Original research article

Exudation of fumarate from roots contributes to high aluminum resistance in *Melaleuca cajuputi*

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Abstract: Two *Melaleuca* species, *M. cajuputi* and *M. bracteata*, were compared to identify the factors determining their distinct aluminum (Al) resistance levels. The presence of Al in a liquid culture medium (maximum tested concentration, 2 mM) did not affect the growth of *M. cajuputi*, but severely inhibited the growth of *M. bracteata*. The Al content in the roots was 50% higher in Al-sensitive *M. bracteata* than in Al-resistant *M. cajuputi*. Al penetration and tissue damage were obvious in the roots of *M. bracteata*, but only mild in the roots of *M. cajuputi*. Relatively high levels of fumarate were released by the roots of *M. cajuputi*, but not by those of *M. bracteata*. Supplementation of Al-containing liquid media with fumarate resulted in a reduction of Al toxicity on *M. bracteata*. These results suggest that Al-resistant *M. cajuputi* releases fumarate from its roots, thereby detoxifying Al.

Keywords: aluminum, fumarate, *Melaleuca cajuputi*, *Melaleuca bracteata*, resistance, sensitivity

Introduction

Plants encounter several growth constraints in acidic soils, such as hydrogen cation (H^+) toxicity, aluminum (Al) toxicity, and manganese toxicity, as well as deficiencies of magnesium, calcium, potassium, phosphorus, and molybdenum (Marschner 1991). Of these, Al toxicity is considered a major growth constraint (Ma 2000; Samac and Tesfaye 2003), and damage to plants under acidic conditions appears to be attributed to Al^{3+} rather than H^+ (Konishi 2002). Al is the most abundant metal in the earth's crust, and because it is insoluble under normal conditions, its toxicity is negligible. However, soluble Al cation (Al^{3+}) is present in acidic soils and inhibits root cell division and membrane function after being absorbed via the roots.

Several species are Al-resistant via a mechanism involving: 1) extracellular detoxification of Al; 2) binding to the cell wall and cellular membrane; and 3) intracellular detoxification (Ezaki 2002). In recent years, the capacity of the roots to release organic acids has been implicated in Al resistance. *Melastoma malabathricum* and *Melaleuca cajuputi* are known to be Al resistant (Watanabe et al. 1998). On the other hand, we previously discovered that *M. bracteata* is much less resistance to Al and demonstrated differences in optimal growth pH (5-6 versus 5-7) and absolute growth pH (4.5-6.5 versus 4.5-8.5) between Al-resistant *M. cajuputi* and Al-sensitive *M. bracteata* (FAO 2007). In this study, we compared the growth response and root exudates of organic acids among *M. cajuputi* and *M. bracteata* after treatment with Al. In addition, we report the presence of fumarate in root exudates of Al-resistant *M. cajuputi*, which to date has not been well studied with regard to Al resistance.

Materials and Methods

Cultivation of M. cajuputi and M. bracteata in Al-containing media and Al absorption

Seedling pre-cultivation

Seeds of *Melaleuca cajuputi* (collected in Narathiwat Province, Thailand) and *M. bracteata* (obtained from the Australian Tree Seed Centre, CSIRO, Australia)

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were placed in moist sand and grown for one month at 25 °C under a 12 h/12 h light/dark cycle. Six seedlings were planted in an expanded polystyrene container (21.0 x 27.3 x 12.6 cm), filled with 6 L of a liquid culture medium (pH 4.5, which is the lowest absolute pH for both species (FAO 2007)), and grown for one month at 30 °C under a 16 h/8 h light/dark cycle with aeration. The culture medium was changed every four days. The composition of the culture medium is shown in Table 1.

Al treatment

$AlCl_3 \cdot 6H_2O$ was added to the liquid culture medium to make final Al concentrations of 0, 0.1, 0.5, 1, and 2 mM (pH 4.5). Six seedlings were then grown in each container for 21 days at 30 °C under a 16 h/8 h light/dark cycle with aeration in the presence of Al, and the culture medium was changed every four days. Fifteen containers (three containers per each Al concentration) were assembled in three rows.

Analysis of root Al content

After Al treatment, roots were washed with pure water, separated from shoots, and dried for 48 h at 70 °C. A predetermined amount of dried roots was wet-digested at 250 °C with 95% sulfuric acid and 30% hydrogen peroxide by using a dry block bath (AL-1000, Scinics Corp., Japan), and the Al content was measured by a flameless atomic absorption method (Z-5010, Hitachi Ltd., Japan was used).

Hematoxylin staining for examining localization of Al in the roots

The basic method was based on Polle et al. (1978). Apical pieces (length, 4 cm) of roots were prepared from the fresh roots of Al-treated *M. cajuputi* and *M. bracteata* and incubated in a 0.2% hematoxylin monohydrate ($C_{16}H_{14}O_6 \cdot H_2O$) solution containing an oxidizing agent (0.02% $NaIO_3$) for 20 min. The surface and cross-sections of stained roots were observed under a stereomicroscope (SZH-ILLB, Olympus Optical Co Ltd., Japan).

Aniline blue staining for examining callose formation in the roots

Apical pieces (length, 4 cm) of roots were similarly prepared and incubated in a commercially available aniline blue solution (aniline blue, 1%) for 20 min based on Yim and Bradford (1998). The surfaces and cross-sections of stained roots were observed under a stereomicroscope (the same as above).

Al-induced release of organic acids by roots of M. cajuputi and M. bracteata

A 0.35 mM $CaCl_2$ solution was prepared because Al toxicity observed in *M. bracteata* might have resulted from Ca deficiency (Tahara et al. 2008), and $AlCl_3 \cdot 6H_2O$ was added to give final Al concentrations of 0, 0.1, 0.5, 1, and 2 mM. A single seedling of *M. cajuputi* or *M. bracteata* was grown in a glass jar filled with a 0.35 mM $CaCl_2$ solution containing one of the five Al concentrations for seven days. The culture media were recovered and purified, based on the method of Tahara et al. (2008), by passing the sample solutions consecutively through a cation-exchange column and an anion-exchange column. The anions retained on the anion-exchange resin were eluted with 9 ml of 2 M HCl. The eluate was concentrated to dryness, and the residue was then redissolved in 200 µl of 10 mM NaOH. Organic acids therein were analysed by high-performance liquid chromatography (HPLC, Agilent1100, Agilent Technologies, Germany) using a Shim-pack SCR-102H column (300 mm × 8 mm; Shimadzu GLC Ltd., Japan) with a Shim-pack SCR-102H (G) guard column (50 mm × 6 mm; the same as above). The following HPLC conditions were used: flow rate, 1 mL min^{-1}; mobile phase, 3 mM perchloric acid; column temperature, 40 °C; sample injection volume, 20 µL; UV detector wavelength, 210 nm.

Effect of fumarate in Al-containing culture medium on the growth of Al-sensitive M. bracteata

The Al-free liquid culture medium (Table 1) and the medium containing 0.1 mM Al were supplemented with 0, 0.6, 1.2, or 120 µM of fumarate. Culture media containing different levels of fumarate were placed into expanded polystyrene containers

Table 1. Composition of liquid culture media

Salt	Concentration (µM)
NH_4NO_3	2000
$NaH_2PO_4 \cdot 2H_2O$	100
KCl	600
$CaCl_2 \cdot 2H_2O$	350
$MgSO_4 \cdot 7H_2O$	250
$FeSO_4 \cdot 7H_2O$	10
H_3BO_3	20
$MnCl_2 \cdot 4H_2O$	3
$ZnSO_4 \cdot 7H_2O$	1
$CuSO_4 \cdot 5H_2O$	0.5
$Na_2MoO_4 \cdot 2H_2O$	0.5

(6L/container, see section "Seedling pre-cultivation"). Six seedlings of *M. bracteata* were grown in each container according to the method described in section "Seedling pre-cultivation", but for only 21 days. The culture medium was changed every three days. Fifteen containers (three containers x five fumarate concentrations) were arranged in three rows. The concentration of fumarate in each culture medium was determined to be 50-1000-fold that in the culture media containing root exudates of *M. cajuputi* (0.012 μM).

Results

Growth response of M. cajuputi and M. bracteata after Al treatment and absorption

Growth

Figure 1 shows the growth response of *M. cajuputi* and *M. bracteata* after Al treatment. Regardless of the concentrations tested, Al treatment did not have a marked impact on the growth of *M. cajuputi*. Rather, a higher number of healthy white roots were observed in the presence of 0.5-1 mM Al than in the absence of Al. On the other hand, the growth of *M. bracteata* was impaired, and the number of leaves was decreased in an Al concentration-dependent manner. *M. bracteata* formed fewer lateral roots than *M. cajuputi*, even under an Al-free condition. The difference in lateral root formation between the two species became more prominent after Al treatment, after which root necrosis was found in *M. bracteata*. These results confirm that *M. cajuputi* is Al resistant while *M. bracteata* is Al sensitive.

Figure 2 shows the change in the dry weight of specimens after Al treatment, which had only a minor effect on the dry weight of *M. cajuputi*. In particular, the dry weight of *M. cajuputi* roots was almost unaltered. On the other hand, even the lowest tested concentration (0.1 mM) of Al reduced the dry weight of both shoots and roots by approximately 50% in *M. bracteata*. This effect became severer as Al concentration increased.

Al content in the roots

Figure 3 shows changes in Al content in the roots of *M. cajuputi* and *M. bracteata*. Al content increased as the Al concentration in the culture medium increased, but the increments became smaller in both species as the Al concentration exceeded 0.5 mM. When the Al concentration in each culture medium was the same, the Al content was approximately 50% higher in *M. bracteata,* the species prone to Al-toxicity, than in *M. cajuputi*.

Localization of Al in the roots

M. cajuputi and *M. bracteata* roots grown in the presence of Al were stained with hematoxylin to reveal Al localization in response to 5 concentrations (Fig. 4). The roots in neither species stained when grown in the absence of Al. Staining intensity

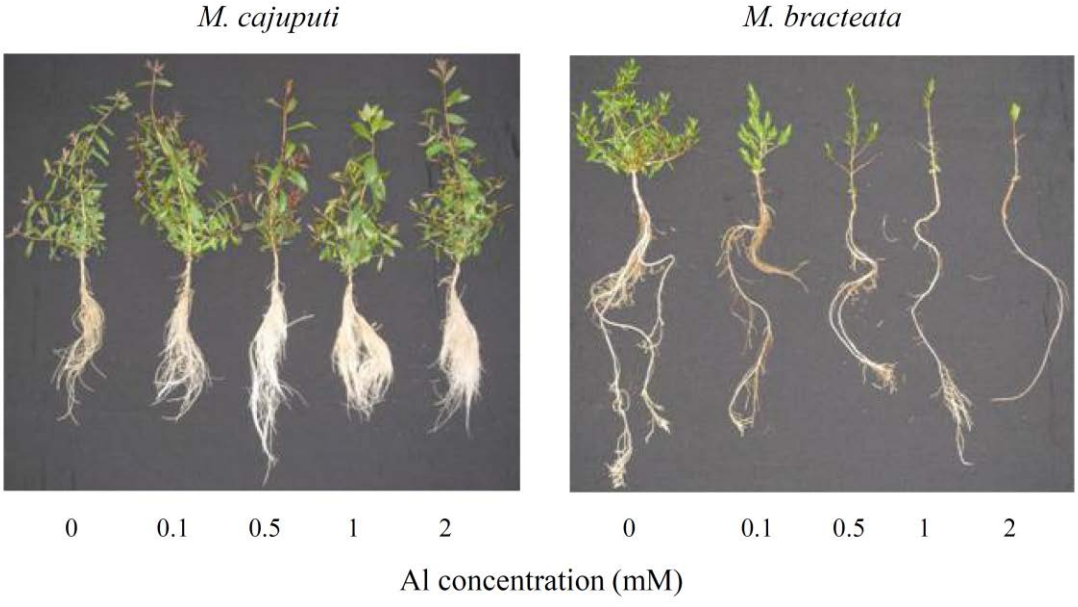

Fig. 1. Growth response of *Melaleuca cajuputi* and *M. bracteata* seedlings after Al treatment.

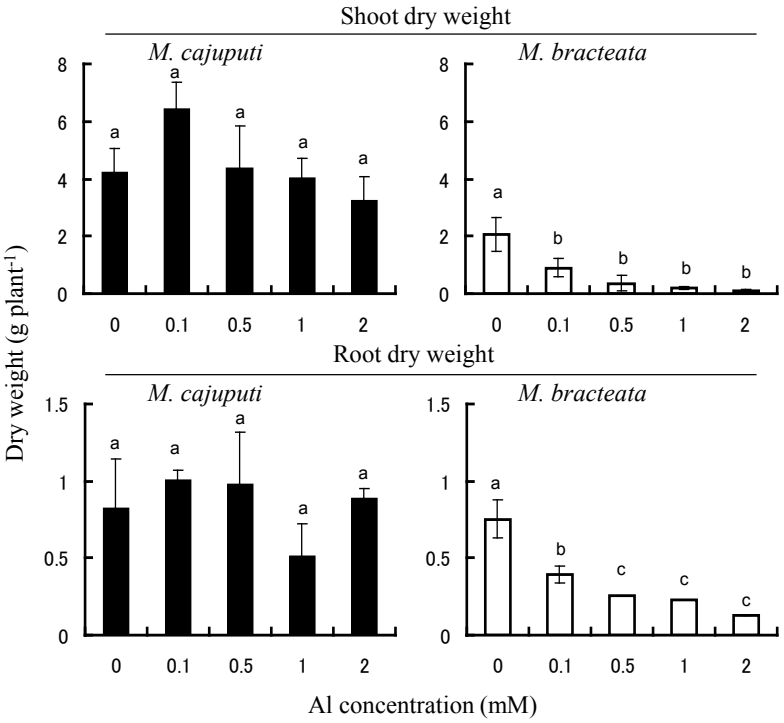

Fig. 2. Effect of Al treatment on the dry weight of *M. cajuputi* and *M. bracteata* seedlings. Values are means ± SE of three replicates. Bars marked with the same letter did not differ significantly at $P < 0.05$ according to LSD test.

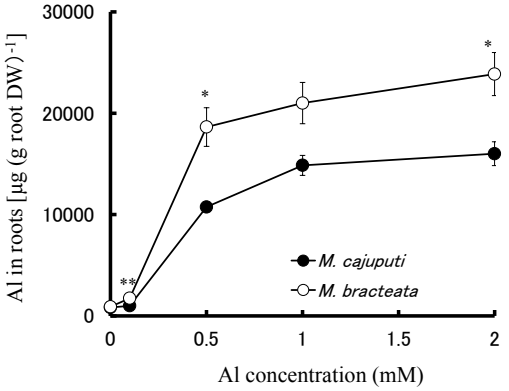

Fig. 3. Effect of Al treatment on Al content in the roots of *M. cajuputi* and *M. bracteata* seedlings. Values are means ± SE of three replicates. Two and single asterisks denote significant differences between species at $P < 0.01$ and 0.05, respectively according to LSD test.

increased in both species as Al concentration in the media incr eased, albeit more prominently in Al-sensitive *M. bracteata*. When root cross sections were examined, staining was found to be restricted to the outer layer and rarely found in the inner area in Al-resistant *M. cajuputi*. On the other hand, strong staining permeated towards the inner area as Al concentration increased in Al-sensitive *M. bracteata*, indicating penetration of a large amount of Al. This penetration became prominent when the Al concentration exceeded 0.5 mM.

Callose formation in the roots

Callose deposition occurs upon plant tissue damage (Chen and Kim 2009). *M. cajuputi* and *M. bracteata* roots grown under different concentrations of Al were stained with aniline blue, and localization and intensity of staining were examined to identify the sites of Al-induced injury (Fig. 5). The roots of neither species stained when grown in the absence of Al, indicating no callose formation under this condition. The surface of roots grown in the Al-containing medium stained blue, and staining intensity was stronger in Al-sensitive *M. bracteata* than in Al-resistant *M. cajuputi*. Root tips stained strongly in *M. bracteata*, and staining intensity increased as Al concentration increased. When Al concentration was increased to 2 mM, the entire root of *M. bracteata* stained, suggesting Al induced extensive tissue damage.

Al-induced release of organic acids by roots of M. cajuputi and M. bracteata

The concentration of organic acids in each liquid culture medium was measured and converted to concentration per gram (dry weight) of the roots (Fig. 6). Malate release was observed in the media containing exudates from roots of neither *M. cajuputi* nor *M. bracteata*. Citrate and fumarate were detected in those containing exudates from *M. cajuputi* roots, while citrate and oxalate were found in those con

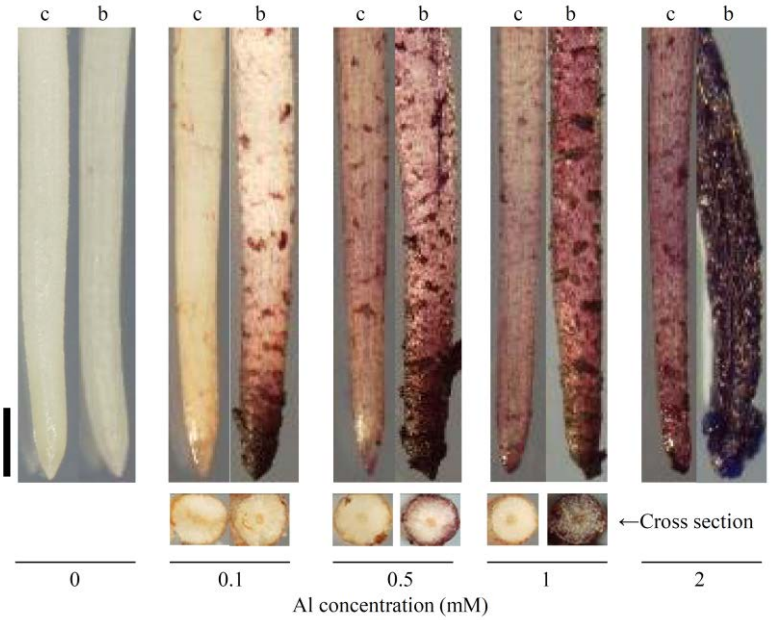

Fig. 4. Localization of Al in the roots of *M. cajuputi* and *M. bracteata* seedlings. The roots of *M. cajuputi* (c) and *M. bracteata* (b) seedlings are stained with hematoxylin. Scale bar = 1 mm.

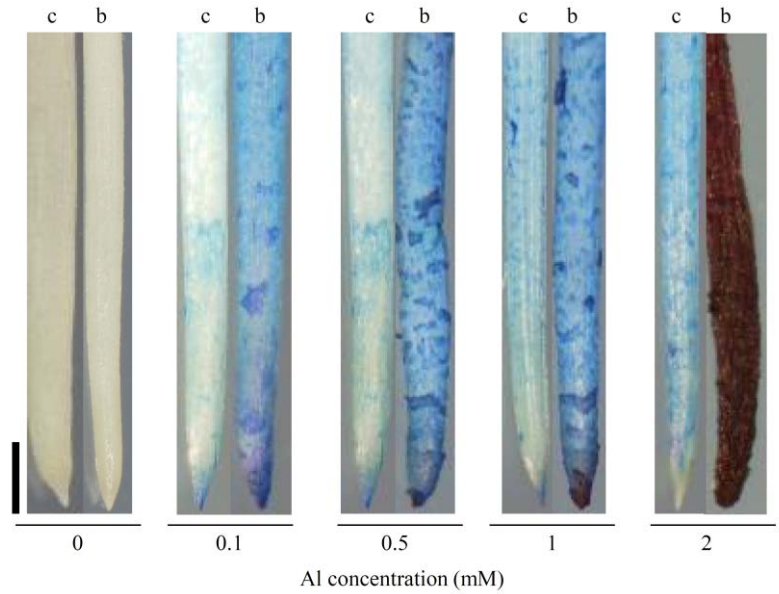

Fig. 5. Sites of callose formation in the roots of *M. cajuputi* and *M. bracteata* seedlings. The roots of *M. cajuputi* (c) and *M. bracteata* (b) are stained with aniline blue. Scale bar = 1 mm.

taining exudates from *M. bracteata* roots. For *M. cajuputi* roots, the secretion volume of citrate increased as the Al concentration increased. In contrast, the secretion volume of fumarate is not dependent on Al concentration, and a relatively large amount of fumarate was released from *M. cajuputi* roots, even in the absence of Al.

M. bracteata roots released citrate and oxalate, albeit in low levels, when the medium contained 0.5 mM Al or higher. The concentration of fumarate was 10-fold higher in root exudates of *M. cajuputi* than in those of *M. bracteata*, suggesting the involvement of fumarate in the Al resistance mechanism.

Effect of fumarate in Al-containing culture medium on the growth of Al-sensitive M. bracteata

Addition of fumarate (up to 120 μM) to Al-free media did not alter the growth response of *M. bracteata*. In particular, change in root weight was negligible. The presence of 0.1 mM Al in the culture medium reduced the dry weight of both shoots and roots by 50% (Fig. 7), in good agreement with the results shown in Fig. 2. The dry weight of roots grown in Al-containing media which were supplemented with 120 μM of fumarate became significantly larger than those grown in them without fumarate. There was similar tendency on the soot dry weight. These results indicate that supplementing the culture medium with fumarate reduces Al toxicity in Al-sensitive *M. bracteata*.

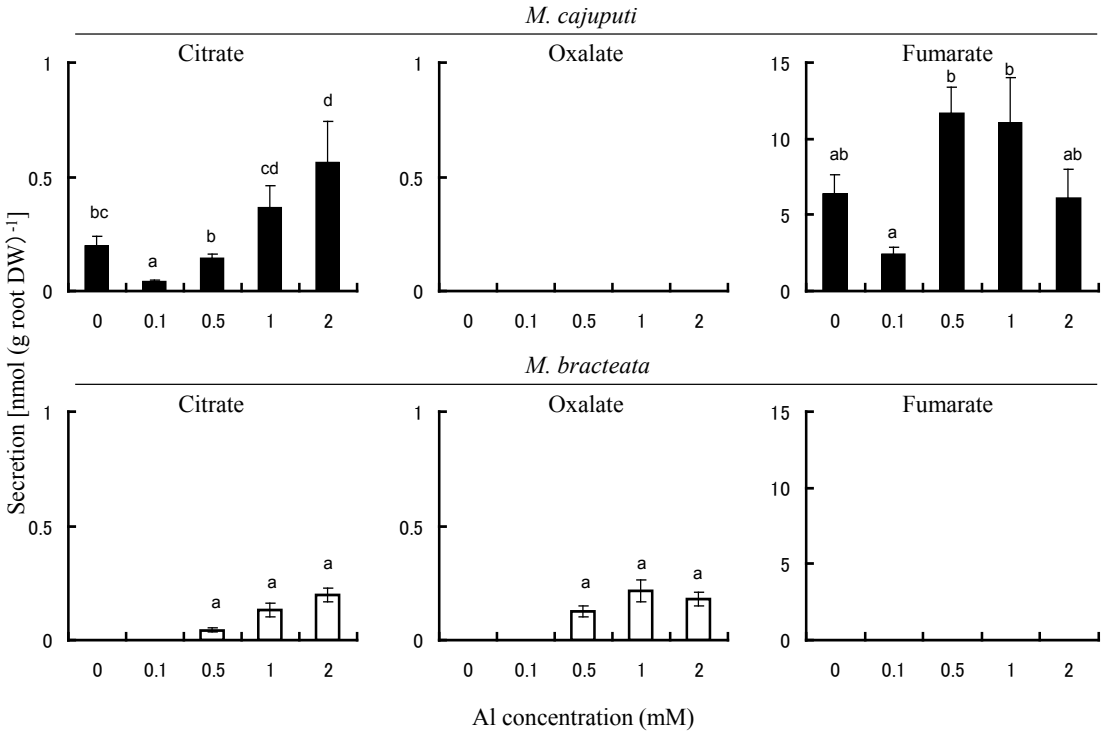

Fig. 6. Al-induced organic acid release from the roots of *M. cajuputi* and *M. bracteata* seedlings. Values are means ± SE of three replicates. Bars marked with the same letter did not differ significantly at $P < 0.05$ according to LSD test.

Discussion

Response to stress, such as excess Al toxicity, varies across closely related plant species and even between those in the same genus. Differences in the capability of roots to release organic acids have been implicated in the differences in response to excess Al. For example, the levels of Al resistance vary among isogenic wheat lines, depending on the level of malate released in response to Al stress (Delhaize et al. 1993). Pellet et al. (1995) showed that the release of citrate by the root apex is induced by exposure to Al in Al-resistant but not in Al-sensitive maize genotypes, indicating the important role of organic acid release by the root apex for surviving Al stress. Further, Mimmo et al. (2009) suggested that *Phaseolus lunatus* L. externally detoxifies Al via a mechanism involving oxalate release from roots, while *P. vulgaris* L var. Red Kidney internally detoxifies Al via a mechanism involving fumarate release.

M. cajuputi has been well studied, whereas *M. bracteata* remains poorly understood. According to the Food and Agriculture Organization of the United Nations Ecocrop database (FAO 2007), both species grow in the northern region of Australia, but *M. cajuputi* inhabits lower latitudes and elevations than *M. bracteata*. The optimal and absolute soil pH ranges for *M. cajuputi* are 5-6 and 4.5-6.5, respectively, while the optimal and absolute pH ranges for *M. bracteata* are 5-7 and 4.5-8.5, respectively. *M. cajuputi* is the dominant species in low-pH soils (Osaki et al. 1998) and is strongly Al resistant (Watanabe and Osaki 2001). It also grows well under flooded conditions (Yamanoshita et al. 1999) and seems to have a mechanism that enables it to survive with low oxygen levels in the rhizosphere (Yamanoshita et al. 2001). Meanwhile, *M. bracteata* has been shown to be salt resistant (Dunn et al. 1994), and this may be explained by its high absolute growth pH (high-end pH, 8.5).

We found that the response to Al exposure differed between *M. cajuputi* and *M. bracteata* (Figs. 1 and 2), and the addition of up to 2 mM Al to the culture medium did not inhibit the growth of *M. cajuputi*. Furthermore, there was no visible damage induced by excess Al, and the amount of healthy white roots was actually increased by a small amount of Al in the medium. Several studies have also reported growth enhancement by Al, which is considered toxic. For example, an increase in the amount of healthy roots was found in tea plants in the presence of Al, and Al-induced activation of antioxidants was implicated in this change of growth (Ghanati et al. 2005). Watanabe and Osaki (2001) demonstrated that Al enhanced the growth of *Melastoma malabathricum* L.,

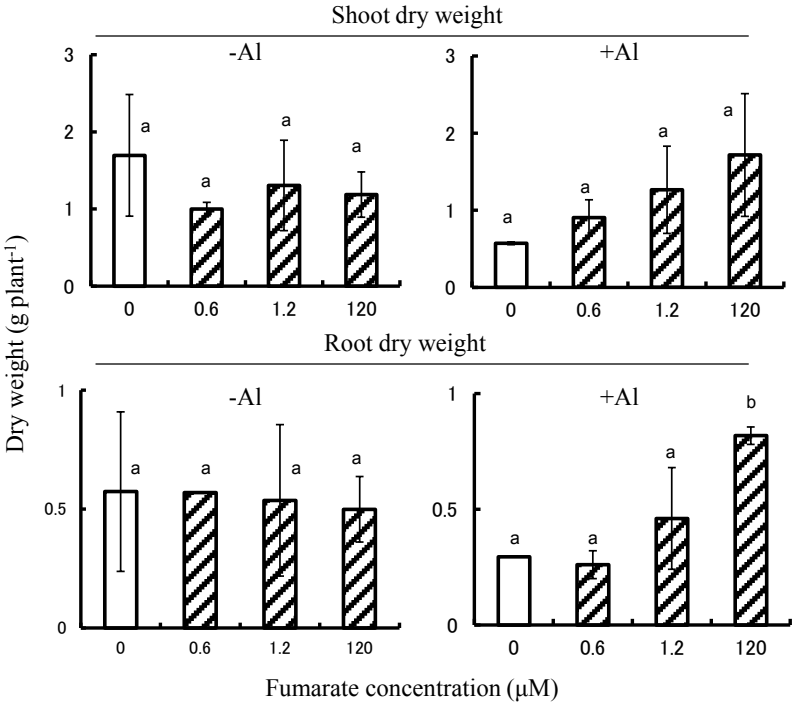

Fig. 7. Changes in the growth response of Al-sensitive *M. bracteata* seedlings grown in the Al-containing culture medium supplemented with fumarate. Values are means ± SE of three replicates. Bars marked with the same letter did not differ significantly at $P < 0.05$ according to LSD test.

indicating the beneficial effect of Al on growth of this species. *M. cajuputi*, as well as tea plants and *Melastoma malabathricum* L., grows in acidic soils, and such species might adopt mechanisms wherein Al is utilized for their growth. In contrast, the growth of *M. bracteata* was inhibited by Al in the culture medium at the lowest tested concentration (0.1 mM), resulting in a 50% reduction in dry weight of shoots and roots. The inhibitory effect of Al was elevated in a concentration-dependent manner, and root necrosis and loss of leaves were observed in *M. bracteata* grown in the presence of a high Al concentration.

When grown under the same condition, the Al content in the roots of Al-sensitive *M. bracteata* was approximately 50% higher than that of Al-resistant *M. cajuputi* (Fig. 3). In addition, compared with *M. cajuputi*, *M. bracteata* showed stronger hematoxylin staining on root surfaces in the presence of Al in the medium and also in the inner tissues of the roots as the Al concentration increased (Fig. 4). These results suggest that levels of adsorption and penetration of Al into the roots are markedly higher in *M. bracteata* than in *M. cajuputi*. Similarly, callose formation was severer in *M. bracteata* than in *M. cajuputi* (Fig. 5), and the level of damage caused by the same amount of Al in the roots was markedly greater in *M. bracteata* than in *M. cajuputi*. Tahara et al. (2005) also showed Al-induced callose formation can be used as an interspecific indicator of Al sensitivity according to their research with nine Myrtaceae species including *M. cajuputi* and *M. bracteata*. In that study, *M. bracteata* induced the largest amount of callose among the nine species. Taken together, the roots of *M. bracteata* are poorly protected from Al penetration, while *M. cajuputi* appears to have a mechanism that limits Al from penetrating the roots as well as a mechanism that detoxifies the Al that penetrates the roots.

Here we identified the type and level of organic acids in root exudates of two species to determine the factor that causes such differences in response to Al. Because malate release was detected from roots of neither *M. cajuputi* nor *M. bracteata*, it was excluded from a target organic acid to discuss. We found that Al-sensitive *M. bracteata* released citrate and oxalate, albeit at low levels, while Al-resistant *M. cajuputi* released a small quantity of citrate and a large quantity of fumarate (Fig. 6). The level of fumarate released by *M. cajuputi* was more than 20-fold that of citrate released by the same species and 20-fold that of citrate and oxalate released by *M. bracteata*. The level of fumarate released by *M. cajuputi* was high even when seedlings were grown in an Al-free medium.

Release of organic acids such as citrate, oxalate,

and malate from roots has been implicated in Al resistance in plants. For example, Rangel et al. (2010) reported that Al-resistant *Phaseolus vulgaris* L. maintains high citrate synthesis activity and restores the internal citrate pool. Al-induced oxalate release from the root was found in taro (Ma and Miyasaka 1998) and buckwheat (Ma et al. 1998; Zheng et al. 1998) and an Al detoxifying mechanism via formation of Al-oxalate complexes was indicated by Ma et al. (1998). Furthermore, Ryan et al. (1995) reported a significant correlation between the levels of Al resistance and the amounts of Al-induced malate release from root apices in wheat.

On the other hand, fumarate has rarely been discussed in the context of Al resistance in plants. Mimmo et al. (2009) examined *Phaseolus* species with different Al absorbency and found that fumarate is involved in the internal detoxification of Al in *P. vulgaris* L., which showed relatively high Al absorbency, while oxalate inhibits the absorption of Al in *P. lunatus* L., resulting in low levels of Al absorption in this species.

Although citrate was released from both the roots of *M. cajuputi* and *M. bracteata* and its concentration was depend on that of Al in the culture solution, *M. bracteata* had no Al-tolerance. We found fumarate release only in the root exudates of *M. cajuputi*, which is an Al-resistant and low Al-uptake species, and the concentration is more than 20-fold of citrate. Furthermore, supplementing the Al-containing medium with fumarate impaired Al-induced growth inhibition in Al-sensitive *M. bracteata*, the species incapable of releasing fumarate. These findings suggest an important role for fumarate in the acquisition of Al resistance in *Melaleuca*. Two mechanisms are proposed for the detoxification of Al by organic acids released from roots: one is via formation of chelate with Al in the rhizosphere, and the other is via internal processes involving Al complex formation (Ma et al. 2001). It is likely that *M. cajuputi* detoxifies Al by constitutive release of fumarate from its roots, thereby inhibiting Al absorption. We also found that when roots of these two species contained the same amount of Al, the level of growth inhibition was greater in *M. bracteata* than in *M. cajuputi*, suggesting that fumarate may have been involved in the internal detoxification process as well. Although our findings indicate a dual role of fumarate by forming complexes with Al internally and in the rhizosphere, the level of fumarate used for supplementation of the Al-containing medium was much higher than that detected in the culture media containing root exudates. Thus, further study is necessary on the details underlying the detoxifying mechanism and the ratio of Al to fumarate in complexes.

Acknowledgements

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