HAYATI Journal of Biosciences March 2012
Vol. 19 No. 1, p 37-43
EISSN: 2086-4094

Available online at:
http://journal.ipb.ac.id/index.php/hayati
DOI: 10.4308/hjb.19.1.

# Physiological and Biochemical Responses to Aluminum Stress in the Root of a Biodiesel Plant *Jatropha curcas* L.

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**Received October 27, 2011/Accepted March 26, 2012**

**We investigated *J. curcas* responses to aluminum stress, histochemically and biochemically. Histochemical stainings were observed to analysis aluminum accumulation, lipid peroxidation and the loss of plasma membrane integrity on the surface and tissue of the root apex. Enzymatic analysis was conducted to measure malate content in leaf, root and malate efflux in the medium. We used *M. malabathricum* as a comparison for Al-tolerance plant. *J. curcas* root elongation was inhibited by 0.4 mM $AlCl_3$, while *M. malabathricum* root elongation was inhibited by 0.8 mM $AlCl_3$ treatment. Inhibition of root elongation has high correlation with Al accumulation in the root apex, which caused lipid degradation and cell death. Generally, malate content in *J. curcas* leaf and root was higher than that in *M. malabathricum*. In the contrary malate efflux from the root into the medium was lower. *J. curcas* root has a different pattern compared to *M. malabathricum* in malate synthesis and malate secretion when treated with a different Al concentration. We categorized *J. curcas* acc IP3 as more sensitive to aluminum than *M. malabathricum*.**

Key words: *Jatropha curcas*, aluminum stress, malate content

## INTRODUCTION

Due to severe energy crisis and escalation of petroleum price, alternate energy sources are gaining importance (Ragauskas *et al*. 2006). One alternative energy source available is *J. curcas* seed oil. *J. curcas* is easy to propagate, has a high content oil seed, a short gestation period, rapid growth, and can be adopted into a wide variety of agro-climatic condition (Divacara *et al*. 2010). Nevertheless, *J. curcas*should not be cultured in large scale fertile soil. These lands should be allocated for more valuable food-crops. The alternative area for *J. curcas* plantation is the marginal areas, for examples some part of Sumatra Island and Kalimantan Island, which have acidic soil with Al toxicity as a major problem.

Al toxicity mostly affects root growth inhibition of Al-treated plants (Nagy *et al*. 2004), and also reduces root/shoot biomass (Poschenrieder *et al*. 2008), and in the long term, of course, it will affect crop yield. *Pisum sativum* treated with aluminum show various responses to Al stress such as root growth inhibition, Al accumulation, lipid peroxidation, and loss plasma membrane integrity (Yamamoto *et al*. 2001). Reactive oxygen species (ROS) appears to be the primary factors that cause growth inhibition in Al-stressed roots (Yin *et al*. 2010).

Several methods have been developed to identify mechanism of aluminum toxicity in root. The staining of Al-stressed root apices using hematoxylin appears to be a powerful tool to assist in Al-tolerance selection in plant breeding programs (Cançado *et al*. 1999). Localization of Al in the root could be rapidly observed using whole root or anatomy section (Yamamoto *et al*. 2001; Tamas *et al*. 2006). The lipid peroxidation is a relatively early symptom induced by the accumulation of aluminum (Yamamoto *et al*. 2001), and it increases with increasing Al concentration (Achary *et al*. 2008). Peroxidation of lipid can be detected by Schiff's reagent staining (Yamamoto *et al*. 2001), which detect peroxide-derived aldehydes, formed downstream of reactive oxygen species (Yin *et al*. 2010). The late biological symptom of Al toxicity is the loss of plasma membrane integrity which causes the inhibition of root elongation (Yamamoto *et al*. 2001). The loss of plasma membrane integrity affect the viability of cells which could be determined by Evan's blue staining (Tamas *et al*. 2006).

Organic acids with Al-chelating ability play a role in detoxification, externally and internally (Ma *et al*. 2000). Exudation of organic acid such as citrate, malate and oxalate into the rhizosphere has been proposed as tolerance mechanism to avoid Al toxicity in many plants

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(Ryan *et al*. 1995). Al stimulates citrate, malate, and succinate efflux from intact roots, but citrate release exhibited greatest Al-induced respond (Osawa & Kojima 2006). The organic acid in rhizosphere may chelate Al outside the membrane plasma, thereby preventing its uptake (Miyasaka *et al*. 1991). In vitro, organic acids do indeed decrease Al toxicity; citrate is more effective than succinate and malate (Hue *et al*. 1986). Tolerance varieties of *Oriza sativa,* have higher citrate synthesis than in those of sensitive varieties (Kang & Ishii 2006). Citrate chelates Al and makes an Al-citrate complex that is not phytotoxic (Ma 2000). Also, citrate has been shown previously to enhance the availability of phosphorus (P) from insoluble Al phosphates (Miyasaka *et al*. 1991). In wheat (*Triticum aestivum* L.), Al-tolerance seedling secreted malate four times higher than Al-sensitive seedling after a 2-h exposure to 200 µM Al (Delhaize *et al*. 1993).

Overexpression of enzymes involved in organic acid metabolism citrate synthase in transgenic tobacco, *Arabidopsis*, and alfalfa (De la Fuente *et al*. 1997), and *Daucus carota* L. (Koyama *et al*. 1999) can, in some cases, result in increased root organic acid content and exudation, as well as enhanced Al tolerance. Expression of *ALMT1*, a malate transporter that is associated with malate efflux and Al tolerance in Al- sensitive barley can increased malate efflux with properties similar to those of Al-tolerance wheat (Delhaiez *et al*. 2004).

The response of *J. curcas* to aluminum stress is not yet observed in detail. An understanding of Al toxicity and mechanism of tolerance is important to develop future breeding and cultivation protocol in *J. curcas*. The objective of this study is to confirm physiological and biochemical characteristic of *J. curcas* under Al stress condition.

## MATERIALS AND METHODS

*Jatropha curcas* variety IP-3 was used for our experiment. Seeds were submerged with tap water for 1 hour and then sterilized with Na hypochlorite solution (1 ml Na hypochlorite, 10 ml Tween-20, and 20 ml water) for 10 minutes. After rinsed with sterile water, the seeds were grown in isolite for 10 days. High vigour of seedlings was selected for Al treatment in hydroponic culture containing half strength of Murashige and Skoog (MS) medium (1962). An Al-tolerant species, *Melastoma malabathricum,* was used as a comparison plant. *M. malabathricum* seedlings were grown in the soil for 1 month, and transferred to hydroponic medium pH 5.8 for a week before treated with different Al concentration.

**Root Growth Inhibition.** The ten-day old *J. curcas* seedling were treated with different concentrations (0, 0.2, 0.4, and 0.8 mM $AlCl_3$) in the half strength of MS medium, and adjusted at pH 4 for 7 days. Each treatment consisted of five seedlings with four replicates. The media were renewed with fresh media on the fourth day. All lengths of the primer roots were measured with a ruler before treatment and after treatment.

**Al Accumulation.** To determine histological and physiological aspect, we used two Al-treatments with and without 0.8 mM $AlCl_3$ for 7 days in hydroponic culture. The longest root of a seedling was rinsed extensively in distilled water for 1 hour, and submerged in a solution consisting of hematoxylin staining [0.2% w/v hematoxylin in 0.02% (w/v) $KIO_3$] for 10 minutes. The roots were then washed with repeated changes of distilled water for 1 hour, and evaluated for the degree of hematoxylin staining in the root tips. The root tips staining pattern were photographed with a photomicroscope (Tamas *et al*. 2006). The transversal sections of the first cm of the root apex were prepared with free hand section.

**Injury of Plasma Membrane.** Injury of plasma membrane by several reactive oxygen species were detected using Schiff's reagent (Yamamoto *et al*. 2001). The roots were submerged in Schiff's reagent for 10 min, and rinsed with 0.5% (w/v) $K_2S_2O_5$ in 0.05 M HCl. The stained roots were kept in sulphite solution to retain the staining color. The stained roots were observed under a light microscope.

**The Localization of the Loss of Plasma Membrane Integrity.** The roots were submerged in 10 ml of Evan's Blue solution [0.025% (w/v) Evans blue in 100 mM $CaCl_2$, pH 5.6] for 10 min. The stained roots were then washed three times with 200 ml of 100 mM $CaCl_2$ (pH 5.6). The root tips were observed under a light microscope.

**Organic Acid Content in the Root, Leaves and Media.** Organic acid extraction followed the method described by Delhaize *et al*. (1993). Roots and fully opened leaves were washed gently to remove salt and blotted dry with paper towels. About 0.3-0.5 g of the first cm of root tips and leaf were collected from 10 mm length root tips, frozen in liquid nitrogen, and stored at -80 $^{o}$C until extracted. The samples were grounded by mortar and pestle in the presence of liquid nitrogen, and added 1 ml of 0.6 N perchloric acid and then grounded further. The extract was transferred to a 1.5 ml plastic tube, centrifuged at 15,000 g for 5 minutes, then 0.9 ml of the supernatant was collected to a new plastic tube. The supernatant was neutralized by adding 80 ml of $K_2CO_3$ (69 g/100 ml). The neutralized solution was centrifuged at 15,000 rpm for 5 minutes. For malic acid assay, 0.25 ml of the sample was incubated with 0.75 ml of buffer (0.5 M glycine, 0.4 M hydrazine, pH 9.0), and 0.05 ml of 40 mM NAD. The reaction mixture was pre-incubated for 30 minutes to obtain a stable $A_{340}$ reading before the addition of 5 µl of malate dehydrogenase. The increase in $A_{340}$ was equal with the production of NADH and directly proportional with amount of malic acid content in the sample (Delhaize *et al*. 1993).

## RESULTS

**Root Growth Inhibition.** After the 7$^{th}$ day cultured in nutrient containing differential Al level, the growth of primary roots were reduced. At the level Al was 0.2 mM, the root growth were not significantly inhibited compared

to roots without Al treatment, but when the Al level was increased to 0.4 and 0.8 mM Al, *J. curcas* showed 36.17 and 48.02% inhibition of root elongation, respectively (Figure 1a). The negative effect of Al did not occur in the Al tolerance plant, *M. malabathricum*. Al treatment of 0.4 mM had a positive effect on *M. malabathricum* root growth while adding 0.8 mM of Al also did not reduced its root elongation (Figure 1b).

**Al Accumulation.** By using hematoxylin staining, Al accumulation was detected in the *J. curcas* roots. The Al accumulation was intensively localized in the first cm of the root tip; where the upper region of the first cm of the root tip saw a significant decrease in Al accumulation (Figure 2a). Hematoxylin staining of transversal section at the first cm of the root apex was intensive in almost all of the root tissue; the epiderm and the cortical cell wall were penetrated as well as the endoderm, xylem and phloem (Figure 2d). In the *M. malabathricum* root tip treated with 0.8 mM Al, the hematoxylin staining was colorless for the entire root surface (Figure 2g).

**Injury of Plasma Membrane.** After grown in the nutrient solution containing 0.8 mM Al for seven days and then stained with Schiff's reagent, the *J. curcas* root was clearly pink and homogenous in the first cm root apex, which represented aluminum distribution (Figure 2b). Transversal section view of the root treated with Al also showed a clear pink color for the entire root tissue, mainly around the epiderm and cortext (Figure 2e). Schiff's reagent staining didn't show in *M. malabathricum* root apex (Figure 2h).

**The Localization of the Loss of Plasma Membrane Integrity.** Blue dye around the *J. curcas* root apex showed that extensive loss plasma membrane integrity was induced by Al treatment (Figure 2c). Evans blue staining was intensively obtained at the first cm of the root tip surface and by the transverse section, the infiltration of the Evan Blue dye occured in all parts of the root tissue (Figure 2f). There was no blue color in the root apex surface of *M. malabathricum* after treated with 0.8 mM Al for 7 days (Figure 2j).

**Organic Acid Content in the Root, Leaves and Media.** Malate content in the *J. curcas* leaf without aluminum stress was fifteen times higher than that of *M. malabatrichum*, and the content was 61.7 μmol $g^{-1}$ FW (24.6%) down by increasing Al concentration. Without Al stress, the malate content in *M. malabathricum* leaf was 16.74 μmol $g^{-1}$ FW and decreased by 46.44% for 0.2 mM of Al stress. It was stable when Al stress was increased (Figure 3a,b).

Without Al stress, malate content in *J. curcas* root was 24.56 μmol $g^{-1}$ FW, or 2.78 times higher than that of *M. malabathricum* root. The respond of root apex to Al stress in *J. curcas* and *M. malabathricum* was quite different, where Al-induced malate in the *J. curcas* root was significantly increased (66.01 μmol $g^{-1}$ FW) in 0.8 mM Al stress for 7 days, while in the *M. malabatrichum* root, the Al-induced malate reached peak (19.6 μmol $g^{-1}$ FW) at 0.4 mM Al treatment, and tended to decrease in 0.8 mM Al stress (Figure 3c,d).

Both *J. curcas* and *M. malabathricum* secreted malate to the medium without Al stress, each 0.102 and 0.174 μmol $g^{-1}$ FW, respectively. The peak of malate exudation in *J. curcas* root occurred at 0.4 mM AlCl3 treatment (0.357 μmol $g^{-1}$ FW) and then significantly decreased under 0.8 mM $AlCl_3$ treatment. In *M. malabathricum* root, the highest number of malate exudation occurred at 0.8 mM $AlCl_3$ treatment (0.466 μmol $g^{-1}$ FW) (Figure 3e,f).

## DISCUSSION

Aluminum inhibits *J. curcas* root elongation in a relative high concentration, more than 0.2 mM. In other sensitive species, such as *Triticum aestivum* variety Scout 66 (Ma *et al.* 2004; *Leucaena leucocephala* (Osawa & Kojima 2006), exposure to 10 mM Al, significantly inhibited

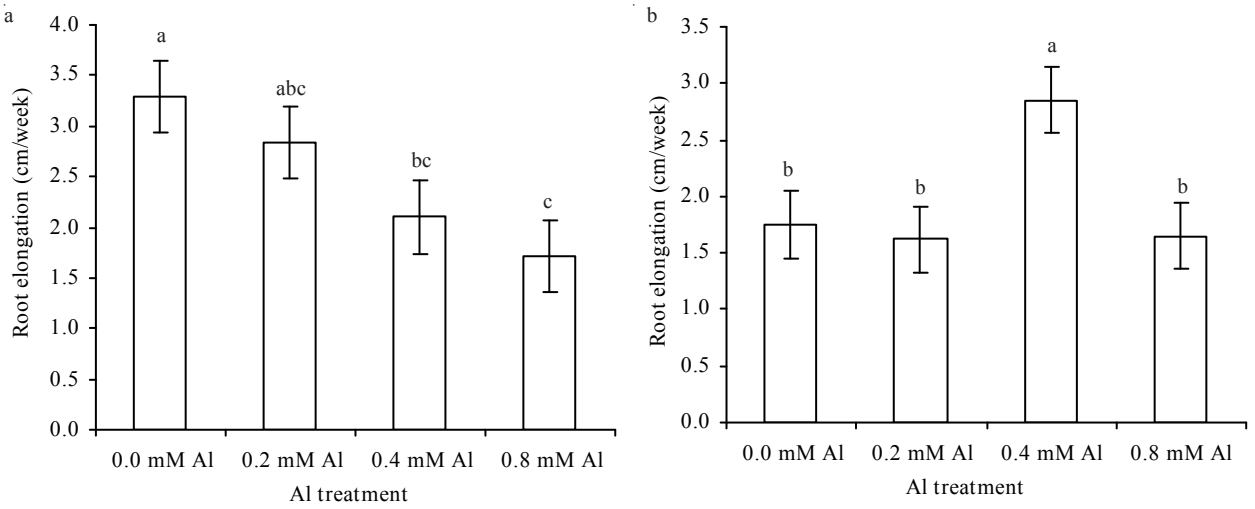


Figure 1. Effect of Al concentration on root elongation of *J.curcas* (a) and *M. malabathricum* (b) after growed on the nutrient culture for 7 days in 250 ml of nutrient solution that contained various concentration of Al. The error bars denote the SE values of the mean of ten seedlings.

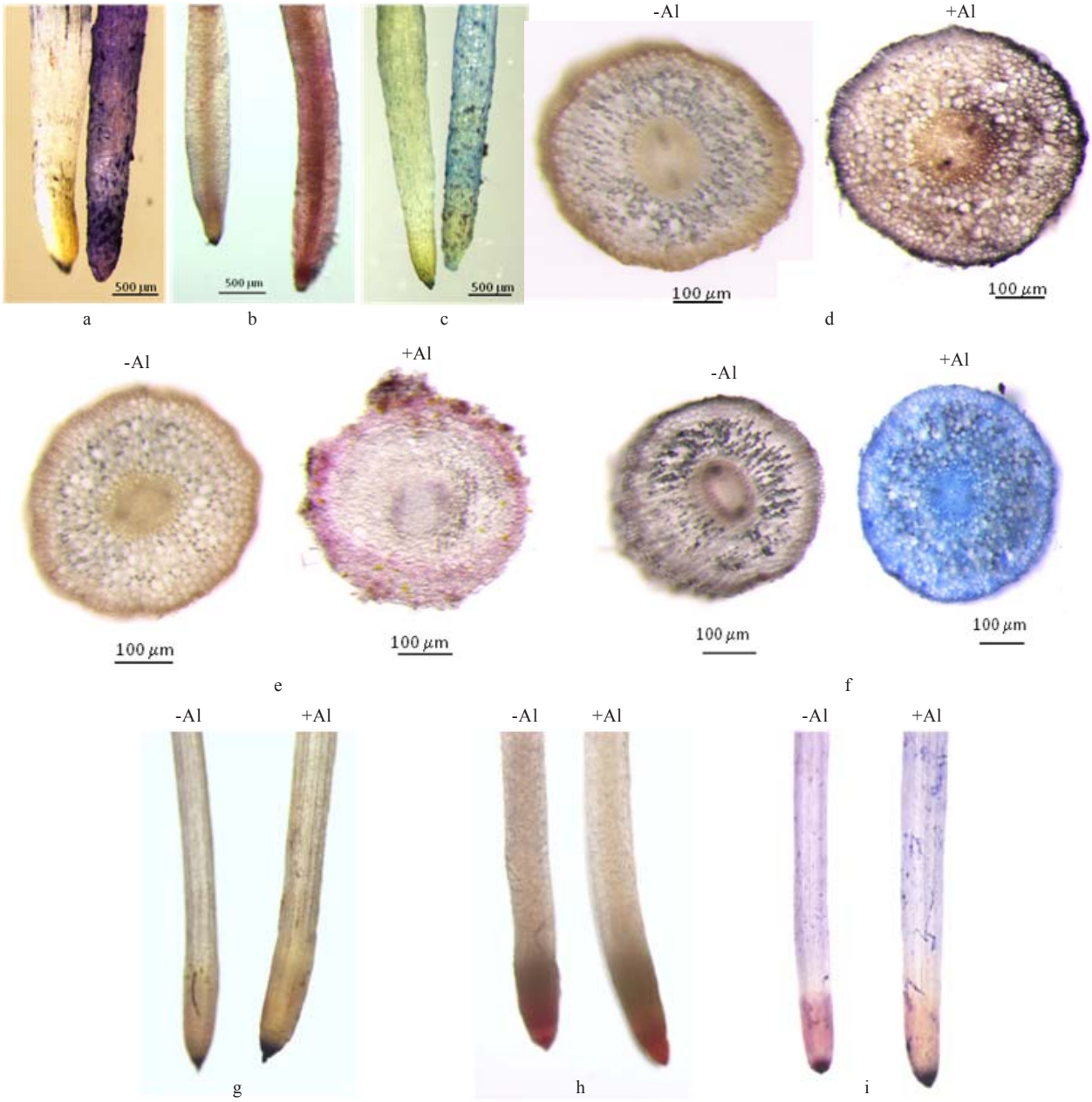


Figure 2. Comparing of root staining between *J. curcas* roots with *M. malabathricum* under with and without Al stress. a. *J. curcas* root was stained with hematoxylin, b. Schiff's Reagent, c. Evan's Blue, d. transversal section of the roots stained with hematoxylin, e. Schiff's Reagent, f. Evan's Blue. As a comparison plant, g. *M. malabathricum* roots were also stained hematoxylin, h. Schiff's Reagent, i. Evan's Blue.

(about 40% inhibition) the root elongation. Some paper reported to only use Al concentration less than 0.2 mM to categorize whether the plants were categorized as Al sensitive or tolerant (Silva *et al*. 2010). In Al-tolerance *Picea abies*, the root growth decreased about 40% after treated with 0.5 mM Al for 2 days (Nagy *et al*. 2004), while in several *Eucaliptus* sp., root inhibition occured only at concentrations larger than 0.6 mM Al (Silva *et al*. 2004). In several soybean genotypes evaluated under 0.7 mM Al stress, root inhibition of the sensitive genotypes and tolerant genotypes were 46.5 and 19.6%, respectively (Sopandie *et al*. 2003). Referring to this experiment; the *J. curcas* IP3 was more sensitive to Al than *M. malabathricum*.

The root elongation inhibition had high correlation with Al concentration in the *J. curcas* root tips, as had been reported in *Pisum sativum* (Yamamoto *et al*. 2001), *Picea abies* (Nagy *et al*. 2004), and barley (Tamas *et al*. 2006). Accumulation of aluminum in *J. curcas* root occurred in the first cm of root apex, but did not occur in *M. malabathricum*. This meant that there was not a mechanism of Al transport to the leaf of *J. curcas*. *M. malabathricum* can accumulate Al into the leaf by Al-citrate binding in the xylem (Watanabe & Osaki 2002). *M. malabathricum* also secreted high density of mucilage which can chelate aluminum (Watanabe *et al*. 2008). The Al localization in the root's first cm was relatively homogenous, with no visually significant differences in the Al content. Pineros *et al.* (2005) also reported that the Al content among the three discrete root sections analyzed (0-0.25, 0.25-0.5, and 0.5-1 cm) had no significant differences.

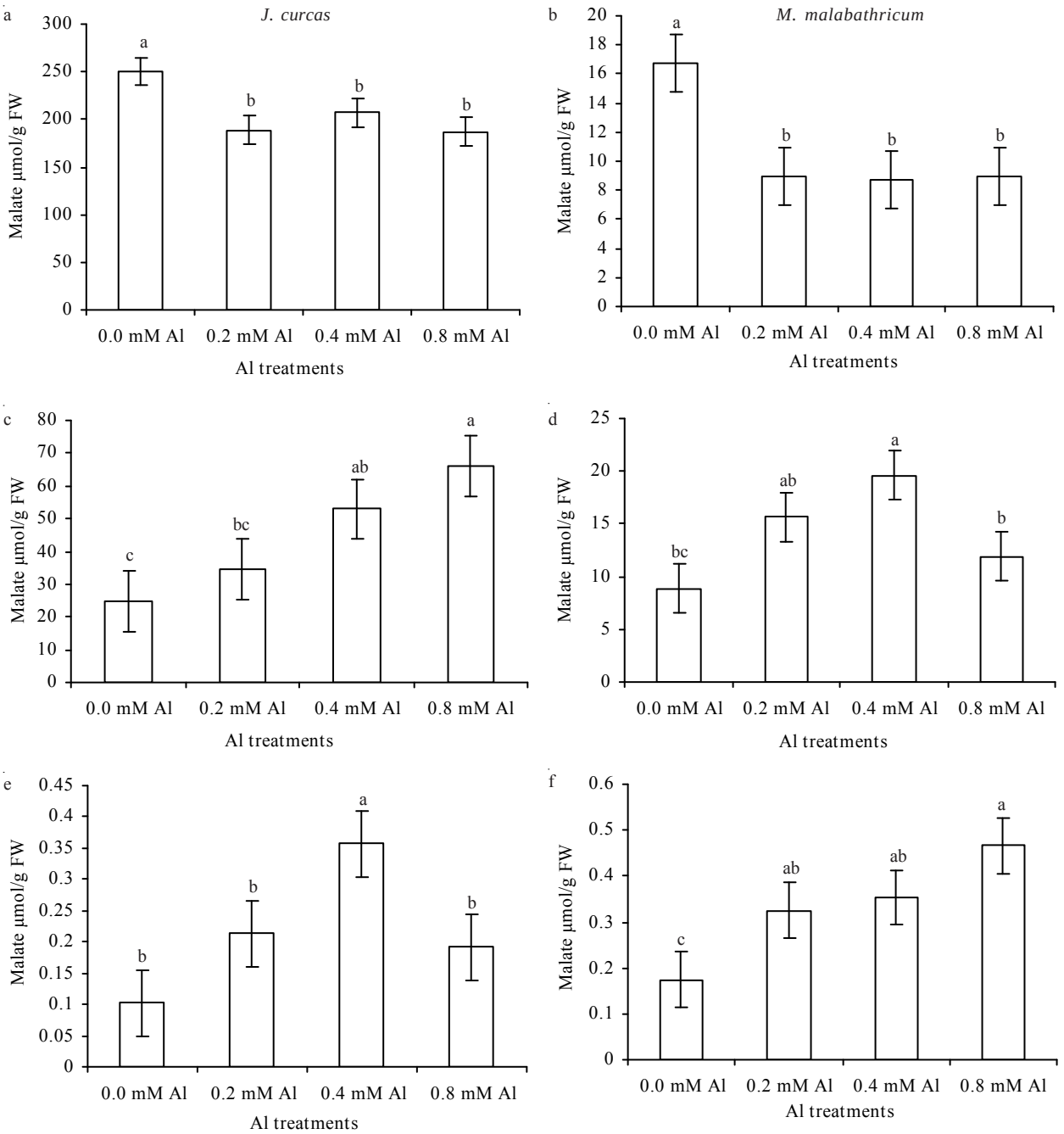


Figure 3. Malate concentration of *J. curcas* and *M. malabathricum* in leaf (a, b), root (c, d), and media (e, f) treated with different concentration of $AlCl_3$ (pH 4) for 7 days. The error bars denote the SE values of the mean of three seedlings.

Peroxidation lipid in plant tissues can be visualized with Schiff's reagent by the development of pink dye (Yamamoto *et al*. 2001; Yin *et al*. 2010). By using Schiff's reagent, the lipid peroxidation was known to spread homogenously in all root apex. In *J.curcas*, the seven day treatment with 0.8 mM Al has caused degradation of plasma membrane lipid throughout the root apex tissue, mainly in the epidermis and cortex. The type of membrane damage is a typical symptom under oxidative stress (Yin *et al*. 2010). Al triggered reactive oxygen species (Yamamoto *et al*. 2002; Jones *et al*. 2006), which induced peroxidation of membrane lipid and symplastic space of the cells (Yamamoto *et al*. 2001).

Degradation of plasma membrane, especially in the lipid structure, caused disturbing results in membrane permeability, so a specific dye for cell viability, Evan's blue can cross the membrane into the cytoplasm. Aluminum induced cell death is indicated by extensive blue dye found in all parts of the root apex tissue (Yamamoto *et al*. 2001). Al accumulation on the outer plasma membrane affects mitochondrial functions by an unknown signal transduction pathway or Al transported across the plasma membrane directly interfere with the mitochondrial functions (Yamamoto *et al*. 2002).

Aluminum stress affected organic acid metabolism in plant tissue (Ma *et al*. 2001). The malate content both in the *J. curcas* or *M. malabathricum* leaf was affected by Al treatments. A decrease of malate content in the leaf up to 60% has been reported for *M. malabathricum* (Watanabe & Osaki 2001). In the root, the influence of Al stress on the increase of malate content in both plant species was significant. The ability of organic acids to chelate and render Al is well established, and it has been speculated for some time that Al-tolerant plant use organic acids to detoxify Al either internally or in the rhizosphere (Delhaize *et al*. 1993).

Ryan *et al*. (2001) emphasized on organic acid exudation as an important factor in the Al-tolerance mechanism. The malate content in *J. curcas* root reached peak when treated with 0.8 mM Al, while malate exudation in *J. curcas* reached the peak in the 0.4 mM Al treatment, and then decreased in the 0.8 mM Al treatments. It meant that *J. curcas* exuded organic acid in response to aluminum stress, but when the stress reached 0.8 mM, it caused damage to the system of transporter in the plasma membrane. This caused a decrease in the malate exudation to the media. Yamamoto *et al.* (2001) reported that the high Al accumulation in the apoplast region and the high affinity of aluminum ions for the negatively charged membrane plasma seemed to contribute to the damage of membrane plasma. The damaged membrane plasma of *J. curcas* roots occurred in the root tips tissue when exposed with 0.8 mM Al for 7 days (Figure 2f).

*M. malabathricum* roots has a different pattern compared to *J. curcas* regarding malate content and malate exudation. The malate content in the *M. malabathricum* roots reached the peak in the 0.4 mM Al treatment, and decreased in the 0.8 mM Al treatment, while the peak of malate exudation occurred in the 0.8 mM Al treatment. The exudation of organic acids may be one mechanism of tolerance in addition to leaves accumulation. In the histological preparation of *M. malabathricum* root, there was no Al accumulation and damage of membrane plasma. This occurrence was caused by the ability of *M. malabathricum* to transport Al to the leaves (Watanabe & Osaki 2002) and *the ability of M. malabathricum* to exude malate to chelate Al in the medium. *M. malabathricum* has a transport system from the root to the leaf facilitated by citrate which is highly expressed in the xylem, and also is able to exude oxalate and citrate in high concentration from the root apex into the medium (Watanabe & Osaki 2002). The avoidance mechanisms of Al accumulation in the root apex is an importance point for Al tolerance, such as a transport system to the leaf and accumulation in the vacuola (Watanabe & Osaki 2002) or exudation of Al-carboxylate into the medium (Kochian *et al*. 2005). Pineros *et al*. (2005) reported that Aluminum resistance in maize cannot be solely explained by root organic acid exudation.

In conclusion, *J. curcas* used malate synthesis and malate exudation as one of its tolerance mechanism to aluminum. *J. curcas* root has a different pattern with *M. malabathricum* in malate synthesis and malate exudations when treated with different Al concentrations. We categorized *J. curcas* acc IP3 as more sensitive to aluminum than *M. malabathricum*.

## ACKNOWLEDGEMENT

This research was supported by the bilateral exchange Program, JSPS-PPGHE Joint Research Project 2010 entitled: "Molecular adaptation of *J. curcas* to acid soil for reforestation of tropical wasteland" and Hibah Kompetensi (Competence Grant) Project entitled: "Isolation and expression of genes in the frame of obtaining plants tolerant to acid and aluminum stresses".

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