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The use of colchicine as an elicitor increases the accumulation of phenolics and flavonoids in Java cardamom (*Amomum compactum*) leaves

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Abstract. *Nurcholis W, Aisyah SI, Komala N, Djamaludin H, Mahendra FR, Putra RP. 2026. The use of colchicine as an elicitor increases the accumulation of phenolics and flavonoids in Java cardamom* (Amomum compactum) *leaves. Biodiversitas 27 (3): d270326. https://doi.org/10.13057/biodiv/d270326*. Java cardamom (*Amomum compactum*) is a medicinal plant valued for its secondary metabolites. This study examined the effects of colchicine treatment on plant morphology, physiology, and phytochemical content using vegetative buds collected from a wild population in West Java, Indonesia. A total of 80 buds were treated with colchicine at 0% (control), 0.1%, 0.2%, and 0.3% concentrations. Morphological parameters—including plant height, leaf number, and tiller count—were measured at 18 weeks after planting. Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant capacity were evaluated using ABTS, DPPH, FRAP, and CUPRAC assays. Colchicine treatment caused dose-dependent reductions in plant height and tiller number, with mild leaf shape alterations, but did not induce significant changes in chlorophyll content or stomatal density. TPC and TFC were significantly increased across all treated groups, with the highest TPC at 0.1% colchicine and the highest TFC and ABTS antioxidant activity at 0.3%. Flow cytometry confirmed that colchicine did not induce polyploidy in *A. compactum*. Bioinformatic analysis based on *Arabidopsis thaliana* orthologs revealed upregulation of genes involved in cytokinin signaling (RR17, RAP2.4, GIS2) and phenolic biosynthesis (LAP5, KMD3), supporting a possible hormonal mechanism underlying phytochemical enhancement. The results suggest that colchicine acts as a chemical elicitor, enhancing secondary metabolism without chromosomal duplication. These findings support the potential of colchicine for improving medicinal quality in Java cardamom. Further studies are recommended to optimize treatment parameters and validate gene-level responses directly in *A. compactum* for targeted phytochemical enhancement.

Keywords: Colchicine, elicitor application, flavonoid accumulation, Java cardamom, phenolic content

INTRODUCTION

Indonesia is home to a diverse range of economically important spice crops, including cardamom, nutmeg, and star anise (*Illicium verum*), which contribute significantly to the country's exports of medicinal plants (Nurhayati et al. 2019). Among these, Java cardamom (*Amomum compactum*) stands out for its adaptability to local agroclimatic conditions and cultural relevance. Compared to the introduced *Elettaria cardamomum*, *A. compactum* is more robust in tropical environments but produces relatively lower essential oil yields, typically ranging between 2.02% and 3.16% depending on cultivation practices (Juliana et al. 2023; Octavia et al. 2024). Nevertheless, it contains a high proportion of 1,8-cineole (50.78%) in the stem oil, which is recognized as a potent bioactive constituent (Arista et al. 2023).

In addition to essential oils, *A. compactum* accumulates non-volatile secondary metabolites, particularly phenolic compounds. These compounds play important roles in antioxidative defense and exhibit pharmacological properties, including anti-inflammatory, anticancer, and antimicrobial activities (Amma et al. 2010; Putri et al. 2016; Nurcholis et al. 2022). These phytochemicals also contribute to the traditional use of *A. compactum* in Indonesian herbal medicine (*jamu*) for maintaining general health and treating various ailments (Kurniawan et al. 2021). Accordingly, enhancing the concentration of phenolic and flavonoid compounds has become a primary target in breeding and phytochemical improvement efforts for this species.

The levels of these bioactive metabolites are influenced by genotype, environmental factors, and postharvest handling (Kumar et al. 2022; Cho et al. 2025). Among the available strategies to increase secondary metabolite production, chemical induction through mutagenic agents such as colchicine has gained attention. Colchicine is a well-known alkaloid that disrupts microtubule polymerization during mitosis, often resulting in chromosome doubling (polyploidy) and associated morphological or biochemical changes (Singh et al. 2025). Induced polyploidy can enhance desirable traits, including organ size, biomass accumulation, and the biosynthesis of secondary metabolites (Boonyawiwat et al. 2023; Moroșan et al. 2023). However, the effects of colchicine are not always positive, as several studies have

reported reductions or alterations in secondary metabolite content depending on species, dosage, and physiological conditions (Divekar et al. 2022; Ma et al. 2025).

Several studies have demonstrated colchicine's effectiveness in enhancing phytochemical content in medicinal plants. In *Zingiber officinale*, colchicine-induced tetraploidy increased essential oil yield and gingerol content (Kun-Hua et al. 2011). Similarly, in *Physalis peruviana* and *Cichorium intybus*, colchicine treatment led to elevated levels of phenolic and flavonoid compounds, along with improved antioxidant activity (Ravandi et al. 2013; Çömlekçioğlu and Özden 2020). These examples suggest that colchicine may act not only through chromosomal alteration but also as a biochemical elicitor capable of modulating secondary metabolism.

However, while colchicine is widely used in other crops for polyploidy induction and metabolite enhancement, its effect on *A. compactum* remains largely unexamined. Previous research has reported significant variation among accessions of *A. compactum* in terms of flavonoid content and antioxidant capacity, indicating that the species possesses high breeding value for bioactive compounds (Nurcholis et al. 2021). Yet, it is not well understood whether colchicine can enhance phytochemical production in *A. compactum* independently of polyploidy.

Moreover, emerging evidence points to colchicine's ability to influence plant metabolism via stress responses and hormonal signaling, such as cytokinin pathways, even without altering ploidy (Obando-González et al. 2025). This dual capacity—as both a cytological and biochemical modulator—makes colchicine a compelling tool for improving secondary metabolite profiles in non-model species with limited genomic resources.

To address this gap, the present study aimed to investigate the influence of colchicine treatment on morphological traits, phytochemical content (total phenolics and flavonoids), antioxidant activity, and ploidy status in *A. compactum*. We hypothesized that colchicine may act as a metabolic elicitor, capable of enhancing the accumulation of bioactive compounds without inducing polyploidy. In addition, we employed transcriptomic data from *Arabidopsis thaliana* to explore potential molecular pathways involved in colchicine response, focusing on genes related to cytokinin signaling and phenolic biosynthesis.

This study offers insight into the potential use of colchicine as a non-genetic, elicitation-based strategy to enhance the medicinal value of Java cardamom. It also contributes to broader efforts in the phytopharmaceutical domain by supporting secondary metabolite enhancement through biochemical modulation rather than genomic manipulation.

MATERIALS AND METHODS

Research location

The research was conducted at the Leuwikopo Experimental Farm, Department of Agronomy and Horticulture, Faculty of Agriculture, Institut Pertanian Bogor, Bogor, West Java, Indonesia. The site is located at an altitude of approximately 183 m above sea level, with geographical coordinates of 6°33′50″S and 106°43′36″E. All planting, colchicine treatment, and phenotypic observations were performed under controlled greenhouse conditions to ensure environmental consistency throughout the experiment.

Plant materials

The plant material was collected from a wild population and taxonomically identified at the Tropical Biopharmaca Research Center, Institut Pertanian Bogor (ID: BMK0472052020). As the plant material was obtained from a single wild population, the generalizability of the results may be limited; therefore, future studies should consider using plant samples from multiple regions or agroecological zones to improve broader applicability. A total of 80 vegetative buds of Java cardamom (*A. compactum*) were prepared for experimentation. Soil, compost fertilizer, and burnt husk were mixed in a 2:1:1 ratio as the planting medium. Prior to colchicine treatment, plants were protected using Mancozeb 80% (fungicide) and prophenophos 500 g/L (insecticide), with applications completed two weeks in advance to avoid interference with metabolite assessment. Colchicine (Applichem A4082) served as the mutagenic agent, while all reagents for phytochemical assays, including Folin-Ciocalteu reagent and aluminum chloride, were sourced from Sigma-Aldrich.

Colchicine-induced mutation treatment

Fresh vegetative buds of *A. compactum* (2-3 cm in length, uniform physiological age) were directly treated with colchicine solutions without prior in vitro preculture. Each treatment group consisted of 20 buds (n = 20), with each bud representing one individual plant (i.e., one bud developed into one production plant). The buds were submerged in colchicine solutions at concentrations of 0.1%, 0.2%, and 0.3% (w/v) for 24 hours under dark conditions, while being agitated at 100 rpm to ensure uniform exposure. The selected concentration range was based on previous studies demonstrating the efficacy of 0.1-0.3% colchicine in inducing polyploidy or modulating metabolic activity in Zingiberaceae and other medicinal plants, including *Z. officinale* and *Citrus* spp. (Zhang et al. 2007; Gantait et al. 2011; Habibi et al. 2022). Lower concentrations are generally favored to minimize cytotoxic effects while still allowing sufficient mutagenic potential (Dhooghe et al. 2011). After colchicine exposure, the buds were rinsed three times in 500 mL of distilled water for 5 minutes each, air-dried at ambient temperature, and then transplanted into polybags containing prepared growing media. Control buds were also immersed in distilled water under identical conditions to match handling exposure.

Experimental design and growth parameter observation

The experiment followed a Randomized Complete Block Design (RCBD) with three biological replications per treatment, a design commonly used in plant experiments to reduce environmental variability and improve statistical precision (Lohmor et al. 2017). A total of 80 vegetative buds of *A. compactum* were prepared, consisting of 20 buds for each colchicine treatment group (0.1%, 0.2%, 0.3%) and the untreated control (0%). Each treatment was divided

into three replications, using five uniform and viable buds per replicate, resulting in 15 plants per treatment group (n = 15) for morphological observations. The remaining five buds per treatment group were allocated for biochemical analysis.

Morphological traits assessed at 18 Weeks After Planting (WAP) included plant height, number of leaves, number of tillers, leaf length (from base to lamina tip), leaf width (at the widest point), and stem diameter (measured at the pseudostem midpoint using a digital caliper). Chlorophyll content was recorded at 18 WAP using a SPAD meter on the darkest green leaf of each plant. Leaf and pseudostem colors were evaluated visually using the Royal Horticultural Society Colour Chart (RHSCC), while stomatal density was analyzed microscopically using epidermal imprints from fully expanded leaves.

For phytochemical assays, leaves were harvested at 18 WAP, pooled per replicate (n = 3), and used for biochemical measurements. Here, "n" represents the number of individual plants per treatment used for morphological analysis (n = 15), and pooled replicate samples used for biochemical analysis (n = 3).

Extract preparation

Leaves from each treatment group were collected, air-dried, and subjected to extraction with 80% ethanol. Two grams of the dried sample were dissolved in 10 mL of ethanol and subjected to a single sonication-assisted extraction for 30 minutes in a dark environment, followed by centrifugation at 10,000 g at 4°C for 15 minutes (Nasution et al. 2021). The supernatant was concentrated with a rotary vacuum evaporator to a final concentration of 200 mg/mL in ethanol. Extraction yield was recorded at approximately 12.5% (w/w). These extracts were utilized to assess total phenolic content, total flavonoid content, and antioxidant activity.

Assessment of total phenolic and flavonoid concentrations

The Folin-Ciocalteu method was used to determine Total Phenolic Content (TPC) (Yodi et al. 2023). In a 96-well microplate, 20 µL of extract was combined with 120 µL of Folin-Ciocalteu reagent and incubated for 5 minutes at ambient temperature. After that, 80 µL of 10% sodium carbonate was added, and the mixture was left in the dark for another 30 minutes. A nano-spectrometer (SPECTROstar Nano BMG LABTECH) was used to measure absorbance at 750 nm. TPC values were expressed as mg Gallic Acid Equivalents (GAE) per gram fresh weight (mg GAE/g FW).

Total Flavonoid Content (TFC) was determined using the aluminum chloride colorimetric method (Asyhar et al. 2023). In a 96-well plate, 10 µL of extract was mixed with 120 µL distilled water, 10 µL aluminum chloride, 10 µL glacial acetic acid, and 50 µL ethanol. After 30 minutes of incubation in the dark at room temperature, absorbance was measured at 415 nm. TFC values were expressed as mg quercetin equivalents per gram fresh weight (mg QE/g FW).

Antioxidant activity assays

Four standard assays—DPPH, ABTS, CUPRAC, and FRAP—were used to evaluate antioxidant activity, as Calvindi et al. (2020) explained. In each assay, 20 µL of extract sample was used in the reaction mixture. The DPPH assay measured the decrease in absorbance of the purple DPPH radical solution at 515 nm after 30 minutes of incubation in the dark. This assay measured the radical-scavenging activity of the sample. In the ABTS assay, potassium persulfate was mixed with ABTS to make the $ABTS^{\bullet+}$ radical cation. Then, the sample was added, and the absorbance was measured at 734 nm after 6 minutes of incubation. The CUPRAC assay was performed by mixing the sample with Cu(II)-neocuproine and ammonium acetate buffer at pH 7. After 30 minutes of incubation, the absorbance of the Cu(I)-neocuproine complex was measured at 450 nm. The FRAP assay assessed the reduction of Fe(III)-TPTZ to Fe(II)-TPTZ by antioxidants in the sample, indicated by the increase in absorbance at 593 nm following 5 minutes of incubation. All results were standardized and expressed in µmol Trolox equivalents per gram of fresh weight (µmol TE/g FW), based on calibration curves constructed for each assay (Apak et al. 2016).

Flow cytometry analysis

The ploidy level was assessed through flow cytometry. Young leaves (0.5 × 0.5 cm) were minced in a Petri dish with 250 µL of nucleic acid extraction buffer supplemented with 1% (w/v) Polyvinylpyrrolidone (PVP). The filtrate was subjected to filtration using a 30 µm nylon mesh, followed by the addition of 350 µL of RNase solution (50 µg/mL) and propidium iodide (50 µg/mL final concentration). Samples were examined with a BD Accuri C6+ flow cytometer to analyze nuclear DNA fluorescence intensity histograms. Nuclear DNA content was evaluated based on the position of the G0/G1 peaks and their Coefficient of Variation (CV) values relative to the untreated control to infer ploidy levels.

Bioinformatic analysis

Bioinformatic analysis was performed to explore regulatory networks potentially associated with colchicine-induced changes in secondary metabolism. *Arabidopsis thaliana* was used as a model organism due to its well-annotated genome, availability of extensive genetic resources, and its frequent application in elucidating secondary metabolic pathways, including flavonoid biosynthesis and trichome-associated metabolite regulation (Chalvin et al. 2020; Feng et al. 2023; Saito 2025). The genetic insights gained from *A. thaliana* are widely transferable to non-model species such as *A. compactum*, for which genomic resources remain limited (Hua et al. 2021; Zhang et al. 2021).

Transcriptomic data were retrieved from the Gene Expression Omnibus (GEO) under accession GSE6828, which includes both colchicine-treated and untreated *A. thaliana* samples. Specifically, control samples GSM157347 and GSM157348, and colchicine-treated samples GSM157355, GSM157356, and GSM157357 were selected. Differential expression analysis was conducted

using a Log_2 fold change (LogFC>1) and adjusted p-value (FDR<0.05) as thresholds, following the limma package method (Ritchie et al. 2015). Enrichment analysis was conducted using ShinyGO v0.80 (Ge et al. 2020), and visualized through a Lollipop plot displaying significantly enriched biological processes based on LogFC, FDR, and gene counts.

For gene network analysis, Cytoscape Version 3.8.2 (Liu et al. 2022a) was used to visualize functional associations among differentially expressed genes, particularly those related to cytokinin signaling and phenolic biosynthesis. The resulting networks helped to infer regulatory modules potentially responsive to colchicine, supporting experimental hypotheses on metabolic elicitation. This integrative approach aligns with previous work where Arabidopsis-based models have been leveraged to understand stress responses and secondary metabolite regulation in medicinal and crop plants (Xing et al. 2022; Battlay et al. 2023).

Data analysis

All statistical analyses were performed using IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA). Prior to analysis, data normality was assessed using the Kolmogorov-Smirnov test, and homogeneity of variances was verified using Levene's test. One-Way Analysis of Variance (ANOVA) was conducted to evaluate the effects of colchicine treatment on morphological traits, chlorophyll content, stomatal density, antioxidant activity, total phenolic content, and flavonoid concentration. Post-hoc comparisons between treatment groups were performed using Tukey's Honestly Significant Difference (HSD) test when ANOVA results were significant ($p<0.05$). Results are reported as means±Standard Deviation (SD), and statistical significance was accepted at $p<0.05$. Data visualizations, including bar charts and line plots, were generated using GraphPad Prism version 10.6.1 for macOS (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Effect of colchicine on plant growth and morphology

Colchicine treatment induced observable yet generally non-significant changes in the vegetative morphology of Java cardamom. As shown in Figure 1, treated plants tended to exhibit reduced plant height and leaf density, particularly at 0.2% and 0.3% concentrations, although these trends were not statistically supported. Quantitative analysis (Figure 2) confirmed that only the number of leaves showed a significant difference across treatments ($p<0.05$), with lower values observed at higher colchicine concentrations (Figure 2.B). Other parameters—including plant height, number of tillers, leaf length, leaf width, and stem diameter—did not differ significantly ($p>0.05$; Figures 2.A, 2.C-2.F), indicating that colchicine effects on growth were limited under the tested conditions. Despite visual impressions of leaf narrowing at higher concentrations (Figure 1.E), morphometric measurements did not support a measurable shift in leaf shape. Leaf color remained unchanged (137 A) across all treatments, but colchicine influenced pseudostem pigmentation, with a shift toward RHS 143 B observed in the 0.3% group (Table 1). Interestingly, natural variation was already present in the control group, which exhibited both 143 B and 143 C, suggesting that colchicine-induced pigmentation changes were mild and potentially physiological rather than structural. These results align with reports in *P. peruviana* and *Citrus* × *limon*, where colchicine caused growth suppression and foliar variation (Bhuvaneswari et al. 2020; Çömlekçioğlu and Özden 2020). Comparable qualitative leaf modifications without significant morphometric changes have also been noted in *C. intybus* (Ravandi et al. 2013), supporting the interpretation that colchicine-induced morphological changes in *A. compactum* are subtle, largely non-quantitative, and likely reflect physiological or anatomical responses rather than overt developmental alterations.

Effect of colchicine on chlorophyll content and stomatal density

Colchicine treatment did not significantly affect chlorophyll content or stomatal density in Java cardamom at 18 weeks after planting. The SPAD index showed a slight but non-significant increase at 0.1% colchicine (63.64±5.84) compared to control (59.52±3.56; $p = 0.328$), while stomatal density varied modestly across treatments ($p = 0.843$), indicating that physiological responses were minimal (Figures 3.A-3.B). These observations are consistent with previous reports of increased plastid abundance without strong phenotypic effects (Galatis 1977; Yulianti et al. 2015). Although studies in other species like kenaf (*Hibiscus cannabinus*) report either increased or decreased chlorophyll content following colchicine treatment (Kasmiyati et al. 2020; Chen et al. 2025), such outcomes appear to be species-specific. Similarly, alterations in stomatal traits such as density and size have been noted in colchicine-induced polyploids (Shrestha et al. 2022), but such patterns were not evident in *A. compactum* under current conditions. Overall, the results suggest that colchicine, at the tested concentrations, elicited minimal physiological effects on leaf chlorophyll or stomatal traits in Java cardamom.

Table 1. Distribution of leaf and pseudostem colors in Java cardamom under colchicine treatments, based on RHSCC classification

Treatment	Leaf color (RHSCC)	Pseudostem color (RHSCC)
Control (0%)	137 A	42.86% 143 B; 57.14% 143 C
0.1% Colchicine	137 A	14.29% 143 A; 71.42% 143 B; 14.29% 143 C
0.2% Colchicine	137 A	42.86% 143 B; 42.86% 143 C; 14.29% 143 D
0.3% Colchicine	137 A	75.00% 143 B; 25.00% 143 C

Note: RHSCC: Royal Horticultural Society Colour Chart. 137 A: Moderate olive green, 143 A-C: Strong yellow-green, 143 D: Moderate yellow-green. Values represent the percentage of plants (n = 7 per treatment) exhibiting each pseudostem color, as classified using RHSCC standards

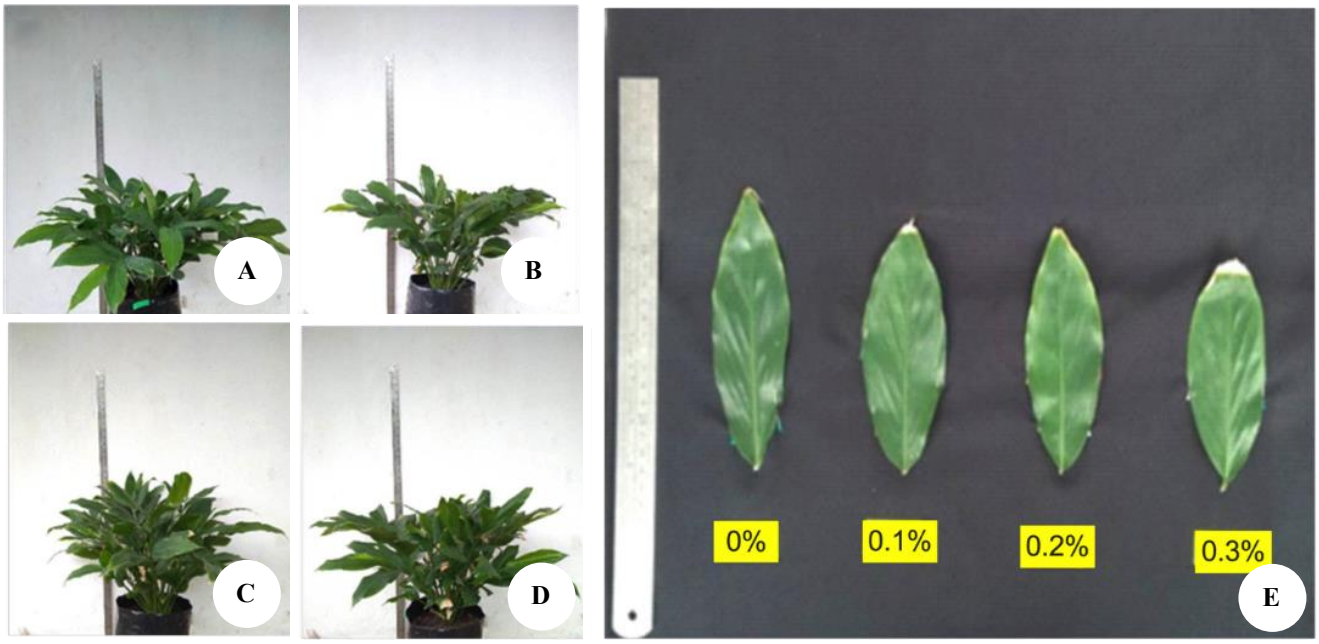

Figure 1. Effect of colchicine on growth and leaf morphology in Java cardamom. Representative plants under colchicine concentrations of: A. 0% (control), B. 0.1%, C. 0.2%, and D. 0.3%, photographed at 18 weeks after planting. Treated plants show a trend of reduced plant height and leaf density at higher concentrations. Panel E. shows representative leaves under each treatment, indicating minor qualitative changes in leaf shape. All photographs were taken under identical lighting and distance

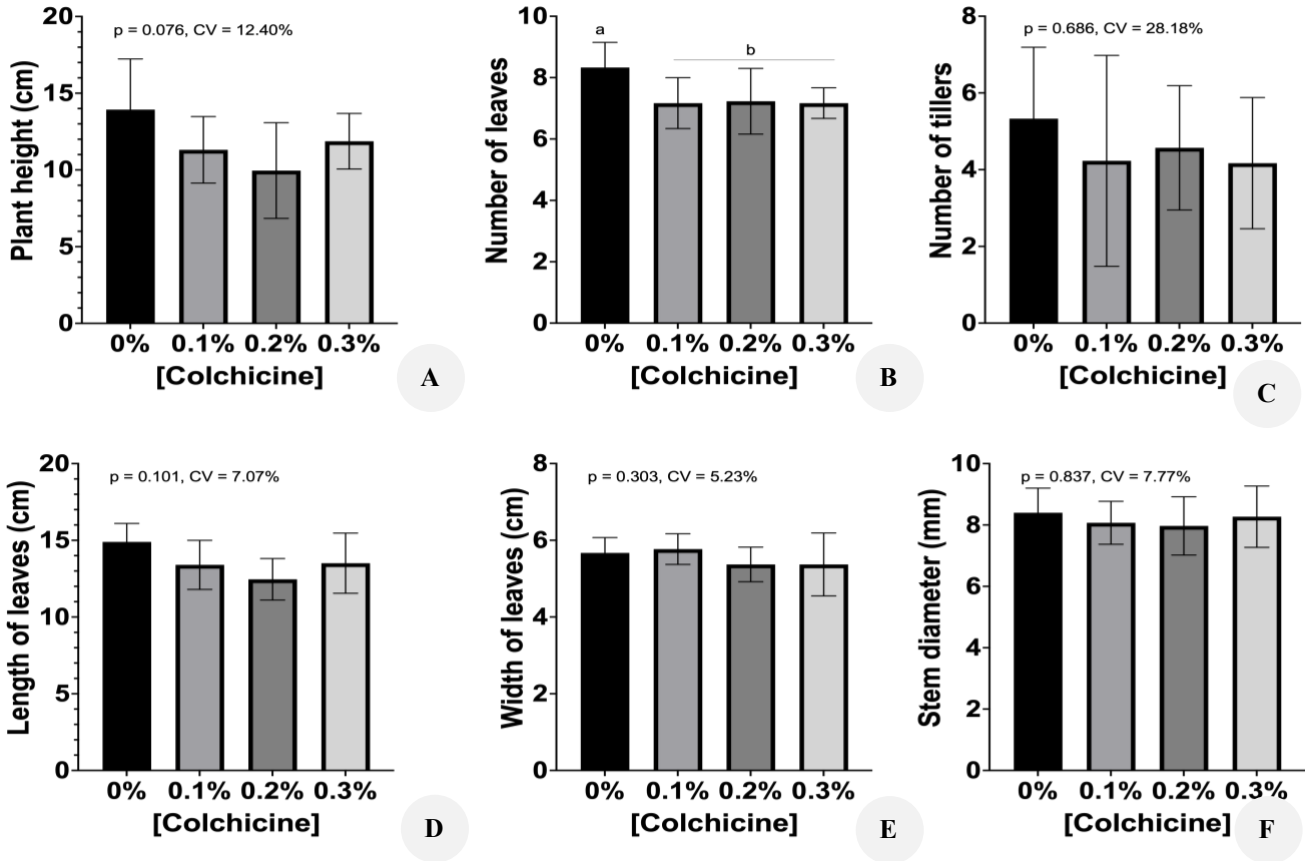

Figure 2. Quantitative effects of colchicine treatment on morphological traits of Java cardamom at 18 Weeks After Planting (WAP). Bar plots showing the effects of colchicine concentrations (0%, 0.1%, 0.2%, 0.3%) on: A. Plant height, B. Number of leaves, C. Number of tillers, D. Leaf length, E. Leaf width, and F. Stem diameter. Data are presented as mean±standard deviation (n = 15). Different letters (e.g., a, b) indicate significant differences between treatments based on Tukey's HSD test at $\alpha = 0.05$. p-values and Coefficients of Variation (CV) are shown for each trait

Effect of colchicine on phenolic content and antioxidant activity in Java cardamom

Colchicine treatment increased Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant activity in Java cardamom leaves compared with the untreated control (Figures 4.A-4.F). The highest TPC was observed at 0.1% colchicine (76.42±0.33 mg GAE g^{-1} FW), significantly higher than control (62.87±1.10 mg GAE g^{-1} FW) (Figure 4.A), while TFC increased in a concentration dependent manner and reached its maximum at 0.3% colchicine (30.78±0.57 mg QE g^{-1} FW) (Figure 4.B). Similar enhancements of TPC and TFC following colchicine treatment have been reported in medicinal and spice plants, where colchicine acts as a chemical elicitor that stimulates secondary metabolite biosynthesis (Hartati et al. 2020; Rosiak et al. 2022). Such increases have been linked to stress induced activation of phenolic biosynthetic pathways, particularly enzymes such as Phenylalanine Ammonia Lyase (PAL), which play a central role in phenolic accumulation (Sanghamitra et al. 2022).

Consistent with the increased phenolic and flavonoid levels, the ABTS assay showed significantly higher antioxidant activity in all colchicine-treated plants, with the highest scavenging capacity at 0.3% colchicine (78.52±1.29%) compared to the control (66.25±0.47%) (Figure 4.C). In contrast, DPPH radical scavenging activity did not differ significantly among treatments (Figure 4.D), a discrepancy that has been widely reported and is attributed to differences in assay sensitivity and the chemical specificity of phenolic compounds produced (Abdullah et al. 2020; Irawan et al. 2022; Muhammad et al. 2023).

The CUPRAC and FRAP assays further indicated moderate but significant increases in reducing power at 0.1-0.3% colchicine (Figures 4.E and 4.F), supporting the view that phenolic accumulation enhanced by colchicine treatment contribute more strongly to electron-transfer-based antioxidant mechanisms than to DPPH radical scavenging. Overall, these results confirm that colchicine functions as a chemical elicitor enhancing phenolic and flavonoid accumulation in Java cardamom, while the magnitude of antioxidant response depends on both colchicine concentration and the assay employed, as also observed in other medicinal plants (Abdoli et al. 2013; Ravandi et al. 2013; Çömlekçioğlu and Özden 2020). Notably, comparative studies in *Salvia officinalis* have demonstrated that polyploidization may differentially influence phenolic composition and antioxidant activity between diploid and autotetraploid genotypes, highlighting that changes in ploidy do not uniformly enhance antioxidant capacity (Tuğlu et al. 2025).

Colchicine did not induce polyploidy in Java cardamom

Flow cytometric analysis of colchicine-treated Java cardamom confirmed that no polyploidy was induced, as all samples displayed singular peaks indicative of diploid nuclear DNA content, with Coefficient of Variation (CV) values between 4.89% and 5.94% (Figure 5). No additional peaks or peak broadening indicative of mixoploidy were detected in any of the analyzed samples, suggesting cytological uniformity of diploid nuclei within the examined tissues. This outcome suggests that under the tested concentrations (0.1%-0.3%) and treatment durations, colchicine was ineffective in triggering chromosomal duplication. Although colchicine is widely recognized as an antimitotic agent that disrupts mitotic spindle formation to induce polyploidy, its success is highly dependent on variables such as concentration, exposure time, and explant type (Zakizadeh et al. 2020; Tsai et al. 2021; Wen et al. 2022). The absence of polyploidy in this study mirrors findings in other species, such as *Chrysanthemum* and tomato, where colchicine-induced phenotypic changes occurred without genome duplication (Lertsutthichawan et al. 2017; Obando-González et al. 2025).

Interestingly, despite retaining diploid status, *A. compactum* plants exhibited altered leaf morphology, increased chlorophyll content, and enhanced levels of phenolic and flavonoid compounds. These physiological and biochemical modifications point to non-genomic mechanisms of colchicine action, likely involving stress responses and hormone-regulated signaling pathways that reprogram secondary metabolism (Liu et al. 2022b; Rosiak et al. 2022). Previous research suggests that colchicine may upregulate key biosynthetic enzymes such as Phenylalanine Ammonia-Lyase (PAL), Chalcone Synthase (CHS), and Flavonoid 3-Hydroxylase (F3H), all of which play critical roles in the biosynthesis of phenolics and flavonoids (Sanghamitra et al. 2022; Mitic et al. 2025). These results support the interpretation that colchicine can act as a metabolic elicitor without inducing changes to chromosomal structure. For future studies, it would be valuable to test a broader range of colchicine concentrations and exposure durations, and to complement flow cytometry with additional cytogenetic verification techniques such as chromosome counting or fluorescence in situ hybridization (FISH), to accurately determine conditions that may induce stable polyploidy in Java cardamom while disentangling metabolic responses from genomic alterations.

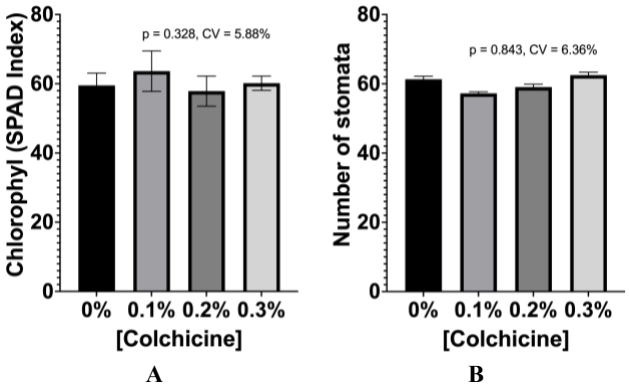

Figure 3. Effects of colchicine treatment on chlorophyll content and stomatal density in Java cardamom at 18 Weeks After Planting (WAP). A. SPAD index values representing relative chlorophyll content in the youngest fully expanded leaf per plant, B. Stomatal density based on microscopic counts from epidermal imprints of fully expanded leaves. Data are expressed as mean±standard deviation. No statistically significant differences were detected between treatments (Tukey's HSD, $\alpha = 0.05$). p-values and Coefficients of Variation (CV) are provided for each parameter

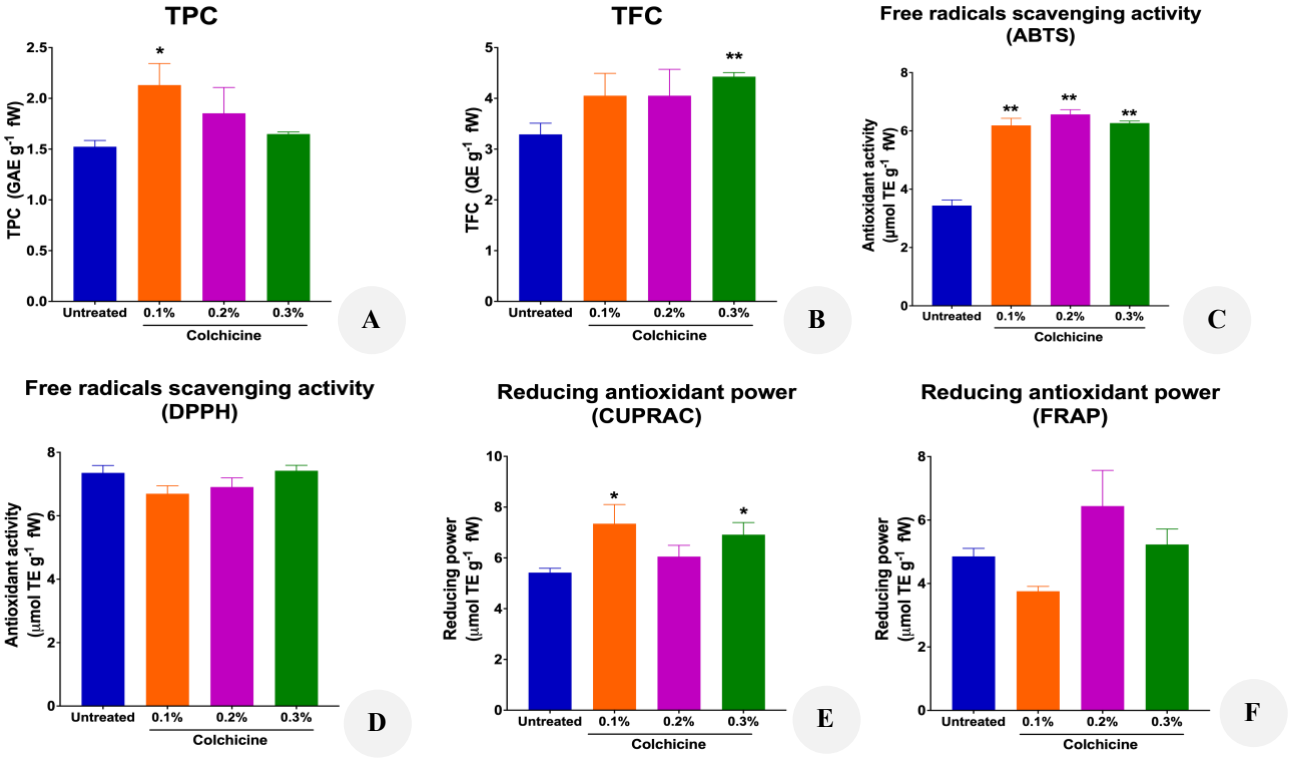

Figure 4. The impact of colchicine treatments at different concentrations on the phenolic and flavonoid content, as well as the antioxidant activity, of Java cardamom leaf extract. A-B. The TPC and TFC of Java cardamom leaves extract were determined using A. Folin-Ciocalteu, and B. Aluminum chloride reagents. C-D. The scavenging activity of Java cardamom leaves was assessed using the C. ABTS, and D. DPPH methods. E-F. Diminishing antioxidant capacity of Java cardamom leaves assessed by E. CUPRAC, and F. FRAP methodologies. n = 3 for each group, and the statistical analysis was done using an unpaired t-test. The comparison was conducted between untreated and colchicine-treated groups. *p<0.05, **p<0.01

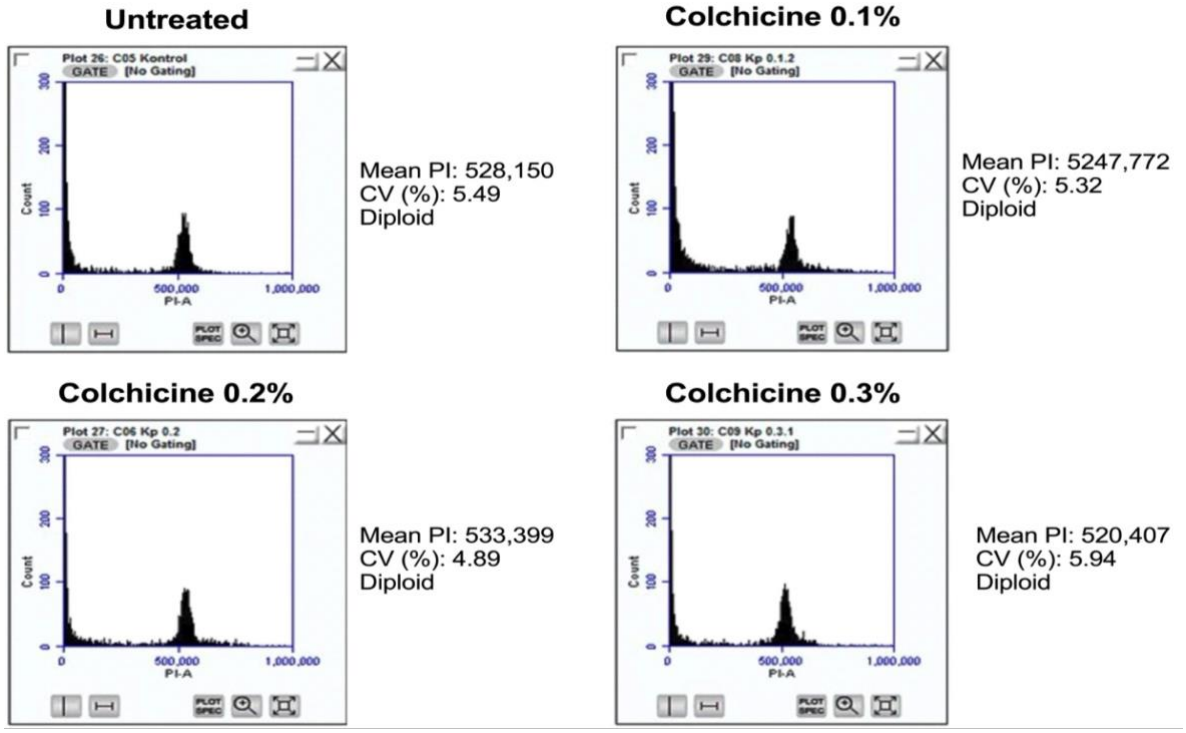

Figure 5. Identification of polyploidy in *Amomum compactum* subjected to varying concentrations of colchicine via flow cytometry. All treatments exhibited singular peaks, signifying diploid status, with Coefficient of Variation (CV) values ranging from 4.89% to 5.94%

Bioinformatic analysis linking cytokinin signaling and phenolic biosynthesis

Bioinformatics analysis using the model organism *A. thaliana* revealed that colchicine treatment induces the upregulation of genes associated with cytokinin signaling and polyphenol biosynthesis, notably GIS2 and LAP5 (Table 2), providing a potential molecular explanation for the increased phenolic and flavonoid accumulation observed in colchicine-treated Java cardamom leaves (Figure 4). Gene Ontology (GO) enrichment results indicated that these upregulated genes are involved in key biological processes including secondary metabolite biosynthesis (e.g., polyketide and phenylpropanoid pathways), hormone response, and signal transduction (Figure 6), suggesting that colchicine-induced metabolic changes may be mediated through hormone-regulated secondary metabolism pathways. Many genes were also associated with phosphorelay systems and transferase activities at the molecular function level. Gene network mapping demonstrated a clear distinction between cytokinin-related genes (red nodes) and polyphenol biosynthesis genes (green nodes), connected through intermediate biological processes (blue edges) (Figure 7), indicating coordinated regulatory interactions between cytokinin signaling pathways and phenolic biosynthesis networks. These interactions suggest that colchicine may stimulate secondary metabolism indirectly via cytokinin-mediated signaling cascades that regulate phenylpropanoid and flavonoid biosynthetic pathways. Key regulatory genes such as RR17, HP6, RAP2.4, RR18, and GIS2 were found to be upregulated, highlighting their potential role in activating cytokinin signaling that may subsequently influence secondary metabolite production in plants. This is consistent with previous studies reporting their involvement in stress response and plant development (Veerabagu et al. 2012; Rudnik et al. 2017; Chang et al. 2019). In parallel, genes involved in phenolic biosynthesis pathways, such as LAP5, LAP6, KMD1, and KMD3, were also significantly upregulated, supporting the hypothesis that colchicine may enhance phenylpropanoid metabolism through coordinated hormonal and metabolic regulation (Kim et al. 2011; Zhang et al. 2015; Wang et al. 2022). These findings reinforce the hypothesis that colchicine enhances the biosynthesis of phenolics and flavonoids in *A. compactum*, either by directly triggering biosynthetic genes or through hormonal regulatory circuits, thereby linking the observed phytochemical enhancement with cytokinin-regulated gene networks.

Bioinformatic analysis using *A. thaliana* orthologs offers predictive insight into the molecular mechanisms underlying the phytochemical responses observed in Java cardamom following colchicine exposure, integrating transcriptomic predictions with the experimentally observed increases in phenolic and flavonoid content (Figure 4). Gene Ontology (GO) enrichment highlighted biological processes related to cytokinin-activated signaling, phenylpropanoid and polyketide biosynthesis, and transferase activity (Figure 6). These annotations align with the observed increases in total phenolic content, total flavonoid content, and antioxidant capacity in colchicine-treated Java cardamom (Figure 4), suggesting a mechanistic link between cytokinin signaling pathways and secondary metabolite accumulation. However, it is important to emphasize that these predictions are based on a model species and should be interpreted cautiously until experimentally validated in Java cardamom.

Among the differentially expressed genes, several cytokinin-associated regulators such as RR17, RAP2.4, and GIS2 were upregulated (Table 2). These genes have been previously implicated in the regulation of secondary metabolism and abiotic stress responses in *Arabidopsis* and other species (Ojeda-Martinez et al. 2021; Argueso and Kieber 2024; Zhao et al. 2024). Additionally, genes involved in flavonoid and polyketide biosynthesis—including LAP5 and LAP6 (Quan et al. 2023)—were also upregulated, supporting a role for colchicine in modulating these pathways.

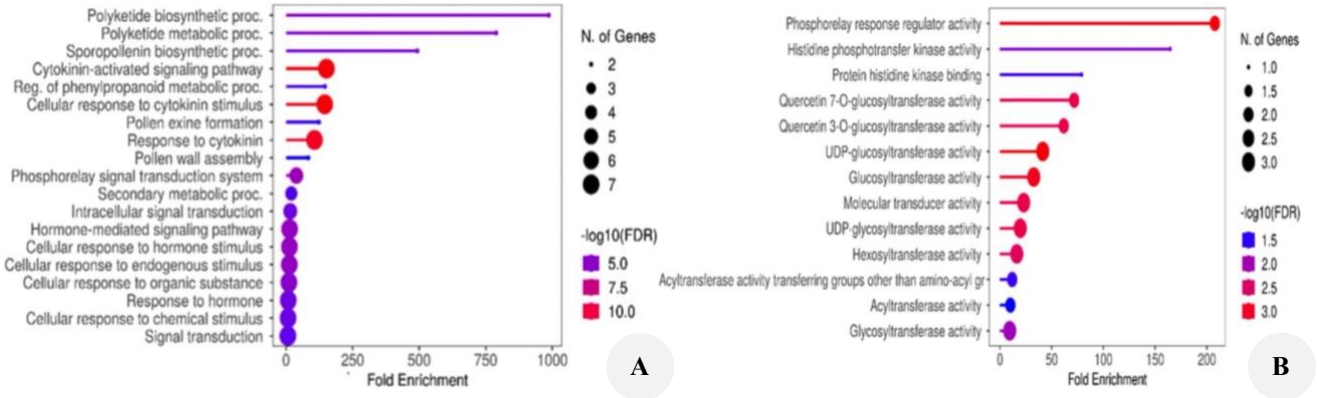

Figure 6. Gene Ontology (GO) enrichment analysis of cytokinin- and polyphenol-related gene pathways potentially involved in colchicine-induced metabolic responses (based on *Arabidopsis thaliana* orthologs). Dot-plot visualization of GO terms associated with the upregulated genes linked to: A. Hormonal signaling and secondary metabolism, including cytokinin response and phenylpropanoid biosynthesis, and B. Transferase activities involved in polyphenol biosynthesis (e.g., quercetin glycosylation). The analysis is based on orthologs identified in the model plant *A. thaliana*, and supports the hypothesis that colchicine may activate metabolic pathways without inducing polyploidy

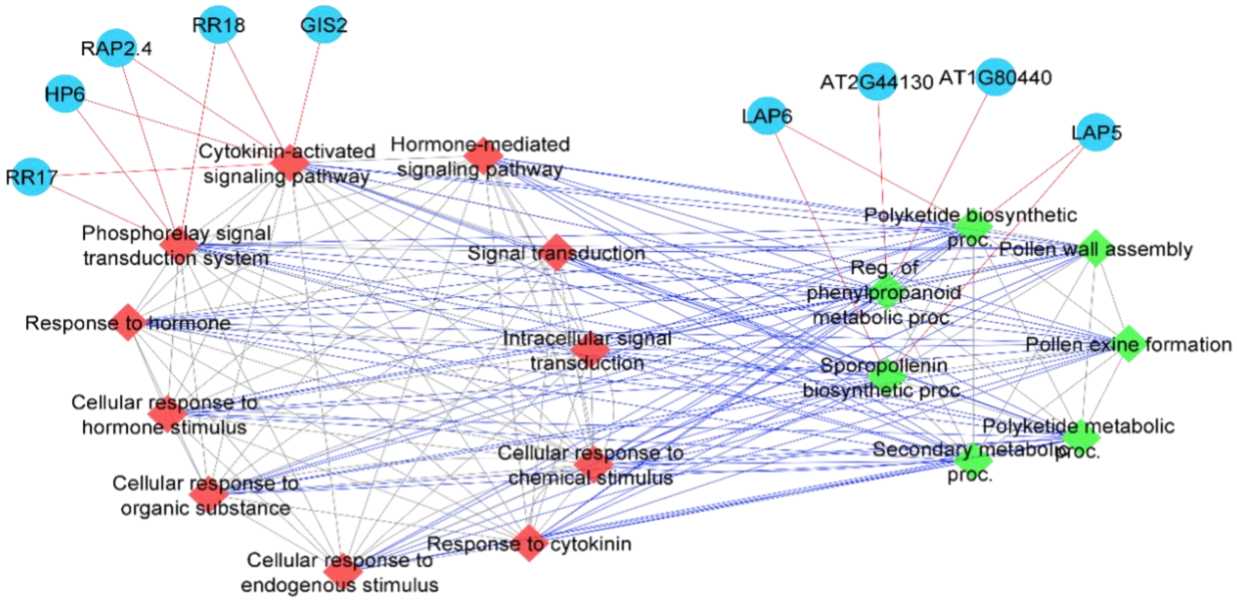

Figure 7. Network interactions between genes involved in the synthesis of cytokinins and polyphenols. Genes are represented by elliptical nodes colored blue, Biological Processes (BP) are represented by red diamond shapes (secondary metabolite biosynthesis), and green color (cytokinin regulation)

Table 2. Gene upregulation upon colchicine treatment in the model organism *Arabidopsis thaliana* is related to the production of cytokinins and polyphenols

LogFC	P.value	Genes	Gene title
2.73	9.60e-03	LAP5	Protein less adhesive pollen 5
2.31	2.87e-02	GIS2	Protein glabrous inflorescence stems 2
1.74	8.54e-07	AT1G80440 (KMD1)	F-box/kelch-repeat protein
1.41	1.61e-01	UGT76E2	UDP-glucosyl transferase 76e2
1.35	1.65e-01	RR23	Protein response regulator 23
1.35	3.31e-05	RAP2.4	Ethylene-responsive transcription factor RAP2-4
1.32	2.37e-01	LAP6	Polyketide synthase a
1.30	2.34e-01	RR18	Response regulator 18
1.22	2.21e-04	AT2G44130 (KMD3)	F-Box/Kelch-repeat protein
1.21	2.29e-01	UGT84B1	UDP-glucosyl transferase 84b1
1.18	2.89e-01	KUOX1	KAR-up oxidoreductase 1
1.17	3.23e-01	RR17	Two-component response regulator ARR17
1.11	2.95e-01	HP6	Histidine phosphotransfer protein 6
1.09	2.43e-01	AT5G54010	UDP-glycosyltransferase 79b6

The gene network visualization (Figure 7) reveals a modular architecture, with cytokinin-responsive genes (red nodes) and phenolic biosynthesis genes (green nodes) connected via intermediate biological processes (blue edges), suggesting indirect regulatory interactions. Glycosyltransferases such as UGT76E2 and UGT84B1, known to modify phenolic compounds for enhanced solubility and bioactivity (Quan et al. 2023), were also represented, pointing to a broader remodeling of flavonoid structure and function.

Furthermore, the detection of cytokinin-related F-box proteins such as KMD1 and KMD3, which mediate the degradation of PAL—a key enzyme in the phenylpropanoid pathway—indicates possible hormonal control over phenolic flux (Zhao et al. 2024). Notably, these bioinformatic predictions are consistent with experimental findings showing that colchicine enhanced phenolic and antioxidant profiles (Figure 4) without inducing polyploidy, as confirmed by flow cytometry (Figure 5), indicating that colchicine may function primarily as a metabolic elicitor rather than a genome-modifying agent in this species. This reinforces the interpretation that colchicine functioned primarily as a chemical elicitor, triggering cytokinin-mediated metabolic reprogramming without structural genome alteration (Zhang et al. 2023; Ding et al. 2025; Yaowachai et al. 2025). Future validation in Java cardamom—e.g., through transcriptomics or targeted qPCR—will be necessary to confirm these candidate genes' roles and to understand the cross-talk between hormonal and metabolic responses in this species.

In conclusion, this study demonstrates that colchicine treatment, up to 0.3%, induces subtle morphological and physiological changes in Java cardamom, including reduced plant height, altered leaf and pseudostem coloration, and minor shifts in chlorophyll content and stomatal density. Although colchicine significantly increased total phenolic and flavonoid contents, along with antioxidant activity as measured by multiple assays, flow cytometry confirmed that polyploidy was not induced under the tested conditions. Bioinformatic analysis based on *A. thaliana* orthologs supports a plausible role for cytokinin-associated regulators—such as RR17, RAP2.4, GIS2, LAP5/6, and KMD1/3—in modulating phenolic biosynthesis in response to colchicine, suggesting that colchicine may act as a

chemical elicitor triggering metabolic reprogramming through hormonal signaling rather than chromosomal duplication. However, despite its elicitation potential, colchicine may not be suitable for broad or large-scale application due to its relatively high cost and well-documented toxicity as a potent alkaloid, which restrict its use primarily to controlled experimental conditions. Further studies are warranted to validate gene-level responses in *A. compactum*, optimize application parameters, and explore safer alternative elicitors for phytochemical enhancement and breeding innovation.

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