BIOTROPIA ISSN: 0215-6334 | e-ISSN: 1907-770X
The Southeast Asian Journal of Tropical Biology
Vol. 32 No. 1, 2025: 47 - 56
DOI: 10.11598/btb.2025.32.1.2354

Research Article

METABOLITE PROFILING OF MORINGA USING PY-GCMS AND TOLERANCE EVALUATION TO ALUMINUM ON IN VITRO CULTURE

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ARTICLE HIGLIGHTS

- Four accessions of *Moringa oleifera* Linn. from different regions in Indonesia were evaluated to identify with tolerance to aluminium (Al) stress
- Eight metabolites show a very high correlation with acetic acid (one of the metabolites associated with $AlCl_3$ stress) including cyclopentene, 2-allyphenol, 4-ethynyl-6-8-dioxane, vinyl ether, ethanone 1-oxiranyl, 2-methylpyridine, 2-butanone, and ethanesulfonic acid.
- This research makes a significant contribution to understanding aluminium tolerance in *M. oleifera* by identifying tolerant accessions, clustering relevant traits, and highlighting key metabolites to in vitro culture.

Article Information

Received : 17 September 2024
Revised : 9 March 2025
Accepted : 14 March 2025

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Reviewers:
Hasyyati shabrina & Rani Agustina Wulandari

ABSTRACT

Moringa oleifera Linn. has received substantial scientific interest due to its numerous bioactive compounds and its function as a nutritional resource. The absorption of aluminum by plants hinders several metabolic and physiological processes, leading to inhibited plant development and decreased agricultural output. Some accessions from different regions in Indonesia were evaluated to identify those with tolerance to aluminum (Al) stress. Al-tolerant selection was carried out in vitro through the selection method for Al stress by adding 0, 50, 100, 250, and 500 mg/L of $AlCl_3$ to the media. Furthermore, identifying the metabolite profile of four *M. oleifera* accessions from four distinct regions in Indonesia: Blora, Bogor, Enrekang, and Bima, has been done using Py-GCMS. Specific metabolites associated with tolerance to Al stress and organic acids need to be identified. The highest survival rate was observed in the Bogor and Blora accessions when exposed to $AlCl_3$ at concentrations ranging from 0 to 250 mg/L, demonstrating greater tolerance to $AlCl_3$ than other accessions based on various variable, such as shoot height, number of shoots, number of petioles on a medium containing 100 to 250 mg/L of $AlCl_3$. The mean value was not statistically different from the control. Acetic acid was identified as one of the metabolites associated with $AlCl_3$ stress. A total of 21 metabolites were specifically correlated with acetic acid in a positive manner, among which 8 metabolites including cyclopentene, 2-allyphenol, 4-ethynyl-6-8-dioxane, vinyl ether, ethanone 1-oxiranyl, 2-methylpyridine, 2-butanone, and ethanesulfonic acid exhibited a very high correlation.

Keywords: accession, aluminum tolerant, in vitro culture, metabolite profiling, *Moringa oleifera*

INTRODUCTION

Aside from its role as a nutritional resource, *Moringa oleifera* Linn. has received significant scientific interest for having numerous bioactive compound. These substances, contained in seeds, pods, and leaves, exert significant physiological effects on humans. *Moringa* exemplifies a food source that is rich in essential elements, such as minerals, vitamins, and proteins, making it a reliable and long-lasting source of nourishment (Yuniati *et al*. 2022).

According to Srivastava *et al*. (2023), the environmentally conscious cultivation of *Moringa* is in line with the ideas of sustainable farming, which makes it a sustainable choice. Humans consume the entire *M. oleifera* plant, including its leaves, fruits, immature pods, and flowers, as traditional food. Various countries, including India, Hawaii, the Philippines, and other African countries, utilize the flowers, fruits, foliage, and early seed pods of this tree as exceptionally nutritious vegetables (Yamaguchi *et al*. 2021).

Moringa tree produces between 43 and 115 tonnes of biomass per hectare annually. Its leaves contain high levels of dry matter, crude protein, essential amino acids, and phytoactive substances, such as phenolic acids and flavonoids. According to Kholif *et al*. (2016) and Nouman *et al*. (2016), they also contain high levels of vitamins A, B, C, and E (Zheng *et al*. 2022).

Conventional propagation techniques for *M. oleifera* predominantly involve the cultivation through seedlings or vegetative cuttings. Debnath and Juran (2020) have identified several obstacles associated with conventional propagation methods, including a limited quantity of explants, lethargic growth, and heightened vulnerability to pests and diseases. Using in vitro micropropagation, a widely applied technique that has significantly improved the quality of several plant propagations, would be an alternative method (Zheng *et al*. 2022).

The reduction of arable land from industrialization and conversion has posed a significant obstacle to propagating *M. oleifera*. To address this challenge, using suboptimal land becomes an alternative option. According to Fanindi *et al*. (2020), Indonesia has approximately 108.8 million ha of suboptimal land area as acid dry land, with 62.6 million ha still having the potential for agricultural use. However, inefficient land use is constrained by several abiotic issues, such as low pH soil and high aluminum (Al) saturation. These characteristics inhibit the photosynthetic process and plant growth, resulting in reduced agricultural yields (Xu *et al*. 2018; Fanindi *et al*. 2020).

Acid-dry land is defined as soil with a pH below 5, indicating acidity. The majority of agricultural land in Indonesia has acidic soil conditions. High rainfall is one contributing factor. Increased rainfall can accelerate the degradation of soil minerals. In moist tropical regions, intensive weathering and nutrient leaching processes cause the soil to be acidic and have high aluminum saturation. Soil acidity can result from prolonged land use and excessive application of chemical fertilizers (Mulyani & Sarwani 2013).

Acid soils make up approximately 40% of the world's potentially cultivable areas. Aluminum ranks as the third most plentiful element, behind oxygen and silicon, according to Riaza *et al*. (2018). Plants absorption of aluminum hinders several metabolic and physiological processes, resulting in inhibited plant development and decreased agricultural output (Liu *et al*. 2023).

Soil pH significantly influences plant metabolism, affecting nutrient availability, enzyme activity, and overall biochemical pathways. In acidic soil conditions, essential nutrients, such as phosphorus, calcium, and magnesium become less available, potentially altering the secondary metabolite profile of plants (Sanches *et al*. 2006). To comprehensively understand these chemical alterations, a precise and sensitive analytical technique is required.

Pyrolysis gas chromatography and mass spectroscopy (Py-GCMS) is a direct method that analyzes the pyrolytic behavior of biomasses and characterizes their volatile products. Recently, there has been a significant increase in interest in this technique due to its ability to identify all pyrolytic products more efficiently than the GCMS of condensable products (liquid fraction). Identification of these products under various operating conditions necessitates complete condensation (Bensidhom *et al*. 2021).

According to Inostroza-Blancheteau *et al*. (2012), organic acids are very important for both tolerance and avoidance pathways because they make stable complexes with Al^{3+} inside and outside of cells that neutralize it. Various crops excrete distinct varieties of organic acids. For instance, studies have shown that the roots of wheat (Yang *et al*. 2011) and eucalyptus (Li *et al*. 2021) secrete large levels of malic acid and acetic

acid. Furthermore, researchers have identified citric acid secretion in maize, common bean, and cassia. Additionally, oxalic acid secretion has been discovered in buckwheat (Liu *et al.* 2023).

The objective of this research was to characterize the metabolite profiles of four *M. oleifera* accessions obtained from four distinct regions in Indonesia, i.e., Blora, Bogor, Enrekang, and Bima, utilizing Py-GCMS. The research further aimed to assess the responses of these accessions to aluminum chloride ($AlCl_3$) stress to in vitro culture. Additionally, to identify specific metabolite molecules, excluding organic acids, that are linked to tolerance to Al stress.

MATERIALS AND METHODS

Materials and Experimental Design

The materials used were shoot cultures of 4 accessions of *M. oleifera* at 6 weeks after culture (WAC). The basal medium used was DKW medium (Driver & Kuniyuki 1984), supplemented with $AlCl_3$ according to the treatment tested. The experimental design was a completely randomized factorial design, with two factors tested: 1) The type of *M. oleifera* accessions: Blora (Java Island), Bogor (Java Island), Bima (Lombok Island), and Enrekang (Sulawesi Island); 2. Concentrations of $AlCl_3$ (0, 50, 100, 250, and 500 mg/L). This experiment used 12 replications and 240 experimental units. The variables observed were: shoot height, number of shoots, number of petioles, and number of roots, which were observed between 0 and 6 WAC, whereas metabolite profiling was done at 6 WAC.

Py-GCMS Analysis

The samples were dried *M. oleifera* explants derived from stem and leaves obtained from in vitro culture at 6 WAC. The sample weight was 20 mg. The specimen was placed inside an SF PY1-EC50F eco-cup and subsequently sealed with glass wool. The Eco cup underwent pyrolysis at a temperature of 500 °C for 0.1 minutes using a multi-shot pyrolizer (EGA/PY-3030D). The pyrolizer was connected to a GC/MS QP NX system (Shimadzu, Japan) equipped with an SH column with an interface 300 °C of temperature. The column of Rxi-5Sil MS, manufactured by Restek in Germany, was used with electron impact at 70 eV and helium as the carrier gas. The applied pressure was 20.0 kilopascals (with a total flow rate of 15.9 millilitres per minute and a column flow rate of 0.61 millilitres per minute). The temperature of the GC was as follows: it was initiated at 50 °C for 1 minute, then increased to 300 °C (5 °C every minute), and the stable temperature was adjusted for 13 minutes. Pyrolysis metabolites were determined by comparing the retention time and mass spectrum data with the NIST LIBRARY 2017.14.

Data Analysis

The quantitative data from observations were analyzed using the Analysis of Variance (ANOVA) F Test at $\alpha \leq 5\%$ significant level. When there were substantial differences between treatments tested, the analysis was further conducted using Duncan's Multiple Range Test (DMRT) using DSAASTAT V.1.1 (Microsoft Excel Plugins). Hierarchical cluster heatmap analysis, partial least squares discriminant analysis (PLS-DA), and principal component analysis (PCA) were analyzed using Metaboanalyst 6.0 (Pang *et al.* 2024).

RESULTS AND DISCUSSION

The survival rates of four accessions of *M. oleifera* shoot grown on DKW medium supplemented with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$ are shown in Figure 1. The highest survival rate was observed in the Bogor accession, with 100% survival being reached when exposure was given to $AlCl_3$ at concentrations ranging from 0 to 250 mg/L. A 100% survival rate was exhibited by the Blora accession when exposed to 0 and 50 mg/L of $AlCl_3$. Higher susceptibility to $AlCl_3$ stress was exhibited by the Bima and Enrekang accessions, as indicated by their poor survival rates in the 100–500 mg/L $AlCl_3$ treatment. The greater susceptibility of these two accessions to aluminum stress was suggested by their poor survival rates in comparison to the Bogor and Blora accessions.

A rapid decline of all treated shoot explants was led by exposure to high concentrations of $AlCl_3$ within a 6 WAC, illustrating the acute sensitivity of *M. oleifera* to intense $AlCl_3$. Conversely, a gradual effect on plant mortality was demonstrated by lower doses (from 0 to 100 mg/L), with a complete mortality rate being observed by the sixth week. Interestingly, a remarkable resilience was exhibited by the lowest $AlCl_3$ concentrations tested (0 to 100 mg/L), with all explants surviving for up to 6 weeks post-treatment. The mortality rates for these explants were observed at the 6-week mark, ranging between 42% and 48%, underscoring a potential threshold for *M. oleifera's* tolerance to $AlCl_3$ exposure (Fig. 1).

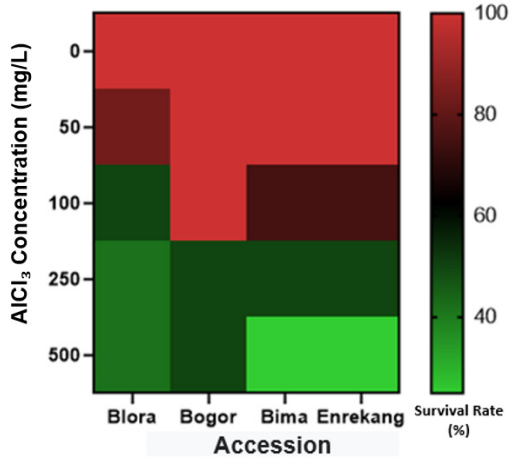

Figure 1 The survival rate of four different accessions of M. oleifera cultured on DKW medium supplemented with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$ at 1 - 6 weeks after culture (WAC)

To investigate the response of four different accessions of *M. oleifera* cultured on DKW medium supplemented with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$, an analysis of variance (ANOVA) was used to analyze the data, as displayed in Table 1. It was indicated by the analytical data that different accessions of *M. oleifera* have a significant impact on shoot height, number of shoots, and number of roots, but no significant effect was observed on the number of shoots. On the other hand, a notable impact on all variables tested was observed with the application of $AlCl_3$. An interaction was found to occur between accessions and $AlCl_3$, affecting all variables tested except the number of shoots (Table 1).

The mean values of shoot height, number of shoots, number of petioles, and number of roots of four accessions of *M. oleifera* on DKW medium supplemented with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$ at 6 WAC is presented in Table 2. On the shoot height variable, the highest mean value was found in the Bogor and Bima accessions on DKW medium with an addition of 50 to 100 mg/L of $AlCl_3$, and was not found to be significantly different from the control medium. Meanwhile, in the Enrekang and Blora accessions, the value of the shoot height variable was observed to be lower than that in Bogor and Bima accessions. In media with 250 mg/L of $AlCl_3$, the average shoot height value was observed to have decreased, and the lowest shoot height value was found in the medium supplemented with 500 mg/L of $AlCl_3$ in all accessions tested (Table 2).

On the number of shoot variables, the highest value was observed in the Blora accession, which was cultured on DKW medium supplemented with 50 mg/L of $AlCl_3$ and was found to be significantly different from the others. Meanwhile, the lowest number of shoots was recorded in the Bogor accession, which was cultured on DKW medium with the addition of 500 mg/L $AlCl_3$. For the number of petiole variables, the highest value was observed in the Bogor accession on medium with 100 mg/L $AlCl_3$, while the lowest number of petioles was recorded in the Enrekang accession on medium with 500 mg/L $AlCl_3$. Regarding the number of roots, it was observed that the higher the concentration of $AlCl_3$ given, the less the number of roots increased. The highest number of roots was found in the control medium or without the addition of $AlCl_3$, especially in the Bima accession, which was found to be significantly different from the others. In the medium supplemented with 500 mg/L $AlCl_3$, almost no roots were formed; only a few roots were observed in the Bogor and Bima accessions (Table 2).

Table 1 Analysis of variance (ANOVA) of four different accessions of *M. oleifera* on shoot height, number of shoots, number of petioles and number of roots at at 6 weeks after culture (WAC)

No	Variable	F value & significance			CV(%)
		Accession	$AlCl_3$	Accession x $AlCl_3$	
1.	Shoot height	16.60**	38.95**	2.74**	32.85
2.	Number of shoots	1.84ns	3.81**	1.08ns	41.92
3.	Number of petioles	3.99**	25.92**	2.71**	24.63
4.	Number of roots	15.89**	25.76**	2.77**	59.15

Notes: * = significant at α ≤ 5%; ** = very significant at α ≤ 1%; ns = not significant.

Table 2 Average shoot height, number of shoots, number of petioles, and number of roots of four different accessions of *M. oleifera* at 6 WAC

Accession	$AlCl_3$ (mg/L)	Shoot height	Number of shoots	Number of petioles	Number of roots
Blora	0	6.58 ± 0.69^{bcde}	1.38 ± 0.18^{abc}	7.63 ± 0.53^{cde}	2.13 ± 0.72^{defg}
	50	6.99 ± 1.01^{bcd}	**2.00 ± 0.38^{a}**	8.88 ± 1.04^{abcd}	1.00 ± 0.33^{ghij}
	100	6.35 ± 1.42^{cde}	1.25 ± 0.16^{bc}	7.00 ± 0.85^{def}	2.75 ± 0.49^{bcdef}
	250	5.50 ± 0.59^{def}	1.63 ± 0.18^{abc}	6.88 ± 0.35def	2.75 ± 0.41^{bcdef}
	500	2.34 ± 0.12^{g}	1.06 ± 0.06^{bc}	5.00 ± 0.46^{fgh}	0.13 ± 0.13^{j}
Bogor	0	8.21 ± 0.49^{abc}	1.25 ± 0.16^{bc}	7.38 ± 0.38^{de}	4.25 ± 0.65^{ab}
	50	6.34 ± 0.73^{cde}	1.75 ± 0.25^{ab}	9.50 ± 0.89^{abc}	4.25 ± 0.75^{ab}
	100	**9.16 ± 0.71^{a}**	1.50 ± 0.19^{abc}	**10.25 ± 1.00^{a}**	2.88 ± 0.23^{bcdef}
	250	6.86 ± 0.45^{bcd}	1.75 ± 0.37^{ab}	8.88 ± 0.61^{abcd}	3.50 ± 0.63^{abcde}
	500	2.21 ± 0.05^{g}	1.00 ± 0.00^{c}	4.63 ± 0.26^{gh}	0.13 ± 0.13^{j}
Bima	0	8.66 ± 0.55^{ab}	1.16 ± 0.13^{bc}	9.75 ± 0.37^{ab}	**4.50 ± 0.42^{a}**
	50	8.45 ± 0.45^{abc}	1.14 ± 0.12^{bc}	8.00 ± 0.27^{bcde}	3.63 ± 0.56^{abcd}
	100	8.21 ± 0.76^{abc}	1.38 ± 0.18^{abc}	8.00 ± 0.57^{bcde}	3.75 ± 0.45^{abc}
	250	4.58 ± 0.93^{ef}	1.25 ± 0.16^{bc}	6.25 ± 0.82^{efg}	2.50 ± 0.76^{cdefg}
	500	2.40 ± 0.25^{g}	1.13 ± 0.13^{bc}	5.00 ± 0.27^{fgh}	0.50 ± 0.27^{ij}
Enrekang	0	5.65 ± 0.86^{def}	1.25 ± 0.16^{bc}	9.00 ± 0.93^{abcd}	2.38 ± 0.50^{cdefg}
	50	3.76 ± 0.43^{fg}	1.50 ± 0.27^{abc}	8.00 ± 0.57^{bcde}	1.75 ± 0.45^{fghi}
	100	4.58 ± 0.48^{ef}	1.50 ± 0.27^{abc}	7.00 ± 0.50^{def}	0.63 ± 0.26^{hij}
	250	3.69 ± 0.08^{fg}	1.13 ± 0.13^{bc}	6.00 ± 0.60^{efgh}	2.00 ± 0.38^{efgh}
	500	2.24 ± 0.16^{g}	1.13 ± 0.13^{bc}	4.00 ± 0.60^{h}	0.00 ± 0.00^{j}

Notes: Numbers followed by the same letter in the same column are not significantly different based on Duncan's multiple range test at $\alpha \leq 5\%$; presented values are means ± standard error (SE) of 12 replicates.

The performance of four accessions of *M. oleifera* (Blora, Bogor, Bima, and Enrekang) is displayed in Figure 2, where they were cultured on DKW medium supplemented with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$ at 6 WAC. Remarkable tolerance to $AlCl_3$ concentrations up to 250 mg/L was exhibited by the Bogor and Blora accessions, as evidenced by their robust shoot growth compared to the other accessions (Bima and Enrekang). This suggested that a higher adaptive capacity to moderate aluminum stress was possessed by Bogor and Blora accessions likely due to more effective physiological mechanisms that mitigate toxicity effects. However, at 500 mg/L, a drastic decline in growth was observed, indicating that this concentration has exceeded beyond the accessions' tolerance threshold, leading to severe stress, toxicity, or inhibition of essential physiological processes. In comparison, weaker performance even at lower concentrations (50 - 250 mg/L) was shown by the Bima and Enrekang accessions, suggesting that their stress-response mechanisms we re less effective, possibly due to lower antioxidant activity or impaired nutrient uptake under high aluminum conditions. The shoot quality of the Bogor and Blora accessions at 0 - 250 mg/L was observed to be superior, with more vigorous growth and longer shoots than that of the other accessions. However, at 500 mg/L, a sharp decline was seen in the Bogor and Blora accessions, with significantly lower shoot quality, possibly due to the rapid accumulation of toxic effects beyond their detoxification limit (Fig. 2).

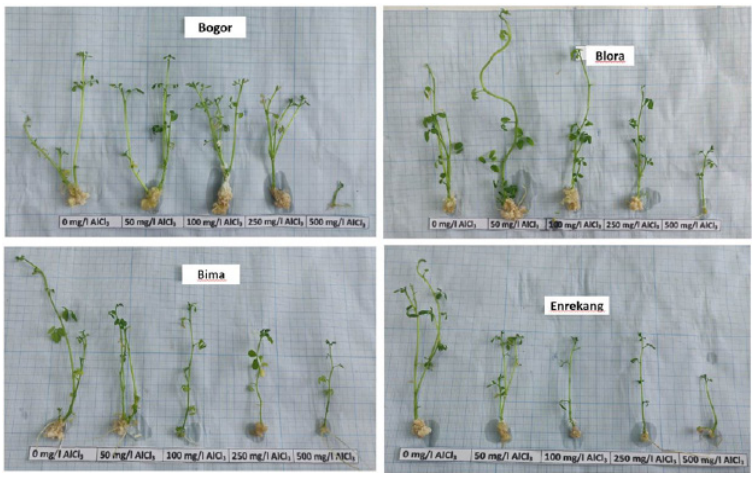

Figure 2 Performance of four accessions of *M. oleifera* (Blora, Bogor, Bima, and Enrekang) cultured on DKW medium supplemented with 0, 50, 100, 250, and 500 mg/L of $AlCl_3$ at 6 WAC

This tolerance to $AlCl_3$ in the Bogor accession was reflected in the shoot heights, number of petioles, and roots, which was aligned with the findings of Peixoto *et al.* (2002), where adaptability to aluminum stress was demonstrated by the photosynthetic apparatus of two sorghum cultivars, exhibiting differences in physiological responses between Al-tolerant and Al-sensitive varieties. In our study, the plant's capability to sustain shoot growth and maintain root development under combined abiotic stresses was hinted at, suggesting that intrinsic mechanisms were conferred for tolerance, perhaps similar to the progressive, sustained adjustments in the photosynthetic apparatus observed by Peixoto *et al.* in sorghum (Table 2; Fig. 2).

Abiotic stresses are responded by plants through the sharing of a diverse range of molecular compounds. Basically, two principal strategies are recognized in response to abiotic stress. The first strategy involves the synthesis of osmoprotectant molecules by the plant to prevent cell damage, while the second strategy is based on a repair mechanism during rehydration (Martin *et al.* 2018). The metabolite profile of four accessions of *M. oleifera* cultured on DKW medium for six weeks is shown in Figure 3.

Pyrolysis Gas Chromatography-Mass Spectrometry (Py-GCMS) was used to identify the samples, yielding distinct chemical groups that were produced from hydrocarbon breakdown. These chemicals included n-heptane and 2-methyl hexane. Besides this, some types of chemicals were capable of breaking down carbon chains, for example, p-methyl guaiacol and 4-allyl-2-methoxy acetate. The latter category refers to a common aromatic chemical that is most likely derived from the breakdown of polyphenolic structures.

The analysis of GCMS data is conducted by categorizing chromatogram peaks that are observed to exhibit alterations (Fendi & Kurniaty 2016). The color variations are used to indicate different levels of correlation between metabolites. Positive correlations are represented by red shades, meaning that when one metabolite increases, the other also increases. In contrast, negative correlations are indicated by blue shades, where an increase in one metabolite corresponds to a decrease in the other. White or lighter shades suggest weak or no correlation between the metabolites. The clustering observed in the heatmap implies that certain metabolites are exhibited in similar behavioral patterns (Fig. 3A).

Acetic acid is identified as one of the metabolites associated with $AlCl_3$ stress. Dolui *et al.* (2023) reported that acetic acid can improve aluminum tolerance in plants by regulating water content, proline accumulation, and oxidative stress defense. This suggests that acetic acid may act as a signaling molecule enhancing plant adaptation to Al stress. A total of 21 metabolites have been found to have a specific positive correlation with acetic acid. A very high correlation is shown by eight metabolites,

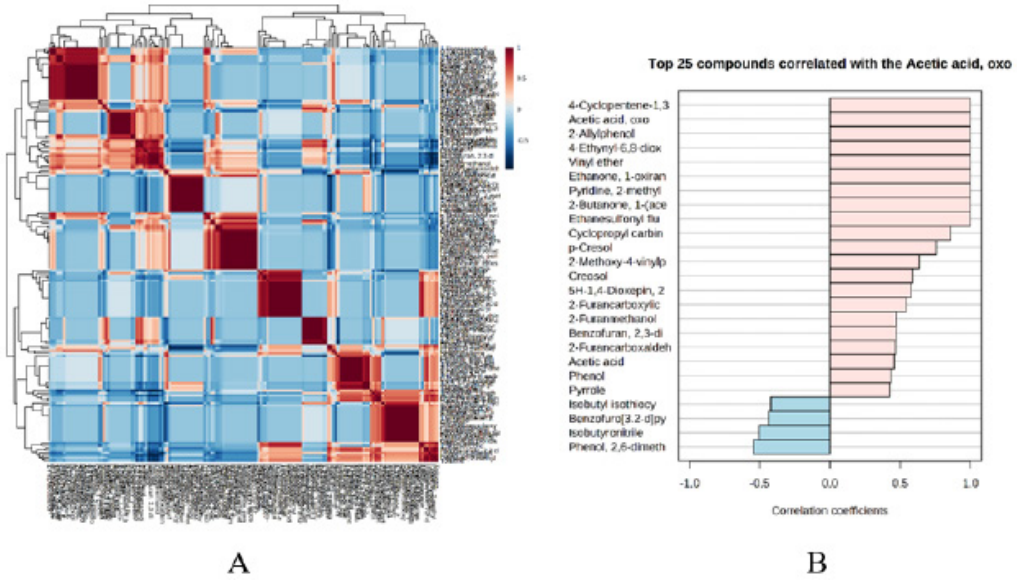

Figure 3 (A) Correlation analysis between the metabolites of four accessions of *M. oleifera*, cultured on DKW medium at 6 WAC; (B) the organic acid acetic acid, which serves as an indicator of $AlCl_3$ tolerance, correlates with 25 metabolites

including cyclopentene, 2-allylphenol, 4-ethynyl-6-8-dioxane, vinyl ether, ethanone 1-oxiranyl, 2-methylpyridine, 2-butanone, and ethanesulfonic acid. Some of these compounds (2-methylpyridine and 2-butanone) are involved in organic chemistry reactions that could potentially influence stress response mechanisms in *M. oleifera*. In addition, a negative relationship with acetic acid is exhibited by four metabolites. These are identified as isobutyl isothiocyanate, benzofuro (3,2-d) pyridine, isobutyronitrile, and 2,6-dimethylphenol. Metabolites that are positively correlated with acetic acid are represented by pink bars, meaning that their levels increase as acetic acid levels rise, while negative correlations are indicated by blue bars, where these metabolites are observed to decrease as acetic acid levels increase. The correlation coefficients on the x-axis are used to indicate the strength of these relationships. This analysis suggests that a role is played by acetic acid in multiple metabolic pathways, with some metabolites responding positively and others negatively, providing insights into the metabolic adaptations of *M. oleifera* under $AlCl_3$ stress (Fig. 3B).

Acetic acid (CH_3COOH) is a colorless liquid substance which has a strong odor, and has a distinctive taste. It is soluble in water, alcohol, glycerol, and ether. At standard atmospheric pressure, the boiling point is about 118.1 °C. *M. oleifera* extract produces acetic acid at a concentration of approximately 2.35%. Acetic acid is a non-flammable chemical. In the pyrolysis system, oxidation produces this acetic acid molecule from ketone compounds that easily turn into acids through oxidation (Emi *et al*. 2014).

The chelating efficacy of amino acids is shown in mitigating heavy metal stress. These chelators, particularly acetic acid, play a critical role by forming stable complexes with Al^{3+} ions, a physiological response noted for mitigating the detrimental effects of Al in plants (Vega *et al*. 2022). When acetic acid is exuded by plant roots, it dissociates into acetate ions (CH_3COO^-), which can bind to Al^{3+}, forming soluble aluminum-acetate complexes, such as $Al(CH_3COO)_3$. This complexation reduces the free Al^{3+} concentration in the soil solution, thereby lowering its toxicity and preventing aluminum from binding to root cell walls. Additionally, some plants enhance their resistance by upregulating genes responsible for organic acid transporters, increasing the secretion of acetic acid and other chelators under aluminum stress (Sun *et al.* 2020).

The Partial Least Squares Discriminant Analysis (PLS-DA) is shown in Figure 4A. The metabolites Ethanol, 1-eth, Nitrous Oxide, Acetaldehyde, 2-Propanone-1, (E)-4-(3-Hydro), and 2-Pyrrolidinone were exhibited with significant variability in their significance in projection (VIP) values. These findings suggested that the most significant influence on the four *M. oleifera* accessions was exerted by these five metabolites.

Klau *et al*. (2022) said that observation metrics with high VIP values may be used as differentiating features among the assessed treatments. The size of the VIP value is directly proportional to its capacity to distinguish among the tested samples. Nitrouse oxide (N_2O) is a versatile, highly diffusible bioactive gaseous free radical that influences various biological processes in plants, alleviating biotic stress by inducing host resistance and abiotic stress by acting as an antioxidant (Kalra & Babbar 2010). The VIP scores, derived from Partial Least Squares Discriminant Analysis (PLS-DA), underscore the contributory metabolites that distinguish between treatment groups. A parallel can be drawn to the findings of Matsuse *et al*. (2022), who utilized similar approaches to elucidate key metabolic changes in response to stress conditions. Furthermore, the variations in metabolite profiles, which are pivotal in understanding the plant's tolerance capabilities, have been illuminated by the robust chemometric analysis (Fig. 4A). The distinct clusters and VIP scores from the PLS-DA analysis have been illustrated to demonstrate the discriminatory power of the metabolites, offering a predictive understanding of the potential effects of different treatments.

In the PCA plot (Fig. 4B), three prominent clusters are observed. Bima and Enrekang accessions were grouped within the same cluster and shown to exhibit convergence, indicating that several observable characteristics were shared by the two accessions, along with a significant correlation. The Bogor and Blora accessions were positioned in distinct clusters, although some overlap was observed. A clear separation between the Bima and Enrekang accessions on one side and the Bogor and Blora accessions on the other was identified, suggesting that metabolite composition was significantly influenced by geographical location. Bima (Lombok Island) and Enrekang (Sulawesi Island) are both situated in the eastern part of Indonesia, where tropical monsoon climates with distinct wet and dry seasons are prevalent. These environmental conditions, including variations in

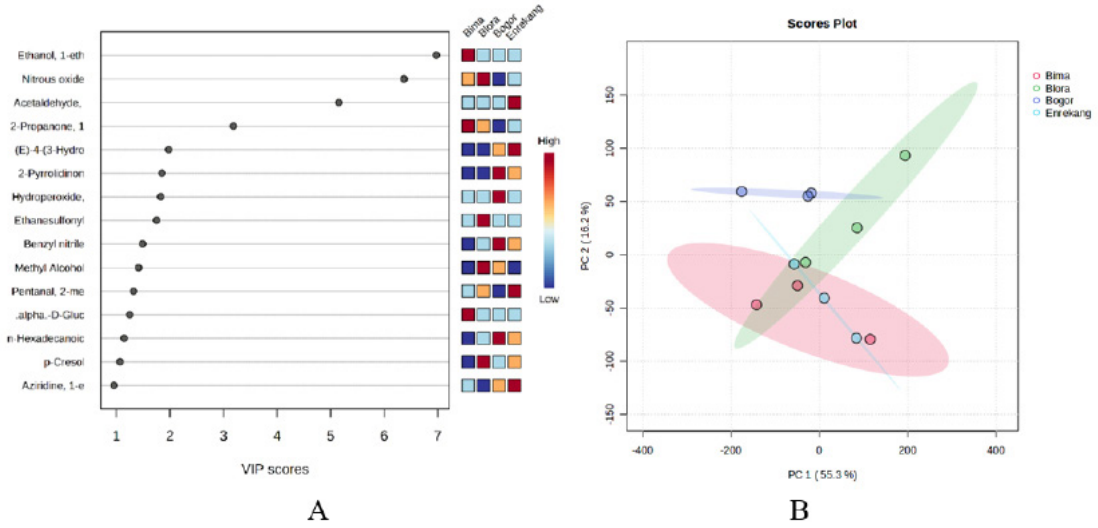

Figure 3 (A) Partial Least Squares Discriminant Analysis (PLS-DA); (B) Principal Component Analysis (PCA) between metabolite compounds on four accessions of M. oleifera: Blora (Java Island), Bogor (Java Island), Bima (Lombok Island), and Enrekang (Sulawesi Island) at 6 WAC

soil type, rainfall, and temperature, are likely to contribute to their similar metabolic profiles, as indicated by their clustering in the PCA plot. In contrast, Bogor and Blora, both are located on Java Island, experience relatively stable temperatures and high humidity, which may explain why their metabolite compositions are more closely related. The clear separation between these two groups suggested that geographical factors, such as climate, soil nutrients, and elevation, played a crucial role in shaping metabolite diversity.

Furthermore, the variations in metabolite profiles, which are pivotal in understanding the plant's tolerance capabilities, have been illuminated by the robust chemometric analysis from Figure 4. The discriminatory power of the metabolites has been illustrated by the distinct clusters and VIP scores from the PLS-DA analysis, offering a predictive understanding of the potential effects of different treatments.

Concomitantly, the metabolomic diversity was mirrored by the distinct clusters observed in Figure 4B, aligning with the metabolite variances and the physiological stress responses reported by Klau *et al.* (2023). The specific metabolic adaptations of *M. oleifera* were revealed through these clusters, similar to the patterns observed in different varieties of *Curcuma zanthorrhiza* grown across various regions, where unique metabolite profiles were found to correspond to distinct environmental conditions. Such clustering is emphasized as an indicator of the potential of *M. oleifera* to modulate its metabolic pathways, which, as posited by Matsuse *et al.* (2022), may be regarded as a survival mechanism under the selective pressures of aluminum toxicity.

CONCLUSIONS

The Bogor and Blora accessions showed higher tolerance to AlCl3 treatment to in vitro culture, as indicated by their relatively high value on shoot height, number of shoots, and number of petioles even when exposed to 100 - 250 mg/L AlCl3 in DKW medium culture, with no significant difference compared to controls. Bima and Enrekang accessions were in the same cluster and demonstrated convergence, suggesting that the two accessions had commonalities in various observable traits and showed a statistically significant association. Acetic acid is one of the metabolites associated with $AlCl_3$ stress. There were 21 metabolites that had a specific positive correlation with acetic acid. Eight metabolites showed a very high correlation including cyclopentene, 2-allyphenol, 4-ethynyl-6-8-dioxane, vinyl ether, ethanone 1-oxiranyl, 2-methylpyridine, 2-butanone, and ethanesulfonic acid. This research contributed significantly to understanding aluminum tolerance in plants by identifying tolerant accessions, clustering relevant traits, and highlighting key metabolites to in vitro culture. These findings pave the way for future research in order to verify this finding in field conditions and the development of innovative agricultural strategies to mitigate aluminum toxicity in plants.

ACKNOWLEDGMENTS

We thank BRIN Talent Management, which has provided funding support and research scholarships and Dr. Ir. Tri Muji Ermayanti (BRIN) as Institutional Supervisor, who has provided a lot of guidance and assistance.

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