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Metal Accumulation and Tolerance in *Artemisia indica* var. *maximowiczii* (Nakai) H. Hara. and *Fallopia sachalinensis* (F.Schmidt) Ronse Decr., a Naturally Growing Plant Species at Mine Site

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Abstract: For growing plants at mine sites, plant species that accumulate metals in tissues and are tolerant to high metal concentrations should be selected from the perspective of phytostabilization. However, the eco-chemical or elemental information of the plant species at the mine sites is limited. The purpose of this study was to identify plants that can adapt to natural growth at mine sites, via: (1) vegetation survey, (2) elemental analysis in soil and plants, and (3) detoxicant detection in plant cells. Our vegetation survey indicated that plants growing at our study site are consistent with plant species confirmed at other mine sites in previous reports. *A. indica* var. *maximowiczii* and *F. sachalinensis*, present at the mine site, highly accumulated Fe, Al, and Cu in the roots, indicating their metal tolerance. Furthermore, *A. indica* var. *maximowiczii* produced detoxicants such as chlorogenic acid and 3,5-dicaffeoylquinic acid in the roots, which exhibited high antioxidative activity that would play an important role in metal tolerance in *A. indica* var. *maximowiczii*. This study will be effective in providing fundamental information on phytostabilization at mine sites.

Keywords: mine site; heavy metal; iron; aluminum; *Artemisia indica* var. *maximowiczii*; *Fallopia sachalinensis*; metal tolerance; detoxicant

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1. Introduction

During Japan's period of rapid economic growth after World War II, many mines were operating to refine metals from ores; nowadays, most mines are closed. At several areas in mine sites, the soil still contains high concentrations of metals, showing acidity; therefore, plants cannot grow easily due to high concentrations of bioavailable metals, which can damage plant growth [1]. In order to grow plants at mine sites, it would be beneficial to consider "phytostabilization", which is a type of phytoremediation method that uses plants [2–4]. For phytostabilization, the following plant characteristics would be useful: plants that are capable of accumulating metals in the roots or the rhizosphere, and do not cause soil run off from the mine sites [3,5]. Therefore, plant species that can accumulate metals in the tissues and tolerate high concentrations of metals should be selected; however, there is limited eco-chemical and elemental information on the plant species suitable for phytostabilization at mine sites.

Apparently, plants that naturally grow on soil concentrated with metal have evolved their diverse metal-tolerant abilities [6,7]. Generally, metal tolerance mechanisms in plants are explained as follows [8]: (a) inhibition of metal ions entry into the cell by binding

to the cell wall, (b) reduction in metal ions across the plasma membrane, (c) production of phyto-chelatins, organic acids, and amino acids to chelate metals in the cytosol; (d) sequestration of metal-chelating complex into the vacuole; and (e) active exclusion of metal ions from the cell. Recently, several studies have emphasized that excess heavy metals cause oxidative injury in plants due to free radicals, thereby stimulating antioxidant defense of plants via antioxidant chemicals and enzymes, such as ascorbate, carotenoids, glutathione, superoxide dismutase, and catalases [9–11]. Therefore, it is important to identify the metal tolerance of plant species growing naturally at mine sites for phytostabilization.

The purpose of this study was to confirm plants that can adapt to natural growth at mine sites from the viewpoint of ecology in combination with phytochemistry via: (1) vegetation survey, (2) elemental analysis in soils and plants, and (3) detoxicant detection in plant cells. Based on the results of preliminary elemental analysis in plants, we selected *Artemisia indica* var. *maximowiczii*, and *Fallopia sachalinensis* as the target plants. Moreover, according to the results of elemental analysis, we selected *A. indica* var. *maximowiczii* to analyze the detoxicants in its roots. In addition, we discuss the importance of *A. indica* var. *maximowiczii*, and its effectiveness in phytostabilization at mine sites.

2. Materials and Methods

2.1. Study Site and Vegetation Survey

Our study site was located at a gold, silver, and copper mine, an abandoned area after ore mining in Akita Prefecture, in the northern part of Japan. The study site (north–south: 15 m, east–west: 20 m) is located at a flat area in an abandoned concentrator including an old chimney, which is surrounded by several small mountains (Figure 1). Several plant species grow naturally. In July 2020, a vegetation survey was conducted using the line transect method. Three lines 1, 2, and 3 were set up, considering the micro-topographical differences; therefore, each length differed. Line 1 (11 m in length) and line 2 (12 m in length) were separately set up in the same horizontal area near an abandoned chimney (the space between the lines was 5 m), and line 3 (15 m in length) was set up along a brook, showing acidity. There were old artifacts, such as steps in the middle of the study site, and the brook flows from the east to the west. Along each line, the plant cover of each species in a quadrat (1 m × 1 m) was recorded. The number of quadrats in lines 1, 2, and 3 were 11, 12, and 15, respectively. The average plant cover rates of species detected in each line were calculated.

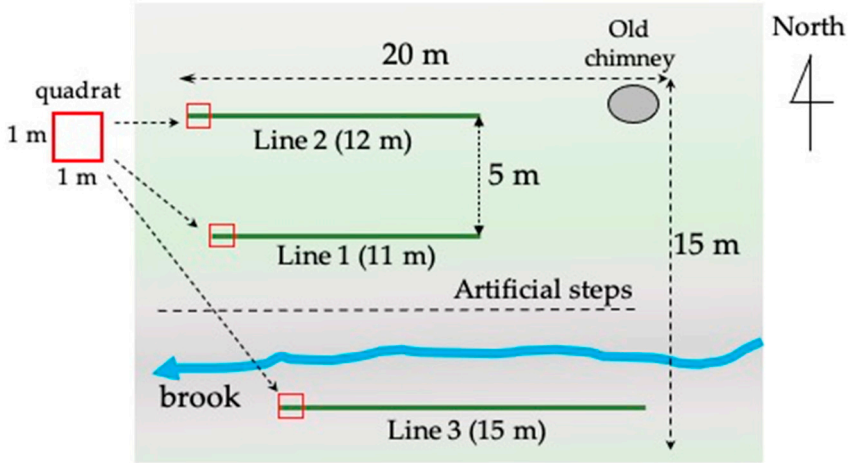

Figure 1. Schematic diagram for vegetation survey at our study site located in the abandoned concentrator.

2.2. Sampling and Analysis of Root-Zone Soil and Plant Tissues and Calculations of Translocation Factor and Bioconcentration Factor

According to the vegetation survey results and preliminary analysis of element concentrations in five plant species (*Miscanthus sinensis*, *Artemisia indica* var. *maximowiczii*, *Fallopia sachalinensis*, *Pinus densiflora*, and *Trifolium repens*) (unpublished data), we selected *F. sachalinensis* and *A. indica* var. *maximowiczii* for further analyses, because *F. sachalinensis* exhibited a high cover rate at the study site, and *A. indica* var. *maximowiczii* accumulated high concentrations of Al, Fe, and Pb in the tissues. In July 2020, which is the the best growing season for the plants at our study site, 5 individuals of *F. sachalinensis* were collected near lines 1 and 2, and 5 individuals of *A. indica* var. *maximowiczii* were sampled near line 3 in the study area; the numbers of plants collected from mine sites were decided according to [12,13]. We collected our plant species out of the vegetation survey area because we will continue to observe the vegetation in a future study. Simultaneously, root-zone soil was collected. The root-zone soil for *F. sachalinensis* was collected from an area of 200 mm × 200 mm × 100 mm deep, and for *A. indica* var. *maximowiczii*, root-zone soil was collected from an area of 100 mm × 100 mm × 50 mm deep.

After the confirmation of damages in plant tissues caused by high concentrations of metals [14,15], the plant roots and root-zone soil were carefully separated, and the root-zone soil was air-dried under the shade at room temperature (23–25 °C). The air-dried soil was passed through a 2 mm sieve. The soil pH (H_2O) was determined using a pH meter (F-71, HORIBA, Kyoto, Japan) with a water to soil ratio of 2.5:1. The air-dried root-zone soil was acid-digested using concentrated perchloric and nitric acids ($HClO_4$-HNO_3) in a volume ratio of 4:1, and the total concentrations of elements (Al, Fe, Cu, Zn, Pb, and Cd) in the root-zone soil were measured using inductively coupled plasma optical emission spectroscopy (ICP-OES; Agilent 5100, Agilent Technologies, Santa Clara, CA, USA).

The plants were carefully washed with tap water followed by Millipore water. *A. indica* var. *maximowiczii* (5 individuals) was divided into three parts: roots, stems, and leaves, and *F. sachalinensis* (5 individuals) was separated into four parts: roots, rhizomes, stems, and leaves. The cleaned plant tissues were dried at 80 °C for 48 h and each plant tissue was ground to a powder using an electric mill (IFM-650D, Iwatani, Tokyo, Japan), and the plant powder was re-dried at 80 °C overnight before digestion. The dried plant powder was weighed and pyrolyzed using concentrated HNO_3. The concentrations of elements (Al, Fe, Cu, Zn, Pb, and Cd) in each plant tissue were measured using ICP-OES. The calibration curve for quantification was prepared using a series of corresponding metal standards at concentrations ranging from 0.03 mg/L to 4.00 mg/L. The results of the 5 replications were averaged, and standard errors (SEs) were calculated.

We calculated translocation factors (TFs) and bioconcentration factors (BCFs) using elements concentrations in each plant tissue and in root-zone soil collected from each plant individual via each formula according to [5,16] as follows;

$$\mathrm{TF} = \frac{\text{Elemental concentration of stems or leaves } (\text{mg kg}^{-1})}{\text{Elemental concentration of roots } (\text{mg kg}^{-1})}$$

$$\mathrm{BCF} = \frac{\text{Elemental concentration of particular plant organs } (\text{mg kg}^{-1})}{\text{Total elemental concentration in soil } (\text{mg kg}^{-1})}$$

The results of the 5 replications were averaged, and standard errors (SEs) were calculated.

2.3. Analysis of Phenolic Compounds in Roots of A. indica var. maximowiczii

Fresh roots of *A. indica* var. *maximowiczii* were used for phenolic analyses. The fresh roots were weighed and cut into several small sections in a methanol solution for extraction of phenolic compounds. Samples were extracted for 5 d in the dark at room temperature (23–25 °C). The root extract solution was filtered and concentrated in vacuo at 35–40 °C. The concentrated sample was dissolved in 1 mL of 50% methanol and analyzed using a high-performance liquid chromatography (HPLC; Prominence UFLC series, Shimadzu,

Kyoto, Japan), and spectral characteristics were analyzed using a diode array detector (DAD; SPD-M20A, Shimadzu, Kyoto, Japan). The gradient eluent solvents used were methanol, and 1.5% tetrahydrofuran and 0.25% phosphoric acid in Millipore water. The HPLC conditions were as described by Yamaji and Ichihara [17]. For the identification of chemicals in the root extracts, the spectral characteristics from 220 to 400 nm, and the retention times of chlorogenic acid (MP Biomedicals LLC., Santa Ana, CA, USA) and 3,5-dicaffeoylquinic acid (Cayman Chemical Company, Ann Arbor, CA, USA) were compared with those of the peaks in the root extracts. Further identification was performed using a liquid chromatograph-mass spectrometer (LCMS-2020; Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source. The conditions for mass detection were as follows: total ion counting (TIC) in the negative mode, 100–1000 *m/z*; nebulizer gas (N_2), 1.5 L/min; drying gas, 15 L/min; interface voltage, −4.5 kV; interface temperature, 350 °C; dissolving line (DL) temperature, 200 °C; and heat block temperature, 200 °C. The MS spectra of the target peaks in the root extracts were compared with those of the standard chemicals as described above.

For quantification of chemicals, standard curves were prepared as follows: chlorogenic acid (25, 50, 100, 200, and 400 µg in 1 mL of 50% methanol) and 3,5-dicaffeoylquinic acid (50, 100, 200, and 400 µg in 1 mL of 50% methanol), were analyzed thrice per concentration at 320 nm using HPLC-DAD as described above, and the values were averaged. The quantification results of the root extracts of the five species were averaged, and SEs were calculated.

2.4. Statistical Analysis

The results were analyzed using SPSS Statistics software (ver. 25.0, IBM, Armonk, NY, USA). The differences between the concentrations of elements (Al, Fe, Zn, Cu, and Pb) in each plant tissue were evaluated by a one-factorial analysis of variance (one-factorial ANOVA) with the non-parametric Scheffé post hoc test. The differences in TFs or BCFs between plant species were evaluated by Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. The Vegetation Survey

The results are summarized in Table 1. The average cover rates of *M. sinensis* and *T. repens* were high (over 20% in line 1), followed by *F. sachalinensis* (6.0% cover rate). In line 2, *P. densiflora* and *M. sinensis* exhibited high cover rates at 20.0% and 14.3%, respectively, and *F. sachalinensis* presented the fourth highest cover at 3.3%. In line 3, near a brook, *Phragmites australis* was the dominant species with a 10% cover rate. *A. indica* var. *maximowiczii* growing at the study site, revealed a similar cover rate in lines 1 and 3, with 3.9% and 4.7%, respectively.

Table 1. Vegetation survey results in the study site.

Plant Species [1]	Average Cover Rate (%)
Miscanthus sinensis	22.5
Trifolium repens	20.7
Fallopia sachalinensis	6.0
Robinia pseudoacacia	5.2
Euonymus spp.	4.4
Artemisia indica var. *maximowiczii*	3.9
Quercus crispula	1.8
Pinus densiflora	1.5
Salix spp.	1.2
Plantago asiatica	1.0

Table 1. *Cont.*

Plant Species [2]	Average Cover Rate (%)
Pinus densiflora	20.0
Miscanthus sinensis	14.3
Euonymus spp.	7.1
Fallopia sachalinensis	3.3
Weigela hortensis	2.5
Salix chaenomeloides	2.5
Populus tremula var. *sieboldii*	1.9
Toxicodendron trichocarpum	1.3
Acer rufinerve	0.4
Tripterospermum japonicum	0.4
Trifolium repens	0.4
Plant Species [3]	**Average Cover Rate (%)**
Phragmites australis	10.0
Fallopia sachalinensis	7.1
Miscanthus sinensis	5.3
Artemisia indica var. *maximowiczii*	4.7
Trifolium repens	3.4
Typha latifolia	2.8
Equisetum arvense	2.5
Carex spp.	1.7
Eragrostis ferruginea	0.5
Salix spp.	0.3

[1] shows result of line 1; [2] shows result of line 2; [3] shows result of line 3.

3.2. pH and Total Element Concentrations in Root-Zone Soil

The pH (H_2O) values of root-zone soil were 7.51 ± 0.15 and 6.56 ± 0.43 for *F. sachalinensis* and *A. indica* var. *maximowiczii*, respectively. The total concentrations of elements in the root-zone soils are listed in Table 2. The data of total Cd concentrations in root-zone soil were not shown in the Table 2 because total Cd concentrations were under the detection limit. Compared to the levels in general soil [8], the total concentrations of Fe in the root-zone soil of *F. sachalinensis* were higher. In addition, both *F. sachalinensis* and *A. indica* var. *maximowiczii* root-zone soils contained much higher concentrations of Zn, Cu, and Pb compared to the levels in general soil.

Table 2. Total element concentrations in root-zone soils of the target plants.

Elements	Root-Zone Soil		Unpolluted Soil in Japan *	General Soil **
	A. indica* var. *maximowiczii	***F. sachalinensis***		
Fe	30,616.1 ± 904.0	46,640.9 ± 11,356.1	–	40,000
Al	8699.5 ± 564.7	8490.1 ± 862.5	–	70,000
Zn	226.4 ± 15.1	624.3 ± 165.7	60	90
Cu	390.5 ± 21.1	1291.5 ± 263.2	19	30
Pb	209.6 ± 31.8	1367.5 ± 523.2	17	30

The results are expressed as mean (mg/kg) ± SE (n = 5). * Unpolluted soil data in Japan were obtained from [18]. ** General soil data were obtained from [8]. – indicates no data were shown in the reference.

3.3. Element Concentrations in the Target Plant Tissues

Compared to the non-toxicity concentration levels in plants [1,19] (Al, around 200 mg/kg dry weight (DW); Fe, less than 229 mg/kg DW; Cu, less than 20 mg/kg DW), *F. sachalinensis* and *A. indica* var. *maximowiczii* accumulated high concentrations of Al, Fe, and Cu in the roots (Figure 2). The data of Cd concentrations in plant tissues are not shown in Figure 2 because they were under the detection limit. Plant tissues were not damaged due to the toxicity caused by high concentrations of metals; damages caused by metal toxicity, such as chlorosis of leaves and necrosis of roots [14,15], were not observed.

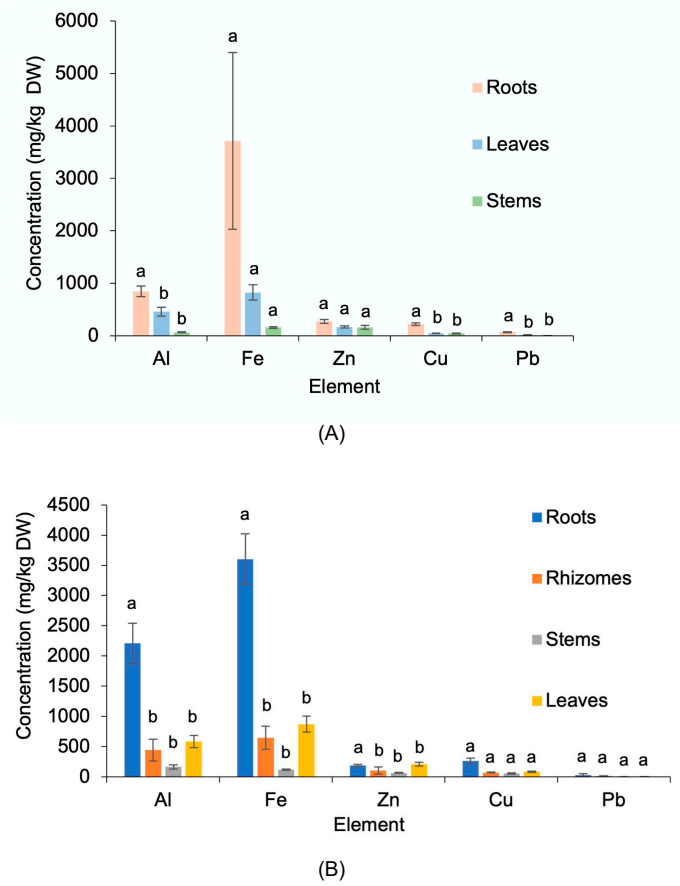

Figure 2. Element concentrations in plant tissues. (**A**) Element concentrations in plant tissues of *A. indica* var. *maximowiczii* and (**B**) element concentrations in plant tissues of *F. sachalinensis*. The results are expressed as means ± SEs (n = 5). The differences between the concentrations of elements (Al, Fe, Zn, Cu, and Pb) in each plant tissue were evaluated by one-factorial ANOVA with Scheffé test. Differences were considered significant at $p < 0.05$, and different letters indicate a statistically significant difference among the tissues.

3.4. Translocation Factors (TFs) and Bioconcentration Factors (BCFs)

The results are listed in Tables 3 and 4. Metals TFs and BCFs in *A. indica* var. *maximowiczii* and *F. sachalinensis* were lower than 1, except for leaves/roots TF of Zn in *F. sachalinensis* (Table 3) and Zn BCF of roots in *A. indica* var. *maximowiczii* (Table 4). In addition, leaves/roots TF of Cu in *F. sachalinensis* was significantly higher than those in *A. indica* var. *maximowiczii*. For Zn, stems/roots TF, and BCFs of roots and leaves in *A. indica* var. *maximowiczii* were higher than those in *F. sachalinensis.* On the contrary, Al BCF in *F. sachalinensis* was higher than *A. indica* var. *maximowiczii*.

Table 3. Translocation factors (TFs) of elements in *A. indica* var. *maximowiczii* and *F. sachalinensis*.

Elements	Stems/Roots		Leaves/Roots	
	A. indica* var. *maximowiczii	***F. sachalinensis***	***A. indica* var. *maximowiczii***	***F. sachalinensis***
Al	0.08 ± 0.01	0.07 ± 0.01	0.56 ± 0.10	0.31 ± 0.08
Fe	0.07 ± 0.01	0.04 ± 0.01	0.36 ± 0.01	0.26 ± 0.05
Zn	0.66 ± 0.16 *	0.38 ± 0.07	0.70 ± 0.15	1.19 ± 0.22
Cu	0.21 ± 0.06	0.23 ± 0.03	0.21 ± 0.03	0.39 ± 0.10 *
Pb	0.07 ± 0.00	–	0.19 ± 0.03	–

The results expressed as means ± SEs (n = 5). – indicates TF values could not be calculated because the element concentrations in stems and leaves were under the detection limit. * $p < 0.05$. Differences between plant species were evaluated by Student's t-test.

Table 4. Bioconcentration factors (BCFs) of elements in *A. indica* var. *maximowiczii* and *F. sachalinensis*.

Elements	Roots		Stems		Leaves	
	A. indica* var. *maximowiczii	***F. sachalinensis***	***A. indica* var. *maximowiczii***	***F. sachalinensis***	***A. indica* var. *maximowiczii***	***F. sachalinensis***
Al	0.10 ± 0.01	0.27 ± 0.04 *	0.01 ± 0.00	0.02 ± 0.00	0.05 ± 0.01	0.08 ± 0.02
Fe	0.13 ± 0.06	0.09 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.03 ± 0.01	0.02 ± 0.00
Zn	1.26 ± 0.24 *	0.37 ± 0.06	0.72 ± 0.14	0.13 ± 0.03	0.71 ± 0.06 *	0.45 ± 0.12
Cu	0.58 ± 0.08	0.25 ± 0.05	0.11 ± 0.02	0.06 ± 0.02	0.12 ± 0.01	0.08 ± 0.03
Pb	0.35 ± 0.07	–	0.02 ± 0.00	–	0.06 ± 0.01	–

The results expressed as means ± SEs (n = 5). – indicates TF values could not be calculated because the element concentrations in roots, stems and leaves were under the detection limit. * $p < 0.05$. Differences between plant species were evaluated by Student's t-test.

3.5. Phenolic Compounds in the Roots of *A. indica var. maximowiczii*

Based on the HPLC-DAD, three peaks (peak 1, 2 and 3) were mainly found in the root extract (Figure 3A). Peaks 1 and 2 from the HPLC-DAD results were identified as chlorogenic acid and 3,5-dicaffeoylquinic acid, respectively, according to the retention times (R_t) and UV spectra (220–400 nm) (Figure 3) of the corresponding standard compounds (chlorogenic acid, R_t = 10.01 min, λ_{max} = 218, 236 and 326 nm, λ_{min} = 230 and 263 nm; 3,5-dicaffeoylquinic acid, R_t = 16.53 min, λ_{max} = 219, 242 and 328 nm; λ_{min} = 230 and 264 nm). In the negative mode of MS detection, peak 1 showed an $[M\text{-}H]^-$ ion at m/z 353 and peak 2 showed an $[M\text{-}H]^-$ ion at m/z 515; similar ions were detected in the respective standard compounds. From the quantification results, the concentrations of chlorogenic acid and 3,5-dicaffeoylquinic acid were 1.01 ± 0.13 mg/g fresh weight (FW) and 2.31 ± 0.52 mg/g FW, respectively. Peak 3 might be a chlorogenic acid derivative due to UV spectrum, however, it was not identified via the comparison of standard chemicals of chlorogenic acid derivatives.

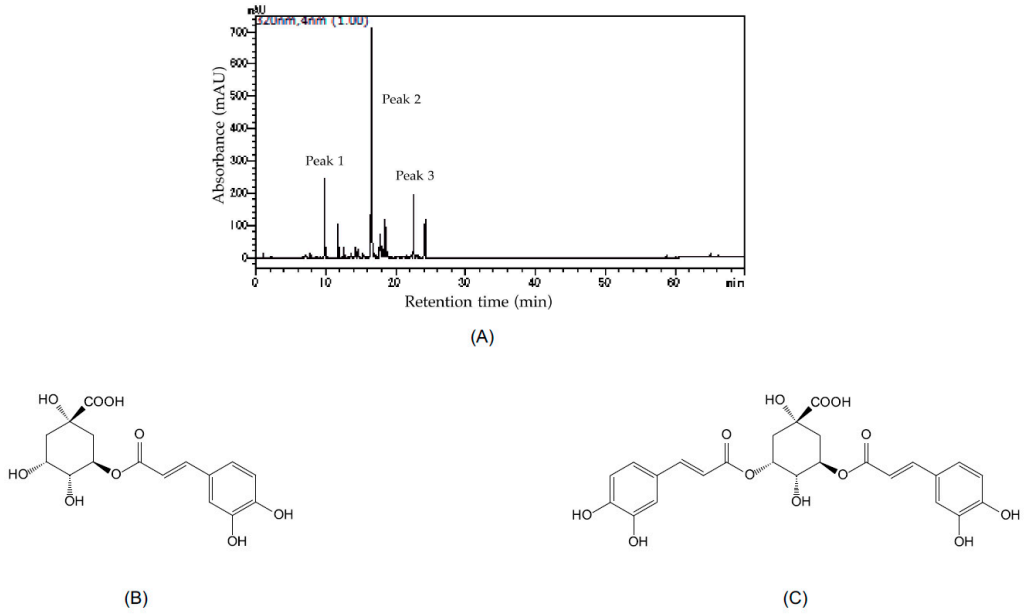

Figure 3. HPLC-DAD results of *A. indica* var. *maximowiczii* root extract. (**A**) HPLC chromatogram at 320 nm of root extracts of *A. indica* var. *maximowiczii.;* retention times of peaks 1, 2 and 3 are 9.86 min, 16.52 min and 22.83 min, respectively; (**B**) chemical structure of chlorogenic acid; (**C**) chemical structure of 3,5-dicaffeoylquinic acid.

4. Discussion

In general, distinctive vegetation has been established on a heavy metal-concentrated soil [20,21]. In fact, previous reports [22,23] conducted surveys at several mines in Japan and observed common vegetation, such as *M. sinensis*, *Fallopia japonica*, and *F. sachalinensis*.

M. sinensis and *F. sachalinensis* were also observed in high frequency in our vegetation survey. In addition, at our study site, high incidences of *T. repens* and *P. densiflora* were confirmed. *M. sinensis* that grows at the mine site is recognized as a metal-tolerant perennial herb, which can accumulate high concentrations of Al in the roots via production of detoxicants and cell-wall adsorption of Al, thus inhibiting its entry into the cytosol [24]. In a previous study [25], *T. repens* growing in metal-concentrated soil accumulated high concentrations of Cd, Pb, and Zn in the roots than in the shoots. In a pot experiment using metal-concentrated soil, *P. densiflora* seedlings accumulated Cd, Cu, Pb, and Zn in the roots [26]. In addition, *Phragmites australis*, which is the main aquatic plant species located along a brook, is also known to accumulate metals in the roots and is used in rhizofiltration [27,28]. According to our survey results and previous reports [22,23], plant species mainly discovered at our study site would be categorized as heavy metal-tolerant species.

From the preliminary elemental analysis of five plant species, including *M. sinensis*, *T. repens*, *P. densiflora*, *F. sachalinensis*, and *A. indica* var. *maximowiczii*, we selected *A. indica* var. *maximowiczii* and *F. sachalinensis* as the target plants, because both plants accumulated higher concentrations of Al and Fe in the roots compared to the others. In the analysis of root-zone soil, which was collected simultaneously with *A. indica* var. *maximowiczii* or *F. sachalinensis*, each soil contained high total concentrations of Zn, Cu, and Pb, exhibiting neutral pH, compared to the average total concentrations of soil suggested by [8]. Elemental analysis of *A. indica* var. *maximowiczii* and *F. sachalinensis* showed higher accumulations of Fe, Al, and Cu in the roots compared to the acceptable concentration range in plants [1,19]. *A. indica* var. *maximowiczii* (synonyms: *Artemisia princeps* Pamp.) is a plant species distributed from Japan and China to Korea. According to Morishita and Boratynski [12], and Kim et al. [29], *A. indica* var. *maximowiczii* growing at metal smelters and mining areas contained high concentrations of heavy metals, such as Cu, Pb and Cd in the roots, and accumulated a high Zn concentration in the roots and shoots. In addition, several studies also reported that other *Artemisia* species growing in heavy metal-concentrated sites accumulated Zn, Pb, and Cd in the roots and Zn in the aboveground portion [30–32]. However, there was not previous research showing the metal tolerance mechanism in *A. indica* var. *maximowiczii*. For *F. sachalinensis*, it also accumulated Fe, Al, and Cu in the roots compared to the acceptable concentration range in the plant [1,19]. *F. sachalinensis* belongs to the Asian weed of *the Fallopia* genus, and its congener *F. japonica* accumulates Cu, Cd, Zn, and Pb in the roots [33,34]; in particular, Cu bound to the cell wall enhances metal tolerance in *F. japonica* [33]. TF and BCF are the value of measuring the ability of plant species for phytoremediation [35]. The TFs and BCFs in *A. indica* var. *maximowiczii* and *F. sachalinensis* were lower than 1, except for leaves/roots TF of Zn in *F. sachalinensis* (Table 3) and Zn BCF of roots in *A. indica* var. *maximowiczii* (Table 4). Even though the TF and BCF of roots values in *A. indica* var. *maximowiczii* and *F. sachalinensis* were low, both plants accumulated high concentration of metals Al, Fe and Cu in the roots compared to normal plants [1,19]. Mendez and Maier [36] indicated that shoots/roots TFs of metals or BCFs of shoots should be less than 1 for plants selection in phytostabilization. The results suggested that both plants would be possible candidates for phytostabilization at our study site. From the results of our elemental analysis and calculations of TFs and BCFs, *A. indica* var. *maximowiczii* was selected to confirm one of the heavy metal tolerance mechanisms, that is, detoxicant production in the roots, because the metal tolerance mechanism of *A. indica* var. *maximowiczii* is not clarified yet.

A. indica var. *maximowiczii* produces several types of chlorogenic acid derivatives [37–39]. According to our HPLC and LC/MS analyses, the main phenolic compounds in *A. indica* var. *maximowiczii* roots were identified as chlorogenic acid and 3,5-dicaffeoylquinic acid (Figure 3). Chlorogenic acid and 3,5-dicaffeoylquinic acid have been identified in the leaves of *A. indica* var. *maximowiczii* [39], and these chemicals show high antioxidative activities [40], which can eliminate reactive radicals released under heavy metal stress in plant cells [9]. High concentrations of metals cause damage to plants, resulting in growth inhibitions [1,11]; this has been attributed to toxic reactive radicals [11]. Therefore,

chlorogenic acid and 3,5-dicaffeoylquinic acid are related to metal tolerance in *A. indica* var. *maximowiczii*.

In conclusion, our vegetation survey revealed that plants growing at our study site are consistent with plant species confirmed at other mine sites [22,23]. Naturally growing plant species under the soil including high concentrations of metals have evolved their diverse metal-tolerant abilities [6,7]. In addition, *A. indica* var. *maximowiczii* and *F. sachalinensis* accumulated Fe, Al, and Cu present in the heavy metal-concentrated soil at the mine site in the roots, indicating their metal tolerance. Furthermore, *A. indica* var. *maximowiczii* produced detoxicants in the roots, such as chlorogenic acid and 3,5-dicaffeoylquinic acid, which exhibited high antioxidative activities; this plays an important role in metal tolerance in *A. indica* var. *maximowiczii*. Our results would increase the value of *A. indica* var. *maximowiczii* for phytostabilization because *A. indica* var. *maximowiczii* is an endemic plant species that has been used in slope revegetation in Japan [41,42]. Recently, it has been observed that root endophytic microbes can enhance metal tolerance in several plants [24,43–46]; therefore, in future studies, we will elucidate the effects of root endophytes on metal tolerance in *A. indica* var. *maximowiczii* via the isolation and identification of microbes. Fundamental knowledge of metal tolerance in *A. indica* var. *maximowiczii* will signify the importance of this plant for phytostabilization at mine sites.

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