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The potential of three endemic *Alocasia* species in Indonesia: Insights from advancing in vitro propagation methods and comparative metabolites analysis

Fitri Rachmawati[a], Dewi Pramanik[a,c], Lily Ismaini[b], Eka Fibrianty[a], Herni Shintiavira[a], Taufiq Hidayat Rahman Side[a], Sri Rianawati[a], Muhammad Prama Yufdy[a], Laela Sari[b], Sri Nopitasari[d], Budi Winarto[a,*]

[a] Research Center for Horticultural, National Research and Innovation Agency Republic of Indonesia (BRIN), Cibinong Science Center, Jl. Raya Jakarta, Bogor KM 46, Cibinong, Bogor 16911, Jawa Barat, Indonesia
[b] Research Center for Applied Botany- National Research and Innovation Agency Republic of Indonesia (BRIN), Cibinong Science Center, Jl. Raya Jakarta, Bogor KM 46, Cibinong, Bogor 16911, Jawa Barat, Indonesia
[c] Evolutionary ecology, Naturalis Biodiversity Center, Darwinweg 2, 2333 CR Leiden, South Holland, the Netherlands
[d] Research and Development of Tissue Culture, GS Biotech-CV. Ranata Nursery, Lembang, Bandung Barat, 40391, Jawa Barat, Indonesia

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ABSTRACT

Alocasia species, endemic ornamental plants in Indonesia, are of significant economic value due to their rarity, slow growth, and challenges in propagation. Our study investigates the in vitro propagation via direct organogenesis of *Alocasia cuprea, Alocasia reversa,* and *Alocasia reginula*, alongside bioactive metabolite analysis using GC/MS. The in vitro propagation process for these species encompasses stages including sterilization, shoot initiation, proliferation, regeneration, and acclimatization. Combining pre-sterilization techniques with sterilization methods, including rifampicin treatment (0.1 %), alcohol, and clorox, shows promise in reducing contamination rates. Various media and TDZ-BAP concentrations were tested for shoot initiation, with *A. cuprea* showing optimal response to the MS medium with 1.0 mg/L TDZ and 0.5 mg/L BAP. Shoot proliferation varied by genotype and medium, with *A. cuprea* exhibiting the highest proliferation on MS medium with 1.5 mg/L BAP. During the shoot regeneration stage, *A. reversa* displayed the highest leaf and root formation, particularly on MS medium with 0.5–1 mg/L BAP, which also proved effective for *A. cuprea*. Acclimatization results indicated that *A. cuprea* had the highest plantlet survivability, especially in a mixture of sand and water hyacinth organic manure medium. Metabolite analysis identified 69 compounds revealing significant variations among roots, tubers, and leaves of in vitro plantlets and acclimatized plants. Key metabolites, such as 9,12-Octadecadienoic acid, Tetrahydrofuran-2-one, and n-Hexadecanoic acid, were found in varying abundances across species and organs. These findings provide valuable insights for enhancing propagation techniques and exploring the bioactive potential of *Alocasia* species, thereby laying a foundation for their sustainable cultivation and exploitation.

1. Introduction

Alocasia spp. is a genus from the Araceae family that exhibits a wide range of leaf shapes and patterns (Boyce, 2008). It comprises approximately 100 species, with over 50 species being found in Indonesia (Kurniawan and Boyce, 2011; Wong and Boyce, 2016). *Alocasia cuprea* K. Koch, *A. reversa* N.E.Br, and *A. reginula* A. Hay are members of the Indonesian endemic *Alocasia* family, which have high economic potential as deciduous ornamental plants (Indrajati, 2021; Indrajati and Asniawati, 2022). *A. cuprea* is prized for its stunning, metallic-looking and glossy finish leaves that appears almost iridescent, with deep green to reddish-brown hues as extraordinary foliage. *A. reversa* is an attractive foliage plant, having variegated in a manner and dark ground colour with the course of the midrib and veins marked with a light tint. *A. reginula* is stunning Alocasia black velvet, a plant celebrated for its striking, velvety leaves and unique apperrance (Boyce, 2004; Widiyanti et al., 2017; Asih and Lestari, 2022; Jensen, 2023; Kelvine, 2024). *Alocasia* exports totaled 16.9 thousand

* Corresponding author.
E-mail addresses: budi079@brin.go.id, budi.winarto4867@gmail.com (B. Winarto).

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sticks worth IDR 1.6 billion (IQFAST, 2020), with the global ornamental plant market valued at US $22.32 billion, where Indonesia's contribution is only 0.1 % (Pancawati, 2021; Windiarto, 2021), highlights the significant unexplored potential for the local and global development of this plant family.

The availability of high-quality, sustainable seeds produced through established protocols is key to both expanding exports and meeting the high market demand for *Alocasia. Alocasia* is typically propagated through the traditional methods of dividing offsets, tubers, corms, and rhizomes (Jo et al., 2008, 2009; Bhatt et al., 2013; Abdelbaset et al., 2020; Raju et al., 2022). Due to the slow growth and multiplication rate of *Alocasia*, applying these conventional methods proved time-consuming and inefficient and resulted in a low yield of regenerants (Jo et al., 2008, 2009). Therefore, establishing an in vitro mass propagation protocol is crucial for facilitating the production of high-quality seedlings for commercial use in *Alocasia*. Specific challenges are generally associated with establishing an aseptic culture, initial callus and/or shoot formation, proliferation, regeneration and plantlet acclimatization (Jianfen et al., 2015; Abdulhafiz et al., 2020; Raju et al., 2022).

Several recent in vitro mass propagation protocols have been developed for *Alocasia* species. (Jianfen et al., 2015) presented a successful method for *A. micholitziana*, utilizing leaves and petioles as explants. Optimal conditions included sterilization with 0.1 % $HgCl_2$ solution for 8 min, a combination of 2 mg/L 6-benzyladenine (BA) and 0.4 mg/L α-naphthalene acetic acid (NAA) for adventitious bud induction, and root induction in half-strength MS medium with 0.5 mg/L indole-3-butyric acid (IBA), achieving a post-acclimatization survival rate of up to 98 %. Abdulhafiz et al. (2020) developed a micropropagation protocol for *A. longiloba* using seeds as explants, achieving optimal seed germination of up to 87 % with 30 % H_2SO_4 treatment and yielding up to 18 shoots per explant with an average height of 7.3 cm using MS medium supplemented with 3 mg/L N6-benzylaminopurine (BAP). Root production from regenerated shoots was successful on MS medium supplemented with 0.5 mg/L indole-3-acetic acid (IAA), resulting in a 90 % survival rate post-acclimatization. Additionally, Raju et al. (2022) reported an effective in vitro regeneration procedure for *A. amazonica* via indirect organogenesis, achieving a 90 % survival rate.

Beyond their ornamental value, *Alocasia* species hold promise for medicinal applications, with extracts from the roots, tubers, and leaves of *Alocasia indica*, as well as the leaves juices and rhizome extract of *Alocasia mycorrhiza*, exhibiting hepatoprotective, antihyperglycemic, antioxidant, and cytotoxic properties (Mulla et al., 2009; Patil et al., 2012; Rahman et al., 2012; Islam et al., 2013). Compounds like alocasin A, hyrtiosin B, hyrtiosulawesine, p-hydroxycinamic acid, and myristic acid have been isolated from *Alocasia odora* rhizomes, showcasing the chemical diversity within the genus (Das et al., 2022). Furthermore, the presence of alkaloids, flavonoids, terpenoids, and phenols in various *Alocasia* species underscores their pharmacological significance and traditional medicinal uses (Mulla et al., 2009). So far there are few researches carried out in exploring phytochemical contents in *A cuprea, A reversa* and *A. reginula*. According to William et al. (1981) *A. cuprea* has apigenin 5C-glycoside, kaemferol, quercetin and cyanidin. Apigenin has several effects as an anti-inflammatory for cancer, diabetes, obesity, depression, insomnia, infection, and respiratory, cardiovascular, hepatoprotective, neurodegenerative, and skin diseases (Chen et al., 2018; Salehi et al., 2019; Mushtaq et al., 2023; Allemailem et al., 2024), cell cycle arrest, apoptosis, and antioxidant function (Salehi et al., 2019), antimicrobial effects (Wang et al., 2019). Furthermore, kaemferol and quercetin have a high potential to be applied as neuroprotective agents in neurodegenerative diseases (Jin et al., 2023), antimicrobial activity, bioavailability and bioactivity in the human body (Jan et al., 2022). Examining plants for drug discovery is crucial, as numerous species have yet to be thoroughly investigated for their medicinal potential.

Modern biotechnological methods, such as plant cell culture, offer promising avenues for secondary metabolite production, complementing the traditional methods of seed propagation. It enabled the synthesis of valuable secondary metabolites, including therapeutic agents that have clinical utility in the treatment of diverse ailments (Bhatia et al., 2015; Rao et al., 2016), including antiviral, anti-cancer, anti-inflammatory, and antioxidant attributes (El-Sherbiny et al., 2016). It also provides advantages such as superior quality control, rapid and efficient production, and year-round availability (Gonçalves and Romano, 2018). Successful finding the in vitro culture stage and product shape that produces high secondary metabolites will support their production commercially in the next step.

In light of these considerations, this study establishes reliable in vitro propagation protocols for *A. cuprea, A. reversa*, and *A. reginula* through direct organogenesis using micro bulbs as explant sources. By optimizing sterilization methods, genotype responses, and media formulations, we seek to streamline the initiation, proliferation, regeneration, and acclimatization stages. Furthermore, we aim to conduct a comparative metabolite profiling analysis of in vitro and acclimatized plantlets of these *Alocasia* species using GC/MS. Ultimately, our findings aspire to foster sustainable seedling production while revealing the bioactive potential of *Alocasia* spp. metabolites for broader applications

2. Materials and methods

2.1. In vitro propagation

2.1.1. *Preparation* of explant

Donor plants provided by CV. Ranata Nursery, West Java, Indonesia. Three Indonesian endemic *Alocasia* species, *A. cuprea, A. reversa*, and *A. reginula* (Fig. 3A, 3B, and 3C), used in this experiment were planted in a plastic pot (Ø 20 cm in diameter) containing row rice husk and bamboo moss (2;1, v/v). The plants were watered regularly and sufficiently twice a week. The micro bulbs were harvested from the donor plants using sterile tissue culture blades.The healthy micro bulbs, measuring between 0.8 to 1.0 cm in size, were collected from mature donor plants aged over 1 year (Fig. 3D). The preparation of donor plants as explants source followed the method described by Rachmawati et al. (2023) (Fig. 3E).

2.1.2. *Incubation of cultures*

During all stages of the experiment, a fluorescent lamp emitting light at an intensity of 2407 Lux was utilized for 16 h daily (SL-Shinyoku, Indonesia; measured using a lux meter-Lutron LX 101, Taiwan). The experimental setup involved maintaining environmental conditions with a temperature range of 23.5 ± 1 °C and a relative humidity between 60 % and 70 %.

2.1.3. *Sterilization of micro bulbs explant from three* Alocasia *spp. using different methods*

Randomized Completely Block Design (RCBD) with three genotypes of *Alocasia* spp. i.e., *A. cuprea, A. reversa*, and *A. reginula* as the first factor and second factor was a combination of pre and sterilization methods (M1, M2, M3, M4, and M5) (Table 1). After sterilization, micro bulbs are cut into two parts (top and bottom). The parts were grown individually in bottles consisting of MS medium enriched with 20 g/L sugar, solidified with 7 g/L agar (Winarto and Rachmawati, 2013; Rachmawati et al., 2023), without the addition of plant growth regulators (PGR), and maintained for four weeks to establish an aseptic culture. Variables observed in the stage percentage of contamination (%), browning explants (%), explant growth potential (%), and

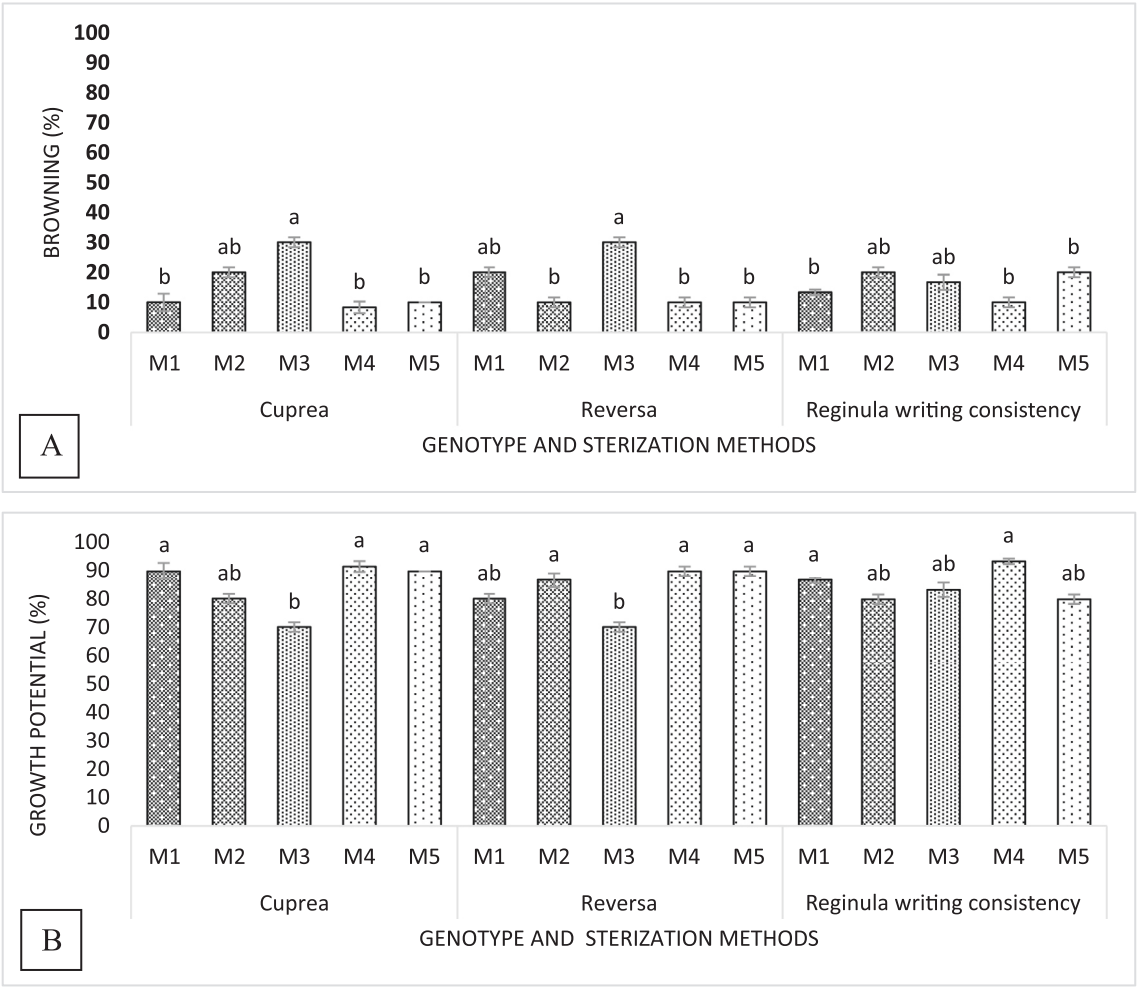

Fig. 1. The efficacy of sterilization techniques on three *Alocasia* spp. to mitigate the browning of explant and to enhance explant potential growth.

percentage of explant shoot initiation (%). We calculated all percentages using Eqs. (1)-(4):

$$\text{Percentage of contamination } (T_c) = \frac{N_{ce}}{N_{explant}} \times 100\% \quad (1)$$

$$\text{Percentage of browning } (T_b) = \frac{N_{be}}{N_{explant}} \times 100\% \quad (2)$$

$$\text{Percentage of growth potential } (T_{gp}) = \frac{N_{gpe}}{N_{explant}} \times 100\% \quad (3)$$

$$\text{Percentage ofinitiated shoots } (T_s) = \frac{N_{ise}}{N_{explant}} \times 100\% \quad (4)$$

where, $N_{explants}$ signifies the number of total explants cultured, N_{ce} represents number of contaminated explants, N_{be} indicates number of browned explants, N_{gpe} refers to the number of explants with growth potential, and N_{ise} denotes the number of initiated shoots.

2.1.4. Shoot initiation of three Alocasia *spp. on various MS media and TDZ-BAP concentrations*

Aseptic micro bulbs derived from the previous experiment were used. The micro bulbs were pre-sterilized in 0.5–1 % bactericide-fungicide and 0.1 % (w/v) Rifampicin for 16–18 h. The explants were then sterilized in 70 % alcohol solution for 3 min, 30 % clorox (v/v) for 5 min, and rinsed with sterile destillate water (SDW) 5–6 times (@ 3 min). The RCBD, which consists of two factors with three replications, was applied in the stage. The first factor was three genotypes of

Table 1
Description of pre-sterilization and sterilization methods tested on *Alocasia* spp. micro bulbs.

Code	Pre-sterilization method	Sterilization method
M1	Pre-sterilization-1 (P1): 1–2 % (w/v) bactericide-fungicide for 30–45 min	Sterilization-1 (S1): sterilization in LAF by shaking the explants in 5 % and 10 % (v/v) Clorox solution for 5 and 10 min, respectively
M2	Pre-sterilization-2 (P2): 1–2 % (w/v) bactericide-fungicide for 45 min proceed by immersion in 0.1 % (w/v) Rifampicin for 16–18 h	Sterilization-2 (S2): sterilization in LAF by shaking the explants in a solution of 70 % (v/v) alcohol for 3 min
M3	Pre-sterilization-2 (P2): 1–2 % (w/v) bactericide-fungicide for 45 min proceed by immersion in 0.1 % (w/v) Rifampicin for 16–18 h	Sterilization-3 (S3): sterilization in LAF by shaking the explants in a solution of 0.05 % and 0.01 % (w/v) $HgCl_2$ for 5 min each.
M4	Pre-sterilization-3 (P3): soaking in a solution of 0.5–1 % bactericide-fungicide and 0.1 % (w/v) Rifampicin for 16–18 h	Sterilization-2 (S2): sterilization in LAF by shaking the explants in a solution of 70 % (v/v) alcohol for 3 min and 30 % Clorox (v/v) for 5 min
M5	Pre-sterilization-3 (P3): soaking in a solution of 0.5–1 % bactericide-fungicide and 0.1 % (w/v) Rifampicin for 16–18 h	Sterilization-3 (S3): sterilization in LAF by shaking the explants in a solution of 0.05 % and 0.01 % (w/v) $HgCl_2$ for 5 min each.

Alocasia spp. i.e., *A. cuprea, A. reversa*, and *A. reginula*. The second factor was four initiation media (I), namely: I1 ($^1/_2$ MS medium containing 0.75 mg/L TDZ and 0.25 mg/L BAP), I2 ($^1/_2$ MS supplemented with 1.00 mg/L TDZ and 0.50 mg/L BAP), I3 (MS fortified by 0.75 mg/L TDZ and 0.25 mg/L BAP), and I4 (MS containing 1.00 mg/L TDZ and 0.50 mg/L BAP). These media were added with 30 g/L sucrose, solidified with 1.8 g/L gelrite, and the pH was set to 5.8 (Winarto and teixeira da Silva, 2015; Rachmawati et al., 2023). Shoot initiation was observed in the experiment, starting four weeks after culture (Fig. 1F). At this stage, the following variables were assessed: explant growth potential (%), percentage of shoot initiation (%), time taken for shoot initiation (days), and the number of initial shoots per explant.

2.1.5. Shoot proliferation of three Alocasia spp. on various media formulations with different concentrations of MS and BAP

Individual shoots initiated from the micro bulbs were carefully separated. Their outer layers were then peeled before subculture onto the treatment medium. The experiment used an RCBD consisting of two factors with three replications. Firstly, the response of three *Alocasia* species (*A. reversa, A. cuprea*, and *A. reginula*) to different proliferation media. Secondly, it examined six treatment media (P) consisting of various combinations of MS media (MS and $^1/_2$ MS) and BAP concentrations (0, 1.0, and 1.5 mg/L). All media were added 30 g/L sucrose and 1.8 g/L gelrite with 5.8 pH media (Winarto and Teixeira da Silva, 2015; Rachmawati et al., 2023). Periodical subcultures were carried out once a month until the 3rd subculture on the same media. Variables observed were period of shoot formation (days), number of shoots per explant, shoot height (cm), and clump diameter (cm). Observations were carried out every 2 weeks until 12 weeks.

2.1.6. Shoot regeneration of three Alocasia spp. on various BAP concentrations

The RCBD experiment consisted of two factors with three replications applied. The first factor was three *Alocasia* spp. i.e., *A. reversa, A. cuprea*, and *A. reginula*. The second factor was six regeneration media (RM), a combination of basic media concentrations (MS and $^1/_2$ MS) with BAP (0, 0.5, and 1.0 mg/L). Each treatment consisted of five initial shoots (1–1.5 cm in size) planted in jam bottles (Ø 7 cm; 12 cm high). The media were added 30 g/L of sugar, 7 g/L of agar with pH media adjusted at 5.8 (Jo et al., 2009; Bhatt et al., 2013; Rachmawati et al., 2023). Subcultures were carried out periodically once a month until the 3rd subculture in the similar media. The variables observed were: (1) plantlet height (cm), (2) number of leaves per shoot, (3) number of secondary shoots per shoot, and (4) number of roots per shoot. Plantlet growth and development were periodically observed each month following the subculture.

2.1.7. Hardening, acclimatization and growth of Alocasia spp. plantlets

2.1.7.1. Survivability of plantlets from three Alocasia spp. on indirect aclimatization (compot method). Plantlet hardening was carried out by placing culture bottles containing planlets in a screen house. The plantlets derived from three *Alocasia* spp. in the bottles was put on the table and arranged in similar distances. The bottles were maintained in a screen house in 3 weeks. After plantlet hardening, the plantlets were harvested and prepared for acclimatization process using compot method. Uniform and vigorous plantlets with 2 leaves, 4–5 cm in height, and healthy roots from the three *Alocasia* spp. were used as explant sources. A total of 100 plantlets were grown in compoted and transferred to a plastic tray (29 cm × 23 cm × 7 cm, length, width, and height) containing roasted husk media after preparing plantlets with root cleaning under tap water and immersing them in 1 % pesticide (systemic fungicide + bactericide) solution for ± 30 min and air drying on filter paper for ± 10 min (Fig. 3L and 3M). Following two weeks of acclimatization under a transparent plastic cover, the cover was gradually removed, and the plants were transferred to an area with 35–50 % reduced sunlight. The compot plantlets were observed 2–4 weeks after acclimatization. After two months of acclimatization, the surviving plantlets were transplanted into single pots and maintained under similar conditions for an additional month. The surviving plantlets were transplanted into single pots after two months of acclimatization and kept in similar conditions until three months.

The RCBD experiment consisted of one factors with five replications applied. The factor was the three *Alocasia* spp. i.e. *A. reversa, A. cuprea*, and *A. reginula*. Variables observed were: (1) Plantlet survivability in compot (%), (2) height of plants (cm), (3) number of leaves per plant, (4) leaf width, and (5) leaf length (cm). Observations were carried out every two weeks until the eighth week.

2.1.7.2. The effect of hardening and media on the direct acclimatization and growth of A. cuprea plantlets. Uniform and vigorous plantlets with 2–3 leaves, 6–7 cm in height, and healthy roots from the *A. cuprea* . was used as explant sources. Preparing plantlets with root cleaning under tap water and immersing them in 1 % pesticide (systemic fungicide + bactericide) solution for ± 30 min and air drying on filter paper for ± 10 min (Fig. 3L and 3M).

The plantlets were transplanted into single pots and maintained under similar conditions for an additional month.

The experiment was set up in RCBD and featured two factors and three replications. The first factor was the hardening period of 1, 2, and 4 weeks (Fig. 3J and 3K), and the second factor was four acclimatization media (A), i.e., roasted husks (A1), sand (A2), roasted husks + water hyacinth organic manure (A3), and sand + water hyacinth organic manure (A4). Variables observed were: (1) survivability of plantlets (%), (2) height of plants (cm), (3) number of leaves per plant, (4) leaf width, and (5) leaf length (cm). Observations were carried out every two weeks until the eighth week.

The variables observed during the acclimatization stage included: (1) survivability of plantlets in community pot (compot) (%) (Eq. (5)), (2) plant height (cm), (3) number of leaves per plant, (4) leaf length (cm), (5) leaf width (cm), and (6) number of roots per plant. Plantlet growth and development were periodically monitored each month up to the third month. The calculation of plantlet survivability in compot is presented in Eq. (5):

$$\text{Plantlet survivability in compot } (T_{ps}) = \frac{N_{sp}}{N_{plantlets}} \times 100\% \quad (5)$$

here, $N_{planltlets}$ refers to the total number of acclimatized plantlets, and N_{sp} represents of surviving plantlets under acclimatization conditions.

2.2. Exploration of bioactive metabolites by GC/MS

2.2.1. Preparation of plant extracts

Each part of three *Alocasia* spp. in vitro plantlet and acclimated plant (roots, tubers, and leaves) was cleaned and dried in an oven at 40 °C. The dried samples were chopped into small pieces and then smoothed into powder using an electric blender. Each of 1 gram of *Alocasia* spp. powder was macerated using 10 mL of methanol, then incubated and shaken at 150 rpm for 24 h. After that, the extract was filtered using Whatman No. 1 filter paper. The filtrate was placed in a tube and concentrated by air drying until the weight of the extract was constant. The crude extract was obtained and stored at 4 °C for further use.

2.2.2. GC/MS analysis

Gas Chromatography/Mass Spectrometry (GC/MS) analysis was performed to identify bioactive compounds, including potent volatile

and semi-volatile compounds, in *Alocasia* spp. extract. The retention time was used to differentiate between different chemicals. The 25 mg of concentrated methanol extracts were redissolved in the respective solvents, vortexed properly and filtered through a 0.22 mm syringe filter. A one-microliter portion of the sample solution was injected into the GC/MS system for analysis. The bioactive compounds of methanol plant crude extracts were identified by GC/MS Column type 19091S-433: 93.92873 DB-5MS UI 5 % phenyl methyl silox, dimension: 30 m x 250 μm x 0.25 μm. Initial temperature: 150 °C; hold time: 2 min. Post run, 300 °C with retention time (Rt) total for 30 min. Identification of bioactive compounds The identification of compounds was based on NIST 17 (MassHunter\Library \NIST17.L) by analyzing the mass spectra of the detected compounds through GC/MS.

2.3. Data analysis

2.3.1. In vitro propagation data analysis

The data underwent analysis of variance (ANOVA) using SAS version 9.4 (SAS, 2023). Tukey Honest Significant Difference (HSD) test was employed to further assess the significant difference in the mean values of the treatments, with a confidence level of 95 %.

2.3.2. GC/MS data analysis

The compound name and molecular formula of the individual compounds found from GC/MS were determined further using NIST WebBook and PubChem. The biological activity was determined by comparing with Dr. Duke's Phytochemical and Ethnobotanical database (Duke, 2023) and literature reviews. Finally, the relationships among compounds and plant organs were analyzed using a heatmap, employing average linkage for clustering and Euclidean distance for measurement. The heatmap was created using the online tool http://www.heatmapper.ca/expression/ (Babicki et al., 2016).

2.3.3. Correlation and PCA analysis of bioactive metabolites and various organs of A. cuprea, A. reversa, and A. reginula

Analyses were conducted to explore the relationship between metabolite identified by GC/MS and the various organs from in vitro and acclimatized plantlets of three *Alocasia* spp. Pearson correlation coefficients were calculated using the R Program version R 4.4.0 GUI 1.80 Big Sur Intel build (8376) (R Foundation Team, 2023) within RStudio version Version 2024.04.1 + 748 (2024.04.1 + 748) (RStudio, 2024), employing the format package (Meyer, 2010). The criteria for Pearson correlation were categorized as high ($\geq \pm 0.50$ to ± 1), medium (≥ 0.30 to $< \pm 0.50$), and low ($< \pm 0.30$). Subsequently, Principal Component Analysis (PCA) was conducted using R with the factor extra package Kassambara (Kassambara and Mundt, 2020)

3. Results

3.1. In vitro propagation method of three Alocasia spp

The in vitro culture experiments for the three *Alocasia* species clearly demonstrated that all treatments applied at each stage of the culture process had statistically significant effects ($p = 0.05$). M4 method was the most suitable sterilization method in obtaining low contamination and high shoot initiation. High initiation of shoots, high potential growth of explants, and in a short period was established on I4 medium, successfully proliferated on P6 medium and regenerated on RM-6 medium. In the research was also clear that *A. cuprea* had high shoot initiation, proliferation, regeneration till acclimatization compared to *A. reversa* and *A. reginula*. For *A. cuprea*, three weeks of hardening

3.1.1. Sterilization of three Alocasia spp. micro bulbs using different methods

Based on periodic observations, it was determined that *Alocasia* genotypes and sterilization methods give different results in obtaining an aseptic culture of micro bulbs, and the interaction effect was noted on browning and potential growth of explants. Aseptic shoots cultured continually grew and increased in their size and performance (Fig. 3F and 3G). The optimal results were determined on *A. reginula* with 27.7 % explant contamination and 57.0 % explant shoot initiation and M4 method (Pre-sterilization-3 combined with Sterilization-2) with 6.7 % explant contamination and 83.9 % explant shoot initiation (Table 2). Combining both treatments effectively reduced explant browning to 10 % while achieving a high explant growth potential of 93.3 %. However, this improvement wasn't statistically significant compared to other *Alocasia* genotypes, as shown in Table 2 and Figs. 1A and 1B. The results also confirm that using Rifampicin as a sterilant agent significantly reduced contamination rates to between 49.3 % and 89.6 %, compared to significantly higher rates without Rifampicin. The most effective sterilization was achieved when combining Rifampicin with 70 % alcohol and 30 % Clorox. In comparison, the lowest sterilization micro bulbs of *Alocasia* spp. were recorded using the M1 method.

3.1.2. Shoot initiation of three Alocasia spp. on various MS media and TDZ-BAP concentrations

Different genotypes and media stimulated different responses on shoot initiation; however, there was no interaction effect from either factor. *A. cuprea* showed the best response in shoot initiation with 4.2 shoots per explant, 97.9 % growth potential of explants, 90.8 % explant shoot initiation, and 10.3 days initiation period ($p < 0.0001$). While I4 medium (MS medium supplemented with 1.0 mg/L TDZ and 0.5 mg/L BAP) proved to be the most suitable medium, stimulating 4.2 shoots per explant, 100 % explant growth potential, 95.6 % explant shoot initiation, and a rapid initiation period of only 9.2 days (Table 3; $p < 0.0001$). The low response and low effect in shoot initiation were noted on *A. reginula* and I1 medium ($^1/_2$ MS+ 0.75 mg/L TDZ + 0.25 mg/L BAP).

Table 2
Percentage of micro bulb contamination and shoot initiation in three genotypes of *Alocasia* spp. under five sterilization methods.

Treatments	Explant contamination (%)	Explant shoot initiation (%)
Genotypes		
A. cuprea	30.0 a	55.0 a
A. reversa	33.3 a	50.7 a
A. reginula	27.7 a	57.0 a
P value	*0.1346* ns	*0.0872* ns
Sterilization methods		
M1	64.4 a	21.1 e
M2	30.0 b	54.4 c
M3	33.3 b	41.1 d
M4	6.7 c	83.9 a
M5	17.2 c	70.6 b
P value	*<0.0001***	*<0.0001***

Note:
- Means sharing the same letter in the same column for genotype and sterilization methods indicates no significant difference based on Tukey's test at a significance level of $p < 0.05$ (*) or $p < 0.01$(**).
- ns= not significant.
- The explanation of the sterilization codes is referenced in Table 1.

Table 3
Shoot initiation responses of micro bulbs derived from three genotypes of *Alocasia* spp. on different initiation media.

Treatments	Explant growth potential (%)	Shoot initiation times (Days)	Explant shoot initiation (%)	Number of initial shoots per explant
Genotypes				
A. cuprea	97.9 a	10.3 b	90.8 a	4.2 a
A. reversa	97.9 a	9.1 c	91.2 a	3.0 b
A. reginula	98.3 a	12.7 a	85.4 b	2.4 c
P value	*0.7671* ns	*<0.001* **	*0.0008* **	*<0.0001* **
Initiation media				
I1: $^1/_2$ MS+ 0.75 mg/L TDZ + 0.25 mg/L BAP	94.4 b	11.8 a	81.3 c	2.4 b
I2: $^1/_2$ MS+ 1.0 mg/L TDZ + 0.5 mg/L BAP	98.3 a	10.7 b	91.3 ab	2.9 b
I3: MS + 0.75 mg/L TDZ + 0.25 mg/L BAP	99.4 a	11.0 b	88.3 b	3.2 b
I4: MS + 1.0 mg/L TDZ + 0.5 mg/L BAP	100.0 a	9.2 c	95.6 a	4.2 a
P value	*<0.0001* **	*<0.0001* **	*<0.0001* **	*<0.0001* **

Note:
- Means sharing the same letter in the same column for genotype and media indicates no significant difference based on Tukey's test at a significance level of $p < 0.05$ (*) or $p < 0.01$(**).
- ns= not significant.
- 30 g/l sucrose and 2 g/l gelrite were added to all media, and the pH of the media was adjusted to 5.8. ns= not significant.

3.1.3. *Shoot proliferation of three* Alocasia *spp. on various media formulations with different concentrations of MS and BAP*

The number of shoots produced during proliferation varied significantly depending on both the *Alocasia* genotypes and the specific type of proliferation media used. Differences in shoot proliferation were observed based on initial shoot height, with *A. reversa* showing stronger responses at higher heights. Similarly, *A. cuprea* exhibited increased proliferation with larger clump diameters (Supplement data; Table 1). The highest proliferation of shoots per explant, as high as 14.7 shoots per clump with 0.83 cm initial shoot height (Fig. 2) and 1.6 cm clump size, was noted in *A. cuprea* cultured on P6 (MS medium containing 1.5 mg/L BAP) with the lowest shoot proliferation recorded in all *Alocasia* genotypes planted on P1 (Fig. 3H and 3I). The stage results indicate a positive correlation between clump diameter and the number of shoots regenerated, while shoot height had a negative correlation with both (Supplement data: Table 1; Fig 2). The inclusion of BAP in the proliferation media significantly enhanced shoot production

3.1.4. *Regeneration of shoots of three* Alocasia *spp. on various BAP concentrations*

Different *Alocasia* genotypes cultured in different regeneration media in the shoot regeneration stage resulted in varied responses in all variables observed. *A. reversa* exhibited the highest leaf formation, averaging 3.4 leaves per shoot and also producing an impressive 4.2 roots per shoot. However, the specific regeneration medium used influenced the responses for each variable (Supplement data: Table 2). The combination of *A. cuprea* and RM-6 medium proved to be the most effective treatment, yielding the highest number of secondary shoots per explant (4.6) and tallest shoots (5.1 cm). Conversely, the lowest performance was observed in single-factor treatments: *A. reginula* and RM-1, and the combination treatment of *A. cuprea* and RM-1 (Table 4). Mini-shoots with one leaf and a height of approximately 2–3 cm, derived from the three *Alocasia* species (*A. reversa, A. cuprea*, and *A. reginula*) in the experiment, were then subcultured onto half-strength MS medium enriched with full vitamins for both plantlet enlargement and rooting, in preparation for acclimatization (Rachmawati et al., 2023).

3.1.5. *Hardening and acclimatization of plantlets derived from* Alocasia *spp*

In the acclimatization stage, interesting results were significantly noted due to different acclimatization ways (compot and single) (Table 5, Fig. 3O and Table 6, Fig. 3P); different responses of genotypes on compot method (Table 5), hardening periods and acclimatization media (sand, roasted husk, and water hyacinth) on direct single method (Table 6). The single method was better than compote with a 7.1 to 39.7 % improvement; *A. cuprea* had better performances than *A. reversa* and *A. reginula*. Longer hardening periods and higher plantlet performances were proved; utilizing water hyacinth organic

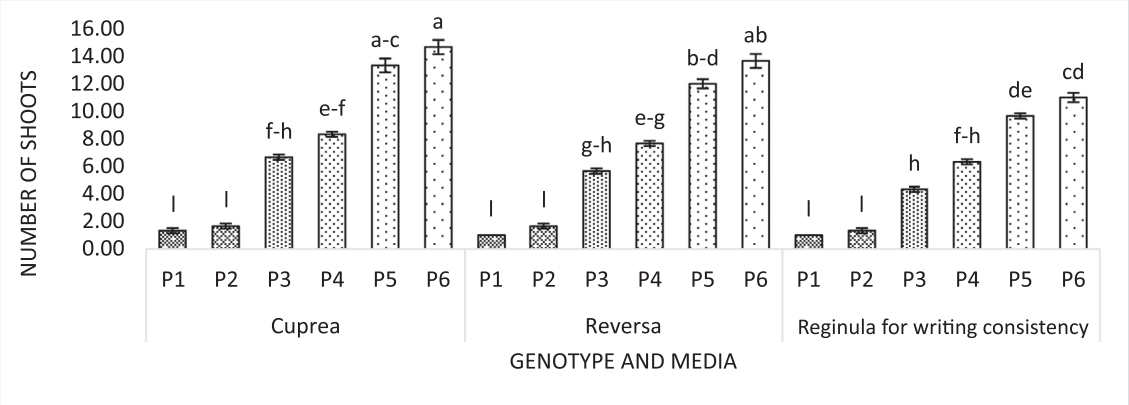

Fig. 2. The influence of different media on the initial shoot number of three *Alocasia* spp. P1: $^1/_2$ MS; P2: MS; P3: $^1/_2$ MS + 1.0 mg/L BAP; P4: $^1/_2$ MS +1.5 mg/L BAP; P5: MS + 1.0 mg/L BAP; and P6: MS + 1.5 mg/L BAP.

Fig. 3. An in vitro propagation protocol was developed for the Indonesian endemic *Alocasia* spp. derived from micro bulbs started from initiation to plant performances derived from the acclimatization stage. A-C) Donor plants of *A. cuprea, A. reversa*, and *A. reginula*) planted in a mixture media of rice husk and bamboo most (1:1 v/v). D) Harvested micro bulbs from donor plants (0.6 – 0.8 cm in diameter). E) Prepared micro bulbs for sterilization stage, F. Top part of micro bulbs shoot 1.5 weeks after culture on MS medium. G) Growth of shoot top part of micro bulbs 3–4 weeks after culture initiation on MS medium containing 1.0 mg/L TDZ and 0.5 mg/L BAP. H) Proliferation of shoots two months after culture on MS medium containing 1.5 mg/L BAP. I) Regeneration of shoots on MS Medium fortified by 0.5–1 mg/L BAP three months after culture. J-K) Rooted shoots in half-strength MS with full vitamin three months after hardening. L) Immersing of plantlets in 1 % pesticide solution (combination of systemic fungicide and bactericide) for ± 30 min. M) Air drying of plantlets on filter paper for 10 min. N) Plantlet performance individually. O) Survive plantlets in roasted rice husks media two months after acclimatization in compote style. P) Survive plantlets in a combination of sand and water hyacinth organic manure (1:1, v/v) 2 months after acclimatization in individual style. Q-S) A Single plant of *A. Cuprea, A. Reversa*, and *A. Reginula* in a mixture of rice husk and bamboo most (1:1, v/v) after ± 2 months plant repotting. Scales: A-C: 10 cm, D-I: 1 cm, and J-S: 5 cm.

Table 4
The effect of genotype and media interaction on plantlet height and shoot number.

Genotypes	Regeneration media	Plantlet height (cm)	Number of secondary shoots per explant
A. cuprea	RM-1: $^1/_2$MS	3.1 k	1.0 h
	RM-2: MS	3.5 ijk	1.9 efgh
	RM-3: $^1/_2$ MS + 0.5 mg/L BAP	5.0 defg	2.8 bcd
	RM-4: $^1/_2$ MS + 1.0 mg/L BAP	4.5 fgh	3.1 bc
	RM-5: MS + 0.5 mg/L BAP	5.0 def	3.7 b
	RM-6: MS + 1.0 mg/L BAP	5.1 def	4.6 a
A. reversa	RM-1: $^1/_2$MS	5.8 abc	1.1 gh
	RM-2: MS	5.0 def	1.2 gh
	RM-3: $^1/_2$ MS + 0.5 mg/L BAP	4.6 fgh	1.4 fgh
	RM-4: $^1/_2$ MS +1.0 mg/L BAP	5.4 bcde	2.2 def
	RM-5: MS + 0.5 mg/L BAP	6.1 ab	2.8 cde
	RM-6: MS + 1.0 mg/L BAP	6.6 a	3.0 bcd
A. reginula	RM-1: $^1/_2$MS	4.5 fgh	1.3 gh
	RM-2: MS	4.9 efg	1.9 efg
	RM-3: $^1/_2$ MS + 0.5 mg/L BAP	3.4 jk	2.5 cde
	RM-4: $^1/_2$ MS +1.0 mg/L BAP	3.9 hij	2.2 def
	RM-5: MS + 0.5 mg/L BAP	4.2 ghi	2.5 cde
	RM-6: MS + 1.0 mg/L BAP	5.7 bcd	3.2 bc
P value		*<0.0001* **	*<0.0001* **

Note:
- Means sharing the same letter in the same column for genotype indicates no significant difference based on Tukey's test at a significance level of $p < 0.05$ (*) or $p < 0.01$(**).
- ns= not significant.
- 20 g/l sugar and 7 g/l agar were added to all media, and the pH of the media was adjusted to 5.8.

Table 5
Survivability of plantlets from three *Alocasia* spp. Genotypes on aclimatization compot method.

Genotypes	Plantlet survivability in compote (%)	Height of plants (cm)	Number of leaves per plant	Leaf length (cm)	Leaf width (cm)
A. cuprea	92.9 a	6.5 a	1.7 a	5.9 a	4.4 a
A. reversa	71.3 b	4.0 b	1.8 a	4.2 b	2.8 b
A. reginula	60.3 c	4.3 b	1.6 a	5.5 a	3.5 ab
P value	*<0.0001***	*<0.0001***	*0.299ns*	*0.0082***	*0.030**

Note:
- Means sharing the same letter in the same column for genotype indicates no significant difference based on Tukey's test at a significance level of $p < 0.05$ (*) or $p < 0.01$(**).
- ns= not significant.

Table 6
Effects of different hardening periods and acclimatization media on the number of leaves per plant, plant height, leaf width, and leaf length of *Alocasia cuprea* on direct acclimatization using a single method.

Hardening periods (week)	Number of leaves per plant	Plant height (cm)	Leaf width (cm)	Leaf length (cm)
Hardening (weeks)				
One week	3.3 b	11.5 b	6.9 a	9.8 b
Two weeks	3.2 b	11.5 b	7.5 a	10.2 ab
Three weeks	4.0 a	13.8 a	7.7 a	11.5 a
P value	*0.0115* *	*<0.0001* **	*0.1176* ns	*0.0384* *
Aclimatization media				
AM-1: roasted husk	3.4 ab	12.3 a	7.8 a	11.2 a
AM-2: sand	2.9 b	9.7 b	5.8 b	7.2 b
AM-3: roasted husk + water hyacinth organic manure (1:1 v/v)	3.8 ab	12.9 a	7.7 a	11.5 a
AM-4: sand + water hyacinth organic manure (1:1 v/v).	3.9 a	14.0 a	8.6 a	12.5 a
P value	*0.0209* *	*<0.0001***	*<0.0001* **	*<0.001* **

Note:
- Means sharing the same letter in the same column for hardening and acclimatization media indicates no significant difference based on Tukey's test at a significance level of $p < 0.05$ (*) or $p < 0.01$(**).
- ns= not significant.

manure improved the growth performances of acclimatized plants compared to roasted husk and sand individually. The existence of water hyacinth organic manure in combination with sand improved growth performances optimally and significantly of plants up to 21.9 % number of leaves per plant, 44.3 % in plant height, 48.3 % leaf width, and 72.6 % in leaf length compared to others (Table 6; Fig. 3Q, 3R and 3S).

3.2. Exploratory analysis of bioactive metabolites by GC/MS

3.2.1. Diversity and distribution of bioactive metabolites in three Alocasia *spp*

A total of 69 different major bioactive metabolites were identified by GC/MS analysis (Fig. 4 and Table S1). Among the roots, tubers, and leaves of in vitro plantlets and acclimatized plants of three *Alocasia*

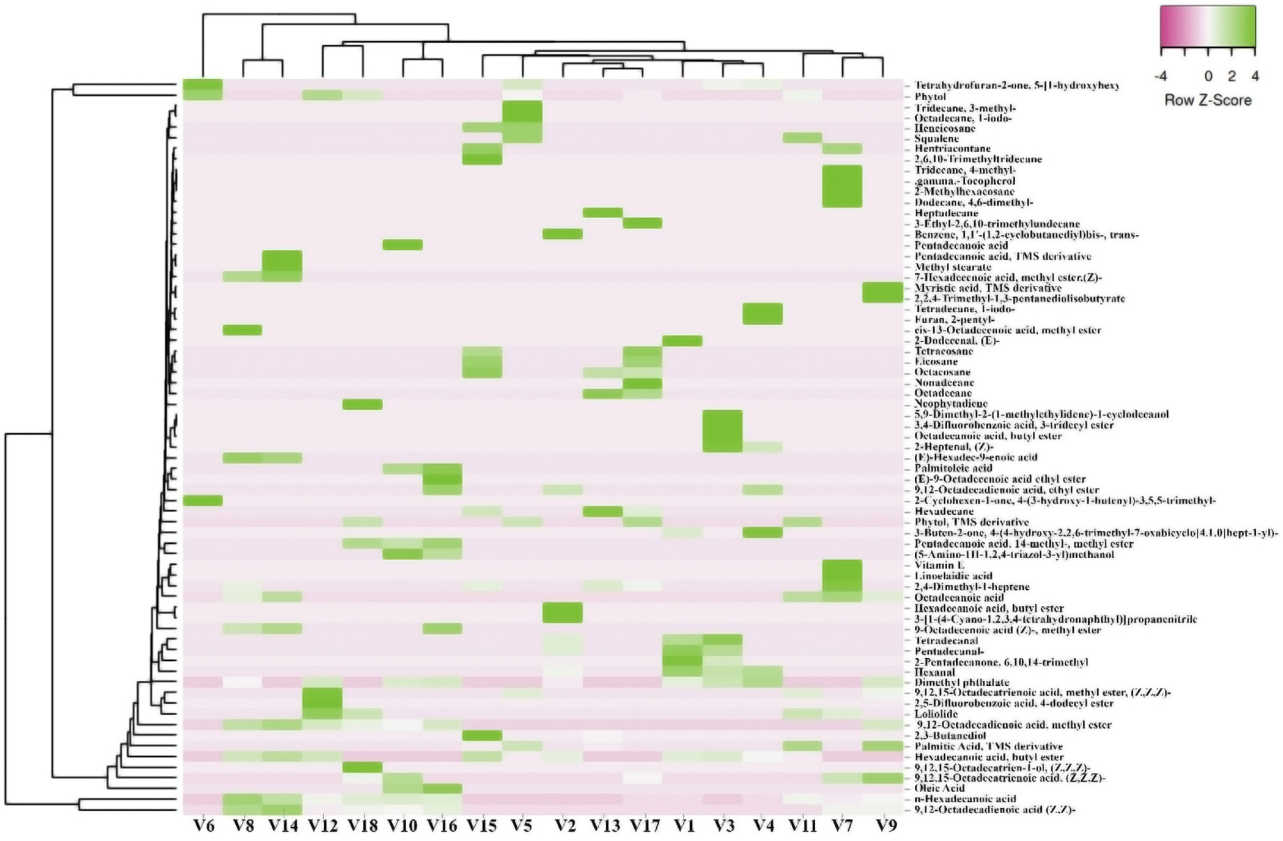

Fig. 4. Bioactive metabolites present in ethyl acetate extracts from the roots, tubers, and leaves of in vitro and acclimatized plants of *A. cuprea, A. reversa,* and *A. reginula*, identified by GC/MS. The results indicate the presence of 69 metabolites, with their relative abundances described by the percentage of peak area. *Abbreviations: V1=* in vitro *roots of A. cuprea; V2= roots of the acclimatized plant of A. cuprea; V3=* in vitro *tubers of A. cuprea; V4= tubers of the acclimatized plant of A. cuprea; V5=* in vitro *leaves of A. cuprea; V6= leaves of the acclimatized plant of A. cuprea; V7=* in vitro *roots of A. reversa; V8= roots of the acclimatized plant of A. reversa; V9=* in vitro *tubers of A. reversa; V10= tubers of the acclimatized plant of A. reversa; V11=* in vitro *leaves of A. reversa; V12= roots of acclimatized plant of A. reversa leaves; V13=* in vitro *roots of A. reginula; V14= roots of acclimatized plant of A. reginula; V15=* in vitro *tubers of A. reginula; V16= tubers of the acclimatized plant of A. reginula; V17=* in vitro *leaves of A. reginula; and V18= leaves of the acclimatized plant of A. reginula.*

species, we observed variations in the type and peak area of metabolites. Notably, 9,12-Octadecadienoic acid was found abundantly in the roots of acclimatized plants of *A. reversa* and *A. reginula*, with peak areas of 26.49 % and 33.14 %, respectively. Similarly, Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]- was abundant in the leaves of in vitro plantlets and acclimatized plants of *A. cuprea*, with peak areas of 14.9 % and 43.1 %, respectively. Interestingly, n-Hexadecanoic acid was present in various organs of *A. reversa* and *A. reginula*, with peak areas ranging from 0 % to 43.1 %, the highest being in the roots of acclimatized plants of *A. reversa*. This compound appeared to be produced in higher quantities in all organs of acclimatized plants compared to in vitro plantlets. Additionally, phytol was detected in the leaves of all tested *Alocasia* species, with the highest production in the leaves of acclimatized plants of *A. cuprea* (25.7 %), followed by *A. reversa* (20.5 %) and *A. reginula* (11.7 %). Lastly, vitamin E was uniquely found in the roots of in vitro plantlets of *A. reversa*, with a peak area of 4.4 % (Fig. 4 and Table S1).

3.2.2. *Correlation of bioactive metabolites and various organs of* A. cuprea, A. reversa, *and* A. reginula, *which were cultured in vitro and/or acclimatized ex vivo*

The correlation matrix was built based on 69 metabolite compounds identified in various organs of *A. cuprea, A. reversa*, and *A. reginula*, which were cultured in vitro and/or acclimatized ex vitro. It illustrates the relationships among different organs within each species and between species, with high coefficients indicating strong associations in metabolite composition and low coefficients suggesting weaker relationships. The results showed some highly significant positive correlations ($r \geq \pm\ 0.50$ *to* $\pm\ 1$; $p < 0.01$) between organs within a single species or between organs of different species. For instance, there were eight highly positive correlations between the organs of *A. reversa* and *A. reginula*, with correlation coefficients ranging from 0.56 to 0.96. The two highest positive correlations were found between the acclimatized roots of *A. reversa* and the acclimatized roots of *A. reginula* ($r = 0.96$; $p < 0.01$) and between the acclimatized tubers of *A. reversa* and the acclimatized tubers of *A. reginula* ($r = 0.88$; $p < 0.01$). Interestingly, there were no correlations between the organs of *A. cuprea* and those of the other two species (Fig. 5; Table S1-S3).

Higher correlations were also found within the organs of *A. cuprea* and *A. reversa*. We identified five highly significant positive correlations within the organs of *A. cuprea*, 14 highly significant positive correlations within the organs of *A. reversa*, and only two highly significant positive correlations within the organs of *A. reginula* (Fig. 1; Tables S1-S3).

3.2.3. *The PCA analysis of bioactive metabolites and various organs of* A. cuprea, A. reversa, *and* A. reginula *from in vitro plantlets and acclimatized plants*

The relationship between organs of *A. cuprea, A. reversa,* and *A. reginula* from in vitro and acclimatized plants and compounds identified by GC/MS can be explained by using a biplot Principal Component Analysis (PCA). We found two PCA factors (Dim1= 31.4 % and Dim2= 16.2 %) with three clusters of variables (Fig. 6 and Table S4).

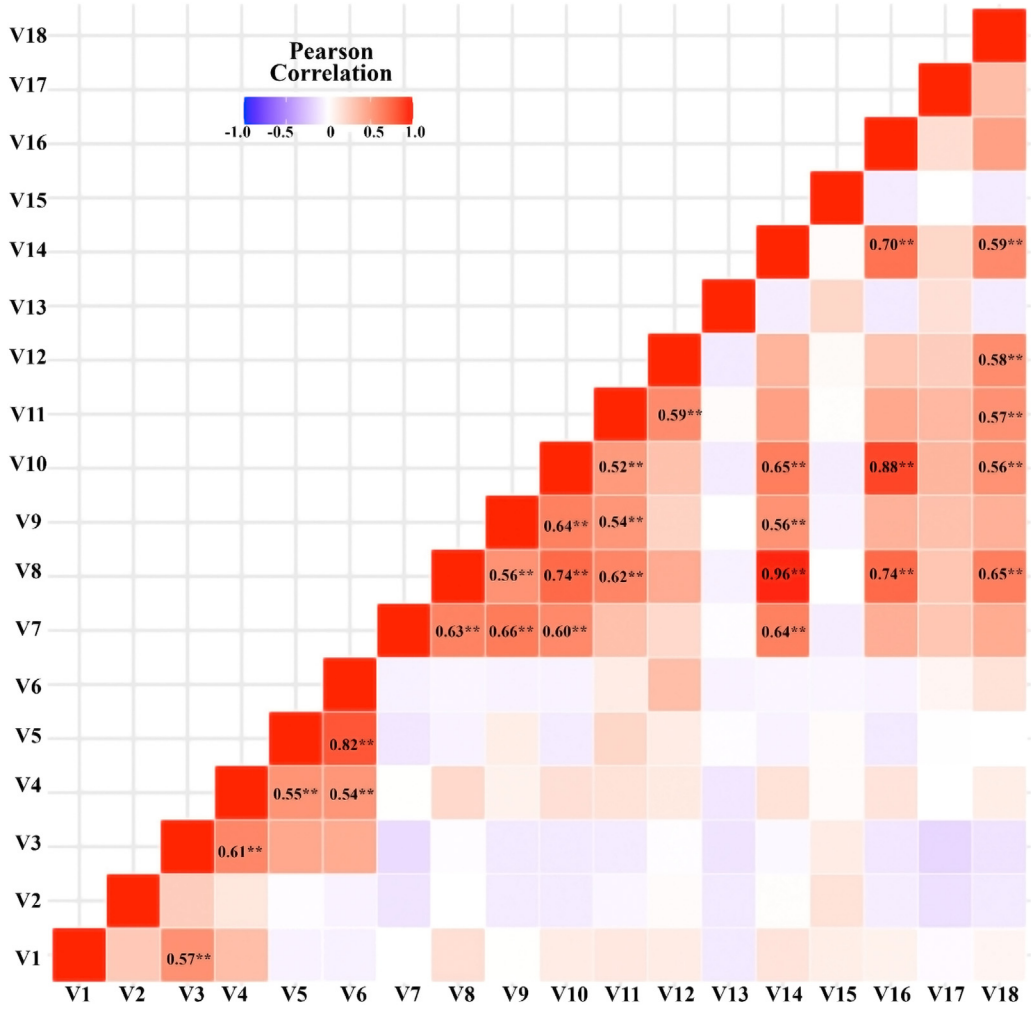

Fig. 5. Pearson correlation plot of plant organs of Alocasia cuprea, A. reversa, and A. reginula, displaying the correlations of 69 metabolite compounds identified from in vitro culture and acclimatization. ** = correlation is significant at the 0.01 level (2-tailed). Person correlation color key: blue = negative correlation (below 0); white = no correlation (0); red= positive correlation (above 0). The degree of the correlation ≥± 0.50 to ± 1 recognized as high correlation. *Abbreviations: V1=* in vitro *roots of A. cuprea; V2= roots of acclimatized plant of A. cuprea; V3=* in vitro *tubers of A. cuprea; V4= tubers of acclimatized plant of A. cuprea; V5=* in vitro *leaves of A. cuprea; V6= leaves of acclimatized plant of A. cuprea; V7=* in vitro *roots of A. reversa; V8= roots of acclimatized plant of A. reversa; V9=* in vitro *tubers of A. reversa; V10= tubers of acclimatized plant of A. reversa; V11=* in vitro *leaves of A. reversa; V12= roots of acclimatized plant of A.reversa leaves; V13=* in vitro *roots of A. reginula; V14= roots of acclimatized plant of A. reginula; V15=* in vitro *tubers of A. reginula; V16= tubers of acclimatized plant of A. reginula; V17=* in vitro *leaves of A. reginula; and V18= leaves of acclimatized plant of A. reginula.*

The first PCA was positively associated with *V7, V8, V9, V10, V14, V16,* and *V17*, and negatively associated with *V12.* Then the second PCA positively associated with *V1, V2, V3, V4, V5, V6, V11, V12, V15* and *V18* negatively associated with other variables. The metabolites distributed in the upper part of the first PCA axis, with higher loadings such as *V7, V8, V9, V10, V14, V16*, and *V18*, were associated with a higher content of 9,12-Octadecadienoic acid (Z,Z)- (metabolite code number 22). This metabolite had the highest percentage area in GC/MS found in the acclimatized roots of *A. reginula* (*V14*) at 33.15 %. Interestingly, the roots of the acclimatized plant of *A. reversa* (*V8)* were also associated with a higher content of n-Hexadecanoic acid (metabolite code number 45), with an area of 43.1 %. In contrast, the organs located in the lower part of the first PCA axis were negatively associated with these metabolites. For instance, *V2, V3, V13*, and *V15* did not contain 9,12-Octadecadienoic acid (Z,Z)- or n-Hexadecanoic acid. Another association was the association between in vitro tubers of *A. cuprea* (*V3*), tubers of acclimatized plant of *A. cuprea* (*V4*), in vitro leaves of *A. cuprea* (*V5*), and leaves of acclimatized plant of *A. cuprea* (*V6)* with the presence of Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]- (metabolite number 66) with area covered from 6.9 %, 8.6 %, 14.9 %, 43.1 %, respectively (Fig. 6; Table S1).

4. Discussion

4.1. *In vitro propagation method of three* Alocasia *spp*

4.1.1. *Sterilization of three* Alocasia *spp. micro bulbs*

Microbial contamination originating from fungi, yeast, bacteria, and viruses is one of the major restrictions in establishing micropropagation systems (Dangariya et al., 2020; El-Banna et al., 2021; Singh et al., 2011). This contamination hinders culture initiation, reduces multiplication and rooting efficiency, and leads to tissue necrosis and culture mortality (Dangariya et al., 2020; Kałużna et al., 2013; Nadha et al., 2012; Odutayo et al., 2007; Singh et al., 2011). The problem is exacerbated when contaminants are plant pathogens, especially when explant materials are obtained from field-grown plants (Chaidir et al., 2018; Sivanesan et al., 2021; Webster and Mitchell, 2003).

In this study, soaking micro bulbs of the three *Alocasia* spp. in a 0.5–1 % bactericide-fungicide solution and 0.1 % rifampicin (w/v) for 16–18 h as pretreatment, shaking the explants in a 70 % alcohol for 3 min and 30 % clorox for 5 min was the most optimal sterilization method in obtaining high aseptic and regenerated explants (Table 2). Utilizing rifampicin has a high effect on reducing gram (+), (-), and

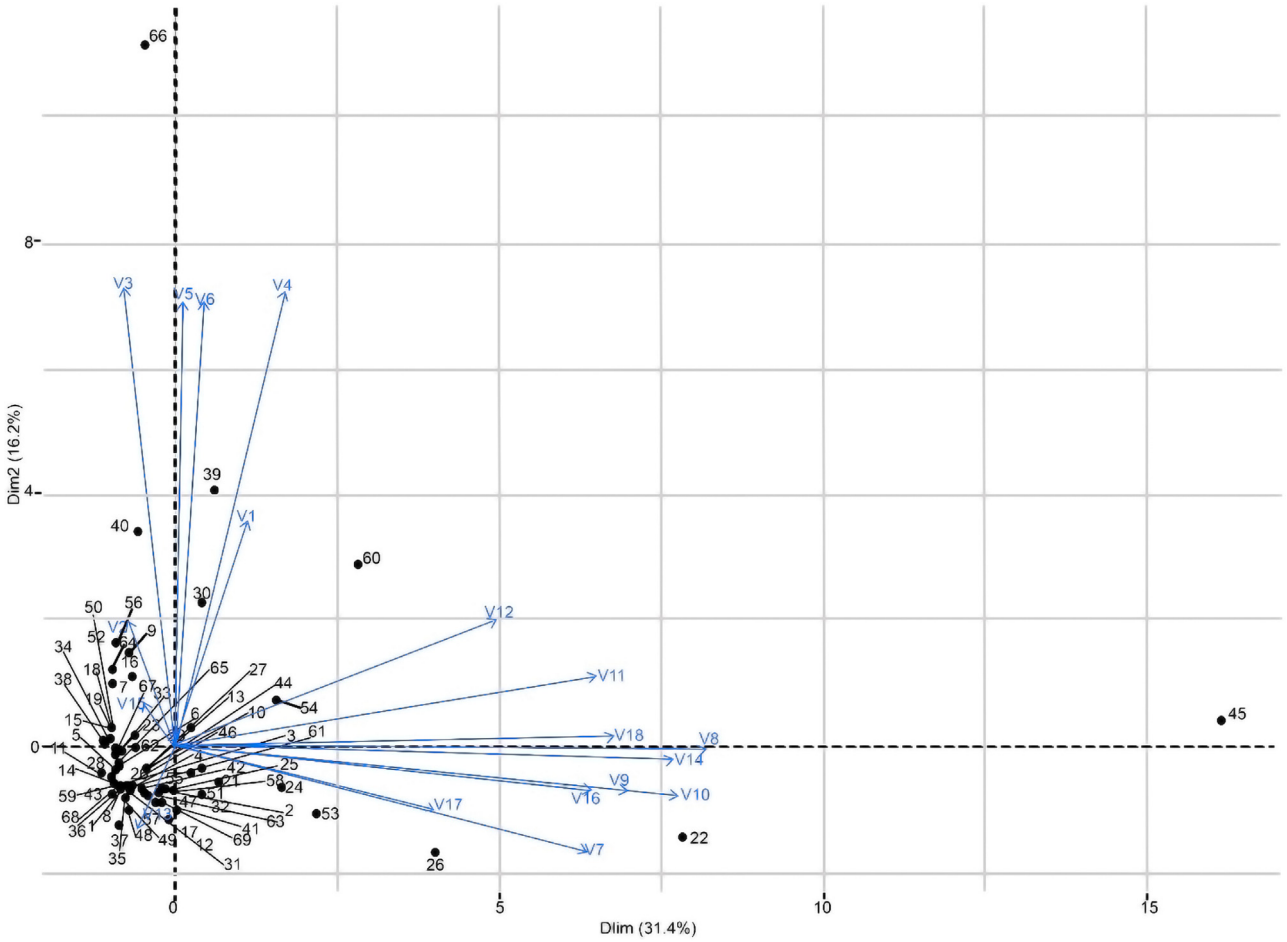

Fig. 6. Principal component analysis of plant organs of *Alocasia cuprea, Alocasia reversa*, and *Alocasia reginula* from in vitro culture and plant acclimatization. The analysis covers 69 metabolite compounds identified in the roots, tubers, and leaves of *Alocasia cuprea, Alocasia reversa*, and *Alocasia reginula*, derived from in vitro culture and plant acclimatization. The corresponding numbers (1 to 69) and variable codes are detailed in Fig. 4 and Table S1.

mycobacteria, binding the β subunit of prokaryotic RNA polymerase, preventing initiation of transcription and blocking a total translocation step that would ordinarily follow the formation of the first phosphodiester bond (Campbell et al., 2001; McClure and Cech, 1978; Węgrzyn et al., 1998) with Clorox (NaOCl) that has a high effect on the broad microbial spectrum, active oxidizing agents and destroy the cellular activity protein (Gallandat et al., 2020; Mcdonnell and Russell, 1999) gave best combination pre and sterilization method to reduce micro bulb contaminants. Optimal application of Rifampicin to reduce contamination with low necrosis was also reported in *Discorea alata* and *Eucalyptus* hybrid (Kuppusamy et al., 2019; Mbah and Wakil, 2012).

4.1.2. *Initiation, proliferation, and plantlet development on in vitro culture protocol of* Alocasia *spp*

Another critical aspect in developing optimal in vitro culture propagation protocol is determining a suitable plant growth regulator (PGR) for each step of propagation (Pasternak and Steinmacher, 2024; Tripathi et al., 2023). Thidiazuron (TDZ), N-phenyl-N′−1,2,3-thiadiazol- 5-ylurea, is an influential PGR with an auxin and cytokinin-like activity with an active role in encouraging shoot formation and promoting axillary shoot proliferation(Desai et al., 2018; Guo et al., 2011). In the research, the application of 1.0 mg/L TDZ in combination with 0.5 mg/L 6-Benzylaminopurine (BAP) having synergistic effect on growth and morphogenesis of shoot with TDZ (Hussain et al., 2018; Sushmarani et al., 2021), produced an impressive 4.2 shoots per explant, with 100 % potential growth and 95.6 % explant shoot initiation within a rapid 9.2 day initiation period in *A. cuprea*. A similar effect of both PGRs was noted in *Caralluma diffusa* (Kalimuthu et al., 2014), *Dendranthema grandiflora* (Sushmarani et al., 2021) and *Rauvolfia tetraphylla* (Hussain et al., 2018). BAP is a broad-spectrum PGR having different effects on shoot formation and proliferation, root and chlorophyll formation, plant rejuvenation, and dormancy breaking(Baymuratova et al., 2023; Singh et al., 2011). A significant effect of the PGR on shoot proliferation was noted on *Alocasia* cv Verde Picante up to 6 shoots per explant on MS medium containing 3.0 mg/L BAP (Guerra et al., 2018) and 13 shoots per explant of *A. amazonica* on similar medium (Abdelbaset et al., 2020). However, the experiment recorded high shoot proliferation as high as 14.7 shoots per clump with 0.83 cm initial shoot height and 1.6 cm clump size in *A. cuprea* cultured on MS medium fortified by 1.5 mg/L BAP. While removing PGR in MS basalt medium was optimal for plantlet development with a half-strength MS medium with full vitamins proved most effective for *A. cuprea, A. reversa*, and *A. reginula*. In different research, MS medium hormone free); regenerated well growth of plantlets of *A. amazonica, A. cuprea, A. robusta, A. longiloba,* and *A. chaii* (Jo et al., 2009; Abdelbaset et al., 2020; Bhatt et al., 2013; Guerra et al., 2018; Abdulhafiz et al., 2020).

4.1.3. Alocasia *spp. plantlet hardening and acclimatization*

In our study, we found that longer hardening periods led to better and higher plantlet performance, likely due to improved adaptation of the plantlets to external environmental conditions. Hardening plantlets by exposing them to the environment with increased light

and temperature, reduced humidity gradually, substituting MS basalt salt with tap water, and utilizing different media improved plantlet capacity to adapt them in an ex vitro environment (Ahmed et al., 2014; Deb and Imchen, 2010; Dev et al., 2019).

Acclimatization is the adaptation of the plantlet derived from tissue culture from heterotrophic conditions (in vitro) to autotrophic conditions (in vivo) (Clapa et al., 2013; Irsyadi, 2021). Paying more attention to several important factors before and during the acclimatization process, such as hardening, acclimatization style, and media, was addressed. In the stage, hardening plantlets for three weeks, a single acclimatization method and utilization of water hyacinth organic manure in combination with sand were the most suitable acclimatization methods for *Alocasia* spp., and the combination was a new finding for *Alocasia* spp.

Applying a single plantlet in acclimatization stimulated plantlet growth maximally with no nutrition and growth space competition and no contamination effect with/from other plantlets. The effectiveness of the method was confirmed in *A. longiloba* (Abdulhafiz et al., 2020) and *A. amazonica* (Raju et al., 2022). Additionally, the utilization of water hyacinth organic manure in acclimatization represents a novel approach for *Alocasia* spp. According to Sukarni et al. (2019) water hyacinth contains a variety of essential nutrients and organic compounds beneficial for plant growth, including carbon, oxygen, sodium, magnesium, aluminium, zirconium, chlorine potassium, calcium, phosphorus, silicon, titanium, and ferum nitrogen. While as compost, it contains nitrogen, carbon, phosphor, methionine, phenylalanine, threonine, lysine, isoleucine, valine, and leucine (Begum et al., 2021; Sahana and Sowmyalatha, 2022). Combining water hyacinth organic manure with sand improves soil characteristics such as porosity, bulk density, cation exchange capacity, and pH, leading to enhanced mineral content and overall growth performance of *Alocasia* spp.

4.2. Exploratory analysis of bioactive metabolites using GC/MS

4.2.1. Bioactive metabolites identified by GC/MS

The identification of 69 different major bioactive metabolites through GC/MS analysis provides valuable insights into the metabolic profiles of three *Alocasia* species across various plant organs and growth conditions. One notable finding is the differential abundance of specific metabolites in different plant organs and species. For instance, 9,12-Octadecadienoic acid (Z,Z)- was found abundantly in the roots of acclimatized plants of *A. reversa* and *A. reginula* or Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]- exhibited high levels in the leaves of both in vitro plantlets and acclimatized plants of *A. cuprea*, indicating roles of these compounds in plantlets and acclimatized growth and development. Meanwhile, there is the presence of n-Hexadecanoic acid across various organs of *A. reversa* and *A. reginula*, particularly in higher quantities in acclimatized plants compared to in vitro plantlets. This variation suggests that different plant organs may have distinct metabolic profiles, which could be influenced by factors like environmental conditions, developmental stages, and genetic backgrounds (Danova and Pistelli, 2022). Another study reported variations in plant secondary metabolites across different plant species and organs, relating to the role of these metabolites in plant defense and stress responses (Isah, 2019).

The class and bioactivity of each compound identified from GC/MS analysis are presented in Table 7. One of the most abundant compounds in our study was 9,12-Octadecadienoic acid, particularly the (Z,Z) isomer. This isomer plays various important roles based on

Table 7
Biological activity of identified compounds from GC/MS analysis of methanol extracts of roots, tubers, and leaves of in vitro plantlets and acclimatized plants of three *Alocasia* spp.

Compounds	Class	Bioactivity
.gamma.-Tocopherol	Vitamin E	anti-inflammatory (Reiter et al., 2007), antioxidant (Brigelius-Flohé, 2021)
(E)-9-Octadecenoic acid ethyl ester	Fatty acids	antibacterial/antibiofilm (Kim et al., 2018; Salem et al., 2022)
(E)-Hexadec-9-enoic acid	Fatty acids	antibacterial/antibiofilm (Kim et al., 2018; Salem et al., 2022)
7-Hexadecenoic acid, methyl ester,(Z)-	Fatty acids	Antibacterial activity (Ghareeb et al., 2022)
9-Octadecenoic acid (Z)-, methyl ester	Fatty acids	antibacterial/antibiofilm (Salem et al., 2022)
9,12-Octadecadienoic acid (Z,Z)-	Fatty acids	antibacterial/antibiofilm (Kim et al., 2018; Salem et al., 2022)
9,12-Octadecadienoic acid, ethyl ester	Fatty acids	antibacterial/antibiofilm (Kim et al., 2018; Salem et al., 2022)
9,12-Octadecadienoic acid, methyl ester	Fatty acids	antibacterial/antibiofilm (Salem et al., 2022)
9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	Fatty acids	antibacterial/antibiofilm (Kim et al., 2018; Salem et al., 2022)
9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	Fatty acids	antibacterial/antibiofilm (Kim et al., 2018; Salem et al., 2022)
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	Fatty acids	Anti-bacterial and Immunostimulant activity (Saha and Bandyopadhyay, 2020)
cis-13-Octadecenoic acid, methyl ester	Fatty acids	Antibacterial, anticancer (Sehim et al., 2023)
Dodecane, 4,6-dimethyl-	Alkanes	Antioksidant, antibacterial (Wiraswati et al., 2023)
Eicosane	Alkanes	Anti-bacterial and Immunostimulant activity (Saha and Bandyopadhyay, 2020)
Heneicosane	Alkanes	Antimicrobial activity (Vanitha et al., 2020)
Hentriacontane	Alkanes	antioxidant (Kim et al., 2011)
Heptadecane	Alkanes	Anti-bacterial and Immunostimulant activity (Saha and Bandyopadhyay, 2020)
Hexadecane	Alkanes	Antibacterial (Nyalo et al., 2023), and antioxidant activities (Tiji et al., 2021)
Hexadecanoic acid, methyl ester	Fatty acids	Antimicrobial (Shaaban et al., 2021)
Loliolide	monoterpene lactone	Anti-aging properties (Cho et al., 2023)
n-Hexadecanoic acid	Fatty acids	Antibacterial (Sehim et al., 2023)
Neophytadiene	Diterpen	Antimicrobial activity (Ruiz-Domínguez et al., 2023), anticancer (Selmy et al., 2023)
Octacosane	Alkanes	Antibacterial activity (Ma et al., 2018)
Octadecane	Alkanes	Antibacterial activity (Kayode et al., 2018)(Kayode et al., 2018)
Octadecanoic acid	Fatty acids	Anti-bacterial and Immunostimulant activity (Saha and Bandyopadhyay, 2020)
Oleic Acid	Fatty acids	Antibacterial/antibiofilm (Kim et al., 2018)
Palmitic Acid, TMS derivative	Fatty acids	Antibacterial/antibiofilm (Kim et al., 2018)
Palmitoleic acid	Fatty acids	Antibacterial/antibiofilm (Kim et al., 2018)
Pentadecanoic acid	Fatty acids	Anticancer activities and antidiabetic activities (Venn-Watson and Schork, 2023)
Phytol	Diterpenoid alcohol	Anti-bacterial and Immunostimulant activity (Saha and Bandyopadhyay, 2020)
Squalene	Triterpene	Hormone precursors in animals and plants, chemopreventive agent, antibacterial, antioxidant, antitumor (Kim and Karadeniz, 2012), anti-cancer (Lozano-Grande et al., 2018)
Tetracosane	Alkanes	Anti-bacterial and Immunostimulant activity (Saha and Bandyopadhyay, 2020)
Tetradecanal	long-chain fatty aldehyde	Antibacterial activity (Bittencourt et al., 2015)
Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]-	lactone	Anti-atherogenic and cardio-protective properties (Adebayo-Gege et al., 2022)
Vitamin E		Anti-inflammatory and antioxidant functions (Li et al., 1998)

research findings. This fatty acid is believed to have multiple health benefits, including analgesic, anti-inflammatory, and antimicrobial properties (Rahman et al., 2014; Mohammed et al., 2016). It interacts with β-ketoacyl-ACP synthase and serves as a potential starting material for tuberculosis drug development (Mtewa et al., 2021). Whereas, Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]- exhibited high levels in the leaves of *A. cuprea.* Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]- is a chemical compound belonging to the class of furanones. The tetrahydrofuran backbone, particularly with a 2,5-trans stereochemistry, is crucial in the synthesis of biologically active compounds like polyether antibiotics and anti-cancer agents (Rabiller, 2009). The abundance of n-Hexadecanoic acid or palmitic acids, across various organs of *A. reversa* and *A. reginula* suggests its potential utility as a source of fatty acids for the cosmetic industry. Furthermore, the consistent detection of phytolin the leaves of all tested *Alocasia* species suggests its ubiquitous role in chlorophyll biosynthesis. Phytol also has been used widely as a geochemical biomarker due to its widespread presence in the biosphere. Its degradation products are used as tracers in aquatic environments (Rontani and Volkman, 2003). In pharmacological, Phytol has been investigated to possess antimicrobial, antioxidant, anti-inflammatory, and anti-arthritic properties, making it a promising compound for biomedical applications offering new therapeutic opportunities (Alencar et al., 2018; Carvalho et al., 2020; Kim et al., 2018).

Although earlier research, particularly that reviewed by Arbain et al. (2022), concentrated mainly on alkaloids, flavonoids, and phenolic compounds linked to medicinal benefits—such as antioxidant, anticancer, anti-inflammatory, antimicrobial, antihyperglycaemic, antidiarrheal, and antidiabetic effects (Karim et al. 2014; Yuliana and Fatmawati 2018; Abdulhafiz et al. 2020), these studies focused on various parts of the *Alocasia* plant, including the leaves, stems, tubers, and roots. Our study expands the scope of investigation by including in vitro plantlets and highlighting the metabolic potential across various plant organs. Importantly, our findings identify several novel bioactive metabolites, including n-Hexadecanoic acid and phytol, which have not been previously reported in the literature, particularly in A. reversa and A. reginula. This contrast emphasizes the need for a more comprehensive analysis of metabolites beyond the traditionally studied parts of the plant.

The identification of n-Hexadecanoic acid (palmitic acid) in *A. reversa* and *A. reginula* highlights its potential in the cosmetic industry due to its skin-smoothing properties, making these species valuable for natural skincare formulations. Additionally, the presence of Phytol in all tested *Alocasia* species suggests its multifunctional potential, particularly in pharmaceutical and nutraceutical applications. Our findings indicate that both *A. reversa* and *A. reginula* species could serve as natural sources for supplements and functional foods that promote overall health.

Although we identify novel bioactive compounds, the study has limitations. The small sample size may not adequately represent the diversity of Alocasia species, potentially biasing results. Genetic diversity among species could yield different metabolic profiles, and a focus on major metabolites might overlook significant minor compounds. Lastly, insufficient assessment of environmental stress factors and variations in sample preparation may impact the reproducibility of the GC/MS results.

4.2.2. Correlation and PCA analyses of organs and bioactive metabolites: intra- and inter-species association and its implication

The correlation analysis of metabolite compounds across various organs of *A. cuprea, A. reversa*, and *A. reginula*, cultured in vitro and acclimatized ex vivo, revealed significant intra- and inter-species correlations and associations. The PCA elucidated the metabolic relationships among organs and species. The identified clusters of variables highlighted distinct metabolic profiles, with certain metabolites associated with specific organs and in vitro or acclimatized conditions. Whereas there were three highlight points noted from the correlation analysis results. First, there are high positive and significant correlations within organs in *A. cuprea* and *A. reversa.* This finding indicates that the various organs (such as roots, stems, and leaves) exhibit similar metabolic profiles within each of these species. This suggests a high degree of metabolic consistency and coordination within the organs of these species, both in vitro and acclimatized. This might be due to shared metabolic pathways and responses to environmental conditions within each species. Second, the lower degree of correlation within the organs of *A. reginula* implies greater metabolic variability within this species. This could be due to a more diverse set of metabolic pathways or a greater capacity for adaptation to varying conditions, resulting in less uniformity in metabolite profiles across different organs.

Lastly, there are positive high correlations between the organs of *A. reversa* and *A. reginula,* but no correlation with the organs of *A. cuprea. A. cuprea* showed no significant correlations with the other two species, suggesting distinct metabolic pathways or adaptations. The lack of significant correlations between *A. cuprea* and the other species may reflect their taxonomic distances. Based on molecular markers, *A. cuprea, A. reversa*, and *A. reginula* are clustered together as clade D, with *A. cuprea* arising in the early Pleistocene, while *A. reversa* and *A. reginula* emerged in the middle to late and late Pleistocene, respectively (Nauheimer et al., 2012). The different emerging times may lead to divergent evolutionary pathways and metabolic profiles, resulting in limited overlap in shared metabolites. Phylogenetic approaches coupled with metabolomics have revealed that related taxa tend to produce similar secondary metabolites, influenced by both genetic relationships and environmental factors (Li et al., 2022). Plants have evolved secondary metabolites as bioactive substances to protect themselves against herbivores and other environmental stresses (Al-Khayri et al., 2023). These metabolites are produced through specialized metabolic pathways that are highly conserved across the plant kingdom, but exhibit vast diversity in their overall structures and biological activities, evolving in different lineages and resulting in the convergent evolution of identical specialized metabolites in phylogenetically distant plants ((Delgoda and Murray, 2017; Duplais et al., 2020; Ono and Murata, 2023; Pichersky and Lewinsohn, 2011; Wink, 2008).

The correlations among metabolite profiles in *A. cuprea, A. reversa*, and *A. reginula* have important implications for further research and practical applications. Understanding how developmental stages, explant sources, and species differences influence bioactive compound production can enhance breeding programs and cultivation practices aimed at maximizing bioactive compound production. Future studies should investigate how environmental conditions affect these correlations, guiding the selection of specific organs for extraction in functional foods and medicinal products. Additionally, using PCA and correlation analyses in plant metabolomics can deepen the understanding of metabolic interactions, helping researchers and practitioners to maximize the health benefits of *Alocasia* species.

5. Conclusion

Our study successfully established efficient in vitro propagation methods for three *Alocasia* spp., enhancing various growth stages through meticulous optimization of sterilization techniques, media formulations, and genotype responses to growth regulators. We also identified novel acclimatization strategies and revealed valuable insights into potential industrial applications through exploratory analysis of bioactive metabolites.

Further and future researches that should be carried out to optimize the propagation techniques holds for sustainable seedling production and species conservation of Allocasia spp. are (1) exploring the functional significance of these metabolites considering factors influencing their production such as environmental cues and genetic

variability, and (2) optimizing metabolite production using elicitors could unlock the full bioactive potential of *Alocasia* spp. for broader applications in various industries.

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Data availability

The supplementary information are available in this manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Fitri Rachmawati reports equipment, drugs, or supplies was provided by GS Biotech-CV. Ranata Nursery. Fitri Rachmawati reports a relationship with GS Biotech-CV. Ranata Nursery that includes: funding grants. Fitri Rachmawati has patent #PROSES PERBANYAKAN MASSAL Alocasia reversa DENGAN KULTUR IN VITRO MELALUI ORGANOGENESIS LANGSUNG MENGGUNAKAN EKSPLAN UMBI MIKRO (Mass Propagation of Alocasia reversa through In Vitro Culture via Direct Organogenesis Using Microtuber Explants), Paten Register Number: P00202314711 pending to -. NO If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Fitri Rachmawati: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Dewi Pramanik:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Lily Ismaini:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Eka Fibriyanty:** Methodology, Investigation, Formal analysis. **Herni Shintiawira:** Validation, Formal analysis. **Taufiq Hidayat Rahman Sude:** Writing – review & editing, Formal analysis. **Sri Ranawati:** Writing – review & editing, Investigation. **Muhammad Prama Yufdy:** Writing – review & editing, Conceptualization. **Laela Sari:** Writing – review & editing, Investigation. **Sri Noptasari:** Investigation. **Budi Winarto:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

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Supplementary materials

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