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Author(s)	Maejima, Eriko; Osaki, Mitsuru; Wagatsuma, Tadao et al.
Citation	Physiologia Plantarum, 160(1), 11-20 https://doi.org/10.1111/ppl.12527
Issue Date	2017-05
Doc URL	https://hdl.handle.net/2115/66334
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	Maejima_et_al-2017-Physiologia_Plantarum.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Physiologia Plantarum
An International Journal for Plant Biology

Physiologia Plantarum 160: 11–20. 2017 ISSN 0031-9317

Contribution of constitutive characteristics of lipids and phenolics in roots of tree species in Myrtales to aluminum tolerance

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Received 29 August 2016;
revised 19 October 2016

doi:10.1111/ppl.12527

High aluminum (Al) concentration in soil solution is the most important factor restricting plant growth in acidic soils. However, various plant species naturally grow in such soils. Generally, they are highly tolerant to Al, but organic acid exudation, the most common Al tolerance mechanism, cannot explain their tolerance. Lower phospholipid and higher sterol proportions in root plasma membrane enhance Al tolerance. Other cellular components, such as cell walls and phenolics, may also be involved in Al tolerance mechanisms. In this study, the relationships between these cellular components and the Al tolerance mechanisms in *Melastoma malabathricum* and *Melaleuca cajuputi*, both highly Al-tolerant species growing in strongly acidic soils, were investigated. Both species contained lower proportions of phospholipids and higher proportions of sterols in roots, respectively. Concentrations of phenolics in roots of both species were higher than that of rice; their phenolics could form chelates with Al. In these species, phenolic concentrations and composition were the same irrespective of the presence or absence of Al in the medium, suggesting that a higher concentration of phenolics is not a physiological response to Al but a constitutive characteristic. These characteristics of cellular components in roots may be cooperatively involved in their high Al tolerance.

Introduction

Aluminum (Al) stress is one of the most severe stresses to plants growing in acidic soil. Aluminum binds tightly to the cell wall and plasma membrane (PM) of plant root cells, resulting in the inhibition of root elongation. Aluminum tolerance mechanisms of plants have long been well studied (Kochian et al. 2015). Plants have developed two different types of Al tolerance mechanisms: Al exclusion and internal Al tolerance. Organic acid anion secretion from root tips has often been reported to be an important Al exclusion mechanism in many plants (Barceló and Poschenrieder 2002, Kochian et al. 2015). However, this mechanism cannot explain the Al tolerance of some woody plants, such as *Melastoma malabathricum* and *Melaleuca cajuputi*, both belonging to the order Myrtales and growing in highly acidic soils in the tropic and subtropic zones (Osaki et al. 1997, Tahara et al. 2005). Root elongation in *M. cajuputi* is not inhibited by Al concentrations as high as 2.5 m*M* Al (Tahara et al. 2008). *Melastoma malabathricum* is known as an Al-tolerant plant and as Al accumulator, which can accumulate more than 10 000 mg kg^{-1} of Al in mature leaves (Watanabe et al. 1998). Although internal

Abbreviations – HC, hemicellulose; Na_2EDTA, disodium salt of ethylenediaminetetraacetic acid; PCV, pyrocatechol violet; PM, plasma membrane.

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detoxification mechanisms in the leaves of *M. malabathricum* have been studied extensively (Watanabe et al. 1998), roots, which are directly exposed to soils, have received little attention.

The proportions of phospholipids and sterols in PM in roots have been suggested to be closely associated with Al tolerance in rice (Khan et al. 2009, Maejima et al. 2014, Wagatsuma et al. 2015). Because Al has a greater affinity for the heads of phospholipids, the major constituent of PM, than for other cations such as Ca, Al can displace other cations that usually bind to phospholipids in PM, maintaining its fluidity and permeability (Chen et al. 1991, Mackinnon et al. 2006). As a result, the phospholipid packing and fluidity of the membrane is altered, increasing the PM permeability. In contrast, the steroid skeleton of sterol causes an increase in microviscosity of the surrounding phospholipid matrix, resulting in decreased fluidity and increased permeability of the PM (Ranadive and Lala 1987). Thus, a higher concentration of sterol in the PM can stabilize membrane permeability (Grunwald 1974, Hartmann 1998). In fact, rice with higher Al tolerance had lower and higher proportions of phospholipids and sterols, respectively, than rice with lower Al tolerance (Khan et al. 2009). Thus, the proportions of phospholipids and sterols in root cells may be associated with a difference in Al tolerance.

The cell wall is the first component of the root that contacts with Al present in the soil solution, and Al binds to root cell wall components, particularly the carboxyl groups of pectin. It replaces Ca and makes the cell wall rigid, thus leading to inhibition of root elongation. Some reports indicate that the characteristics of negative sites in cell wall are involved in Al tolerance. In fact, lower content in the cell wall of uronic acid with a high degree of methylesterification results in fewer carboxyl groups to serve as Al-binding sites, resulting in greater Al exclusion, as observed in Al-resistant rice (Yang et al. 2008). In *Arabidopsis*, hemicellulose (HC) is the main pool for Al accumulation in root cell walls (Yang et al. 2011). Thus, differences in cell wall composition may affect Al accumulation in roots as well as Al tolerance.

Phenolics, abundant secondary metabolites in plants, play various roles including as components of pigments (anthocyanins) and cell walls (lignin), and as antioxidants in wood, bark and seeds (flavonoids). Phenolics in root cells can be responsible for Al tolerance in plants. Ofei-manu et al. (2001) reported that woody plants with higher concentrations of phenolics in their roots showed higher Al tolerance. *Eucalyptus camaldulensis*, an Al-resistant tree, contains oenothein B in its root, a compound having the ability to detoxify Al and possibly contributing to the Al tolerance of this species (Tahara et al. 2014). Furthermore, it has been suggested that some phenolics could detoxify Al in leaves in some Al-tolerant plants such as *Rumex acetosa* and *Camellia sinensis* (Nagata et al. 1992, Tolrà et al. 2005, 2010). Thus, it may be that phenolics as well as lipids and cell walls in root cells are commonly involved in tolerance mechanisms in highly Al-tolerant plant species.

In this study, we analyzed and characterized cell components including lipid, cell wall and phenolics in roots of *M. malabathricum* and *M. cajuputi* to elucidate the mechanisms underlying the high Al tolerance of woody plants.

Materials and methods

Plant materials and growth conditions

Cuttings of *M. malabathricum* were prepared from adult plants and rooted in tap water for 1 month in a greenhouse at Hokkaido University (14 h photoperiod and day/night temperatures of 25–28°C and 18–22°C, respectively). Seeds of *M. cajuputi* were germinated and grown in sphagnum for 1 year. The seedlings were then transferred to a plastic container filled with tap water and grown for about 2 months in a greenhouse. Tap water contained 0.5 m*M* Ca, 0.05 m*M* K, 0.27 m*M* Mg and 0 m*M* Al. Both seedlings were transferred to Al-free nutrient solution containing 1.07 m*M* N (NH_4NO_3), 0.16 m*M* P ($NaH_2PO_4{\cdot}4H_2O$), 0.36 m*M* K (K_2SO_4:KCl = 1:1), 0.6 m*M* Ca ($CaCl_2{\cdot}2H_2O$), 0.41 m*M* Mg ($MgSO_4{\cdot}7H_2O$), 17.5 µ*M* Fe ($FeSO_4{\cdot}7H_2O$), 4.55 µ*M* Mn ($MnSO_4{\cdot}5H_2O$), 23.2 µ*M* B (H_3BO_4), 1.55 µ*M* Zn ($ZnSO_4{\cdot}7H_2O$), 0.008 µ*M* Cu ($CuSO_4{\cdot}5H_2O$) and 0.025 µ*M* Mo ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) and precultured for 1 month. The plants were then transferred to Al treatment solutions (phosphorus free nutrient solution containing 0 or 0.5 m*M* $AlCl_3$) and grown for 1 week. During the preculture treatment and Al treatment, solution pH was kept at 4.0.

Mineral analysis

A lyophilized shoot or root sample (50 mg) was digested in HNO_3–H_2O_2, and mineral concentrations were determined using inductively coupled plasma mass spectrophotometry (ELAN DRC-e; Perkin Elmer, Waltham, MA).

Lipid analysis

Phospholipids and galactolipids were extracted using the method described by Bligh and Dyer (1959) and modified by Uemura and Yoshida (1984). The lyophilized root sample (50 mg) was homogenized three times in a mixture that included 2.5 ml 2-propanol, 2.5 ml chloroform

and 1.25 ml H_2O using a mortar and pestle. The homogenized sample was centrifuged at 1000 *g* for 5 min, and the chloroform layer was separated. Then, 5 ml of chloroform was added to the residue, shaken for 5 min, and centrifuged, and the chloroform layer was removed. This layer was then filtered through filter paper (No. 6; Advantec, Tokyo, Japan) and shaken with the same volume of 0.1 *M* KCl several times to remove protein and water-soluble molecules (e.g. ATP). The chloroform layer then was dehydrated with Na_2SO_4, evaporated at 40°C and resolubilized in 1 ml chloroform:methanol (2:1, v:v). Phospholipids were quantified by measurement of the phosphorus concentration in the lipid extract using the malachite green spectrophotometric method (Vanveldhoven and Mannaerts 1987) after wet digestion with sulfuric acid. The lipid extracts were separated on thin-layer chromatography plates (silica gel 60 F_{254}; Merck, Tokyo, Japan) with chloroform:methanol:water (65:15:2, v:v:v) to determine the galactolipid concentration. Monogalactosyldiacylglycerol (Funakoshi, Tokyo, Japan) and digalactosyldiacylglycerol (Funakoshi) were used as standards. To visualize the lipid bands, the plates were sprayed with 2% (w/v) anthrone–sulfuric acid and heated at 90°C for 5 min. The plates were scanned with a scanner (GT–9300UF; Epson, Tokyo, Japan) and analyzed using ImageJ 1.46 (Ferreira and Raband, National Institutes, of Health, Bethesda, MD; http://rsb.info.nih.gov/ij).

Sterols were extracted as described by Hartmann and Benveniste (1987) with slight modifications. The lyophilized root sample (50 mg) was homogenized three times in 5 ml dichloromethane:methanol (2:1, v:v) using a mortar and pestle. The homogenates were filtered through a filter paper (No. 6) and washed several times with the same solution. The crude lipid extract was purified, dehydrated and resolubilized as described above. The Δ^5-sterol concentration was determined using the method of Zlatkis and Zak (1969). Stigmasterol (Funakoshi) was used as a standard.

Analysis of cell wall in roots

In addition to *M. malabathricum* and *M. cajuputi*, rice (*Oryza sativa*), an Al-tolerant herbaceous plant, was grown for a few months in nutrient solution (pH 4.9) without Al and analyzed for comparison. Crude cell walls were prepared as described by Zhong and Lauchli (1993) with minor modifications. Briefly, approximately 10 mg of a lyophilized root powder sample was homogenized with a mortar and pestle in 75% (v/v) ethanol on ice. The homogenates were centrifuged at 13 000 *g* for 10 min, and the supernatant was discarded. The pellets were washed three times: with ice-cold acetone, methanol:chloroform mixture (1:1, v:v) and methanol. The supernatant of each wash was discarded, and the final pellet was dried under vacuum. The dried cell wall material was treated with 20 units of α-amylase.

Crude cell walls were fractionated according to the procedure described by Nishitani and Masuda (1983), with minor modifications according to Zhu et al. (2012). Pectins were extracted from the crude cell walls by incubating three times with 1 ml of 20 m*M* ammonium oxalate at 70°C for 1 h each. The supernatants containing the oxalate-soluble pectins were collected after centrifugation at 14 000 *g* for 10 min. The pellet was subjected to triple extraction with 1 ml of 4% (w/v) KOH solution at room temperature for a 24 h, followed by a similar extraction with 24% (w/v) KOH. The pooled supernatants from the 4 and 24% KOH extractions yielded the HC1 and HC2 fractions, respectively. The HC2 fraction was neutralized with acetic acid before analysis.

The uronic acid concentration in the pectin fraction was assayed using galacturonic acid as the standard, according to the method of Blumenkrantz and Asboe-Hansen (1973). Briefly, 200 µl of pectin extract was incubated with 1.2 ml of 98% (w/v) H_2SO_4 (containing 12.5 m*M* $Na_2B_4O_7{\cdot}10H_2O$) at 100°C for 5 min. After cooling, 20 µl of 0.15% (w/v) *m*-hydroxy-diphenyl in 0.5% (w/v) NaOH was added to the solution. The sample was allowed to stand at room temperature for 20 min, and the absorbance was spectrophotometrically measured at 492 nm. Because carbohydrates produce a pinkish chromogen with sulfuric acid/tetraborate at 100°C, the absorbance of a blank sample was measured without *m*-hydroxy-diphenyl, which was replaced with 20 µl 0.5% NaOH. The absorbance of the blank sample was subtracted from the total absorbance.

The total sugar concentration in the HC1 and HC2 fractions was analyzed by the phenol–sulfuric acid method, with glucose as the standard (DuBois et al. 1956). Briefly, 200 µl of the HC1 or HC2 extract was incubated with 1 ml of 98% H_2SO_4 and 10 µl of 80% (w/w) phenol at room temperature for 15 min and then at 100°C for 15 min. After cooling, the absorbance was measured spectrophotometrically at 492 nm.

Phenolic concentrations in root cells

Phenolics in roots were extracted as described by Ofei-Manu et al. (2001) with some modifications. Ten milligrams of lyophilized root samples were heated with 5 ml of 50% (v/v) methanol in a glass tube for 30 min in a water bath at 70°C. After cooling on ice, the tubes were centrifuged at 500 *g* for 10 min. The supernatant was filtered through filter paper (No. 5A, Advantec). The extraction procedure was repeated three times. The

concentration of total phenolics was determined by the method of Folin and Denis (1915). Briefly, 0.2 ml of Folin–Denis reagent composed of 2% (w/v) phosphomolybdate, 10% (w/v) tungstate and 5% (v/v) phosphoric acid was added to a tube containing 3.6 ml of diluted extract. Four hundred microliters of saturated Na_2CO_3 was added, the samples were allowed to stand for 30 min and absorbance at 700 nm was measured. Catechin was used as a standard. In addition to *M. malabathricum* and *M. cajuputi*, rice (*O. sativa*), an Al-tolerant herbaceous plant, was also grown for a few months in nutrient solution without Al and analyzed for comparison. Aluminum concentration in the phenolic extract was determined by inductively coupled plasma mass spectrophotometry after evaporation and resolubilization with 2% of HNO_3.

Analysis of organic acids in phenolic extract

Extracted phenolic samples were evaporated and dissolved in 0.02 *M* HCl. The organic acid concentration was determined by capillary electrophoresis (Quanta 4000 CE; Waters, Milford, MA), as described by Watanabe et al. (1998).

Chelating ability of phenolic extract

Phenolic extract samples of −Al treated plants and $AlCl_3$ solution were mixed (phenolic samples were diluted 1:10 in reaction solution to a final Al concentration of 10 μ*M*), and monomeric Al in the mixture was determined by the pyrocatechol violet (PCV) method (Kerven et al. 1989). Three milliliters of the mixture and 200 μl of 0.037% PCV solution were mixed and allowed to stand for 7 min. Then, 1 ml of 15% hexamine buffer (pH 6.2) and 0.5 ml iron interference reagent (0.1% 1,10-phenanthroline and 0.5% ascorbic acid) were added to the solutions. Absorbance at 585 nm was measured after 7 min. Because phenolic extracts contain organic acids that can also form chelation complexes with Al, monomeric Al in 10 μ*M* $AlCl_3$ solution with concentrations and species of organic acids comparable to those in phenolic extracts were also measured as well as simple 10 μ*M* $AlCl_3$ solution for comparison. Organic acid concentrations in phenolic extracts were measured and the highest concentration among replications was selected in each organic acid species to prepare the organic acid mixture solution.

Extraction of phenolics and Al in apoplastic and symplastic fractions

Fresh root samples were cut into 2 cm segments, and phenolics and Al were extracted with 0.1 *M* disodium salt of ethylenediaminetetraacetic acid (Na_2EDTA, apoplastic fraction). Symplastic phenolics and Al were then extracted with 0.1 *M* Na_2EDTA by the freeze–thaw method. The residue was ground and extracted again with 0.1 *M* Na_2EDTA (residual fraction). The concentrations of phenolics and Al in each fraction were determined as described above.

Phenolic profiling

Phenolic profiles were determined by capillary electrophoresis (Quanta 4000 CE, Waters) according to the method described by Wang et al. (2004) with some modifications. Chromatographic data were collected using SMART CHROM software (KYA Technologies, Japan). Compounds were separated in a 50 cm × 75 μm i.d. fused silica capillary. Direct UV detection was performed at 254 nm. Running voltage was 16 kV. Running time was 20 min. Running buffer solution was 10 m*M* NaH_2PO_4, 5 m*M* $Na_2B_4O_7$, 90 m*M* SDS and 10% methanol. Buffer solution was filtered through 0.45 μm membrane filters.

Localization of phenolics in roots

Localization of phenolics in root was determined by Fast Blue BB staining (O'Brien and McCully 1981). After washing with deionized water, roots were sectioned (20 μm thickness) with a cryomicrotome (CM-3050S; Leica Biosystems, Wetzlar, Germany). After sectioning, the transverse sections were stained on a grass slide with Fast Blue BB according to the methods of O'Brien and McCully (1981). Briefly, the sections were stained with 0.05% Fast Blue BB and observed under a light microscope (BX51; Olympus, Tokyo, Japan).

Results

Al concentrations

Fig. 1 shows Al concentrations in leaves and roots of each plant grown in Al-containing nutrient solution for 1 week. Aluminum concentrations of leaves and roots were much higher in *M. malabathricum* than in *M. cajuputi*.

Lipid proportion in roots

The root cells of *M. malabathricum* and *M. cajuputi* contained lower and higher proportions of phospholipid and sterol, respectively, than those of rice (Fig. 2A). Whereas the proportion of sterol was slightly increased with +Al treatment, that of phospholipid did not change between ±Al treatments in either *M. malabathricum* or *M. cajuputi*.

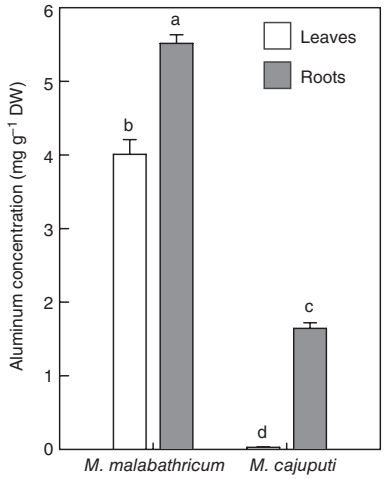

Fig. 1. Aluminum concentration in leaves and roots of *Melastoma malabathricum* and *Melaleuca cajuputi*. Aluminum concentration in leaves and roots of *M. malabathricum* grown in nutrient solution containing 0.5 m*M* of $AlCl_3$ for 1 week. White bars show concentrations in leaves and gray bars show those in roots. Values are means (n = 4), and error bars indicate SE. Different letters indicate significant differences between them.

Concentrations of cell wall components in roots

Fig. 2B shows the concentrations in roots of cell wall components, including pectin and HC (HC1 and HC2). Pectin concentration was significantly higher in roots with +Al treatment than that with −Al treatment in *M. malabathricum*, whereas there was no significant difference between ±Al treatments in *M. cajuputi*. In comparison with rice, pectin concentrations of both *M. malabathricum* and *M. cajuputi* were much higher irrespective of treatment. In contrast, the concentration of total HC was highest in rice. There were no significant differences between treatments in total HC between *M. malabathricum* and *M. cajuputi* or between ±Al treatments in each species (Fig. 2B).

Phenolic concentrations

The concentrations of phenolics in both *M. malabathricum* and *M. cajuputi* were much higher than that in roots of rice grown in nutrient solution for 1 month (Fig. 3). There was no significant difference in phenolic concentrations of roots grown with Al between *M. malabathricum* and *M. cajuputi*, whereas a significantly higher concentration was observed in *M. cajuputi* grown without Al (Fig. 3).

Ability to form chelate with Al

Monomeric Al was estimated by measurement of PCV reactivity with Al in each solution [water (control), water-extracted phenolics or organic acid mixture solution] containing 10 μ*M* of $AlCl_3$. A significantly lower concentration of monomeric Al was detected in the phenolic solution than in the control or the organic acid mixture solution in both species (Fig. 4).

Distribution of phenolics and Al in roots of *M. malabathricum*

More phenolics were distributed in the symplastic than in the apoplastic fraction. In contrast, more Al was distributed in the apoplastic fraction than in other two fractions (Fig. 5); however, a substantial concentration of Al was found in the symplastic fraction.

Profiles of phenolics

Fig. 6 show phenolic profiles of *M. malabathricum* and *M. cajuputi* detected by CE. While phenolic profiles in roots of *M. cajuputi* did not change between ±Al treatments (Fig. 6F, I), those of *M. malabathricum* were completely different (Fig. 6A, D). When Al was added to the phenolic extract of roots of *M. malabathricum* with −Al treatment, its profile changed to one similar to that with +Al treatment (Fig. 6B, D). Likewise, addition of Al to the phenolic extract of *M. cajuputi* with −Al treatment also changed its profile (Fig. 6F, G). Further addition of EDTA to these extracts tended to restore the profiles to those with −Al treatment in both species (Fig. 6A–C, F, G, H) with some peaks disappearing. The phenolic profile in roots of *M. malabathricum* with +Al treatment also became similar to that with −Al treatment following addition of EDTA, but some peaks showed different intensities (Fig. 6A, E).

Localization of phenolics in roots of *M. malabathricum*

Phenolics stained with Fast Blue BB turned red (Fig. 7B), whereas there was no red color without staining (Fig. 7A). Fast Blue BB staining of the root transverse section indicated that phenolics localized not only in epidermal cells but also in the endodermis and stele of *M. malabathricum* (Fig. 7A, B).

Discussion

Melastoma malabathricum and *M. cajuputi* grow naturally in tropical and subtropical zones and are both highly Al tolerant. However, their Al tolerance mechanisms cannot be explained by the exudation of organic acid anions from roots, the most common mechanism of Al tolerance, and have awaited full elucidation. It has been suggested that lower and higher proportions of phospholipids and sterols, respectively in

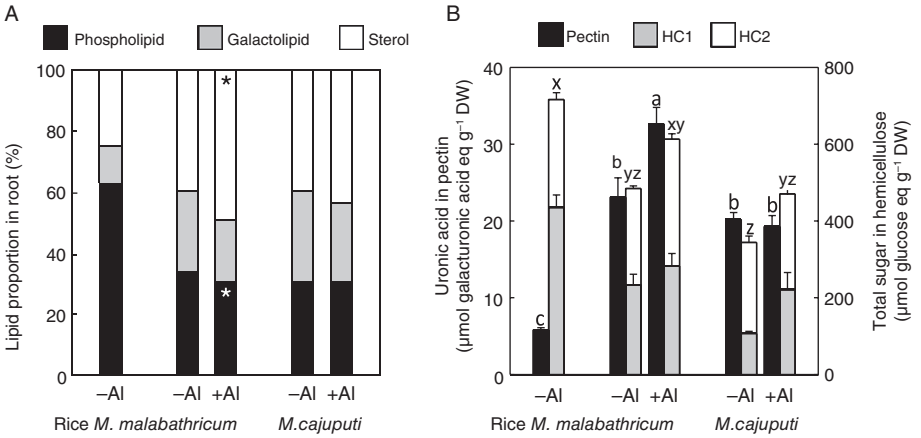

Fig. 2. (A) Lipid proportion and (B) concentration of cell wall components in roots of *Melastoma malabathricum* and *Melaleuca cajuputi*. (A) Phospholipid, galactolipid and sterol were extracted from whole roots. Proportions of each lipid are indicated. Asterisks denote significant differences between ±Al treatments ($P < 0.05$). (B) The concentrations of pectin, HC1 and HC2 in whole roots were analyzed. Galacturonic acid and glucose were used as standards for pectin and HC, respectively. Values are means (n = 4), and error bars indicate SE. a–b and x–z indicate significant differences between pectin concentration and total HC concentration, respectively.

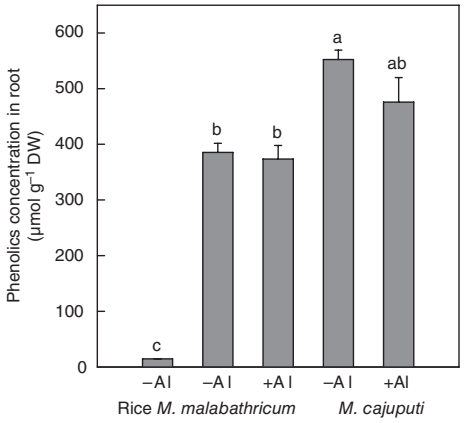

Fig. 3. Phenolic concentrations in roots of *Melastoma malabathricum* and *Melaleuca cajuputi*. Error bars indicate SE and different letters indicate significant differences between them.

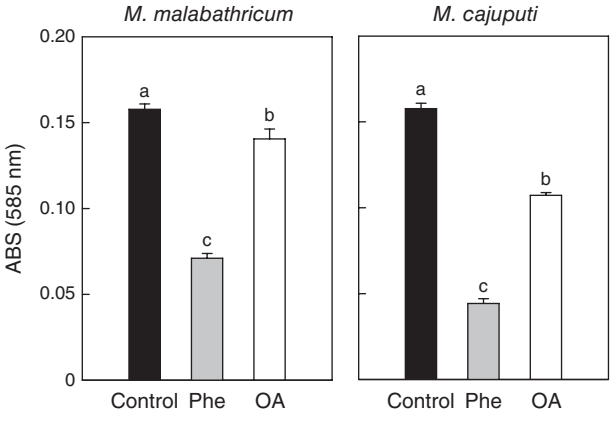

Fig. 4. Pyrocatechol violet reactivity. Monomeric Al was estimated by measurement of pyrocatechol violet reactivity with Al in each solution. Black bars indicate control (10 μ*M* $AlCl_3$), gray bars indicate Phe (water-extracted phenolics solution containing 10 μ*M* $AlCl_3$) and white bars indicate OA (organic acid mixture solution containing 10 μ*M* $AlCl_3$). Values are means (n = 4), and error bars indicate SE. Different letters indicate significant differences between them.

the PM of root cells of rice and higher concentrations of phenolics in roots of some woody plants are involved in their Al tolerance (Ofei-manu et al. 2001, Khan et al. 2009, Maejima et al. 2014). Cell walls of root cells are associated with Al tolerance as a major Al-binding site (Chang et al. 1999, Ma et al. 1999, Hossain et al. 2006). In this study, it was accordingly hypothesized that root cellular components such as lipids, cell wall and phenolics are involved in the tolerance mechanisms of highly Al-tolerant plant species.

Characteristics of Al accumulation and lipid composition in roots

Aluminum concentrations in leaves and roots of *M. malabathricum* were extremely high, and those of *M. cajuputi* were low (Fig. 1). In comparison with rice, phospholipid and sterol proportions were markedly lower and higher, respectively, in roots of both *M. malabathricum* and *M. cajuputi* (Fig. 2A). Phospholipids are a major Al-binding site in PM and sterols function in maintaining PM permeability. Thus, *M. malabathricum* and *M. cajuputi*, which have different characteristics of Al accumulation, have similar PM structures resistant to Al: fewer Al-binding sites and higher retention of permeability in the PM. In roots of *M. malabathricum*, the phospholipid proportion decreased slightly but significantly,

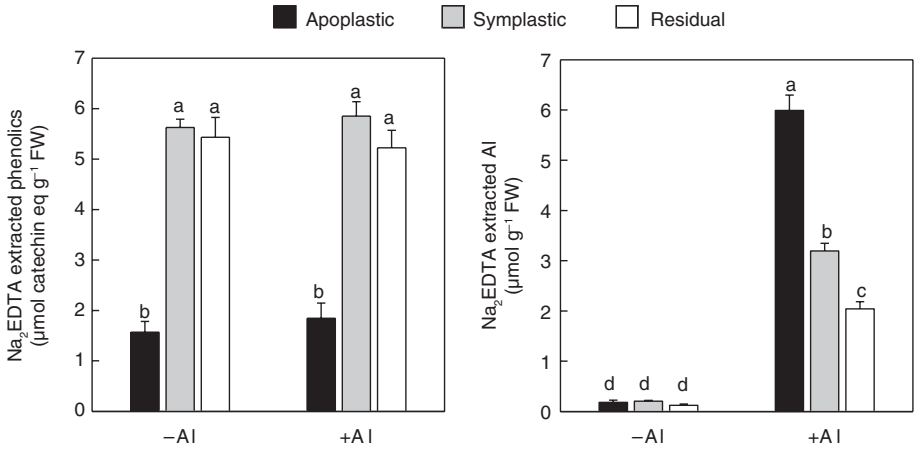

Fig. 5. Na_2EDTA extracted phenolics and Al concentrations in root of *Melastoma malabathricum*. Fresh root samples of *M. malabathricum* were cut into 2 cm segments, and phenolics and Al were extracted with 0.1 *M* Na_2EDTA (apoplastic; black bars). Symplastic phenolics and Al were then extracted with 0.1 *M* Na_2EDTA by the freeze–thaw method (gray bars). The residue was then ground and extracted again with 0.1 *M* Na_2EDTA (residual; white bars). Values are means (n = 4), and error bars indicate SE. Different letters indicate significant differences between them.

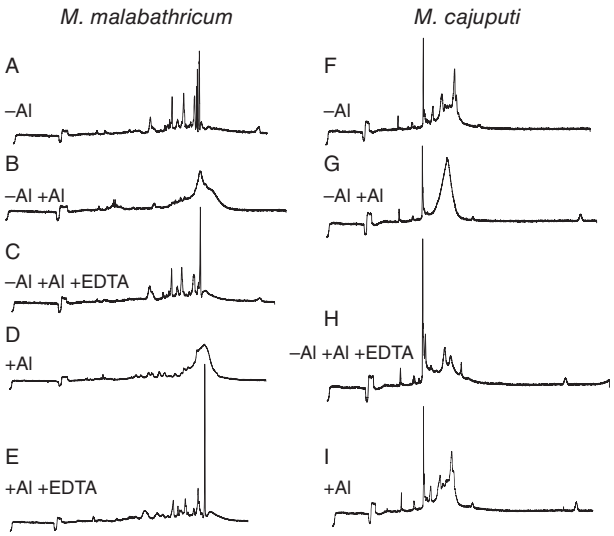

Fig. 6. Profiles of phenolics. Phenolic profiles of *Melastoma malabathricum* (A–E) and *Melaleuca cajuputi* (F–I) detected by CE.–Al (A, F) phenolics extracted from roots under –Al treatment; –Al +Al (B, G) mixture of phenolics extracted from roots under –Al treatment and 2 m*M* $AlCl_3$; –Al +Al +EDTA (C, H) mixture of phenolics extracted from roots under –Al treatment, 2 m*M* $AlCl_3$, and 25 m*M* EDTA; +Al (D, I): phenolics extracted from roots under +Al treatment; +Al +EDTA (E) mixture of phenolics extracted from roots under +Al treatment and 25 m*M* EDTA.

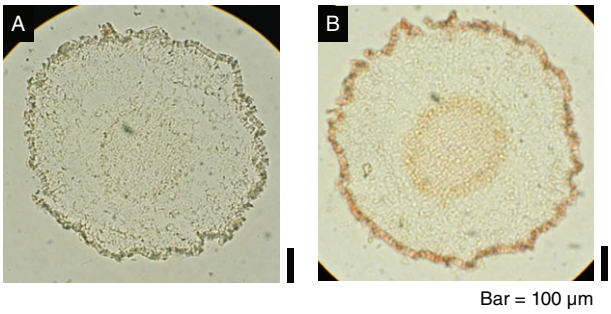

Fig. 7. Localization of phenolics in roots of *Melastoma malabathricum*. Phenolics stained with Fast Blue BB turned red. (A) root tip with –Al treatment without staining; (B) root tip with –Al treatment stained with Fast Blue BB. Bar, 100 μm.

and the sterol proportion increased significantly with +Al treatment. In contrast, the proportion of each lipid did not change between ±Al treatments in *M. cajuputi* (Fig. 2A). Given that *M. malabathricum* contained a high concentration of Al in root under +Al treatment, the lipid composition may have been modified in response to Al accumulation.

Characteristics of the cell wall

Concentrations of pectin, a major Al-binding site, were higher in *M. malabathricum* and *M. cajuputi* than in rice (Fig. 2B). Generally, higher concentrations of pectin can induce higher accumulation of Al in roots. However, Al concentration in roots was lower in *M. cajuputi* than in rice (Osaki et al. 1997). Although methylesterification of pectin, an important factor in Al binding to pectin (Yang et al. 2008), was not determined in this experiment, these results suggest that the composition of the cell wall is not primarily involved in Al exclusion mechanisms in *M. cajuputi*. Likewise, even though pectin concentrations in root of *M. malabathricum* were 1.5 times higher than that of *M. cajuputi* under +Al treatment, Al concentrations in roots of *M. malabathricum* were more than three times higher than those of *M. cajuputi* (Figs 1 and 2B), suggesting that pectin is not the primary factor

in higher Al accumulation in roots of *M. malabathricum*. HC was also reported to be the main site for Al accumulation in cell walls of *Arabidopsis* (Yang et al. 2011). However, HC concentrations in *M. malabathricum* and *M. cajuputi* did not differ from each other and were lower than that of rice (Fig. 2B). This result also suggests that HC concentration in not involved in mechanisms of Al exclusion in *M. cajuputi* and the higher Al accumulation of *M. malabathricum*.

HC concentration in the Al-sensitive wheat Scout 66 increases with exposure to Al, whereas that in the Al-tolerant cultivar Atlas 66 does not change (Tabuchi and Matsumoto 2001). Van et al. (1994) suggested that the reason for increasing HC in Al-sensitive plants in response to Al is that HC turnover, along with cell extension, is inhibited by Al. In this study, however, the concentration of total HC did not change between ±Al treatments in either *M. malabathricum* or *M. cajuputi* (Fig. 2B). Thus, it may be that cell extension and/or cell wall turnover of root cells in these woody plants were not affected by Al.

Characteristics of phenolics

The concentrations of phenolics in roots of *M. malabathricum* and *M. cajuputi* were approximately 8–10 times higher than in those of rice (Fig. 3). Roots of maize exposed to Al exuded high levels of phenolics, and the total phenolic concentration in root of *Pinus sylvestris* increased with the concentration of Al in nutrient solution (Oleksyn et al. 1996, Kidd et al. 2001). These reports suggest that phenolic synthesis is promoted in response to Al treatment. However, high concentrations of phenolics in *M. malabathricum* and *M. cajuputi* might occur irrespective of Al treatment, and phenolics of both species had the ability to chelate Al (Fig. 4). Although phenolic profiles of *M. cajuputi* determined by CE were almost the same between ±Al treatments, those of *M. malabathricum* were sharply different (Fig. 6A, D, F, I). However, the phenolic profile of root of *M. malabathricum* under −Al treatment changed with addition of Al in vitro, showing a profile similar to that under +Al treatment (Fig. 6C, D). Moreover, addition of EDTA to a phenolic extract from root of *M. malabathricum* under +Al treatment changed the profile to one similar to that under −Al treatment (Fig. 6E). These results indicate that the difference in profiles of phenolics in roots of *M. malabathricum* between ±Al treatments is caused by chelate formation of phenolics with Al and not by the alteration of phenolic species synthesized in the root in response to Al. These results suggest that phenolics in roots of *M. malabathricum* could contribute to its Al accumulation and/or Al detoxification mechanisms.

^{27}Al NMR analysis indicated that most Al in tea leaves forms complexes mostly with catechin (Nagata et al. 1992). Tahara et al. (2014) reported that oenothein B binds to Al in *M. cajuputi*. In this study, however, it is likely that almost all phenolic species in roots of *M. malabathricum* have the ability to chelate Al, given that all peaks in the electrogram changed on addition of Al (Fig. 6A, B). In contrast, phenolic profiles of *M. cajuputi* were similar between ±Al treatments, but the profile of *M. cajuputi* under −Al treatment changed on addition of Al to the extract (Fig. 6G). Because in *M. cajuputi*, only a small amount of Al accumulated in roots, owing to its Al-exclusion mechanisms, the phenolic profiles might not change between ±Al treatments.

A higher concentration of phenolics (catechin equivalent) was found in the symplastic than in the apoplastic fraction in roots of *M. malabathricum* (Fig. 5). The concentration of symplastic phenolics was sufficient to chelate all symplastic Al in roots (Fig. 5). Phenolics occurred not only in the epidermis but also the stele of roots of *M. malabathricum* (Fig. 7A, B). This phenolic distribution was nearly the same as that of Al in roots of *M. malabathricum* (Watanabe et al. 1998). Taken together, these results suggest that phenolics detoxify Al primarily in epidermis and endodermis by binding to Al.

Melastoma malabathricum has been investigated for pharmacological use because of its abundant content of phenolics (Joffry et al. 2012). Although it has been suggested that phenolics are involved in Al tolerance mechanisms, the roles of phenolics in Al tolerance mechanisms of *M. malabathricum* have not been investigated. *Melastoma malabathricum* is known as a highly Al-tolerant species, but its Al tolerance mechanisms remain to be fully elucidated. Thus, this is the first report suggesting the roles of phenolics in Al tolerance mechanisms in *M. malabathricum*. However, it has been reported that phenolics are also a component of the PM and stabilize its structure. For example, flavonoids could be embedded in the hydrophobic part of the membrane and stabilize PM structure similarly to sterols (Arora et al. 2000, Tsuchiya and Iinuma 2000). Thus, phenolics may also maintain PM permeability, which is usually disrupted by Al. Furthermore, because the basic skeleton of phenolics has the ability to scavenge free radicals, phenolics in PM can function as antioxidants (Torel et al. 1986, Ioku et al. 1995). Tolrà et al. (2005) suggested that Al-induced increase of anthraquinone, a kind of phenolics, in roots of *R. acetosa* contributes to its Al tolerance, because of its strong antioxidant activities. It has often been reported that Al causes oxidative stress in the PM, resulting in lipid peroxidation. Thus, phenolics could ameliorate

oxidative stress caused by Al in the PM. Although membrane permeability and the level of lipid peroxidation were not measured in this experiment, abundant phenolics in *M. malabathricum* could be one of the most important materials involved in its high Al tolerance mechanism.

In this study, root cellular components such as lipid, cell wall and phenolics were investigated in *M. malabathricum* and *M. cajuputi*, both are highly tolerant to Al. The results suggested that constitutive characteristics of lipid and phenolics could be involved in their Al tolerance mechanisms, given that root cellular components did not show dynamic response or drastic change upon Al treatment. These constitutive characteristics in root cellular components may be responsible for Al tolerance mechanisms in other Al-tolerant plant species, including both woody and herbaceous species, the Al tolerance of which cannot be explained by known mechanisms such as organic acid secretion.

Acknowledgements – This work was partly financially supported by a Grant-in-Aid for Scientific Research (266461, 15H04465, 15K14673) from the Japan Society for the Promotion of Science.

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Edited by J. K. Schjørring