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GROWTH AND ORGANIC ACID EXUDATION OF *Samanea Saman* (Jacq.) Merr. SEEDLING WITH ALUMINUM EXPOSURE

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Al exposure, concentration, growth, organic acids, S. saman

Abstract

This study aimed to examine the effect of aluminium exposure on the growth and exudation of organic acids in *Samanea saman* seedlings. This study was carried out using a one-factor completely randomized design (CRD), namely aluminium concentration with five levels: 0, 2, 4, 6, and 8 mM. *S. saman* seedlings were grown in water culture and after two weeks' adaptation, they were treated with different concentrations of Al for 4 weeks. Plant growth and organic acid content were measured in response to the treatments. The results showed that Al exposure treatment had a significant effect on all growth parameters, and the 2 mM of Al concentration was able to increase all growth parameters, while Al exposure of 6-8 mM caused a bad effect on the growth of *S. saman* seedlings. Al exposure significantly reduced chlorophyll a and b content as well as carotenoids of *S. saman* seedlings. Organic acids secretion which consisted of acetate, lactic, citrate, malic, and oxalate were found at almost all Al concentrations, but malic acid was secreted at Al 4 and 8 mM concentrations, while oxalic acid was only secreted at 8 mM of Al concentrations. Acetic acid and lactic acid were more secreted and accumulated in plants than others, suggesting that acetic and lactic acid probably play an essential role in the defence mechanism of *S. saman* against Al exposure.

INTRODUCTION

Aluminium (Al) is one of the most abundant metals found in the Earth's crust, after oxygen and silicon [1]. Al is abundant and very soluble in acid soils. The high concentration of Al in acid soils is one of the limiting factors to plant growth and productivity. In acidic to very acidic soil conditions (pH <5), Al is present in the form of Al^{3+} which is very toxic and causes stunted root growth [2]. One of the reasons for the inhibition of growth is due to the inhibition of the nutrient absorption process. Al in high concentrations causes inhibition of the absorption of nutrients such as calcium (Ca), magnesium (Mg), and phosphorus (P) [3]; As a result, some nutrients are not available to the plants. In addition, a decrease in plant growth caused by Al exposure is associated with the decrease in chlorophyll and carotenoids content which has an impact on a decrease in the rate of photosynthesis [4, 5]. Heavy metal exposure such as Al also induced lipid peroxidation, which caused oxidative stress to the plants [6].

Plants develop various mechanisms against exposure to Al as a defence strategy against aluminium poisoning, one of which is by secreting organic acids. Plants can create or excrete root exudates and organic acids in response to exposure to heavy metals. Al exposure can stimulate the production of organic acids, such as citrate, oxalate, and

malate, which can facilitate the formation of stable complexes with Al in the cells [7]. Organic acids can become chelating agents to bind heavy metals, especially Al, to increase plant tolerance to Al. The results of several studies indicated that organic acid exudation has a good correlation with Al tolerance, such as malic acid in the Chinese Spring wheat (*Triticum aestivum*) [8], and *Triticum aestivum genotype* Line ET3 [9]. The positive role of organic acids to improve plant tolerance to Al is expected a common response that works not only to the crops but also to the woody plant such as forest trees.

Samanea saman belongs to the Fabaceae family known as rain forest trees and is native to tropical America and grows in many tropical regions [10]. *S. saman* is a fast-growing type with high adaptation to various lands. *S. saman* is widely planted in pasture areas, vacant land, roadsides, and other areas [11]. *S. saman* species is also known as an N-binding tree which is an essential component in achieving sustainable tropical agriculture and forestry, so that it is widely planted in rehabilitation and revegetation activities of marginal lands, such as post-mining land [12]. The experiment of Al exposure to *S. saman* with the focus on organic acid exudation is still rarely carried out and found. Therefore, this research is still important to be carried out to investigate basic information related to the growth response and exudation of organic acids in *S. saman* in response to Al exposure. Therefore, this study aimed to examine aluminium exposure to the growth and exudation of organic acids in *S. saman* seedlings.

MATERIALS AND METHODS

Materials

Materials used in this study include: *Samanea saman* seeds, zeolite, calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$), ammonium nitrate (NH_4NO_3), potassium chloride (KCl), magnesium sulphate heptahydrate ($MgSO_4.7H_20$), potassium dihydrogen phosphate (KH_2PO_4), manganese sulphate monohydrate ($MnSO_4.H_2O$), copper(II) sulphate pentahydrate ($CuSO_4.5H_2O$), zinc sulphate heptahydrate ($ZnSO_4.7H_2O$), boric acid (H_3BO_3), ammonium heptamolybdate tetrahydrate ($(NH_4)_6\ MO_7O_{24}.4H_2O$), ferric EDTA/sodium feredetate (Fe-EDTA), aluminium chloride ($AlCl_3$), potassium hydroxide (KOH), hydrochloric acid (HCl), acetone, distilled water, water, hot water, ethyl acetate, trichloroacetic acid (TCA), thiobarbituric acid (TBA).

Methods

Seed Germination

The seeds were soaked in hot water (80 ^oC) for 15 minutes, then soaked again in water (25-30 ^oC) for 24 hours. The seeds were then germinated by sowing the seeds in the containers that have already been prepared using zeolite media. The seeds were germinated and nurtured until ready to be transplanted to the treatment media.

Preparation of Media and Al Treatment

The experiment was carried out using water culture with the basic media of distilled water supplemented with nutrient solution (macro and micronutrients). The nutrient solution referred to the solution developed by Sopandie [13] which comprised: 5 mM $Ca(NO_3)_2.4H_2O$, 1.0 mM NH_4NO_3, 1.0 mM KCl, 0.4 mM $MgSO_4.7H_20$, 1.0 mM KH_2PO_4, 0.50 ppm $MnSO_4.H_2O$, 0.02 ppm $CuSO_4.5H_2O$, 0.05 ppm $ZnSO_4.7H_2O$, 0.50 ppm H_3BO_3, 0.01 ppm $(NH_4)_6\ Mo7O_{24}.4H_2O$. Aluminum (Al) treatment was applied using $AlCl_3$. Before Al treatment, the *S. saman* seedlings were adapted in nutrient solution (without Al treatment) for ± 2 weeks. During the treatment, the Al treatment was applied with different concentrations of Al i.e.: 0, 2, 4, 6, and 8 mM. The seedlings were maintained for 4 weeks, and every 2 weeks the nutrient solution was replaced to optimize the growth of the seedlings.

Evaluating Parameters and Harvesting

Plant height measurements were carried out weekly to 4 weeks, while root length was measured at week four. Plants were harvested after 4 weeks, then the shoots (leaves and stems) and roots were separated and dried using an oven for 2 days at 80 ^oC, and after that, it was weighed to get the dry weight of the plants.

Chlorophyll and Carotenoid Analysis

Chlorophyll and carotenoid analysis followed the method from Sims & Gamon [14] with modification. Leaf samples were weighed 0.03-0.05 g and ground until smooth using a mortar and added 2 ml of acetone (85: 15%, Trs HCl 1%, pH 8), then centrifuged at 10000 rpm for 5 minutes. A 1 ml supernatant was taken and 3 ml of tris acetone was added and then shaken using a shaker until homogeneous. The absorbance was measured at wavelengths (λ) of 470, 537, 647, and 663 nm using a UV-VIS spectrophotometer. Chlorophyll and carotenoid values were expressed in mg/g. Chlorophyll and carotenoid content were determined based on the [14] equation:

$$\text{Anthocyanin} = 0.08173*A_{537} - 0.00697*A_{647} - 0.002228*A_{663}$$

$$\text{Chl}_a = 0.01373*A_{663} - 0.000897*A_{537} - 0.003046*A_{647}$$

$$\text{Chl}_b = 0.02405*A_{647} - 0.004305*A_{537} - 0.005507*A_{663}$$

$$\text{Carotenoid} = \frac{(A470 - (17.1*(Chla+Chlb) - 9.479*Anthocianin))}{119.26}$$

Where, Ax is absorbance at the wavelengths measured.

Organic Acid Test and Analysis

The organic acid test treatment was carried out under aseptic conditions. The seeds of *S. saman* were sterilized and germinated on fixed media. The seeds were maintained until they were ready to wean (±2 weeks). The ready-to-wean seedlings were transferred to a test tube containing 70 ml of sterilized nutrient solution (pH 5.3). Adaptation of seedlings was carried out for seven days and placed on a shaker at 80 rpm at 23 ºC. The nutrient solution was replaced with a 0.35 mM $CaCl_2$ (calcium chloride), and $AlCl_3$ (aluminum chloride) was added according to the predetermined concentrations. This organic acid analysis refers to the method Baziramakenga et al., [15] with modifications. For the analysis of organic acid secretion, the culture medium was measured. The culture media was extracted with 20 ml of distilled water for 12 hours. Then, the solution mixture was centrifuged for 15 minutes at 3000-5000 rpm. After that, it was filtered using Whatman No. 42 and extracted with 10 ml of ethyl acetate for 5 minutes. For the analysis of organic acids accumulation, all parts of the plant (roots, stems, and leaves) were used as samples. The samples were weighed 0.5-1 gram, then added 10-20 ml m0.1 N NaOH (sodium hydroxide) for 12 hours. The extract samples were filtered using Whatman 42 and centrifuged for 15 minutes at 3000-5000 rpm. The supernatant was acidified using 1 N HCl (hydrochloric acid) to a pH of 2.5 and lasted for 16 hours. The solution mixture was again centrifuged for 15 minutes at 3000-5000 rpm. The supernatant was extracted three times with 10 ml of ethyl acetate for 5 minutes. The solvent was evaporated to dryness in a rotary elevator at a temperature of 40 ºC, and the residue was redissolved with 0.01 N H_2SO_4 (sulfuric acid) solvent. Organic acids were identified and measured by injecting 50 µl in ion chromatography. Each peak was identified with a different retention time based on the known standard solution of organic acids. Organic acid analysis were carried out using HPLC SHIMADZU 20A 2008, with the following conditions: column: BSD Hypersyl C18 15 x 4.6 mm, mobile phase: 0.01 N H_2SO_4, flow rate: 0.5 ml/min, detector: Diode Array. UV-VIS, Nm: 190, and column temperature: 35 ºC.

Data Analysis

The data were analysed using ANOVA test with a confidence level of 95% ($\alpha = 5$). If the ANOVA test showed significantly different, then the analisis was continued with the Duncan Multiple's Range Test (DMRT) at a 95% confidence level ($\alpha = 5$).

RESULTS AND DISCUSSION

Plant Growth

Al exposure significantly affected the growth of *S. saman* seedlings. Al treatment significantly reduced seedling height and root length, especially when Al was applied 4 – 8 mM (Figure 1 and 2). The effect of Al treatment was consistent, which reduced all the parameters of *S. saman* growth, but the reduction was still not significant to the dry weight of shoot as well as roots except at Al concentration of 8 mM to the roots (Figure 3). This probably because the time of measurement (4 weeks) was too short to make the dry weight among the treatment were significantly different in response to Al treatment, even though those effect on the seeling height and root length were already significantly different. Interestingly, Al Application at 2 mM tended to cause improvement of *S. saman* growth based on all growth parameter measured although statistically not significant (Figure 1-3), while other concentration (at 4 – 8 mM) induced reduction of growth. This improvement may be associated with the concentration threshold of Aluminum for *S. saman* which was higher than 2 mM. In addition, this phenomenon may be also indication that *S. saman* is rather tolerant to aluminum toxicity. In line with this. some experiment proved that Al can stimulate the growth of native plants and some plants that tolerant to acid soils at low concentrations, such as *Melaleuca cajuputi*, *Arnica montanam*, *Hydrangea paniculate*, *Melastoma malabatricum*, etc. [16].

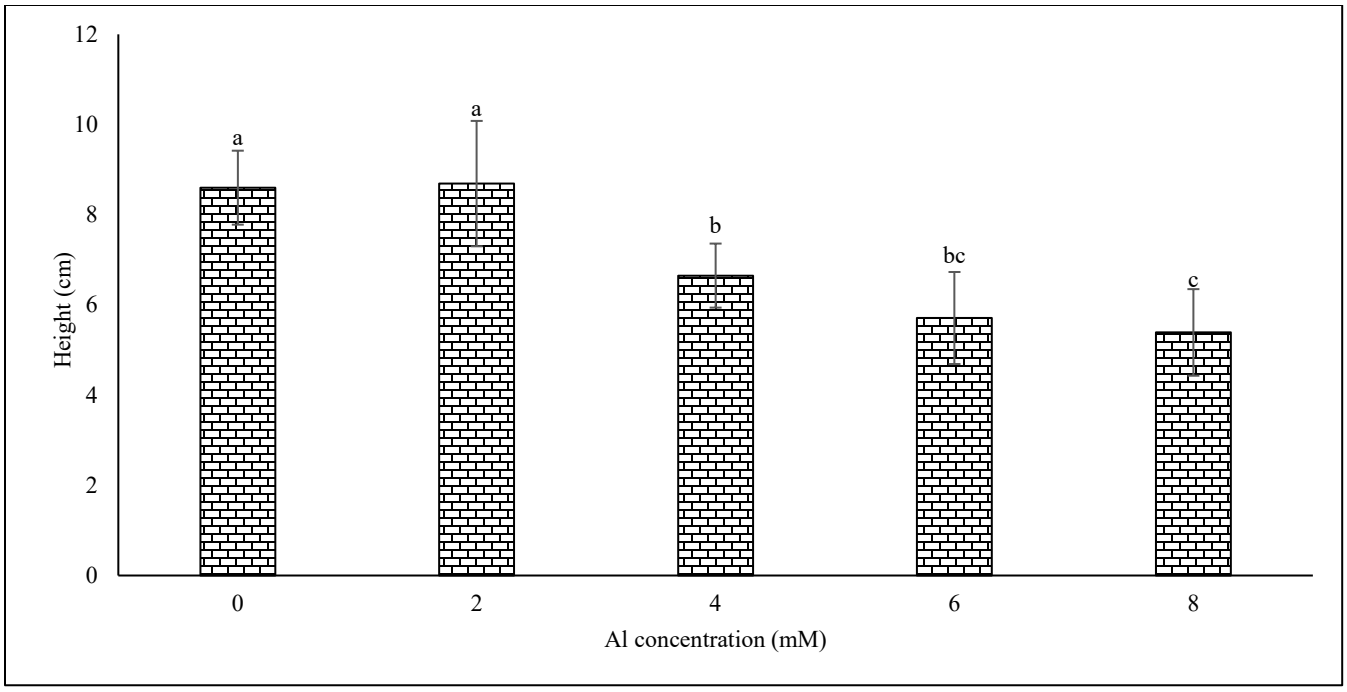

Figure 1. Seedling height of *S. saman* at various concentrations of Al exposed for 4 weeks. Vertical bars indicate standard deviations (n = 3). Different letters show a significant difference in the result of the DMRT test at the 5% level.

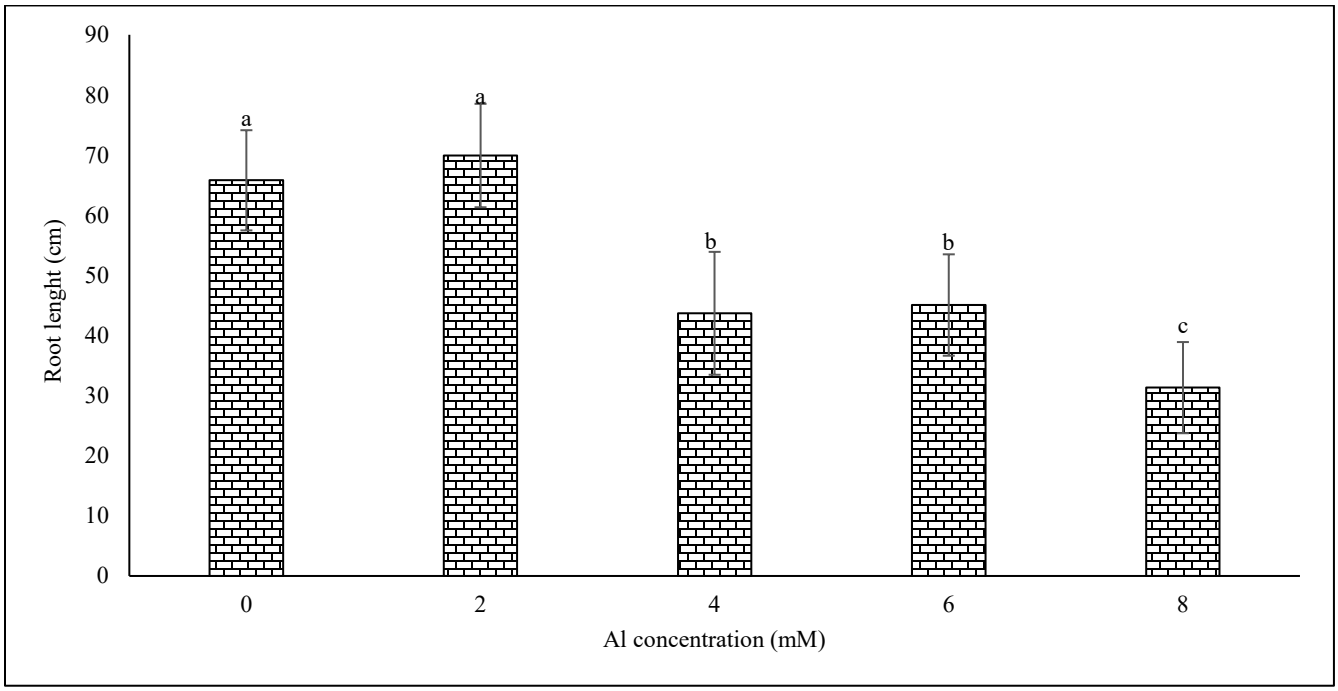

Figure 2. Root length of *S. saman* seedlings at various concentrations of Al exposed for 4 weeks. Vertical bars indicate standard deviations (n = 3). Different letters show a significant difference in the result of the DMRT test at the 5% level.

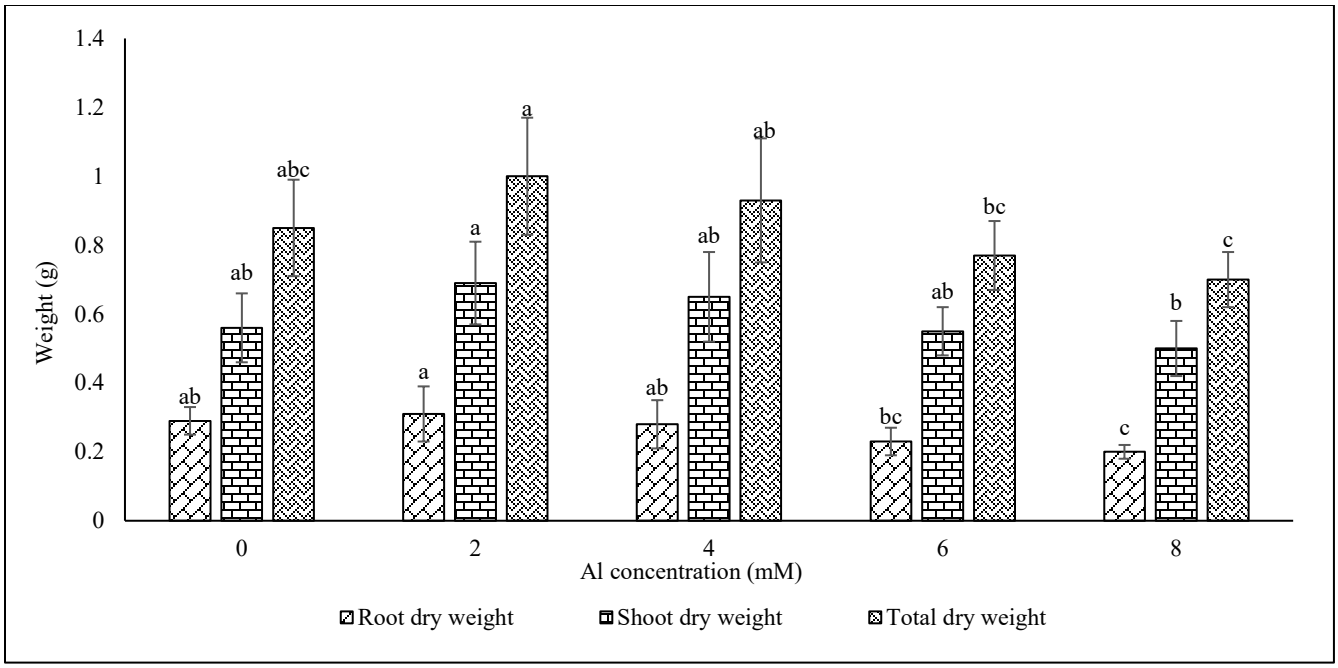

Figure 3. Shoot, root and total dry weight of *S. saman* seedlings at various concentrations of Al exposed for 4 weeks. Vertical bars indicate standard deviations (n = 3). Different letters show a significant difference in the result of the DMRT test at the 5% level.

Aluminum is a non-essential element for plants and has no specific biological function. Each plant may respond to Al toxicity differently. The tolerant plants such as *Melastoma malabatricum* may be still able to grow well under specific Al concentration [16] while it has caused inhibition to others. However, at high concentration Al caused growth inhibition to almost all plants. In this experiment, at the concentration of 4 mM to 8 mM, Al treatment has decreased the growth of *S. saman* seedlings which indicated that 4 mM is toxic concentration for *S. saman*. Some other experiment also found that Al toxicity apparently reduced the biomass growth of shoots, stems, leaf of Cacau plants [17]. Al reduces plant growth by inhibiting plant roots. Al can prevent root elongation, reduce root cell division, inhibit root hair formation and damage the plant root system [18]. The root becomes the most sensitive organ, and Al causes quickly inhibition of cell division in the root tip meristem. So that the reduced root growth is the main symptom of Al poisoning [23]. This also can be seen from the experiment with Al concentration of 4 mM to 6 mM, which apparently decreased the root growth of *S. saman* seedlings (Figure 2).

Chlorophyll and Carotenoid Content

Chlorophyll a and b content in *S. saman* plants, decreased in the presence of Al exposure. The presence of Al caused a significant reduction on the content of chlorophyll a and carotenoids, while the content of chlorophyll b was not influenced by the treatment at all Al concentrations (Figure 4 and 5). The decrease in chlorophyll a and b content started when the plants were exposed to 2 mM Al, but the value was fluctuation among the Al concentration may be due to the variation among the replication, so that only at the concentration of 4 and 8 mM that were significantly different (Figure 4). The content of chlorophyll a was higher than the chlorophyll b at each Al concentration, but chlorophyll b content data were not significantly difference at any Al concentration. It shows that Al exposure did not significantly affect the chlorophyll b content in *S. saman* plants. Plant carotenoids also decreased in the presence of Al exposure with almost had similar content among the treatment from 2 until 8 mM of Al (Figure 5), but the reduction was not significantly different as compared to the control plants (without Al).

Chlorophyll content is an important parameter that is able to predict stress condition experienced by the plants under abiotic stress. For Al exposure this parameter was also pronounced suggesting that the plants underwent stress due to Al exposure (Figure 4). Samad et al. for example [4] reported that Al (100 µM) was able to decrease 9% to 22% of chlorophyll content in rice plants, while Al (150 µM) was able to reduce 11.6% to 30% of chlorophyll content. Al can reduce chlorophyll synthesis by inhibiting the activity of aminolevulinic acid dehydratase (ALA), responsible for the formation of monopirrol profobilonogen [5]. The Al^{3+} ion can hinder the work of the chloroplast enzyme and reduce the absorption of Mg and Fe [19]. The Al treatment was also able to reduce the carotenoid content of *S. saman* seedlings

but the decrease did not significantly different (Figure 3). This was different from Jesus [20] who found that the treatment using Al (50 - 150 µM) caused the reduction of carotenoid content in rice plants significantly.

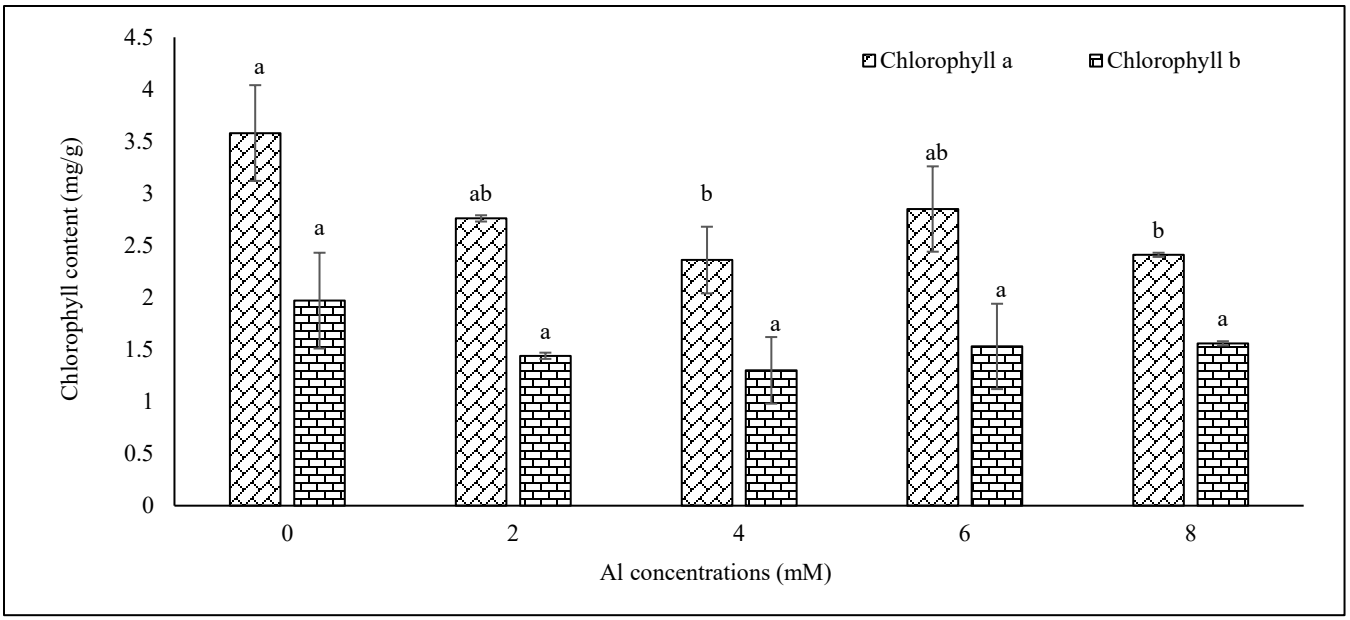

Figure 4. Chlorophyll a and b content at various Al concentrations. Vertical bars indicate standard deviations (n = 3). Different letters show a significant difference in the result of the DMRT test at the 5% level.

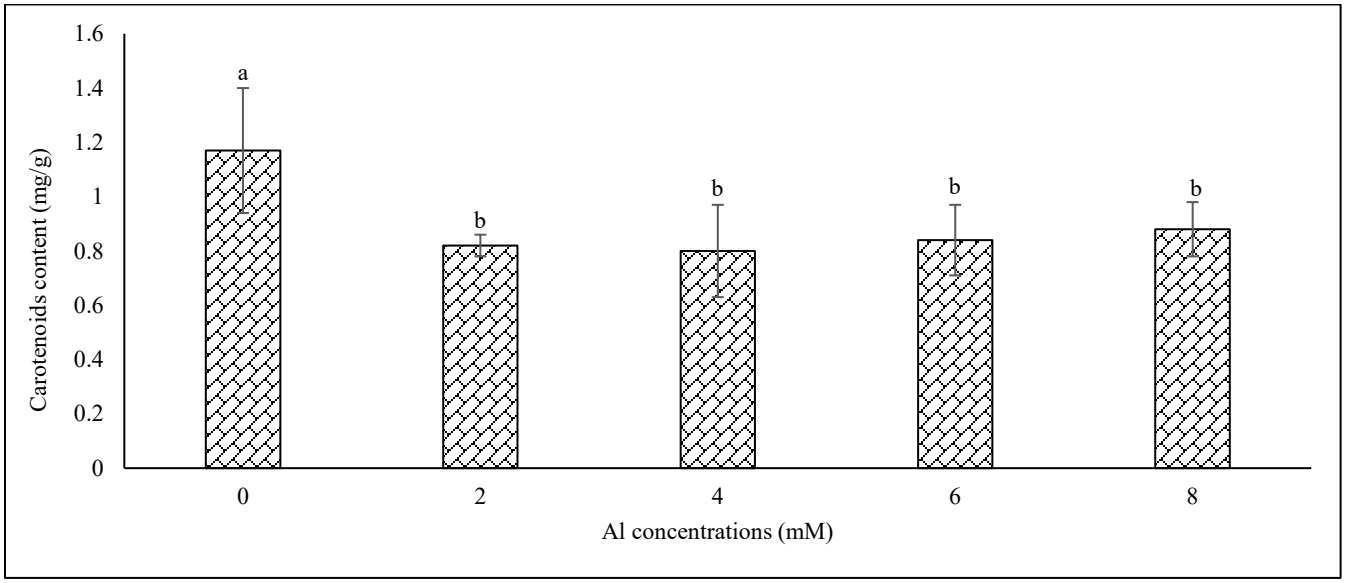

Figure 5. Carotenoid content at various Al concentrations. Vertical bars indicate standard deviations (n = 3). Different letters show a significant difference in the result of the DMRT test at the 5% level.

Secretion and Accumulation of Organic Acids

Plants can excrete organic acids as a defense due to exposure to heavy metals including Al. Plants can secrete organic acids rapidly when exposed to Al. Many organic acids secreted have been documented, but only a few specific ones are induced by Al stress [21]. *S. saman* can excrete several organic acids such as acetate, lactate, citrate, malate, and oxalate, but only acetate and lactate that consistently induced by Al concentration (Table 1). Ryan et al. [22] noted that Al can stimulate various organic acids, such as malate, citrate, and oxalate to increase in the plants. The secretion of acetate, lactate, and citrate increased in the presence of Al exposure. However, the increased secretion of acetate, lactate, and

citrate did not consistently in line with the increase of Al concentrations. Malic acid was expressed when the Al concentration was 4 mM (0.141 mg/L) and 8 mM (0.267 mg/L), while oxalic acid was expressed when the Al concentration was 8 mM (0.026 mg/L). It indicated that malic acid may only secreted when the Al concentration is high enough (8 mM or more).

Table 1. Organic acid secretion of *S. saman* seedlings at various Al concentrations after seven days of treatment.

Al	Secretion organic acid (mg/L)				
(mM)	Acetate	Lactate	Citrate	Malate	Oxalate
0	0.131 ± 0.029 c	0.148 ± 0.043 b	0.006 ± 0.043 a	nm	nm
2	0.180 ± 0.017 c	0.234 ± 0.064 ab	0.007 ± 0.064 a	nm	nm
4	0.429 ± 0.029 b	0.312 ± 0.030 a	0.009 ± 0.030 a	0.141 ± 0.009 b	nm
6	0.409 ± 0.030 b	0.223 ± 0.063 ab	0.009 ± 0.036 a	nm	nm
8	0.591 ± 0.033 a	0.242 ± 0.040 ab	0.009 ± 0.040 a	0.267 ± 0.043 a	0.026 ± 0.004
P-value	**	*	ns	*	-

Note: mean ± standard deviation, the different letters show a significance in the DMRT test results at the 5% level (n = 3). ** significant effect at the 1% level, * significant effect at the 5% level, ns: not significant at the 5% level, and nm (not measurable)

Oxalic, citric, and malic acids are the most effective organic acids to chelate Al and reduce Al toxicity [23]. In this study acetic acid and lactic acids were among the highest organic acids secreted by *S. saman* seedlings in response to Al treatments. It shows that *S. saman* seedlings had organic acid accumulation as a significant role in increasing plant defense against Al exposure. Some author reported that acetic acid and lactic acid were important organic acid secreted by *Pisum sativum* L. exposed to Al [24]. However, it is different from other studies who reported that citric acid has a higher ability to chelate Al, followed by oxalic acid and finally malic acid [25]. It suggests that the different types of organic acid may work dealing with Al exposure in one plants which different from others as well as the differences due to exposure levels. The result of this study indicated that malate and oxalate acid were secreted by *S. saman* when exposed to Al on eight mM (Table 1), suggesting that those organic acids play a role in the constitutive resistance of *S. saman* against Al exposure. Citric and oxalic acids detoxify Al internally and prevent Al to interfere metabolic process in the cytoplasm [26]. Therefore, the malic acid in high concentrations is needed to avoid Al toxicity. Malic acid has been reported to have good correlation with the resistance of Eucalyptus species to Al [27].

Almost similar to organic acid secretion, the accumulation of acetic, lactic, and citric acids also increased in the presence of Al exposure, indicated by the lower concentrations of acetic, lactic and citric acid in the control plants (without Al) compared to those exposed to Al (Figure 6). However, the increase of Al concentration did not always induce the increase accumulation of organic acids in plants, as seen from the fluctuation of organic acid concentrations at several Al concentrations. The concentration of malic acid at various Al concentrations did not show a significant difference. At 4 mM Al concentration, the plant accumulated the highest lactic acid (1.239 mg/L) compared to other concentrations. Different from other organic acid, the significant increase of accumulation of oxalic acid was only at a concentration of 8 mM (0.805 mg/L). It shows that only at high Al concentration can increase the accumulation of oxalic acid in *S. saman*, or oxalic acid content may not sensitive to Al treatment in this species.

The increase in the accumulation of organic acids in plants is related to the synthesis activity of each organic acid. In melastoma root for example, Al application caused significant increase of oxalic acid levels in the apoplast [28]. However, Cárcamo et al., [29] reported that the concentration of malate in the roots decreased in highbush blueberry cultivars after 48 hours of exposure to Al, suggesting that time of exposure may influence organic acid accumulation. In *S. saman* seedlings, the exudation of organic acids especially acetate, lactate and citrate may have important role in defense mechanism against Al toxicity, while malate and oxalate did not have significant role. Meanwhile, some studies also showed that organic acid exudation was not correlated with plant tolerance to Al, such as in maize [30] and weed plant *Brachiaria decumbens* [31].

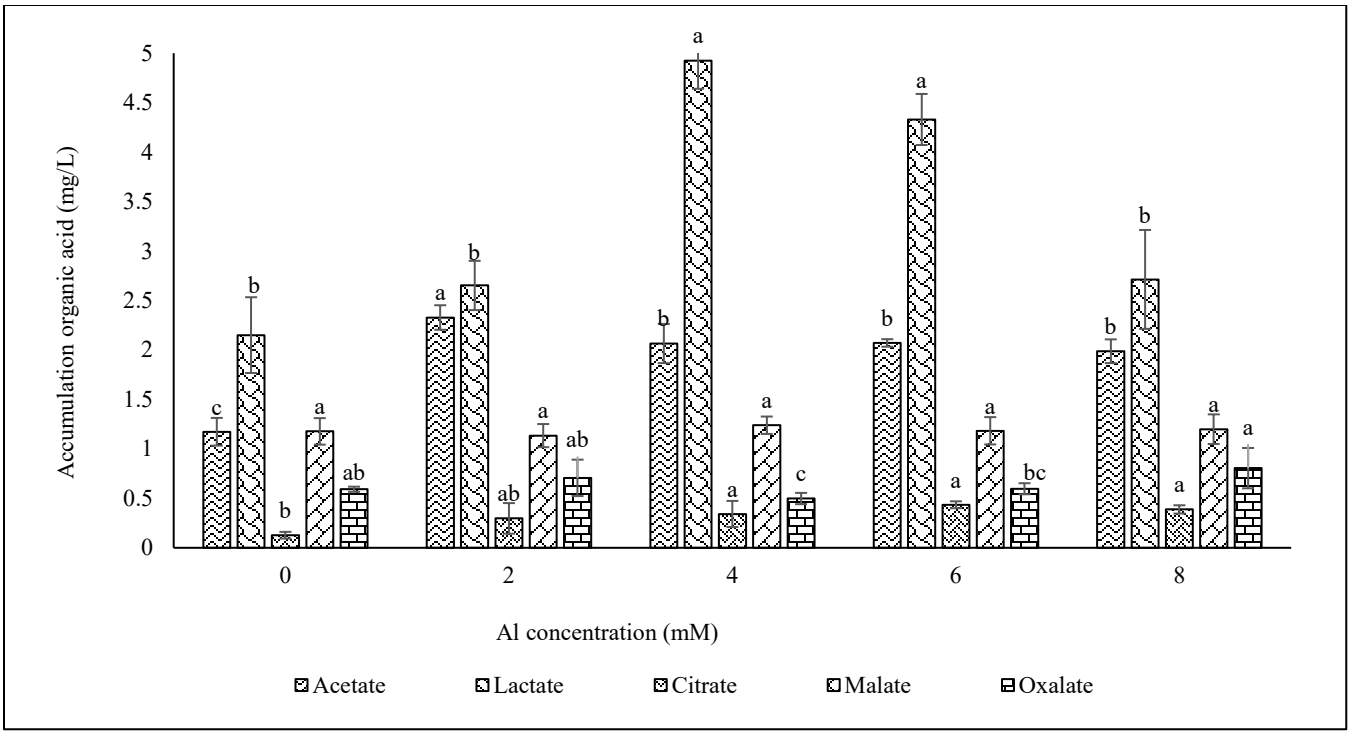

Figure 6. Accumulation of organic acids for *S. saman* seedlings at various Al concentrations after seven days of treatment. Vertical bars indicate standard deviations (n = 3). Different letters show a significant difference in the result of the DMRT test at the 5% level.

CONCLUSION

From this experiment, it is clearly that Al exposure for 4 weeks caused a significant decrease in the growth of *S. saman* seedlings as well as chlorophyll-a and carotenoids content. Al concentration at 2 mM was able to stimulate and increase growth, while Al concentration of 6-8 mM had shown a bad effect on the growth of *S. saman* seedlings. Al treatment induced secretion as well as accumulation of organic acids especially acetic acid, lactic acid and citric acid significantly, while malic acid and oxalic acid were less pronounce, suggesting that acetate, lactate and citrate have important role in the defense mechanism of *S. saman* under aluminum stress.

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CONFLICT OF INTEREST

We have no conflicts of interest to declare.

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