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Investigation of Aluminum in Symplocaceae: Total Content, Histological Structure, and Enzyme Activities from Different Leaf Ages

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Abstract

Since Symplocaceae is known to be a hyperaccumulator of Aluminum (Al), studies on its accumulation capacity and physiological responses have not been widely investigated. The objectives of this research are to investigate the Al content of different leaf ages of *Symplocos fasciculata* and *Symplocos cochinchinensis* using Inductively Coupled Plasma Optical Emission Spectrometry (ICP OES). Moreover, the Al localization within tissues was determined using hematoxylin staining, followed by Scanning Electron Microscopy coupled with Energy-Dispersive X-ray spectroscopy (SEM-EDX) mapping to address the ultrastructural form and determine elemental distribution in the cross-section of the leaf. Additionally, confirmation of the mechanism of Al toxicity in relation to stress-induced oxidative enzymes, including Catalase (CAT), Superoxide Dismutase (SOD), and Peroxidase (POD), as well as the lipid peroxidation product Malondialdehyde (MDA) and Hydrogen peroxide (H_2O_2), serves as stress markers. ICP OES assay reveals a significant influence of leaf age on Al content. *S. fasciculata* old leaves had the highest Al concentration (39,001 ± 343.48 mg kg^{-1}), while *S. cochinchinensis* young leaves had the lowest (16,788 ± 187.98 mg kg^{-1}). The histological and SEM-EDX mapping evidence shows Al accumulation in *S. fasciculata* and *S. cochinchinensis* increases significantly with leaf age. Al^{3+} accumulation is found in cell walls, epidermal tissue, and the palisade layer, with the highest Al distribution in the old leaf lamina. The leaf ages of both *Symplocos* species exhibit a significant influence on CAT, POD, MDA, and H_2O_2, whereas SOD activity shows no significant variation. A positive correlation is observed between SOD, POD, MDA, and H_2O_2 in *S. fasciculata* ($r > 0.7$), similar to *S. cochinchinensis*, except for MDA ($r = -0.6$). This research presents a valuable investigation into the Al accumulation strategies and physiological responses of 2 *Symplocos* species.

Keywords: *Symplocos*, ICP OES, Leaves histology, SEM-EDX mapping, Oxidative stress

Introduction

Aluminum (Al) is relatively stable in neutral pH soil, existing in the form of aluminosilicates and aluminum oxides. Both forms are insoluble, ensuring they do not pose a significant threat to plants and organisms [1-4]. In acidic environments, aluminosilicates and other Al compounds are dissolved into the soil solution as Al ions (Al^{3+}), which are harmful to plants [4]. Several plant species have evolved strategies for Al avoidance or tolerance. Excluder plants limit Al uptake by secreting organic acids that bind with metal ions, forming a barrier layer to prevent entry into plant tissue [3,4]. On the other hand, Al-resistant and accumulator plants form a chelate of organic acids with Al^{3+}, converting them into a harmless form that inhibits their spread and stores them in the vacuole [3,5-7].

Symplocos is a remarkable hyperaccumulator plant known for storing large amounts of Al in its stems and leaves [5]. Based on previous research by Zheng *et al.* [7], *Symplocos sumuntia* leaf contains high concentrations of organic acids and multiple metal ions, particularly Al^{3+}, whose content can reach 23,071.98 ± 11.19 mg kg^{-1}, confirming it as an Al hyperaccumulator plant. Metal and metalloid content were determined by inductively coupled plasma optical emission spectrometry (ICP OES) [8-10]. ICP OES allows precise quantification of metal ions in solutions before and after bioaccumulation, providing complementary data to SEM-EDX analysis [11]. Furthermore, the detection of Al in tissues and organs may vary depending on the method employed. Histochemical techniques typically identify specific compounds through their binding to chemical elements within biological tissues [12]. Al accumulation in leaves, as visualized using hematoxylin staining, results in a purplish hue in several tissues [12,13]. Although the hematoxylin staining method is well-established for detecting aluminum accumulation in plant tissues, it does have limitations, particularly in the scoring range [14]. Scanning electron microscopy and energy-dispersive X-ray spectroscopy (SEM-EDX) can provide more comprehensive insights into the structure and distribution of metal compounds stored within the tissues of hyperaccumulator plants [15]. This method benefits practical applications by determining the elemental composition, making it a vital analytical tool for the morphological and chemical characterization of various materials [16].

Al accumulation in plants at certain levels does not interfere with plant physiological processes; several studies have shown the benefits of low Al levels [3]. In maize, low levels of Al support leaf growth, whereas in tea, Al maximizes macronutrient absorption, increases root elongation, and accelerates metabolic rates, thereby increasing plant biomass levels [17,18]. Nonetheless, high Al levels exert antagonistic effects, notably inhibiting root growth and water absorption, and impairing the uptake of several essential elements, including potassium (K), calcium (Ca), and magnesium (Mg). Additionally, excess lipid peroxidase increases oxidative stress, leading to cell death [18-20]. Moreover, plants under Al stress upregulate their oxidative stress metabolism to counter Al toxicity [12]. Plants maintain their metabolism through enzymatic and non-enzymatic antioxidant defenses to mitigate the imbalance of *Reactive oxygen species* (ROS) mediated oxidative stress, considering the sophisticated strategies plants deploy to counteract a spectrum of combined abiotic stresses [21,22]. Some plant species, however, exhibit enhancement of their intrinsic antioxidant defenses to counterbalance stress induced by ROS accumulation and consequent oxidative damage [23-26]. Balancing ROS in cellular delivery through 2 pathways, including enzymatic antioxidants such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), is essential for mitigating reactive oxygen species (ROS) in cells [27]. Moreover, non-enzymatic antioxidants, including vitamins, flavonoids, tocopherols, carotenoids, and phenolic compounds, play crucial roles in maintaining redox balance and protecting against oxidative stress [11,27,28]. The accumulation strategies and physiological responses of Al in *Symplocos* leaves remains limited. Therefore, understanding the mechanisms of Al tolerance is deemed essential and requires a multidisciplinary approach. To compare the plant responses to Al in 2 *Symplocos* species across various leaf ages, this study employed a novel method that combines elemental analysis, histology, and enzymology.

Materials and methods

Study sites and species

Fieldwork was conducted from August to November 2024 at the Baturraden Botanical Garden, Mount Slamet, Central Java, Indonesia. *Symplocos* exhibits a shrubby to tree habit, reaching heights up to 20 m and is characterized by their hairy twigs. The young leaves of *S. cochinchinensis* are hairy and arranged in an alternate spiral pattern with leaf sheaths. In contrast, *S. fasciculata* also exhibits an alternate arrangement but in odd numbers. Both species possess elliptical or oval leaves with pointed apices, opposite venation, serrate margins, and a paracytic stomata. The primary difference lies in the leaf bases; *S. fasciculata* has rounded leaf bases, while *S. cochinchinensis* has narrowed bases. The general characteristics of the areas where each plant species was sampled, including *S. fasciculata*, located at 7°18’19” S - 109°13’57” E at 1,076 m, lower altitude 7°18’50” S - 109°13’58” E at

1,010 m, is the sampling area for *S. cochinchinensis*. The annual precipitation level record at the site was 5.198.96 mm, with 90% humidity levels for both species. However, these sites still exhibited different light intensities due to the presence of various organisms. *S. fasciculata* site area had a temperature of 23.7 ℃ with an average light intensity of 2.931 Cd, posses pH soil at 6.41, while *S. cochinchinensis* had a warmer temperature at 24.1 ℃ with an average light intensity of 5.658 C, posses pH soil at 6.19.

Collection of leaf material

Leaf samples were classified into 3 developmental stages - young, mature, and old - based on position and color [6,29]. Leaves were classified based on their position on a single twig, which indicated their increasing age from apex to base. Young leaves, which are still at an early developmental stage, exhibited a shoot position (the first leaves of apex) with a reddish to light green color. In contrast to the middle (4th leaves from apex to 5th leaves before basal) branches, dark green color showed mature leaves, and the older leaves were generally at the bottom (4 last leaves of base) with a yellowish green to yellow color [30]. The primary difference lies in the leaf bases; *S. fasciculata* has rounded leaf bases, while *S. cochinchinensis* has narrowed ones. Moreover, the average leaf size of *S. cochinchinensis* is larger than that of *S. fasciculata*, providing a clear visual distinction between the 2 species (**Figure 1**).

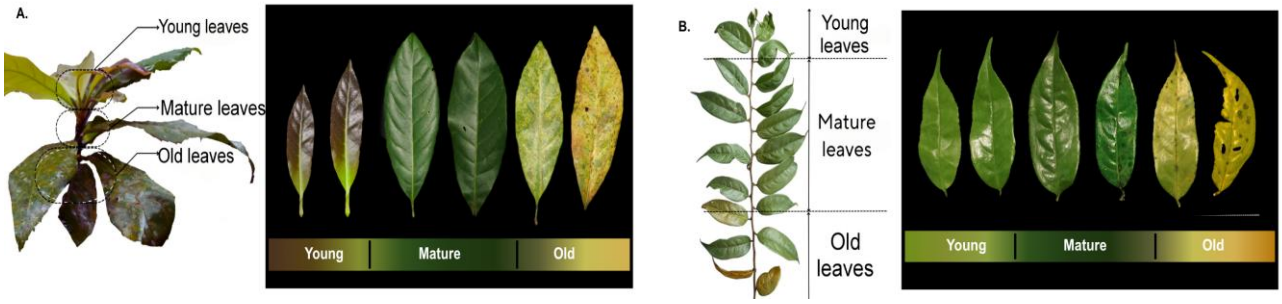

Figure 1 Description of leaf age categories in 2 *Symplocos* species: (A) *Symplocos cochinchinensis*; (B) *Symplocos fasciculata*.

Total Al content by ICP OES assay

The leaves were washed with a 3% hydrochloric acid solution [8], then rinsed sequentially with tap water and deionized water to reduce the potential risk of contamination. The leaves were placed in paper bags and oven-dried (WTC Binder 7200, Tutlingen, Germany) at 60 °C for 72 h. The dried leaves were ground into a fine powder and filtered through 74 µm [31] for subsequent chemical analysis. Powder samples of 1.0 g were transferred into a vessel, HNO_3 was added, and the mixture was allowed to react for 15 min. The mixture was subsequently subjected to microwave digestion. The resulting digested solution was transferred into a 50 mL measuring flask, and 100 mg of yttrium (Y) was added as an internal standard. Distilled water was added to the flask, and the solution was thoroughly homogenized. The solution was filtered through a syringe filter 0.20 µm and measured using an ICP OES (Agilent 5800, Agilent Technologies, USA). The absorbance used for Al detection was 396.152 nm, and for yttrium (Y), 371.029 nm.

Histological preparation by the embedding technique

Fresh leaf samples were cut into 0.2×0.5 cm^2 pieces and fixed with FAA (5% from formaldehyde 37%, 5% glacial acetic acid, 70% ethanol) for 24 h. Samples were dehydrated through a graded ethanol series and cleared in an ethanol–xylene mixture. Infiltrated for 24 h at 57 °C (Memmert oven Incubator, Western Germany) and embedded in paraplast (Merck, CAS No: 64742-51-4, Darmstadt, Germany). Sectioning using a rotary microtome (KD 1508A, Zhejiang Jinhua Kedee Instrumental Equipment Co., Ltd, China) at 8 µm. Remove the paraplast with xylene, then rehydrate it to decrease the ethanol. Furthermore, a solution of 2.0 g of hematoxylin (Merck, C.I. 75290,

Darmstadt, Germany) and 0.2 g of KIO_3 [12] was applied at room temperature to detect Al decomposition in tissue. Samples were mounted using Entellan (Bio Optica, Biomount HM). Samples were examined using an optical microscope (Nikon Eclipse 50i with Nikon camera DS-Fi1, Tokyo, Japan). Color quality from optical microscope capture was accessed by the colorimeter software (Colorimeter vers. 2.25.19, Lab Tools). Color grading was evaluated using the L*a*b* values and the L* value represents light intensity, with a range of 0 to 100, indicating that a higher L* value corresponds to a softer color [32].

Distribution pattern of element by SEM EDX

The fresh leaves were cut fragments measuring 0.5 mm thick×0.5 cm lenght and immersed in FAA (Formaldehyde-Alcohol-Acetic Acid) fixative for 24 h, then dehydrated through graded ethanol, mounted on carbon tape, and loaded into a JEOL JEC-3000FC Auto Fine Coater (Japan), and coated with gold for 120 s at 20 mA under a pressure of approximately 3.2 Pa. After coating, the samples were loaded into a JEOL JSM-6510LA SEM (Japan) and vacuumed for 60 s. The samples were then imaged using an electron beam optimized for the specific element being examined.

Enzymatic assay

Fresh leaves, having been sorted and cleaned, were weighed to a mass of 0.5 g and then crushed by adding 0.1 g of liquid nitrogen. The sample was diluted with 500 µL of 50 mM PBS (pH 7.0). Centrifugation was performed at 10,000 rpm for 15 min at 4 °C to obtain a supernatant. SOD activity was assessed by monitoring the enzyme's ability to inhibit pyrogallol auto-oxidation, a reaction mediated by superoxide free radicals, at 325 nm [33]. CAT activity was evaluated spectrophotometrically by measuring H_2O_2 decomposition at 240 nm every 15 s for 1 - 2 min [34]. POD activity was measured by monitoring guaiacol oxidation at 420 nm [35], with readings taken every 30 s for 2 - 3 min. Enzyme activities were measured using a UV-Vis Spectrophotometer (Genesys UV10, Thermo Scientific, USA). The Thiobarbituric Acid Reactive Substances (TBARS) method was used to quantify MDA levels. This method relies on the reaction between MDA and thiobarbituric acid (TBA), which formed a pink complex measurable with a Microplate Spectrophotometer (Multiskan SkyHigh, Thermo Fisher Scientific, USA) at 532 nm [36].

Statistical analysis

All experiments were conducted in triplicate (n = 3), and the data were presented as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 8.0.2 (GraphPad Software Inc., San Diego, CA, USA). Two-way analysis of variance (ANOVA) followed by Tukey's post hoc test was employed to determine statistical significance between species *Symplocos* and leaf ages. Differences were statistically considered significant at $p < 0.05$. The correlation was examined using Pearson's correlation analysis to investigate the relationship between Al content, L* value, and each enzymatic activity. The value of strong positive correlation was $r \geq 0.7$.

Results and discussion

Total Al content

The total Al content of *S. fasciculata* and *S. cochinchinensis* was analyzed using ICP OES, revealing a significant influence of leaf age on Al content. A significant difference ($p = 0.002$) in Al content was observed between the oldest and youngest leaves in both species. The old leaves of the 2 *Symplocos* species have significantly accumulated more Al than the young leaves. Additionally, the Al element was reported to be deposited and immobilized in older tissues. A non-significant difference was demonstrated in young and mature leaves ($p = 0.06$) vs mature and old leaves ($p = 0.09$) in *S. fasciculata* (**Figure 2**). This condition is assumed to cause the immobility of aluminum once it is bound within the cell walls [2]. There was a lack of a biological mechanism for its efficient remobilization.

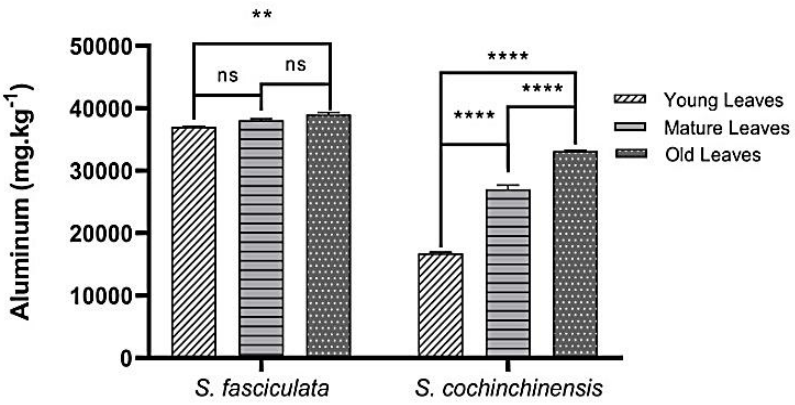

Figure 2 Aluminum (Al) content in 3 different leaf ages from 2 species of *Symplocos* was measured by ICP OES. Data are presented as mean ± SD, $p < 0.05$, ** $p = 0.02$, ****$p < 0.001$.

The typical Al concentration in the tissue of generally non-accumulating plant species is less than 100 mg kg^{-1} of dry weight [6]. Research findings revealed that the highest Al content was observed in the old leaves of *S. fasciculata*, with 39,001 ± 343.48 mg kg^{-1}, followed by the old leaves of *S. cochinchinensis*, with 33,150 ± 147.84 mg kg^{-1}. Furthermore, the mature leaves of *S. fasciculata* contained 38,045 ± 283.39 mg kg^{-1}, whereas those of *S. cochinchinensis* contained 27,013 ± 660.45 mg kg^{-1}. Additionally, the young leaves of *S. fasciculata* contained 37,002 ± 160.66 mg kg^{-1}, and the lowest content among all samples was found in the young leaves of *S. cochinchinensis* 16,788 ± 187.98 mg kg^{-1}.

Leaf age is a key parameter for detecting Al accumulation in tissue [6,12]. This finding aligns with the existing data that the old leaves of *Symplocos* are the highest accumulators of Al. Schmitt *et al.* [6] highlight that the highest Al concentrations were found in old leaves (24,180 ± 7,236 mg kg^{-1} dry weight, mean ± SD). In contrast, young leaves had significantly lower Al levels (20,708 ± 7,025 mg kg^{-1}) in 3 different age groups of *S. odoratissima, S. ophirensis,* and *S. ambangensis* at 3 montane rainforest sites in Central Sulawesi [6]. Furthermore, our results for the old leaves demonstrated a similar trend to those from *S. cochinchinensis* and *S. fasciculata* in East Nusa Tenggara, Indonesia, which have Al contents of 39,311 and 49,775 ppm, respectively [37]. These results provide information on Al content in *Symplocos* leaves in the Indonesian montane rainforest, particularly on Java Island, as well as on the Sulawesi and East Nusa Tenggara Island.

Leaves histology assay

Histological assay revealed Al was detected in the cuticle, epidermis cell walls, palisade mesophyll, and chloroplasts (**Figures 3(A) - 3(C)**). Lamina of old leaves *S. fasciculata* (**Figure 3(C)**) showed dark purple (L* = 33.9 ± 0.854) at epidermal adaxial tissue compared to other leaf ages. This result led to the assumption that most of the Al was stored in old leaves. On the other hand, epidermal adaxial tissue of the young lamina of *S. cochinchinensis* appears light brown (L* = 70.1 ± 0.265) due to low Al content (**Figure 3(D)**). The results were in line with evidence that old leaves are Al storage in hyperaccumulator species [5,28,38,39]. The increasing color that develops from young to older leaves at both species ($p < 0.0001$) as they age suggests that Al^{3+} binds with pectin or hemicellulose, which constitute major components of the cell wall [12,15]. Functional cell walls act as a barrier to prevent Al^{3+} from penetrating the cell compartment and triggering overproduction of ROS within the cell [13,19].

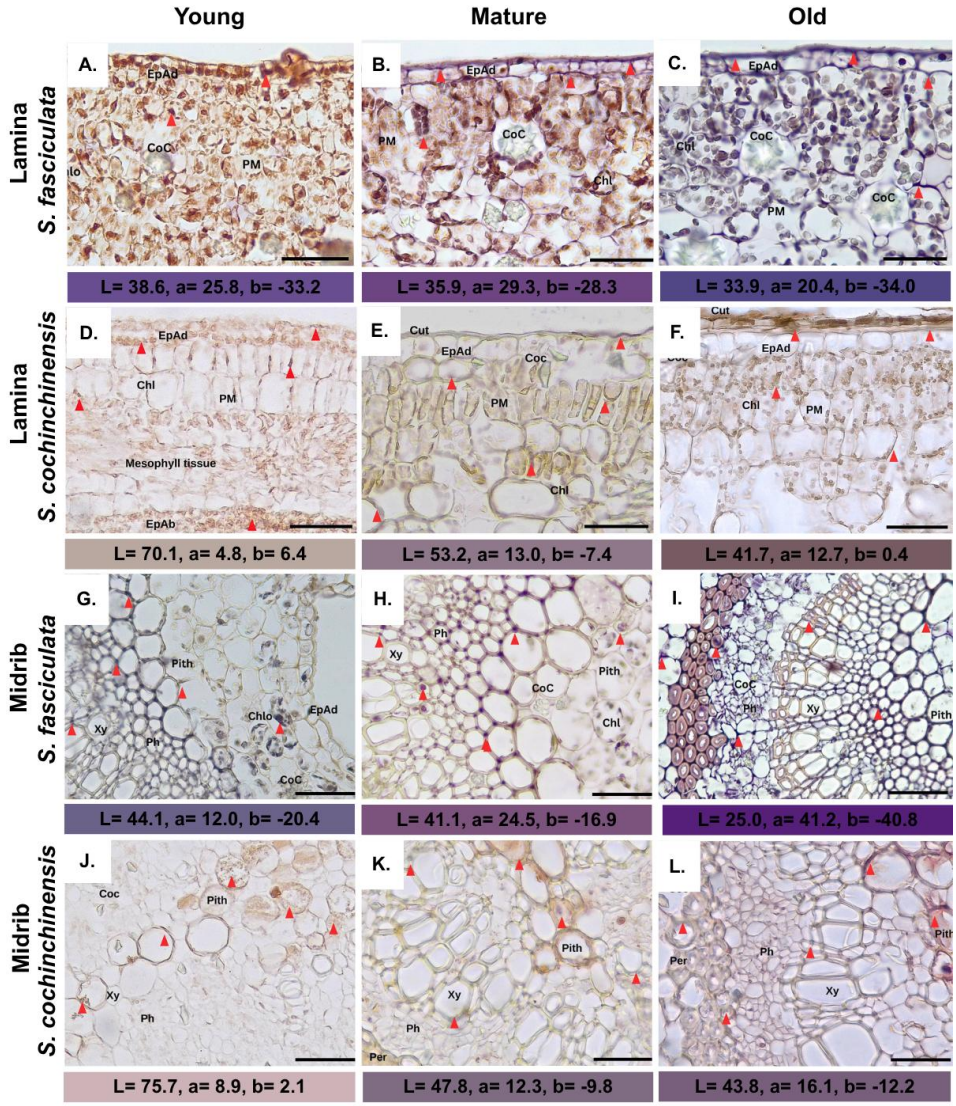

Figure 3 *Symplocos* leaf transverse sections with hematoxylin staining. Abbreviations: Chlo: Chloroplast; COC: Calcium Oxalate Crystal; Coll: Collenchyma; EpAd: Adaxial Epidermis; PM: Palisade Mesophyll; Ph: Phloem; Pith: Pith; Xy: Xylem; Red triangle: Al accumulation. Mag: 10×, scale bar: 200 µm.

The polar nature of the dye and metal cations will form a positively charged hematoxylin - Al (III) bond, allowing interactions with negatively charged components, such as basophilic components, carboxylate groups, and nucleic acids, to produce a brownish to dark purple color [40]. Furthermore, the Al accumulation in *Symplocos* lamina resembles that of Al complexes observed on the blade surface of *Camelia major* [13]. The accumulation of Al in hyperaccumulator plants is more abundant in the epidermal cells of old leaves, supporting the observation that leaf Al content increases with age (p <0.0001), where the primary distribution is in cell walls, especially in pectin or hemicellulose [12,15]. This condition causes the cell walls to act as a barrier, immobilizing Al from penetrating the cell compartment [36,37]. In addition to being in the cell walls, the epidermis and mesophyll parts of the leaves, particularly within the palisade mesophyll tissue, have staining that indicates Al accumulation [3,12,15]. However, sponge cells of both species did not exhibit any dye uptake except for limited staining observed in certain cell walls (**Figures 3(A) -3(F)**). These results contradict those of de Andrande *et al.* [13], who reported purple coloration in the cell walls of sponge parenchyma and absent in palisade mesophyll and chloroplasts. In contrast, palisade parenchyma tissue and chloroplasts were intensely stained purple by

hematoxylin staining, as observed in our recent study of both leaves throughout their lifespan [41], as observed in our recent study of both leaves throughout their lifespan (**Figures 3(A) - 3(F)**), consistent with the results of of Zheng *et al.* [7]; de Andrande *et al.* [13]. The results showed that chloroplasts were capable of absorbing purple coloration, but no damage was observed. Differences in chloroplast size and shape were not observed, indicating that Al did not damage the chloroplasts [13]. Nevertheless, the role of Al in chloroplast metabolism warrants further study.

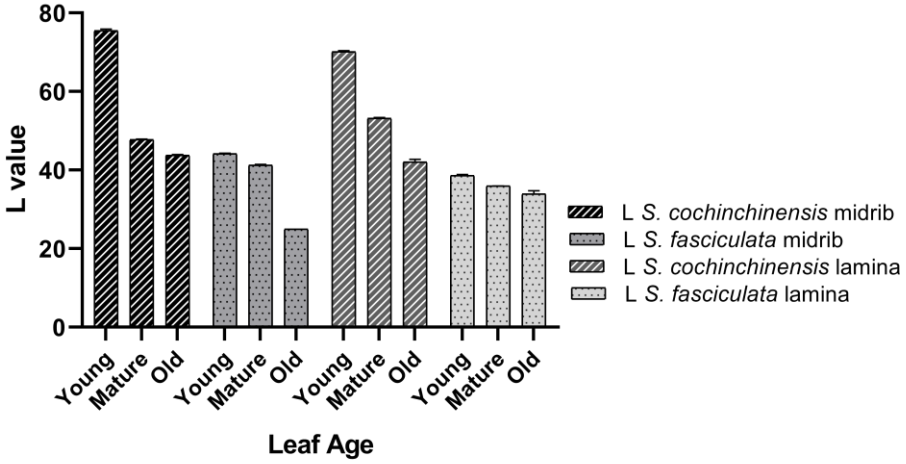

Figure 4 The comparison of L* value ranges from 0 to 100 indicates the light intensity of color as a histological measurement between *S. fasciculata* and *S. cochinchinensis* on the lamina and midrib, performed using a colorimeter. *p*-value < 0.0001.

In the midrib of both older leaves, hematoxylin was clearly visible in the phloem, xylem walls, and cortical parenchyma (pith), presenting a pale brown to purplish hue with red triangles (**Figures 3(G) - 3(L)**). The pericycle *S. fasciculata* (**Figures 3(G) - 3(I)**) presents different shades from light purple (L* = 44.1 ± 0.158) to darkened purple (L*= 25.0 ± 0.112). Moreover, the red triangle indicates the space between cells, which is mainly present outside the vascular bundle and pith. Accordingly, this is also observed in *S. cochinchinensis*; however, the L* value is lighter (**Figure 4**), ranging from nude brown (L* = 75.7 ± 0.141) to light purple (L* = 43.8 ± 0.190), indicating a specific reaction with Al [32]. These results suggest that the distribution of Al throughout the plant occurs through symplastic transport via the vascular tissue, from the root to the shoot, by forming ligand-Al complexes [42,43]. This finding is also in line with the research from de Andrade *et al.* [16], which reported the presence of Al complexes in the vascular bundles, particularly in the xylem region [13]. However, in contrast to the absence of staining in the vascular system.

An inverse correlation between aluminum (Al) content and lightness intensity, as represented by the L* value, has been observed across different leaf ages in *S. fasciculata* lamina ($r = -0.998$) and midrib ($r = -0.919$). Similarly, a phenomenon is also present in the L* value of *S. cochinchinensis* lamina ($r = -0.996$) and midrib ($r = -0.965$). These findings indicate that increasing aluminum content with leaf age is associated with a reduction in the L* value, resulting in a darker color intensity in histological preparations.

SEM EDX mapping assay

The distribution pattern of aluminum accumulation is represented by blue coloration that scatters in the midrib and lamina regions (**Figures 5(A)** and **5(B)**). Notably, the abundant distribution occurs in the epidermis, penetrating the upper and lower epidermal layers, with partial presence in the mesophyll (**Figures 5(A)** and **5(B)**). These findings are consistent with previous research on *C. sinensis* leaves,

where aluminum was predominantly identified in the upper epidermis, with lower concentrations in the lower epidermis, as assessed through proton beam analysis [15]. Conversely, earlier study reported aluminum accumulation in the epidermis cell walls, with minimal detection in the mesophyll cell walls, using X-ray fluorescence [44]. Furthermore, elemental mapping also demonstrates the presence of calcium, represented by green coloration, showing localization in distinct clusters throughout the midrib to the lamina of older *S. fasciculata* leaves (**Figure 5(A)**). In comparison, calcium distribution in older *S. cochinchinensis* leaves is considerably less (**Figure 5(B)**). Additionally, Al is abundant in old leaves, indicating that both species utilize a range of mechanisms, including various minerals, to combat metal stress.

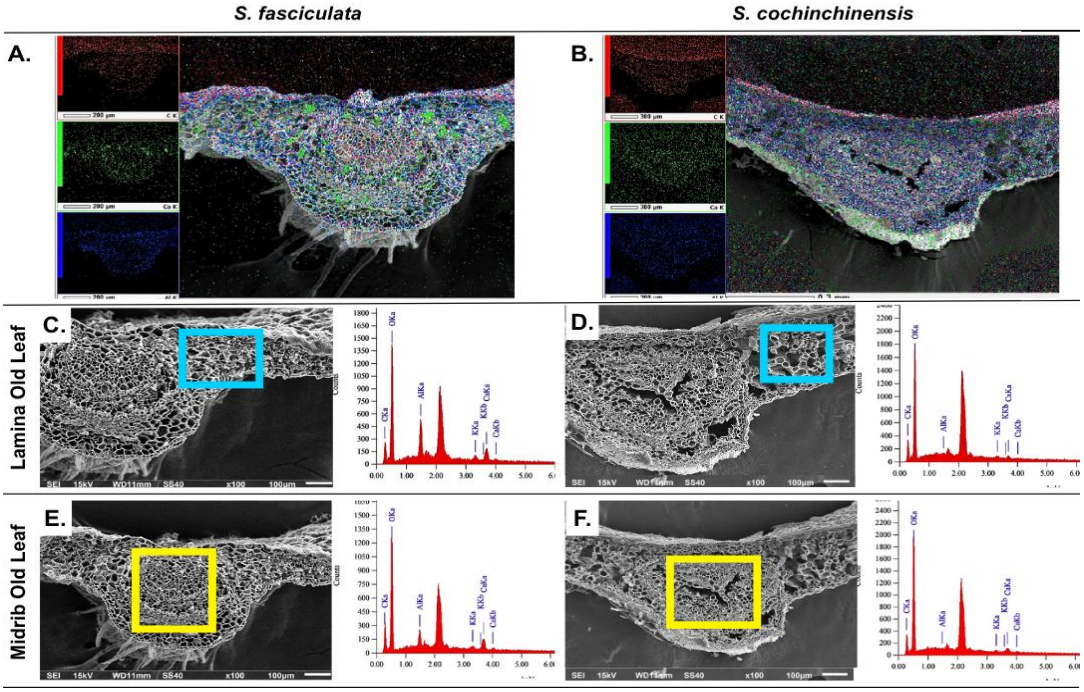

Figure 5 Scanning electron microscope (SEM) mapping element presenting distribution of Al, Ca, and O in old leaves of (A) *S. fasciculata* and (B) *S. cochinchinensis*. Energy-dispersive X-ray spectroscopy (EDX) elements through transverse sections in: (C,E) *S. fasciculata* (D,F) *S.cochinchinensis.* Mag: 150×, scale bar: 200 µm (A); Mag: 100×, scale bar: 300 µm (B); Mag: 100×, scale bar: 100 µm (C-F).

Table 1 EDX mass value (%) of the trace elements from various leaves in midrib (mid) and lamina (lam) of *S. fasciculata* and *S. cochinchinensis.*

Element	*S. fasciculata*						*S. cochinchinensis*					
	Young		Mature		Old		Young		Mature		Old	
	Mid	Lam	Mid	Lam	Mid	Lam	Mid	Lam	Mid	Lam	Mid	Lam
C	40.90	40.65	18.97	13.80	17.08	12.87	50.46	48.31	15.16	12.01	10.55	11.83
O	45.80	37.45	72.93	70.06	72.94	71.45	48.33	49.25	76.08	80.68	80.12	79.50
Al	**1.24**	**2.45**	**2.56**	**6.99**	**3.47**	**7.96**	**0.04**	**0.38**	**0.55**	**0.99**	**0.67**	**1.01**
K	0.02	0.02	0.74	1.57	1.06	1.75	0.08	0.06	2.03	1.61	2.34	2.06
Ca	12.04	19.43	4.80	7.58	5.45	5.97	1.09	2.00	6.18	4.71	6.32	5.60

The Al element exhibited a higher tendency for accumulation in *S. fasciculata*, particularly in the lamina of the old leaves (7.96%) (**Table 1**). Meanwhile, in *S. cochinchinensis*, Al was also most abundant in the lamina of the old leaves (1.01%). Higher Al reflects differences in metal accumulation capacity or physiological adaptations between the 2 species to their environment [15]. The research indicates that Al uptake by Al-accumulating plants is not constrained by the saturation status of other mineral elements, such as Ca, K, and Mg in the soil [45,46]. However, in some cases, Al causes an imbalance in ion homeostasis by increasing the efflux of potassium and an abundant amount of calcium in the cytoplasm [45-47]. Potassium (K), a crucial element in osmotic regulation and metabolic processes [10], exhibited its highest concentration in the midrib of old *S. cochinchinensis* leaves (2.34%).

In contrast to the finding in *S. fasciculata*, the highest potassium content was observed in the lamina of old leaves. The influx of Ca^{2+} into the cytosol helps activate antioxidant enzymes, such as SOD, CAT, and APX [48]. In leaves of *Symplocos*, calcium was predominantly found in the lamina of young leaves of *S. fasciculata* (19.43%), differing from *S. cochinchinensis*, which has the highest calcium observed in the midrib of mature leaves. Aluminum and calcium ions experience competition in binding to the cell wall surface; Ca^{2+} interact with the cell surface, and Donnan's free space in the roots will inhibit the movement of Al^{3+} to the cytoplasm [46]. In addition, Ca^{2+} ions play a pivotal role in increasing the glycolysis process, resulting in an increase in pyruvate and acetyl-CoA, both of which contribute to reducing Al toxicity in cells [47].

Enzymatic assay

Symplocos is commonly known as a hyperaccumulator plant; however, high levels of aluminum (Al) within the cells may not consistently yield positive effects, particularly from a physiological perspective [21]. The presence of aluminum in cells acts as a catalyst that triggers the production of reactive oxygen species (ROS), notably hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl ions (OH^-), resulting in the significant amounts of radicals [49,50]. An imbalance in the production of reactive oxygen species (ROS) can lead to oxidative stress, which subsequently causes lipid peroxidation [17-19].

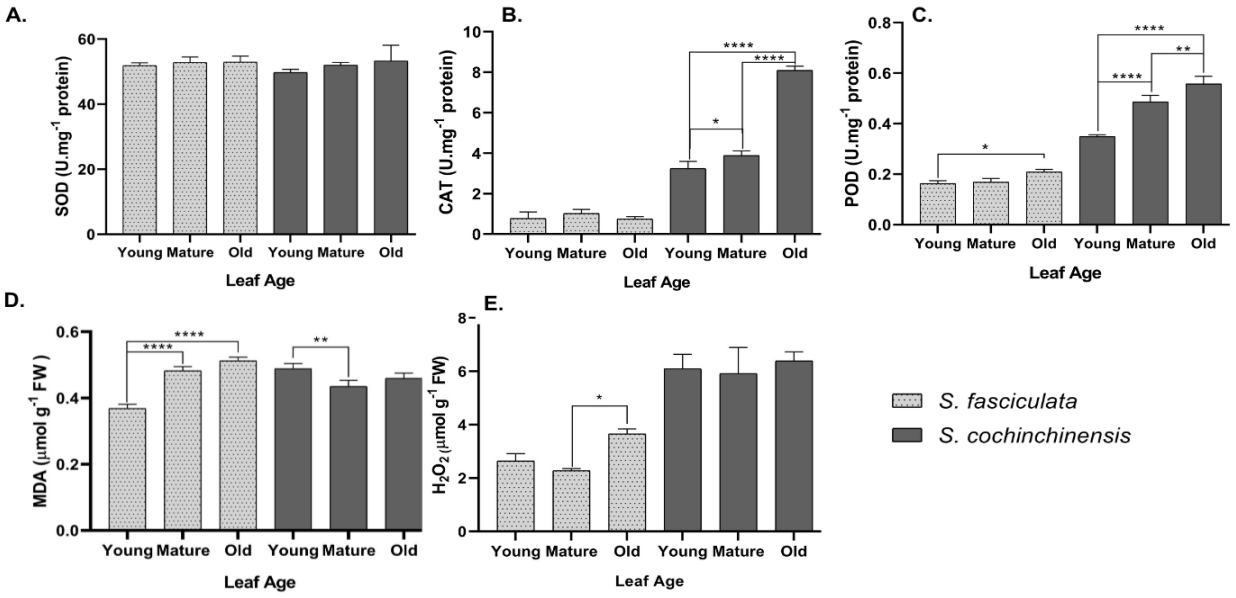

Figure 6 Enzyme activity profiles in *S. cochinchinensis* and *S. fasciculata* leaves at different leaf ages. (A) SOD enzyme activity, (B) CAT enzyme activity, (C) POD enzyme activity, (D) MDA concentration, (E) H_20_2 concentration. Abbreviations: Light gray: *S. fasciculata*, Dark gray: *S. cochinchinensis*, ****: $p < 0.001$, ***: $p < 0.01$, **: $p < 0.03$, *: $p < 0.05$.

Superoxide dismutase (SOD) serves as the primary cellular defense mechanism in stressful oxidative conditions [50]. The results established an increase in SOD activity across leaf ages in both species (**Figure 6(A)**). In contrast to the results of previous studies at the same aluminum accumulator plant, where SOD activity is dominant in young leaves [12], both *Symplocos* species exhibited the highest activity in older leaves [12]. The abundant mass of Al in older leaves has been suggested to influence SOD activity, leading to a significant increase as a response to the mass production of ROS ($p < 0.05$). On the other hand, CAT activity indicated fluctuating activity between the 2 species (**Figure 6(B)**). Both exhibited distinct responses to catalyzed H_2O_2; however, significant differences in CAT activity were detected across leaf ages in *S. cochinchinensis* ($p < 0.0001$). As the presence of Al^{3+} triggers ROS production, younger leaves experience oxidative stress and develop mechanisms to prevent cellular damage [12]. However, although stress levels increase with leaf age, the associated stress-response mechanisms, indicated by elevated catalase activity in mature leaves, appear to remain stable in older leaves of *S. fasciculata* ($p > 0.05$).

Moreover, in high-stress conditions, POD plays a crucial role as a scavenger agent for ROS molecules. Both species demonstrated a significant increase ($p < 0.0001$) in POD activity from young leaves to older leaves (**Figure 6(C)**). *S. fasciculata* exhibited lower POD activity than *S. cochinchinensis*, with activity differing significantly from mature to older leaves ($p = 0.0398$). A subtle defense mechanism in this species may be associated with the activity of other enzymes, such as CAT [12]. In *Vigna radiata,* old leaves exhibit increased oxidative stress, as evidenced by the production of ROS, which in turn leads to higher peroxidase activity and lipid peroxidation [49]. Inversely, vigorous peroxidase activity is recorded in *S. cochinchinensis*, especially in young to mature leaves, then gradually increases to a stable point in old leaves ($p < 0.0001$). According to Lu *et al.* [12], the activity of peroxidase (POD) increases linearly with leaf age. Besides its role as a scavenger enzyme for the H_2O_2 substrate, POD contributes to the lignification of cell walls, where Al^{3+} is mainly stored [51].

Metal stress, in this case, Al, induces lipid peroxidation within the cell, as Al^{3+} binds to the phospholipid surface, replacing other cations such as Ca^{2+} and Mg^{2+}, thereby disrupting the stability of plasma membrane homeostasis [43,44,47]. Furthermore, the degradation of lipids leads to an increasing production of hydrogen peroxide (H_2O_2) by peroxisomes and glyoxisomes under oxidative stress conditions [52]. The H_2O_2 dismutase reaction is a parameter used to assess oxidative stress rates by measuring the catalase activity of CAT and the peroxidation activity of POD, thereby reducing it into water molecules [53,54]. In this research, young leaves exhibited high concentrations of H_2O_2 (**Figure 6(E)**). Over time, the plant develops effective defense mechanisms through enzymatic activity, resulting in a decrease in the concentration in mature leaves [12]. Furthermore, this concentration then increased significantly at its peak in older leaves of both species ($p < 0.0001$). Elevated concentration in old leaves suggests greater Al accumulation, which is associated with heightened oxidative stress [12,15].

Additionally, the product of lipid peroxide, called MDA, is commonly used as a marker for lipid peroxide activity during oxidative stress conditions [22]. In *Vigna radiata* leaves*,* increasing ROS levels lead to higher peroxidase activity and lipid peroxidation [49]. The MDA concentration values exhibited a different pattern between the 2 species, depicting an increasing trend from young to mature leaves in *S. fasciculata* (**Figure 6(D)**), which suggests an accumulation of oxidative stress with leaf age ($p < 0.0001$). Otherwise, in *S. cochinchinensis* (**Figure 6(D)**) exhibited a pattern of higher concentration in young leaves, followed by a decline in mature leaves and stabilization thereafter. Consistent with this pattern, POD and CAT activities increased efficiently from mature to old leaves ($p < 0.0001$), decomposing high concentrations of H_2O_2 in older leaves and thereby reducing lipid peroxidation [50-52]. This suggests that the detoxification mechanism against metal stress in both species is more effective in older leaves. Furthermore, a study by Shahnaz *et al.* [55] indicated that aluminum significantly increased MDA levels, alongside peroxidase and other aldehyde compounds [54,55]. These observations suggest that aluminum stress can

adversely affect metabolism and the activity rate of enzymes involved in breaking down ROS in cells [51,54,55].

Many external factors may disrupt the activity of antioxidant enzymes, such as replacing metal cofactors with aluminum [56]. However, the correlation between aluminum content, enzyme activity (SOD, CAT and POD), lipid peroxidation product (MDA), and oxidative markers (H_2O_2) across leaf age in 2 species is incredibly varied (range $r = -0.6 – r = > 0.7$). A high aluminum content, which significantly increases with leaf age ($p = 0.002$), exhibits a strong positive correlation with SOD activity in *S. fasciculata* ($r = +0.9156$), playing a crucial role as the first line of defense [12]. However, a peculiar result shows a very weak relationship between aluminum content and CAT activity ($r = +0.0247$), as CAT activity across leaf age in *S. fasciculata* yields no significant results ($p > 0.05$). The consequence of this is a robust correlation between aluminum content and MDA levels ($r = +0.9830$), similar to H_2O_2 ($r = +0.7240$), indicating a high level of oxidative stress in *S. fasciculata*. Such elevated stress likely constrains the ability of other enzymes [51-53], such as SOD and POD ($r = +0.9045$), to effectively mitigate the damage. Moreover, a strong defense mechanism against aluminum stress was displayed by *S. cochinchinensis*. The correlation between aluminum content and enzyme activity in this species demonstrates a highly positive correlation for all enzymes SOD ($r = +0.9998$), CAT ($r = +0.9998$), and POD ($r = +0.8327$), and an inverse relationship with the oxidative stress marker MDA, which exhibits a moderate negative correlation ($r = -0.6245$). On the other hand, H_2O_2 concentrations exhibit a mild positive correlation with aluminum content ($r = +0.5427$), indicating that enzyme activity has a practical impact due to its high correlation to maintain oxidative stress in a stable condition (SOD - CAT: $r = +0.8319$, SOD - POD: $r = +0.9997$, CAT - POD: $r = +0.8429$). These mechanisms lead to a speculation pattern that *S. cochinchinensis* adaptively responds to aluminum stress by performing dynamic enzyme activity.

Conclusions

This investigation confirms *S. fasciculata* and *S. cochinchinensis* are Al Hyperaccumulator plants. Accumulating Al in the old leaves of *S. fasciculata* is higher than in *S. cochinchinensis*. The histological assay shows Al accumulates in cell walls, epidermal tissue, mesophyll, and chloroplasts. An inverse correlation between Al content and L* value, indicating that higher al content tends to result in a darker color, approaching purple. Confirming this data, SEM-EDX and mapping clearly show higher Al accumulation in the lamina. Both species exhibit increased SOD activity, CAT activity was significantly different across leaf ages in *S. cochinchinensis*, yet not significantly different in *S. fasciculata.* Additionally, POD activity increases in all leaves from both species. Furthermore, H_2O_2 fluctuates and then stabilizes in old leaves, resulting in MDA consistently increasing in *S. fasciculata*. However, significant difference between young and mature leaves in *S. cochinchinensis*, suggesting that young leaves experience a high level of lipid peroxidation. The correlation between aluminum content and enzymatic activity, lipid peroxidation products, and stress markers shows a strong positive relationship in *S. fasciculata* for SOD, POD, MDA, and H_2O_2; however, a weak correlation with CAT activity suggests a stable enzymatic adaptation. Lastly, *S. cochinchinensis* shows a strong correlation for SOD, CAT, POD, and H_2O_2, except for MDA, which portrays a negative correlation due to highly dynamic stress management.

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Declaration of generative AI in scientific writing

The authors declared that generative AI tools, including Grammarly Edu, were utilized solely to support the writing process, specifically language refinement and grammar correction. These tools were not involved in generating original content, interpreting results, or analyzing data. The authors have thoroughly

reviewed the entire manuscript and take full responsibility for its accuracy and conclusions.

CRediT author statement

Dewi Puspita Sari: Investigation, Visualization, Writing - Original draft preparation. **Bambang Retnoaji**: Data curation; Software. **Nastiti Wijayanti**: Conceptualization, Supervision, Reviewing, and Editing. **Purnomo**: Reviewing and Language editing.

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