Physiological and Genetic Variations of *Amorphophallus variabilis* in Bojonegoro based on Glucomannan Content, Calcium Oxalate and RAPD Markers

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Abstract

This study had purpose to determine the glucomannan and calcium oxalate content in 7 variants of *A. variabilis* and their genetic relationships based on RAPD markers. *Amorphophallus variabilis* Tuber samples were taken from Karangdowo village, Sumberrejo Sub-district, Bojonegoro District, East Java. Each variant was analyzed for its glucomannan content by the spectrophotometric method using 3,5-DNS reagents and calcium oxalate by the 0.1N KMnO4 titration method. Leaf DNA extraction was carried out using the CTAB method. Relationship analysis used RAPD markers with 5 primers (OPA-11, OPC-04, OPU-06, OPC-07, and OPN-1). The obtained data were analyzed using the Numerical Taxonomy and Multivariate System (NTSYS-pc) version 2.1. The grouping of glucomannan content or oxalium oxalate used hierarchical clustering analysis (SPSS 16.0). This research found that the calcium oxalate content in seven variants of *A. variabilis* ranged from 0.01 to 0.03 g, where the variant with the lowest calcium oxalate is V6 with a value of 0.01 g and the highest is the V7 variant with a value of 0.03 g, while the glucomannan content ranges from 9 - 38%. The highest glucomannan content is V3, while the lowest is V6. Phenograms formed based on RAPD markers showed the formation of two groups of *A. variabilis*. Group one has two subgroups. Subgroup one consists of variants V1 and V4, while subgroup two consists of V6 and V7. Meanwhile, the second group consists of variants V2, V3, and V5. The seven variant grouping pattern of *A. variabilis* based on RAPD markers has no similarity to the grouping pattern based on the results of glucomannan or calcium oxalate analysis.

Keywords: *A. variabilis*, Calcium oxalate, Glucomannan, RAPD, Variant.

INTRODUCTION

Amorphophallus variabilis is a native plant of Java, Madura, and Kangean Islands, Indonesia. This plant is located at 700 to 900m above sea level. *Amorphophallus variabilis* is locally called white *iles-iles* or *cocooan oray* in Sundanese. Local people are often confused by the terms *iles-iles* (yellow) for *A. muelleri* and *suweg* for *A. paeoniifolius* Dennst. Nicolson. Morphologically, *A. variabilis* does not have an aerial bulb in rachis, unlike *A. muelleri* [1].

Amorphophallus variabilis is one of Java's traditional foods. *A. variabilis* bulbs contain about 50% glucomannan, so they suitable to be used as raw materials in the food industry. Since the 1960s, rice plants became popular in Indonesia, therefore the utilization of tuber crops, including *Amorphophallus* species, decreased [1]. Besides glucomannan, *Amorphophallus* also contain calcium oxalate, which is a dicarboxylic acid with two C atoms in each carboxylic group. Consumption of oxalate in large quantities can cause kidney stones [2]. In addition, Calcium oxalate is dangerous for human health because this substance is anti-nutrient, which affects the unavailability of calcium in the body. Also, it can cause mechanical abrasion in the digestive tract and various fine tubules found in the kidneys [3].

Bojonegoro Regency is one area at East Java that has a lot of *iles-iles* (*Amorphophallus variabilis*) plants. Bojonegoro is located at 112°25' - 112°09' East Longitude and 6°59' - 7°37' South Latitude. Topographically, Bojonegoro area has land with a relatively flat slope. Existing data of Bojonegoro Regency showed that 91.26% of land in this region has a slope between 0-15%. The surface area of Bojonegoro Regency is relatively low, which is located between 25-500 m above sea level. Based on the location of the topography, Bojonegoro is one of the suitable areas for *A.variabilis* habitat. *A.variabilis* grows well in the area with condition 100-700 m above sea level [4].

From field observations, we found morphological variations of *A. variabilis* in Bojonegoro, especially in the petiole, both color and pattern of its skin. Pattern variations of petiole skin (*Corak*) may affect glucomannan content [5]. As a member of the Araceae family, glucomannan content of *Amorphophallus sp* has varying levels depending on the species.

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Therefore, it was necessary to determine both glucomannan and calcium oxalate content among *A. variabilis* variants in Bojonegoro with the expectation to find candidate variant which has high glucomannan content as well as low calcium oxalate content.

The existence of petiole pattern's skin variation from *A. variabilis* reflects genetic diversity. However, the morphology pattern sometime reflect its ecotype, so it is difficult to know the true diversity within a species. Therefore, we used molecular markers to determine the genetic diversity of plants that have a close genetic relationship [6]. Molecular markers have been used to study variation even among the species members of Araceae, such as molecular analysis, with RAPD markers on the *Amorphophallus* genus. *Amorphophallus* species that have been successfully analyzed by RAPD markers are *A. titanium* [7]. In this study, molecular analysis was performed with RAPD markers to determine whether the grouping of *A.variabilis* based on RAPD markers was similar to grouping based on glucomannan content or calcium oxalate content.

MATHERIAL AND METHOD

Preparation of Glucomannan Analysis

Amorphophallus variabilis tubers used in this research were taken from Bojonegoro, East Java. Glucomannan analysis followed the Chua Method [8], using 3.5 DNS reagents. This reagent will give a yellow color that is proportional to the amount of glucomannan. 3,5-Dinitro Salisilic Acid reagent is made by mixing solution A and solution B. Solution A was prepared by mixing phenol (0.7g), 10% NaOH (1.5 mL), aquadest (5 mL), and sodium bisulphite (0.7 g). Solution B was prepared by mixing potassium sodium tartrate (22.5 g), 10% NaOH (30mL), and 1% dinitro salicylic acid (88 mL).

Extract and Hydrolyzate of Glucomannan

Glucomannan extract is made by dissolving 0.2 g grind tuber-flesh in 100 mL of buffer solution (formic acid-sodium hydroxide), stirred for 4 hours then centrifuged at 4000 rpm for 20 minutes at 25°C; the white liquid produced is glucomannan extract. Glucomannan hydrolyzate was prepared by mixing 5 mL of glucomannan extract with 2.5 mL of 3M Sulfuric Acid, heated in boiling water bath for 1.5 hours, then add 2.5 mL of 6M NaOH and dilute to 25 mL using aquadest. A total of 2 mL of glucomannan extract or glucomannan hydrolyzate added with 1.5 mL of 3.5 DNS reagents, vortexes to homogenize. The absorbance of the solution was measured using a spectrophotometer with a wavelength of 550 nm. Glucomannan content determined according to the following formula.

$$glucomanan\ content\ (\%) = \frac{\varepsilon\ (5T - T0) x50}{m\ x\ (1-W)\ x\ 100} x100$$

Description:

ε : The ratio of glucose molecular weight and mannan residues in glucomannan to glucose and mannan molecular weights produced after hydrolysis, ε = 0.9
T : The amount (mg) of glucose in glucomannan hydrolyzate obtained from a standard curve
T_0 : The amount (mg) of glucose in glucomannan extract obtained from the standard curve
m : Sample mass (g)
W : Moisture content of the sample

Calcium oxalate

Calcium oxalate content determined through three stages, **1) Digestion**, at this stage, 2 g grind-tuber mixed with 190 mL of distilled water in a 250 mL measuring flask, 10 mL of 6M HCl was added, and the suspension digested at 100^0C for 1 hour, then cooled and added distilled water to 250 mL before filtration. **2) Precipitation**, the oxalate of the filtrate from every 125 mL of the suspension was added 4 drops of the red methyl indicator, then added NH4OH to obtain PH 4-4.5. Each part was heated at 90°C, then cooled and filtered to remove iron-sulfur ions. The filtration product reheated at 90°C, when the solution was stirred with a stirrer, 10 mL CaCl 5% was added. After heating, then cooled overnight at 5°C. The solution then centrifuged with a speed of 2500 rpm for 5 minutes. Subsequently, supernatant removed and precipitation result dissolved in 10 mL of 20% H_2SO_4 solution. **3) Permanganate titration ($KMnO_4$)**, the resulting filtrate was made up to 300 mL with water. 125 mL of aliquot filtrate heated to a slight boil, then titrated with standard 0.1N KMnO4 standard until produced a faded- pink color. The color was maintained for 30 seconds [9]. The calcium oxalate content was calculated using the following formula.

$$Calcium\ oxalate\ (g) = \frac{V\ x\ N\ Oxalate\ x\ Mr\ Calcium\ Oxalate}{1000}$$

Description:

V : the titration volume or titer volume = volume of $KMNO_4$ (mL) used in the titration until equilibrium reached or the equivalent point reached.
N : stated that the normality of calcium oxalate, which has been standardized
Mr (calcium oxalate) was 128.

DNA Isolation and Random Amplified Polymorphic DNA (RAPD) Analyzis

DNA isolated with the Doyle and Doyle CTAB method [10]. Leaf of *A. variabilis* was taken fresh

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and weighed 0.1-0.2 g, and crushed using mortal-pestle. Before grinding, the leaf sample poured with enough liquid nitrogen. The results were transferred into the Eppendorf tube (hereinafter referred to as Epitube) and added 700 µL CTAB buffer then vortex. Epitube was incubated at 65°C in a water bath for 10 minutes.

After the incubation time fulfilled, extraction performed by adding phenol (PCI) as much as 700 µL (vortex). Epitube which has received the addition of PCI, centrifuged at 13000 rpm for 10 minutes at 4°C. Then the supernatant transferred to a new Epitube and the CI was added to the same volume as the supernatant volume, vortexed. The extraction was continued with centrifuged 13000 rpm for 5 minutes at 4°C, the supernatant was discarded carefully so that only pellets remained. DNA (pellets) was added with 0.1 volume of ammonium acetate and 2.5 volumes of absolute ethanol (vortex). The epitube containing DNA was then incubated at -20°C overnight. The following day the solution was centrifuged at 12000 rpm for 15 minutes at 4°C. Pellets washed with 70% cold alcohol with centrifuged at 12000 rpm for 15 minutes at 4°C. The pellets were dissolved in 20 µL TE solution (1 mM Tris HCl pH 8, 0.1 mM) for RAPD analysis.

The genomic DNA of *A. variabilis* amplified using 5 RAPD primers (Table 2), which based on previous studies [11]. The reaction mixture of each PCR tube was 10 µL containing 3 µL ddH_2O, 1 µL primary RAPD 10 pmol, 5 µL 2x PCR Intron master mix, 1 µL DNA template 50 ng. PCR reaction was carried out for 45 cycles using a thermocycler (Takara). Initial denaturation was done at 94°C for 5 minutes, then followed by 45 cycles namely denaturation of 94°C for 1 minute, annealing 36°C for 1 minute, extension 72°C for 2 minutes. The final extension is 72°C for 4 minutes and cooling at 4°C for 30 minutes [7].

Table 1. RAPD primers used in research

No.	Primers Code	Nitrogen base sequence 5'-3'
1	OPA-11	CAATCGCCGT
3	OPC-04	CCGCATCTAC
4	OPU-06	ACCTTTGCGG
5	OPC-07	CACACTCCAG
8	OPN-18E	AAGGTGAGGTCA

Data Analyzis

Glucomannan or calcium oxalate data were analyzed by analysis of variance (ANOVA) using the SPSS program followed by the Tukey test with $p < 0.05$ significant. Before conducting the ANOVA test, the homogeneity test was carried out by the Levene test, and the normality of the data was carried out by the Kolmogorov-Smirnov test. Glucomannan or calcium oxalate data grouping performed using Hierarchy Clustering with SPSS program.

The DNA band profile resulting from PCR amplification using RAPD primers converted into binary data with two values, namely one (1) for the presence of DNA bands and zero (0) for the absence of DNA bands. Only the clear band counted. Binary data was used to generate binary data matrices using the Numerical Taxonomy and Multivariate System (NTSYS-pc) version 2.1. The binary data matrix then reduced to a genetic similarity matrix between *A. variabilis* using Jaccard's similarity coefficient. The genetic similarity matrix created using Similarity for Quantitative Data (SIMQUAL). Based on the genetic similarity matrix, Cluster Analysis performed using the Unweighted Pair-Group Method with Arithmetic Average (UPGMA) by the Sequential Agglomerative Hierarchical Nested Cluster Analysis (SAHN) sub-program. The results of UPGMA grouping analysis were made in a cladogram using a tree-display, which is a sub-program Graphics NTSYS-pc Program [12].

RESULT AND DISCUSSION

A. variabilis, which found in the Bojonegoro District, has variations in petiole skin color called *corak* of both color and shape. Petiole skin color variations of seven variants of *A. variabilis* that found included dark green, light green, brownish-green and blackish green, while the colors of the *corak* were white, brownish, greenish-white and black and white. The shape of the *corak* of the seven variants is round, oval, and elliptical (Table 2, Fig. 1). Variations that arise in the skin color of the petiole and the color of the *corak* thought to be due to a combination of pigment levels such as chlorophyll, β-carotene and anthocyanin [13].

Table 2. *A. variabilis* variation

Variant	Petiole color	Pattern color	Shape of corak*
V1	Dark green	White	Irregular round
V2	Dark green	White	Ellipse and oval
V3	Brownish green	Brownish	Small round and tight
V4	Light green	White	Ellipse and big enough
V5	Light green	Greenish white	Round irregular and tight
V6	Brownish green	Light green	Large round and not tight
V7	Blackish green	Black and white	Small round and tight

*pattern-architecture of skin surface

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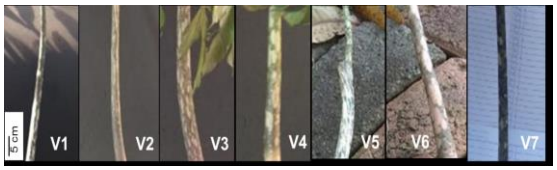

Figure 1. V1-V7: Variation of *A. variabilis*

Tubers are a means of vegetative propagation of plants and plant parts that used as storage for food reserves [14]. Tuber seeds used in this study were taken from the field with an average weight of 70.5-110.5 g. Then the tubers were planted until the aerial shoots part fall. After the aerial shoots fall, the tubers were harvested. Tuber yields of these plants weigh about 196.28-334 g, diameters range from 86.37-115.82 mm and tuber thickness ranges from 42.82-65.18 mm (Table 3).

Diameter and tuber weight of *A. variabilis* correlated very significantly with tuber glucomannan content. It meant that tuber diameter and tuber weight of harvest will influence the increase or decrease of tuber glucomannan content. The heavier tubers in *A. variabilis* may contain greater glucomannan [15]. In this study, however, heavy tubers and wide diameters, for example variant 1 (V1) or variant 2 (V2), did not contain high glucomannan (Table 3).

Table 3. Tuber weight, diameter, and thick of *A. variabilis* variant

Variant	Tuber weight (g)	Tuber diameter (mm)	Tuber thick (mm)
V1	304.60	115.82	48.03
V2	334.56	106.54	65.18
V3	286.24	99.12	55.47
V4	253.36	93.63	56.28
V5	196.28	86.37	42.82
V6	305.88	102.46	60.89
V7	234.65	92.24	59.25

Glucomannan is a type of special polysaccharides found in *Amorphophallus* tubers. The results of glucomannan extraction in seven variants of *A. variabilis* showed different glucomannan contents, ranging from 10-38%. The highest glucomannan content found in variant V3 of *A. variabilis* for 38% and the lowest in variant V6, which was 9% (Fig. 2). In summary, the glucomannan content of seven variants grouped into three categories, namely low, medium, and high categories. Low category comes from variants V2, V4, V5, and V6 (9-12%). Medium categories come from V1 and V7 (15-19%). High category consists of V3 (38%).

Mekkerdchoo *et al*. [16] used the same method (assay using 2,5-DNS) also grouped the glucomannan content of six *Amorphophallus* species (*A. muelleri, A. krausei, A. kachiensis, A. bulbifer, A. Xiei, and A. corrugatus*) into three groups: low (16%), moderate (22-24%), and high (32-42%). Whereas, Dini [5] grouped the glucomannan content of 8 variants of *A.variabilis* into low (16%), moderate (22-24%), and high (32-42) using a different method of centrifugation which was an adaptation of the Tatirat and Charoenrein method [17].

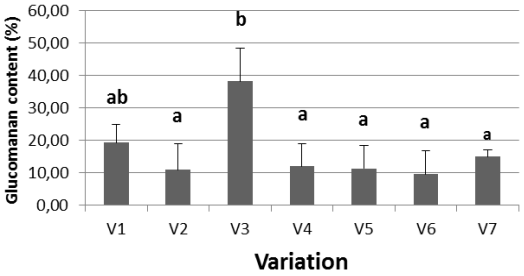

Figure 2. Glucomannan content in seven variants of *A. variabilis*, V1-V7: seven variants of *A. variabilis*. **Note:** the same notation shows no significant difference in the 0.05 Tukey test.

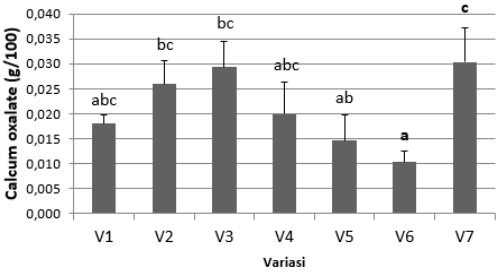

Figure 3. Calcium oxalate content in seven variants of A. *variabilis*, V1-V7: seven variants of A. *variabilis.* **Note:** the same notation shows no significant difference in the 0.05 Tukey test

The calcium oxalate content of seven variants of *A. variabilis* showed variants V1, V4, V5, and V6 have the same letter notation (Fig. 3). Meanwhile, the lowest V4 calcium oxalate content was significantly proven significantly different from V7, which had the highest oxalate content as indicated by different letter notation (c). The calcium oxalate content in *A. variabilis* variant V3 was not significantly different from variant V1 (b). Based on the picture above, it was known that the *A. variabilis* tuber contains different calcium oxalate content, ranging from 0.01 to 0.03 g. In these results, the V6 variant has the lowest oxalate content, while the V7 variant had the highest one, which was 0.03 g. These results showed that calcium oxalate was still safe for consumption (0.60-1.25 g per day) [18].

RAPD on seven variants of *Amophophallus variabilis* using OPA-11, OPC-04, OPU-06, OPC-07, and OPN-18E primers produced 24 amplicons

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ranging from 500 bp to 4000 bp. Sixteen bands (61.54%) were polymorphic bands, and ten bands (38.47%) were monomorphic. A relatively high level of polymorphism with RAPD markers showed a high mark index. The highest number of polymorphic bands (4) was in the OPN-18E primer, while the lowest number (2) was in the OPU-06 primer (Table 6). The high band polymorphism in this study showed the high genetic diversity in variants of *A. variabilis*. Research by Poerba and Martanti [11] on *Amophophallus* muelleri using RAPD also showed genetic diversity. By using five primers, a total of 42 amplicons obtained, 29 bands (60.05%) were polymorphic, and 13 bands (30.95%) were monomorphic.

Table 6. RAPD amplification band

Primer	Polymorphic bands	Monomorphic bands	Total band
OPA-11	3	1	4
OPC-04	4	4	8
OPU-06	4	2	6
OPC-07	3	1	4
OPN-18E	4	0	4
Total	16 (61.54(%)	10 (38.46%)	26(100%)

Phenograms formed based on RAPD markers showed the formation of two groups of *A. variabilis*-variant. Group one has two subgroups. Subgroup one consists of variants V1 and V4 with a similarity coefficient 0.65, while subgroup two consists of V6 and V7 with a similarity coefficient 0.72. Group two consisted of V2, V3, and V5 variants with a 0.68 similarity coefficient (Fig. 4).

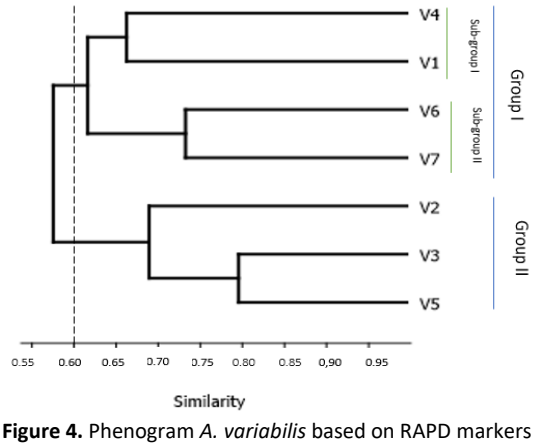

Figure 4. Phenogram *A. variabilis* based on RAPD markers

The results of grouping the seven variants of *A. variabilis* based on the RAPD markers did not show similarity in grouping patterns based on glucomannan or calcium oxalate content (Fig. 5 and 6). For example, in glucomannan, V3 and V7 formed one group, whereas, in phenogram based on RAPD markers, V3 grouped with V5, and V7 formed a group with V6. In calcium oxalate, V3 not only grouped with V7 but also with V2, V6, and V1. It can occur due to various factors, including the result of random amplification of primary RAPD that does not lead to a particular trait. One weakness of the RAPD marker for amplicon identity is unknown, unless the study is supported by pedigree analysis. From the results of the study, it can be said that the grouping based on RAPD markers cannot be used to predict groupings based on glucomannan or calcium oxalate content. Nevertheless, the results of grouping based on RAPD indicated that the seven variants of *A. variabilis* do indeed vary, the genomes were divided into two large groups.

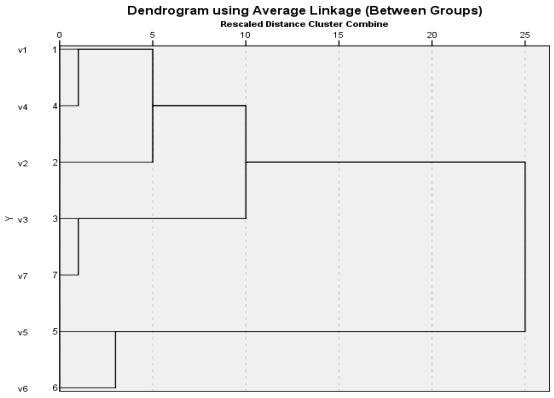

Figure 5. Glucomannan phenogram *A. variabilis* based on Hierarchy Clustering

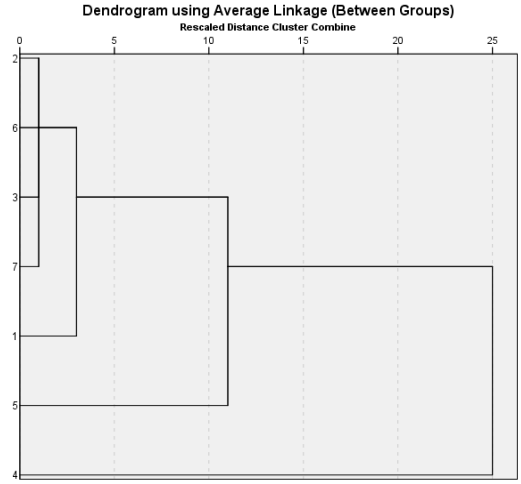

Figure 6. Calcium oxalate phenogram *A. variabilis* based on Hierarchy Clustering

CONCLUSION

The conclusion obtained from this study is that the glucomannan content in seven variants of *A. variabilis* ranges from 9-38%. Classification of variants based on glucomannan content is divided into three categories, namely low,

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consisted of variants V2, V4, V5, and V6 with glucomannan content of 11-12%. The medium category consists of variants V1 and V7 with glucomannan content of 11, 12, and 15%. The high category consists of variants of V3 with 38% glucomannan content, while for the content of calcium oxalate contained in seven variants of *A. variabilis* ranged from 0.01 to 0.03 g. The variant with the lowest calcium oxalate is V6 with a value of 0.01 g, and the highest is the variant V7 with a value of 0.03 g. The range of calcium oxalate content is in a good range for health.

Phenograms formed based on RAPD markers showed the formation of two groups of *A. variabilis*. Group one has two subgroups, subgroups one consisted of variants V1 and V4, while subgroup two consists of V6 and V7. Group two consists of variants V2, V3, and V5. There is no similarity between the grouping of seven variants of *A. variabilis* based on variations in glucomannan or calcium oxalate content with grouping based on RAPD markers.

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