0.08 a-A	1.62 ± 0.10 a-A
	5	2.05 ± 0.16 a-A	1.93 ± 0.04 a-A	1.78 ± 0.12 a-A
	40	2.24 ± 0.11 a-A	2.15 ± 0.17 a-A	1.98 ± 0.15 a-A
As-contaminated site	0	2.24 ± 0.09 b-A	2.22 ± 0.14 a-A	–
	5	2.70 ± 0.14 ab-A	2.43 ± 0.26 a-A	–
	40	2.96 ± 0.20 a-A	2.79 ± 0.18 a-A	–
			Malonic acid	
Uncontaminated site	0	0.22 ± 0.03 a-A	0.19 ± 0.02 a-A	0.18 ± 0.01 a-A
	5	0.27 ± 0.01 a-A	0.23 ± 0.04 a-A	0.22 ± 0.02 a-A
	40	0.30 ± 0.02 a-A	0.25 ± 0.02 a-A	0.21 ± 0.03 a-A
As-contaminated site	0	0.26 ± 0.01 b-A	0.28 ± 0.02 b-A	–
	5	0.39 ± 0.01 a-A	0.39 ± 0.01 a-A	–
	40	0.41 ± 0.02 a-A	0.29 ± 0.03 b-A	–
			Malic acid	
Uncontaminated site	0	3.52 ± 0.23 a-A	3.31 ± 0.27 a-A	3.15 ± 0.26 a-A
	5	3.47 ± 0.35 a-A	3.58 ± 0.38 a-A	3.09 ± 0.11 a-A
	40	3.92 ± 0.29 a-A	3.54 ± 0.22 a-A	3.68 ± 0.16 a-A
As-contaminated site	0	3.51 ± 0.32 a-A	4.16 ± 0.35 a-A	–
	5	4.42 ± 0.30 a-A	4.41 ± 0.26 a-A	–
	40	4.69 ± 0.28 a-A	4.77 ± 0.33 a-A	–
			Citric acid	
Uncontaminated site	0	0.62 ± 0.02 a-A	0.62 ± 0.02 a-A	0.62 ± 0.04 a-A
	5	0.66 ± 0.03 a-A	0.67 ± 0.06 a-A	0.61 ± 0.03 a-A
	40	0.71 ± 0.06 a-A	0.62 ± 0.03 a-A	0.67 ± 0.03 a-A
As-contaminated site	0	0.71 ± 0.08 a-A	0.70 ± 0.04 a-A	–
	5	0.78 ± 0.10 a-A	0.78 ± 0.03 a-A	–
	40	0.88 ± 0.06 a-A	0.83 ± 0.07 a-A	–
			Palmitic acid	
Uncontaminated site	0	0.25 ± 0.02 a-A	0.24 ± 0.02 a-A	0.22 ± 0.01 a-A
	5	0.27 ± 0.02 a-A	0.25 ± 0.01 a-A	0.24 ± 0.01 a-A
	40	0.28 ± 0.03 a-A	0.27 ± 0.01 a-A	0.26 ± 0.02 a-A
As-contaminated site	0	0.26 ± 0.02 b-A	0.29 ± 0.05 a-A	–
	5	0.37 ± 0.01 a-A	0.35 ± 0.02 a-A	–
	40	0.39 ± 0.01 a-A	0.36 ± 0.03 a-A	–
			Linolenic acid	
Uncontaminated site	0	0.11 ± 0.01 a-A	0.10 ± 0.01 a-A	0.08 ± 0.01 a-A
	5	0.13 ± 0.01 a-A	0.12 ± 0.01 a-A	0.10 ± 0.02 a-A
	40	0.15 ± 0.01 a-A	0.14 ± 0.02 a-A	0.12 ± 0.02 a-A
As-contaminated site	0	0.10 ± 0.02 a-A	0.11 ± 0.01 a-A	–
	5	0.14 ± 0.01 a-A	0.14 ± 0.03 a-A	–
	40	0.14 ± 0.01 a-A	0.16 ± 0.01 a-A	–
			Total organic acids	
Uncontaminated site	0	6.620 ± 0.14 a-A	6.23 ± 0.20 a-A	5.87 ± 0.23 a-A
	5	6.85 ± 0.46 a-A	6.77 ± 0.29 a-A	6.04 ± 0.07 a-A
	40	7.60 ± 0.18 a-A	6.98 ± 0.41 a-A	6.92 ± 0.32 a-A
As-contaminated site	0	7.08 ± 0.50 a-A	7.76 ± 0.27 a-A	–
	5	8.79 ± 0.49 a-A	8.50 ± 0.49 a-A	–
	40	9.47 ± 0.32 a-A	9.19 ± 0.57 a-A	–

Note: Different small letters after data indicate significant differences in organic acid concentrations among As concentrations with the same source/population of a plant (column) ($p < 0.05$); different capital letters indicate significant differences in organic acid concentrations among or between plants treated with the same concentrations of As (row) ($p < 0.05$).

and *P. multifida* with and without As treatments are shown in Figures 2 and 3, respectively. The results of two-way ANOVA analysis are presented in Table 3.

The data presented in Figure 2 and Table 3 show that there were no significant differences ($p > 0.05$) in the concentrations of malonic acid, malic acid, citric acid and total organic acids in the fronds of *P. semipinnata* and *P. multifida* when exposed to 20 mg As L^{-1} compared to the control (0 mg As L^{-1}), respectively. There were also no significant differences among the different times in the concentrations of malonic acid, malic acid, citric acid and total organic acids of the two fern species ($p > 0.05$, Table 3). However, there were significant differences ($p < 0.05$) in the concentrations of oxalic acid,

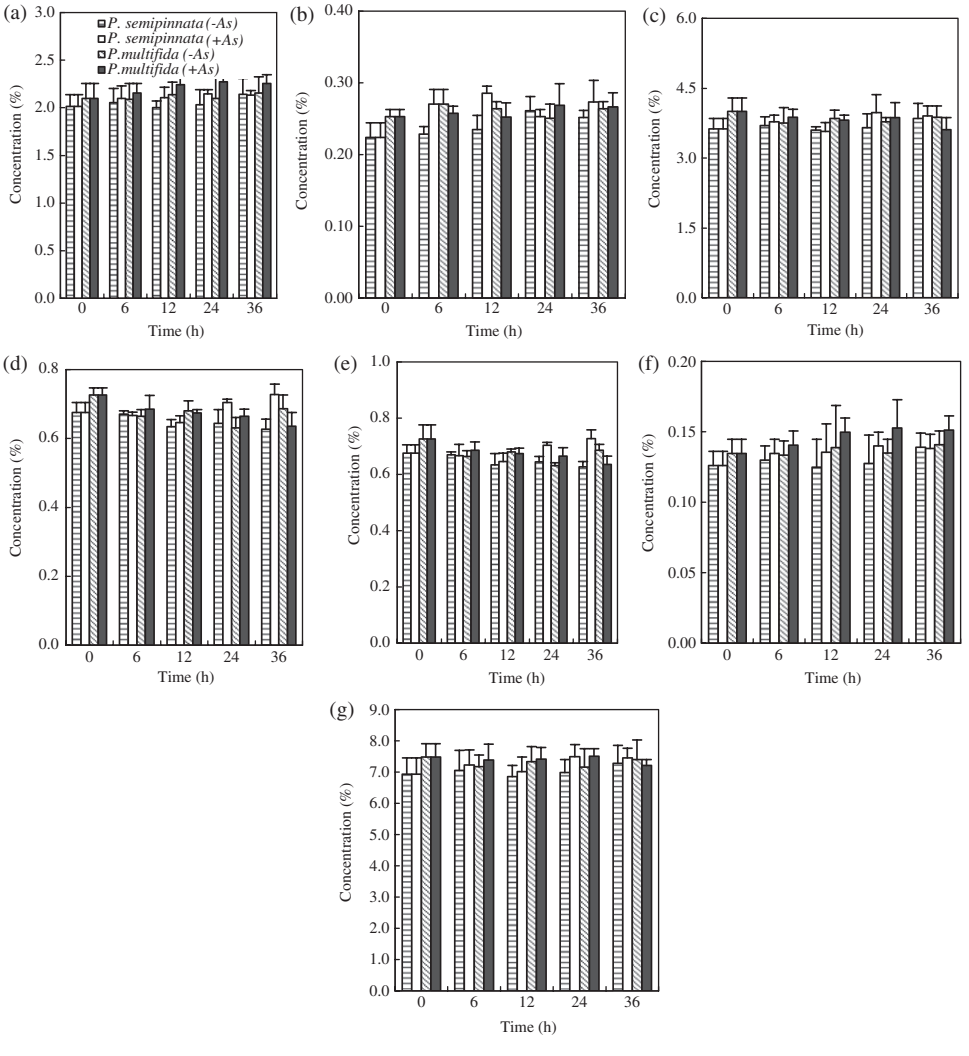

Figure 2. Time-course effect of organic acids in the fronds of *P. semipinnata* and *P. multifida*. Error bars indicate standard errors of the means, $n = 3$. −As: 0 mg As L^{-1}; +As: 20 mg As L^{-1}. (a) Oxalic acid; (b) malonic acid; (c) malic acid; (d) citric acid; (e) palmitic acid; (f) linolenic acid; (g) total organic acid.

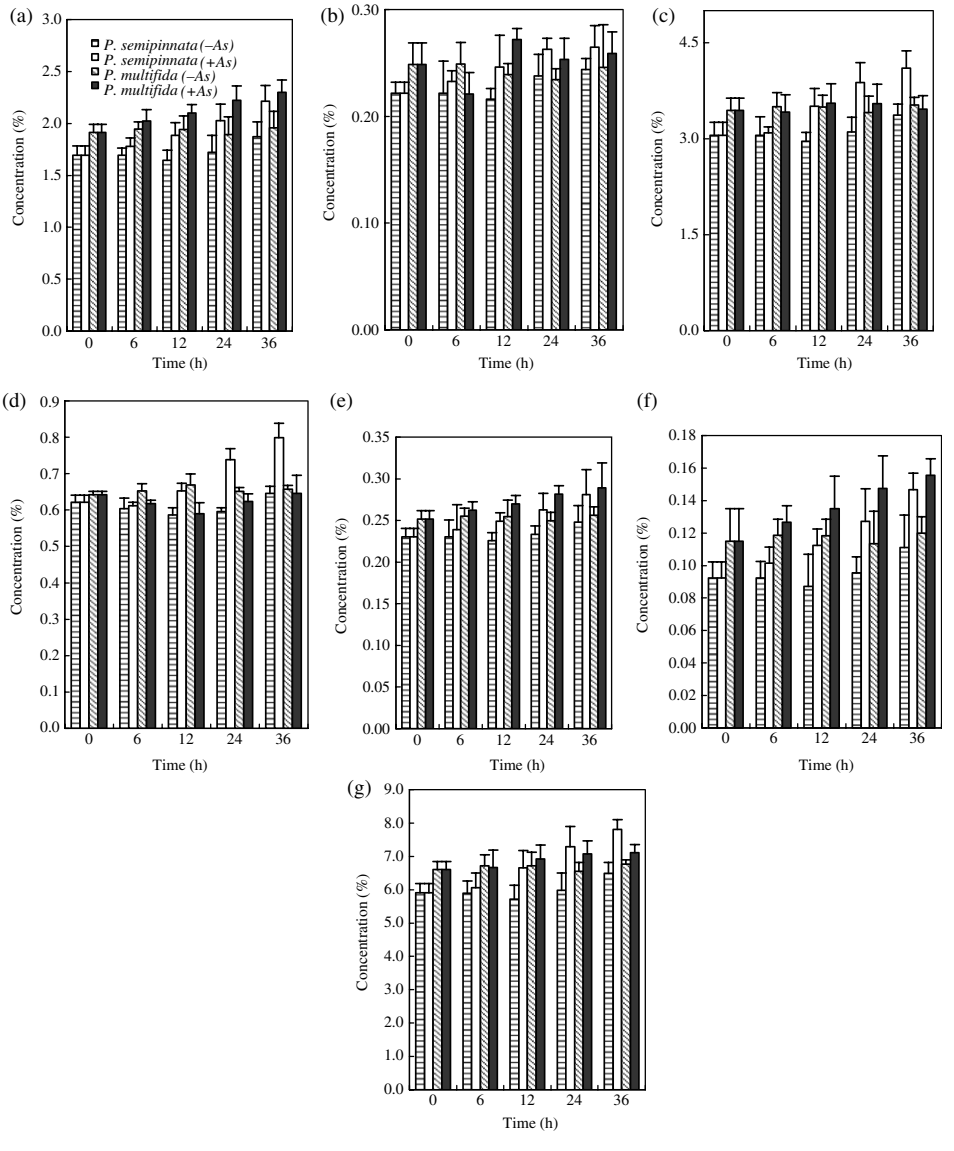

Figure 3. Time-course effect of organic acids in the roots of *P. semipinnata* and *P. multifida*. Error bars indicate standard errors of the means, $n = 3$. −As: 0 mg As L^{-1}; +As: 20 mg As L^{-1}. (a) Oxalic acid; (b) malonic acid; (c) malic acid; (d) citric acid; (e) palmitic acid; (f) linolenic acid; (g) total organic acid.

palmitic acid and linolenic acid in the fronds of *P. multifida* when treated with and without As (20 mg As L^{-1}), but the differences were not significant among the different times ($p > 0.05$).

There were no significant differences ($p > 0.05$) in the concentrations of malonic acid, malic acid, citric acid and total organic acids in the fronds of *P. semipinnata* and *P. multifida* under 20 mg As L^{-1} treatment. Differences in concentrations were also not significant among the different times ($p > 0.05$). However, significant differences were

Table 3. Two-way ANOVA analysis for time-course effect of organic acids in fronds and roots of *P. semipinnata* and *P. multifida* (p value).

Parts	Comparative subject[a,b]	Comparative factors	Oxalic acid[c]	Malonic acid	Malic acid	Citric acid	Palmitic acid	Linolenic acid	Total organic acid
Fronds	sem. (−) – sem. (+)	Between concentrations	0.115^{NS}	0.136^{NS}	0.249^{NS}	0.170^{NS}	0.111^{NS}	0.116^{NS}	0.076^{NS}
		Among times	0.214^{NS}	0.348^{NS}	0.151^{NS}	0.768^{NS}	0.215^{NS}	0.214^{NS}	0.103^{NS}
	mul. (−) – mul. (+)	Between concentrations	0.034*	0.885^{NS}	0.798^{NS}	0.992^{NS}	0.034*	0.034*	0.395^{NS}
		Among times	0.216^{NS}	0.688^{NS}	0.350^{NS}	0.131^{NS}	0.218^{NS}	0.215^{NS}	0.702^{NS}
	sem. (+) – mul (+)	Between concentrations	0.002**	0.880^{NS}	0.637^{NS}	0.807^{NS}	0.003**	0.002**	0.275^{NS}
		Among times	0.014**	0.468^{NS}	0.800^{NS}	0.891^{NS}	0.015*	0.014**	0.693^{NS}
Roots	sem. (−) – sem. (+)	Between concentrations	0.041*	0.032*	0.066^{NS}	0.083^{NS}	0.040*	0.041*	0.056^{NS}
		Among times	0.116^{NS}	0.056^{NS}	0.199^{NS}	0.305^{NS}	0.115^{NS}	0.117^{NS}	0.173^{NS}
	mul. (−) – mul. (+)	Between concentrations	0.055^{NS}	0.516^{NS}	0.826^{NS}	0.084^{NS}	0.056^{NS}	0.055^{NS}	0.133^{NS}
		Among times	0.447^{NS}	0.749^{NS}	0.741^{NS}	0.864^{NS}	0.449^{NS}	0.442^{NS}	0.469^{NS}
	sem. (+) – mul (+)	Between concentrations	0.002**	0.588^{NS}	0.848^{NS}	0.143^{NS}	0.002**	0.002**	0.639^{NS}
		Among times	0.002**	0.181^{NS}	0.402^{NS}	0.349^{NS}	0.003**	0.002**	0.150^{NS}

Notes: [a](−) indicates the 0 mg As L^{-1} treatment (control), and (+) indicates the 20 mg As L^{-1} treatment in this table.
[b]In the column entitled 'comparative subject', 'sem.' represents *P. semipinnata*, and 'mul' represents *P. multifida*.
[c]'*' shows the difference is significant ($p<0.05$); '**' shows very significant ($p<0.01$); and 'NS' shows not significant.

observed in the concentrations of oxalic acid, palmitic acid and linolenic acid in the fronds of the two plants under 20 mg As L^{-1} treatment ($p < 0.01$), and the differences were also significant among the different times ($p < 0.05$).

3.4 *Time-course effects of organic acid induction in roots*

The data presented in Figure 3 and Table 3 show that there were significant differences ($p < 0.05$) in the concentrations of oxalic acid, malonic acid, palmitic acid and linolenic acid in the roots of *P. semipinnata* when exposed to 20 mg As L^{-1} compared to the control (0 mg As L^{-1}). However, there were no significant differences among the different times in the six individual and total organic acids determined in the fern species ($p > 0.05$, Table 3). In the case of *P. multifida*, there were no significant differences ($p > 0.05$) in the concentrations of all six individual and total organic acids in the roots when treated with and without As (20 mg As L^{-1}), and the differences were also not significant among the different times ($p > 0.05$).

There were no significant differences ($p > 0.05$) in the concentrations of malonic acid, malic acid, citric acid and total organic acids in the roots of *P. semipinnata* and *P. multifida* under 20 mg As L^{-1} treatment. There were also no significant differences among the different times ($p > 0.05$). However, significant differences were observed in the concentrations of oxalic acid, palmitic acid and linolenic acid in the roots of the two fern species under 20 mg As L^{-1} treatment ($p < 0.01$), and the differences were also significant among different times ($p < 0.01$).

Organic acids play an important role in plant metabolism [18,19]. It is generally accepted that there are double effects of organic acids on the soil-plant rhizosphere. On the one hand, organic acids can increase desorption of heavy metals and rare earth elements from soil, thus increasing the mobility of metals in the vicinity of roots and enhancing the phytoavailability of metals to plants. On the other hand, organic acids participate in metal absorption by plant roots, long-distance translocation in the xylem and storage in the vacuole of leaf cells [20].

The present results showed that there were no significant differences in the concentrations of six organic acids between As hyperaccumulators and the non-hyperaccumulator exposed to different As concentration treatments. Based on the concentrations of As in both fronds and roots, we conclude that the changes of organic acids in the three plants studied were, to a great extent, a stimulative response to As stress, rather than a direct relation to As hyperaccumulation. However, it has been reported that the amount of oxalic acid exuded by roots of *P. vittata* was 3 to 5 times higher than that of *Nephrolepis exaltata*, a non-As hyperaccumulator [7]. Therefore, further research is needed to elucidate whether organic acids play an important role in external detoxification or metal mobilisation by root exudation in heavy metal hyperaccumulators. There are two main arguments proposed concerning the major role of organic acids such as malate in the phenomenon of metal hyperaccumulation and metal tolerance: (1) many plant species (e.g. crassulacean acid metabolism, CAM) that produce high malate levels are not able to hyperaccumulate metals and (2) metal hyperaccumulation and tolerance are metal-specific, while malate and citrate are good chelators for several metal ions [21]. In addition, organic acid concentrations in *T. caerulescens* were high even under suboptimal Zn supply (1.5 μM), suggesting that the high organic acid concentration in shoots was a constitutive property [10]. Variation of organic acid concentrations appears to be a consequence of the

cation-anion balance rather than a specific Zn tolerance mechanism. It was also documented that when roots of maize took up an excess of K^+ ion, the negative charge required to balance this was often provided by organic acids, such as malate, malonate, citrate and aconitate [22].

Our results also showed that the concentrations of malic, oxalic and linolenic acids in the fronds and roots of two As hyperaccumulators collected from As-contaminated or uncontaminated site and the non-hyperaccumulator collected from uncontaminated site followed the sequence of malic acid > oxalic acid > linolenic acid. However, root exudation was not considered. Root cells may exude organic acids externally, but organic acid exudation was not studied in present research.

Advances have been achieved in understanding the mechanisms of Al-organic acid complex translocation in plants [1]. For example, in an Al-accumulating plant (*Fagopyrum esculentum*), Al^{3+} is chelated with oxalate to form an Al-oxalate complex when it crosses the plasma membrane. When Al is translocated from the roots to the shoots, a ligand-exchange reaction occurs in the xylem to form Al-citrate. Once unloaded from the xylem to leaf cells, another ligand-exchange reaction occurs to reform the Al-oxalate complex, which is then stored in the vacuole [1]. Comparatively, there is limited information available concerning the reaction laws between As and organic acids in As hyperaccumulators. In the phytoremediation process, asparagus fern plants accumulated up to 1400 mg As kg^{-1} when grown on soils containing 1200 mg As kg^{-1} with the addition of 5 mmol kg^{-1} trans-1, 2-cyclobexylenedinitrilotetraacetic acid (CDTA). Compared to the control (0 mmol kg^{-1} CDTA), the As concentration in plants increased to 450 mg kg^{-1} [23]. More organic chelators should be screened to assist phytoremediation of As-contaminated soil.

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