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Metabolite Profiling of Porang (*Amorphophallus muelleri* Blume) After Polyploidization Using Colchicine

Didik Wahyudi[1*)], Suyono[1)], Ruri Siti Resmisari[1)], Bagas Prateso[1)], and Eriyanto Yusnawan[2)]

[1)] State Islamic University of Maulana Malik Ibrahim Malang, Indonesia
[2)] Research Center for Food Crops, Research Organization for Agriculture and Food, National Research and Innovation Agency (BRIN), Indonesia

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*) Corresponding author:
E-mail: didik_wahyudi@bio.uin-malang.ac.id

ABSTRACT

Both chemically and physically, mutation induction is a common strategy to increase the genetic diversity of porang. However, the in-depth evaluation of changes in secondary metabolites after mutation induction has not been widely implemented. This study aimed to profile metabolites in porang after colchicine mutation induction and analyze the relationship between these metabolites and the glucomannan synthesis pathway. Porang explants induced with a variation in colchicine concentration were used for metabolite profiling analysis. The metabolite profile was identified using GC-MS (Gas chromatography tandem-mass spectrometry) and then analyzed using MetaboAnalyst. The metabolite pathway network was carried out using Cytoscape software. Application of 0.02% of colchicine resulted in mixoploid porang and has a different metabolite profile than control porang. In addition, mixoploid porang (0.02% colchicine) has more metabolite content related to glucomannan synthesis. Therefore, mutation induction using 0.02% colchicine concentration is recommended to produce superior porang.

INTRODUCTION

Porang is widely distributed in Indonesia, particularly in the regions of Sumatra (Bidarti et al., 2021), Madura, Bali, West Nusa Tenggara (NTB), and predominantly on Java (Alifianto et al., 2013). This distribution reflects the impact of Indonesian government policies promoting porang cultivation as part of agricultural diversification and increasing export value. However, in Indonesia, porang generally exhibits low genetic diversity (Sulistiyo et al., 2015), which poses a threat to the future sustainability of porang cultivation. Therefore, efforts to enhance genetic diversity through crossbreeding (Arruum & Waluyo, 2021), somaclonal variation (Shamsuzzaman et al., 2021) and polyploidization (Wahyudi et al., 2023) are important to support the development of porang cultivation in Indonesia.

Chemical mutation induction using colchicine (Madani et al., 2021), oryzalin (Bharati et al., 2023) and trifluralin (Karami et al. (2019) is popular in plant breeding. However, colchicine has been widely used to induce plant polyploidy (Eng & Ho, 2019; Madani et al., 2021; Manzoor et al., 2019) and improve plant morphological characteristics (Touchell et al., 2020). In addition, colchicine treatment has proven effective in improving morphological features (Widoretno et al., 2023) and genetic variation (Suyono et al., 2023) in porang. Mutation induction using colchicine effectively enhanced the physiological characteristics (Markova et al., 2022) and photosynthetic efficiency (Ulum et al., 2021). Improving physiological traits might have been triggered by higher genetic material and amplified gene expression in polyploid cells, which enhances metabolic activity and cellular organization (Fang et al., 2022).

The metabolomics approach is the best way to detect metabolite compounds in plants comprehensively and quantitatively (Tugizimana et al., 2019). Relationship between genetic changes

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and secondary metabolites after mutation induction (Sharma et al., 2021) easily detected through metabolomic study. In addition, metabolomic study also provides broad expertise in various fields, including metabolite responses after treatment (Bhattacharyya et al. 2021), biomarker identification (Singh et al., 2021), and understanding the relationship between metabolites, morphological characteristics and gene expression (Liu et al., 2022) in plants after mutation induction (Ku et al., 2020). Understanding metabolic changes after mutation induction provides important information for plant improvement, stress tolerance (Chen et al., 2023) and develop biomarkers for certain genetic traits (Manickam et al., 2023) that can be used as a guide for porang breeding programs in the future.

The effectiveness of polyploidization on metabolic profiles has been reported in *Solanum commersonii* induced by oryzalin which produces more amides, amino acids, and phenylpropanoids than controls (Fasano et al., 2016). Mutation induction using gamma-ray in garlic (MV3) also increase the content of secondary metabolites and produce 5-6 new compounds such as 2-hexanol; 2-hexanol, 3,4-dimethyl-; Cyclopentene, 1,2,3,3,4-pentamethyl-; Furan, 2-(1,1-dimethylethyl)-4-methyl-; and Trisulfide, methyl 2-propenyl (Winarni et al., 2022). Similarly, in *Salvia officinalis*, the metabolomics study can identify gene expression and biosynthesis of terpenoid compounds from various levels of mutation induction with hydrazine hydrate (Ali et al., 2023). However, there has been no research on metabolomics studies on porang after mutation induction using colchicine, especially the effect of mutation on glucomannan synthesis. Therefore, this study aims to identify metabolite profile of porang (*Amorphophallus muelleri* Blume) after mutation induction with colchicine and analyze the relationship between these metabolites and the glucomannan synthesis pathway.

MATERIALS AND METHODS

Plant material and experimental sites

This study was conducted from January to December 2024 at the Plant Tissue Culture and Molecular Genetics Laboratory, Biology Study Program, Faculty of Science and Technology, Universitas Islam Negeri Maulana Malik Ibrahim Malang. Porang shoot explants (2n=26) were cultured on Murashige and Skoog (MS) medium supplemented with 2 mg/L benzyl adenine (BA). The explants were kept at 21 °C under continuous fluorescent light at 1500 lux. The explants were prepared for polyploidy induction after 30 days.

Polyploidy induction

Polyploidy induction was performed by incorporating 0%, 0.01%, and 0.02% colchicine into the culture medium. Colchicine was added to 12.5 ml of MS medium using a 0.22 µm millipore filter. The explants were moved to a colchicine-free MS medium after 7 days of colchicine treatment. After 53 days in the colchicine-free medium, the explants were observed.

Flowcytometry analysis

Porang explant leaves (25 mg) were ground in a mortar containing 100 µL of LBO1 lysis buffer (pH 15 mM Tris, 2 mM Na2EDTA, 0.5 mM permine, 4 HCl, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100). The mixture was then incubated at room temperature for 5 min. The mixture was then filtered using a 30 µm nylon mesh to isolate the nuclei (Parsons et al. 2019) and stained with 25 µL of propidium iodide fluorescent dye solution (10 mg/mL) and 2.5 µl RNAseq (20 mg/mL). The solution was then incubated on ice for 15 min and homogenized by vortexing. The homogenate was then analyzed using a flow cytometer (Guava easyCyte), and the data were analyzed using InCyte 3.1 software.

Metabolite extraction

Metabolite extraction followed the method of (Muthukumaran et al., 2020) with some modifications. All parts of the porang plant were ground into a powder using liquid nitrogen. 300 mg of the porang powder was placed in a 2 mL tube and mixed with 1.5 mL of ethanol. The mixture was left at room temperature for approximately 12 hours. The mixture was then centrifuged at 9,000 rpm for 10 minutes, after which the supernatant was collected and analyzed using GC-MS.

Metabolite profiling using gas chromatography-mass spectrometry (GC-MS)

Metabolic profile identification was performed using the method described by Putra et al. (2024). The supernatant obtained from sample extraction in ethanol was evaporated under a nitrogen stream. The dried sample was methoxylated using 50 µL of methoxamine hydrochloride in pyridine and incubated for 120 min at 30°C for 120 minutes. The sample was then derivatized with 50 µL of N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA),

incubated at 37°C for 90 minutes, and analyzed using GC-MS (Trace 1310-ISQ LT, Thermo Scientific, Massachusetts, USA). The identified metabolites were obtained as raw data from the Xcalibur software and compared with mass spectra and retention indices from the National Institute of Standards and Technology MS Search 2.2 and Wiley databases.

Data analysis

Data processing was performed using Microsoft Excel and MetaboAnalyst 6.0 software (https://www.metaboanalyst.ca/MetaboAnalyst). The data processing workflow includes: (1) alignment, (2) data filtering, (3) missing data estimation, (4) data normalization, (5) chemometric analysis, and (6) metabolic pathway analysis. Data without a definite substance name and no spectral ratio or substances with a missing quantity greater than 50% were removed. The K-nearest neighbor (KNN) algorithm was used to simulate missing values for substances with a missing quantity of less than 50%. Principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and hierarchical clustering (heat map) were used for statistical analysis, as well as metabolic pathway analysis (Yuan et al., 2022). Cytoscape 3.10.3 software was used for metabolic network analysis and construction.

RESULTS AND DISCUSSION

Porang total ploidy after mutation induction using colchicine

Colchicine application at varying concentrations (0%, 0.01%, and 0.02%) significantly influenced the ploidy levels of Amorphophallus muelleri Blume (Figure 1). This treatment induces ploidy transitions, resulting in tetraploid (4n = 4x = 52) and mixoploid porang (Table 1). Higher colchicine concentrations increased the frequency of polyploid cells and the likelihood of mixoploid porang.

The successful polyploidy using colchicine has also been observed in several plants, including some medicinal plants like *Catharanthus roseus* and *Lithospermum erythrorhizon* (Madani et al., 2021) and some horticultural plants (Eng & Ho, 2019). The mixoploid plant after colchicine treatment was also a common phenomenon. Varying responses of cells to colchicine may explain why cells have different ploidy levels (mixoploid) (Widoretno et al., 2023).

Metabolite profile of porang (*Amorphophallus muelleri* Blume) after treatment with colchicine

Colchicine treatment successfully increased the total porang metabolites. A total of 75 compounds were successfully detected in mixoploid (K2) and 53 compounds at tetraploid porang (K1), whereas only 45 compounds were detected in control porang (Fig. 1). These compounds were classified based on their functional groups. Colchicine treatment increased the levels of alkaloids, amino acids, sugars, alcohols, and other compounds, and only aldehydes and ketones were detected to be higher in the control porang. Tetraploid porang (K1) contains other compounds that are higher than those of mixoploid and control porang. Meanwhile, mixoploid porang has higher alkaloids, amino acids, lipids, and fatty acids and amino acids than tetraploid and control porang.

Mixoploid plants often produce higher levels of secondary metabolites compared to diploid and tetraploid. Mixoploid plants of *Cannabis sativa* showed that reducing sugars, soluble sugars, total protein, and total flavonoids increased significantly in mixoploid plants compared with tetraploid and diploid plants (Bagheri & Mansouri, 2015). The unique cellular environment of mixoploids can result in epigenetic changes, including DNA methylation, histone modification, and chromatin remodeling, which upregulate secondary metabolite biosynthesis (Chen, 2007) and enhance the expression of genes involved in secondary metabolism (Song & Chen, 2015).

Table 1. Ploidy of porang after colchicine induction

No	Concentration	Peak	Peak Count	Mean Channel Number (G0/G1 Peak)	Category
1	K0 (0.00%)	R2	1624	6676.86	2n = 2x (Diploid)
2	K1 (0.01%)	R2	306	6399.026	4n = 4x (Tetraploid)
		R3	425	10847.76	
3	K2 (0.02%)	R2	72	6697.502	Mixoploid

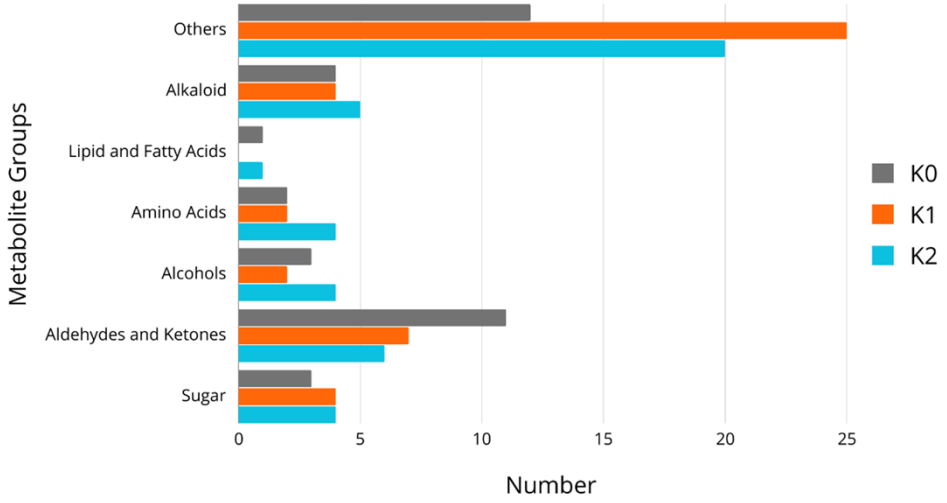

Fig. 1. Composition of porang metabolites after colchicine treatment. K0: Control; K1: Porang induced by 0.01% colchicine; K2: Porang induced by 0.02% colchicine

Increased amino acids were detected after colchicine treatment, 4.5% in tetraploid and 9.09% in mixoploid porang, with the emergence of Gly-Asp-Lys and Trp-Cys compounds. This condition may be caused by genomic instability that leads to cellular stress and finally triggers increased metabolic activity and higher amino acid production. However, further research is needed to prove this hypothesis. Amino acids play an important role in the development, stress response, and plant immune system (Wang et al., 2025). The enhanced sugar and alcohol were also detected in this research. The increase in sugar and alcohol in tetraploid and mixoploid porang may be related to the saccharification process, which can produce sugar and alcohol metabolite precursors (Corneillie et al., 2019).

Compared to other compounds, the aldehyde and ketone compositions were lower in tetraploid and mixoploid porang. The possible reason for this result is the effect of colchicine, which increases the activity of enzymes involved in reduction reactions, such as aldehyde dehydrogenase (ALDH) (Samadi et al., 2022). Aldehyde dehydrogenase (ALDH) reduces aldehydes to carboxylic acids and alcohols (Tola et al., 2020). As a result, the concentration of alcohol increases, whereas aldehydes and ketones decrease.

Colchicine treatment also generated new metabolic pathways in porang, as evidenced by the emergence of new secondary metabolites in tetraploid and mixoploid porang. For example, mixoploid porang has more unique metabolites (57 metabolites) than control porang (37 metabolites) and tetraploid porang (41 metabolites). The primary compounds present in all treatments and controls include acetamide, 2-Pyridinecarboxaldehyde, D-Fructose, and D-(-)-Tagatose. The metabolites that are only present in tetraploid (K1) and mixoploid (K2) porang include ethane, 2-bromo-1,1-difluoro, theobromine, diethyl borane, glucose-O-methane, 2-pyridine carboxylic acid, and S-nitrofuran-2-carbocylic acid (Fig. 2).

The main compounds play an important role in plant growth. Acetamide acts as a source of nitrogen in plants and a precursor for auxin formation in the indole-3-acetate biosynthesis pathway (Pérez-Alonso et al., 2021). D-Fructose and D-(-)-Tagatose are photosynthesis sugar products used as energy sources and metabolic substrates. In addition, D-Tagatose can increase plant immunity to pathogens by inhibiting enzymes that disrupt plant metabolism (Mochizuki et al., 2020).

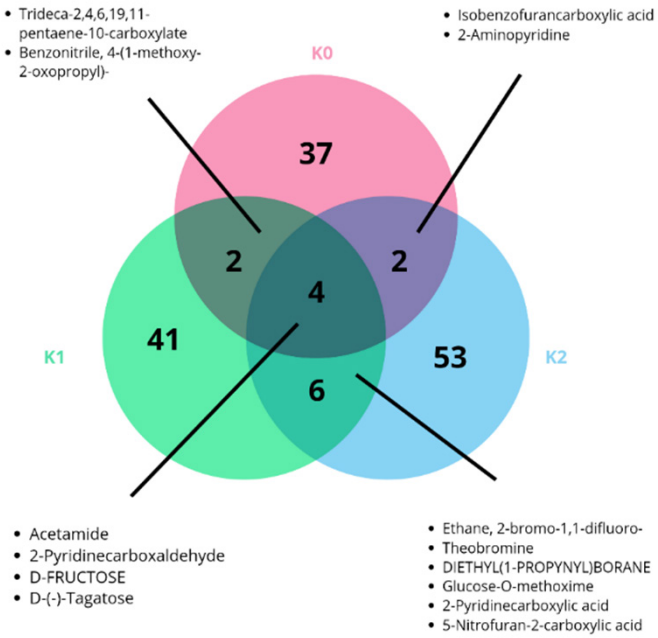

Fig. 2. Venn diagram of the porang metabolite accumulation composition

To visualize the relationship and differences between control and colchicine-treated porang, we used HCA and a heat map. The dendrogram separated the porang into 2 distinct groups (Figure 3). Mixoploid porang (K2) is grouped into a different cluster from control (K0) and tetraploid porang (K1). The mixoploid porang (K2) has relatively higher plant metabolite expression than tetraploid porang (K1). Tetraploid porang (K1) has the accumulation of sugars such as L-sorbose, D-tagatose, D-fructose; and several amino acids such as Grp-cys, Gly-asp-lys, and sulforaphane are marked by darker areas than the control. In addition, Tetraploid porang (K1) also has fewer organic acid compounds such as acetic acid, picolinic acid, 3,5-Dideutero pyridine-4-carboxylic acid; and fatty acids such as arachidoyl, glycerol. Meanwhile, mixoploid porang (K2) showed an increase in the accumulation of several important compounds such as L-Sarbose, D-tagatose, sulforaphane, pyridazine, and dimethylamine, but compounds such as acetaldehyde, aminopyridine and several carboxylic acids decreased (Fig. 3).

The increase in metabolites, such as D-fructose, glucose, 2-pyridine carboxylic acid, benzonitrile, and 4-(1-methoxy-2-oxopropyl), is an effect of colchicine induction. Polyploidy induction often leads to an increase in cell size, which can enhance the storage capacity of carbohydrates in the form of sugars such as D-fructose and glucose (Corneillie et al., 2019). 2-Pyridinecarboxylic acid, also known as picolinic acid, is an important tryptophan metabolism intermediate. This compound has been shown to play a role in various physiological processes in plants (Satoh et al., 2020). Polyploidy-induced plants tend to focus on growth and enlargement, resulting in higher production of primary metabolites (Gupta et al., 2024). Consequently, compounds such as aminopyridine and 2,2,2-trifluoro-N-methyl, which are generally involved in plant defense, are reduced.

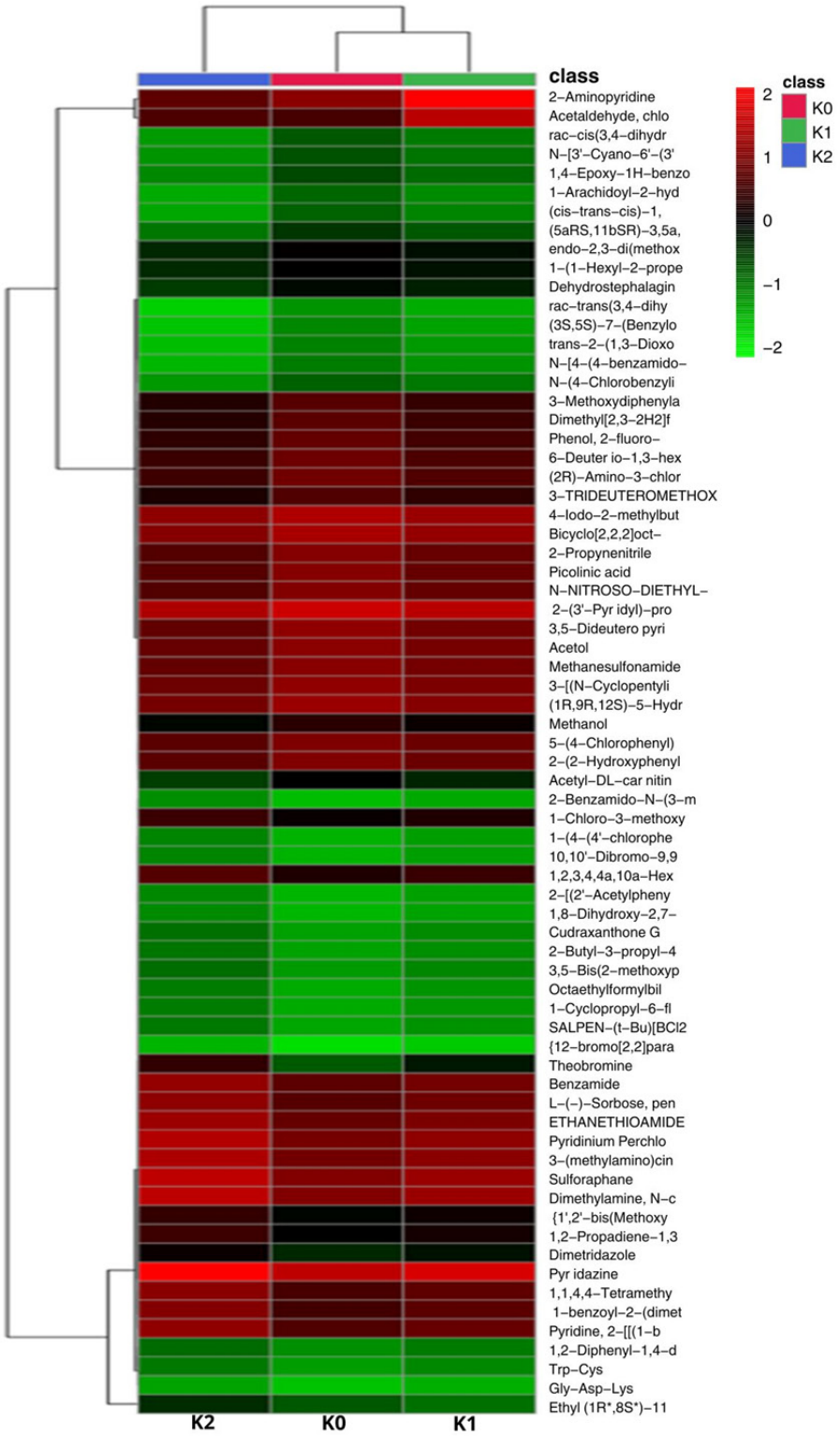

Fig. 3. Heat map of the metabolite content of colchicine-mutated porang K1 (0.01%), K2 (0.02%), and K0 (control)

Colchicine treatment increases glucomannan biosynthesis

Mutation induction using colchicine affects 8 metabolic pathways in control porang, 9 in tetraploid, and 13 in mixoploid porang. Two metabolic pathways showed significant impacts in control porang (sulfur, fructose, and mannose metabolism), two in tetraploid porang (Starch and sucrose and galactose metabolism) and 5 in mixoploid porang (starch and sucrose, pyruvate, glycolysis/gluconeogenesis, galactose, and riboflavin metabolism) (Fig.4). Sulfur, fructose, and mannose metabolism was higher in control porang than in tetraploid and mixoploid porang.

The increasing starch and sucrose metabolism after polyploidization in plants was also detected in lily (Zhang et al., 2022) and cowpeas (Qiu et al., 2024). The upregulation of genes associated with starch and sucrose metabolism is the main factor behind this phenomenon. Upregulation of these genes eventually leads to increased synthesis and accumulation of non-structural carbohydrates (NSCs) like starch and sucrose (Zhang et al., 2022). This theory was also supported by transcriptomic studies where differentially expressed genes (DEGs) reveal that polyploid plants have significant differences in the expression of genes involved in carbohydrate metabolism (Qiu et al., 2024).

Further analysis showed that 25 metabolites correlated with sucrose (Figure 5). Sucrose is positively correlated with several other sugar compounds, such as glucose and D-fructose. In addition, several organic compounds, such as fluvoxamine, 2-propanone, 1,3-benzodioxole, and 2-aminopyridine, have positive interactions with sucrose. However, sucrose is also negatively correlated with ethanethioamide, acetamide, 2,2,2-trifluoro-N-methyl, and pyrazole (Fig.5).

Several compounds that are positively correlated with sucrose are involved in the sucrose metabolic pathway. For example, several pyrazole derivatives act as plant growth regulators, influencing processes degradation of 5-chloro-3-methyl-4-nitro-1H-pyrazole (CMNP), which promotes starch degradation and influences growth regulation in Arabidopsis (Alferez et al., 2007). Other compounds, such as fluvoxamine for response stress (Koch, 2004) and trifluoromethyl-Benzamide for auxin and cytokinin regulation (Winter & Huber, 2000), also affect sucrose metabolism pathways.

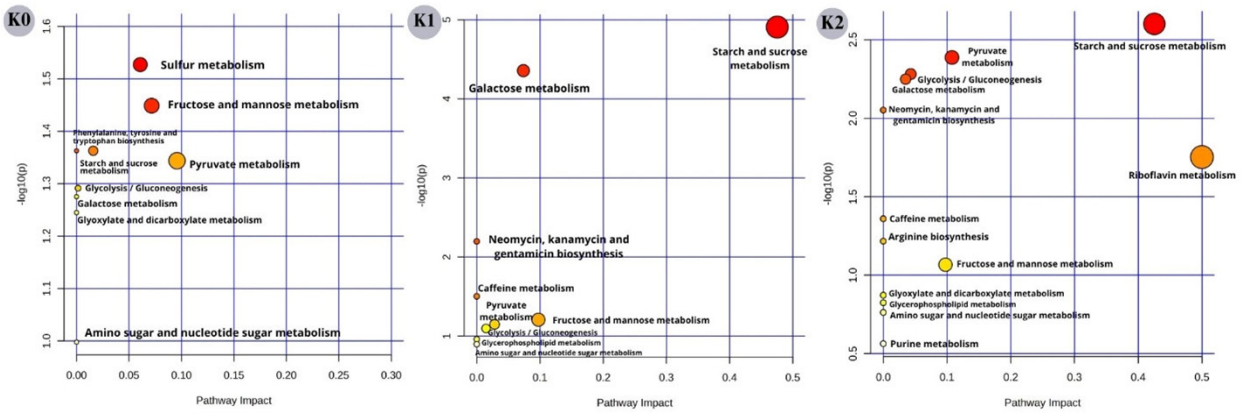

Fig. 4. Colchicine induction increases starch and sugar metabolism -log10(p) values below 1.3 are less significant and highly significant if Larger than 3.X-Axis closer to 1.0 or beyond indicates a greater contribution to the pathway

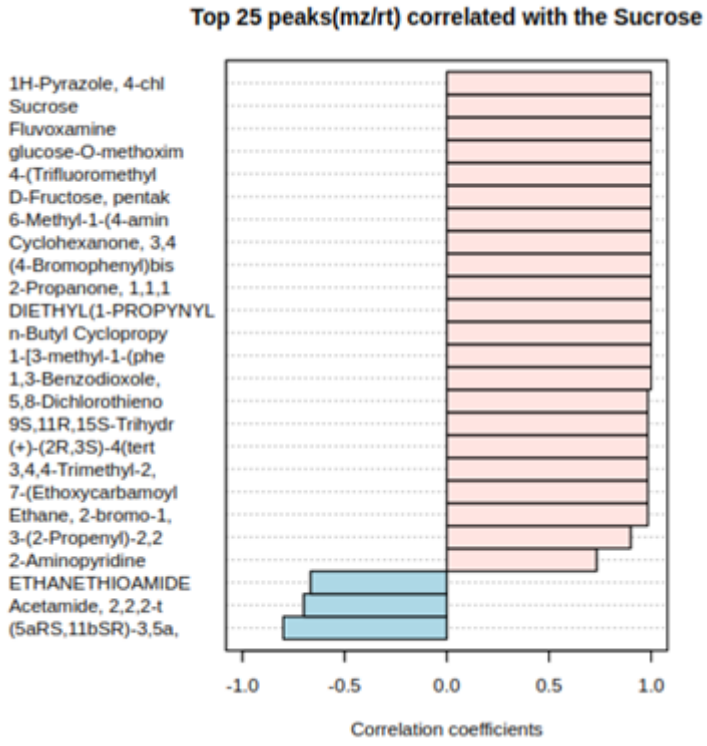

Fig. 5. Bar graph showing the top 25 metabolites correlating sucrose metabolism. Variables with the same distance from zero with similar positions are positively correlated, while those in opposite directions exhibit a negative correlation

Pathway analysis was performed to identify secondary metabolites that are directly related to carbohydrates, especially glucomannan synthesis. The secondary metabolites of tetraploid and mixoploid porang are more involved in genes related to glucomannan synthesis than those of control porang (Fig. 6). The secondary metabolites of tetraploid and mixoploid porang affect sucrose synthase (SuS), invertase (INV), Hexokinase (HXK), Fructokinase (FRK), and GDP mannose pyrophosphorylase (GMPP) (Figure 6).

The carbohydrates present in *Amorphophallus* include glucose, fructose, starch, sucrose, and glucomannan (Qi et al., 2023). Fructose and glucose are either synthesized through photosynthesis during the daytime or derived from the breakdown of sucrose, which is then used to produce starch, glucomannan, and other polysaccharides (Diao et al., 2014). Glucomannan was synthesized via the sucrose and invertase pathways. Glucomannan synthesis begins with the hydrolysis of sucrose into its monosaccharide components, glucose and fructose. The reaction hydrolysis is catalyzed by invertase (INV) and sucrose synthase (SuS), which breaks the glycosidic bond in sucrose, producing free glucose and fructose (Gille et al., 2011).

Several enzymes, such as hexokinase (HYK), fructokinase (FRK), and phosphoglucomutase (PGM), catalyze Glc-6-P to produce glucose-1-phosphate (Glc-1-P) (Granot et al., 2013). GLC-6-P is catalyzed by phosphoglucomutase (PGM) in the synthase sucrose pathway to produce glucose-1-phosphate (GLC-1-P). GLC-1-P is subsequently converted to GDP-Glucose (GDP-GLC) by GDP-GLC pyrophosphosphosphosforilase (GGP) and ADP-Glucose (ADP-GLC) by ADP-GLC pyrophosphorilase (Qi et al., 2023). In the invertase pathway, Fructose-6-phosphate (FRU-6-P) is catalyzed by PMI to produce mannose-6-phosphate (MAN-6-P). MAN-6-P is then converted to Mannose-1-Phosphate (MAN-1-P) by Phosphomannomutase (PMM) and continued by GDP mannose pyrophosphorylase (GMP), which catalyzes the production of mannose GDP (GDP-Man) by GDP-Mannose Pyrophosphoryosphorylase (GMPP). Then, the GDP-Man, GDP-GLC, and ADP-GLC products are transported into the golgi apparatus and used as CSLA- and/or CSLD-forming substrates for glucomannan synthesis (Nurhidayati et al., 2024).

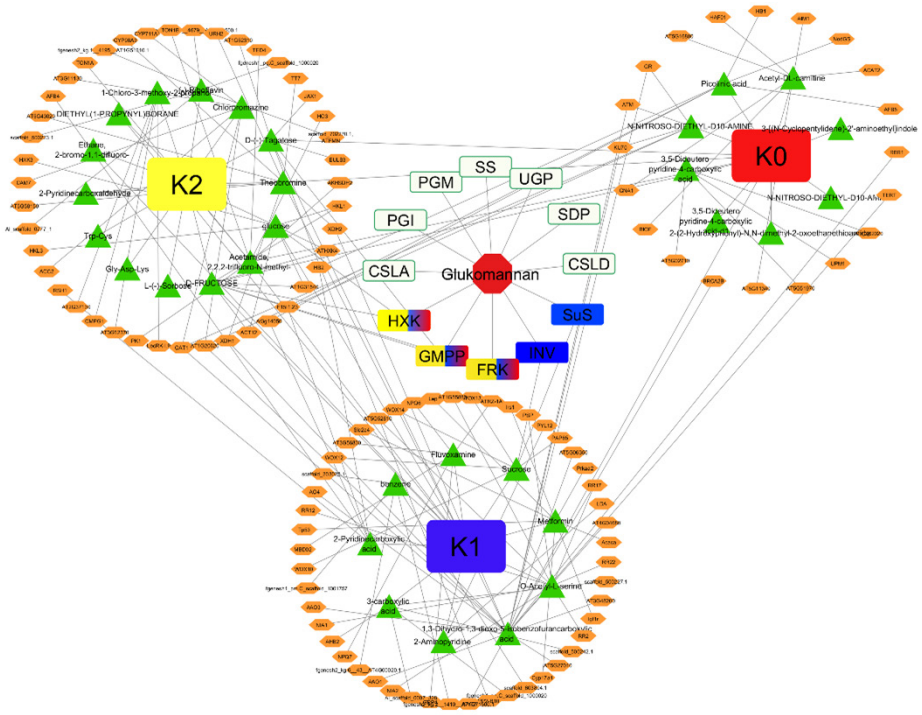

Fig. 6. Profil metabolite of porang related to glucomannan synthesis pathway

CONCLUSION

In the current study, colchicine is effective for inducing polyploidization in porang (*A. muelleri*). Induced polyploid of porang showed a significant increase in metabolite profile. The mixoploid porang (0.02% colchicine induction) has higher various compounds, including sugars, alcohols, amino acids, and alkaloids. Mixoploid porang (0.02% colchicine) also has more metabolite content related to glucomannan synthesis, including HXK, FRK, and GMPP proteins, while 0.01% affect the SuS and INV proteins, both of which play roles in the glucomannan synthesis pathway. Therefore, mutation induction using a colchicine concentration of 0.02% is recommended to produce superior porang.

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