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Research Article

Stomata characters of sugarcane (*Saccharum officinarum* L.) mutants of GMP3 variety at PT Gunung Madu Plantations, Lampung, Indonesia

Mahfut[1]*, Putri Kendari[2], Admi Syarif[3], Sri Wahyunigsih[2], Endah Susiyanti[4]

1)Department of Biology, Faculty of Mathematics and Natural Sciences, University of Lampung. Jl. Prof., Dr. Ir. Soemantri Brodjonegoro, No. 1. Gedong Meneng, Kec. Rajabasa, Bandar Lampung, Lampung, 35145

2)Postgraduate Student of Biology, Faculty of Mathematics and Natural Sciences, University of Lampung. Jl. Prof., Dr. Ir. Soemantri Brodjonegoro, No. 1. Gedong Meneng, Kec. Rajabasa, Bandar Lampung, Lampung, 35145

3)Department of Computer Science, Faculty of Mathematics and Natural Sciences, University of Lampung. Jl. Prof., Dr. Ir. Soemantri Brodjonegoro, No. 1. Gedong Meneng, Kec. Rajabasa, Bandar Lampung, Lampung, 35145

4)Division of Agronomy, Research and Development, PT. Gunung Madu Plantations. KM 90 Terbanggi Besar, Gunung Batin Udik, Terusan Nunyai, Lampung, 34167

* Corresponding author, email: mahfut.mipa@fmipa.unila.ac.id

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ABSTRACT

The induction of colchicine mutations is one method of breeding. PT Gunung Madu Plantations, for example, has induced mutations of commercial sugarcane (*Saccharum officinarum* L.) varieties, however, investigations on the impact of colchicine on stomatal characters have received less attention. Therefore, this study aimed to analyse the stomata character of 21 sugarcane mutants of the GMP3 variety at PT Gunung Madu Plantations, Lampung, Indonesia with a focused look at stomata aperture width, stomata length and width, number of stomata, stomatal density, and stomata index. The collected data were analysed using cluster and Principal Component Analysis (PCA) through MVSP software. This study showed that all GMP3 mutants had Graminae-type stomata. In terms of stomata length and width, the average size of the GMP3 variety mutant was greater than that of the control. The diversity of stomata characters is fairly high due to differences in stomata size between GMP3 and control mutants. With a similarity index of 0.20, the phenetic analysis of 21 mutants of the GMP3 variety revealed that the relationship between mutants and controls was getting further. A six-character principal component analysis revealed that axis I's total variation accounted for 40.54 percent of the variation and had an eigenvalue of 2.43, whereas axis II's contribution to the variation was 19.02 percent and had an eigenvalue of 1.14. The findings indicate that stomata are excellent taxonomic evidence for identifying and analysing sugarcane varieties induced by colchicine-induced breeding.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous plant with has the highest sucrose and the lowest fiber content (Lubis et al. 2015). Therefore, it is widely used as the main raw material in the sugar industry. The consumption of sugar in the country has been continuously increasing as the population grows as well as consumption per capita has increased 1.5 times, reaching 14.5 kg per capita per year. However, the increase in sugar consumption has not been matched by an increase in

sugar production. Based on sugarcane production data from 2017-2021, it is currently around 2.1-2.3 tons/ha with sugar recovery of around 7-8% (Central Bureau of Statistics 2021).

PT Gunung Madu Plantations (PT GMP) is one of the sugar industries attempting to increase production by assembling high-yielding sugar cane varieties through its research and development department, including several commercial varieties such as GMP1, GMP2, GMP3, GMP4, GMP5, GMP6, and GMP7. The GMP3 is the most commercial and dominant variety, comprising approximately 30% of the total land area. However, according to the field data, this variety has a low quality, low sugar yield, an agronomic appearance on small stems, and narrow leaf width (PT Gunung Madu Plantations 2016; Windiyani et al. 2022).

As widely stated in the literature Mugiono (2010), plant breeding is one of the ways to improve the quality of a variety. The primary principle of a sugarcane breeding program is to obtain transgressive segregation that exhibits maximum heterosis from across. Furthermore, adequate genetic diversity is essential to producing superior sugarcane varieties (Carsono et al. 2022). Therefore, plant breeding can be conducted through mutation induction using the chemical mutagen, such as colchicine (Kamwean et al. 2017). Colchicine can be used to induce mutations to obtain polyploid plants. Colchicine can improve the characteristics of plants with better traits in breeding programs (Sivakumar 2018). Sattler et al. (2016), showed that corn induced by colchicine experienced an increase in stomata density, and an increase in stomata length and width. The character of stomata in plants that have experienced polyploidy has a character of larger cell size, especially seen in epidermal cells and cell nuclei (Bagheri & Mansouri 2014).

PT GMP induced mutations with the colchicine mutagen in sugarcane GMP3 variety, but this effect of colchicine has not been tested further, tested whether the induced sugarcane has changed, especially in stomatal characters. So based on these problems, this study aims to analyze stomatal characters in 21 mutant variety of GMP3.

MATERIALS AND METHODS

Plant materials

The plant materials investigated in this study were twenty-one mutants of GMP3 varieties, one control, 0.1% and 0.2% colchicine concentrations. The twenty-one varieties of these mutants are Mutan1, Mutan2, Mutan3, Mutan4, Mutan5, Mutan7, Mutan8, Mutan9, Mutan10, Mutan11, Mutan12, Mutan13, Mutan15, Mutan16, Mutan17, Mutan18, Mutan19, Mutan21, Mutan22, Mutan23, and Mutan24, aquadest, immersion oil, glycerine, and clear nail polish. In each sample mutant taken for observation there is 3 leaf sample. Leaves samples collected from the experimental garden of field-aclimatized PT GMP. Observation of stomatal characters was observed when the sugarcane mutant of the GMP3 variety was 9 months old, and the leaves used were young leaves in the middle of the leaves. This research was conducted at the botanical laboratory of PT GMP in Lampung, Indonesia.

Method

Leaf stomata characters were observed from the paradermal part, making microscopic incisions on the paradermal part of the leaf (Munir et al. 2011; Arzani et al. 2013; Chikmawati 2013; Arofatun et al. 2020; Mahfut et al. 2023). The first step was to take the leaf and clean it using 70% ethanol. Then the bottom of the leaf was placed on a slide, covered with transparent nail polish, and dripped with glycerine and covered with a

cover glass. Light microscopic observations (Model: Olympus, magnification 10 x for the ocular and 40 x for the objective) were used to observe the specimens (Pitoyo et al. 2018). The stomata characters observed were stomata aperture width, stomata length and width, number of stomata, stomata density, and stomata index (Arofatun et al. 2020). In this research, the number of repetitions for each character measured from each mutant was carried out at 5 times the anatomical observations in a broad field of view.

Data analysis

Stomata characters were analysed quantitatively (stomata aperture width, stomata length and width, number of stomata, stomatal density, and stomata index) and qualitatively (stomata type) in a descriptive form to identify variations in stomata characters observed using MVSP (Multivariate Statistics Package) software v.3.2 to construct a phenetic dendrogram and for Principal Component Analysis (PCA). The dendrogram was built using the Jaccard coefficient, and the similarity index for genetic distance analysis used the Unweighted Pair-Group method with the arithmetic average (UPGMA) method, while the PCA Scattered plot was constructed using the Euclidean Distance algorithm.

The formula for calculating stomata density is as follows (Suhaimi 2017):

$$\text{Stomata density } (\mu m) = \frac{\text{Number of stomata}}{\text{Broad field of view}}$$

Meanwhile, the formula for calculating the stomata index is as follows (Tambaru 2015):

$$\text{Stomata index } (\mu m) = \frac{\text{Number of stomata}}{\text{Number of epidermal cells} + \text{Number of stomata}} \; x\ 100$$

RESULTS AND DISCUSSION

Stomata characters

Based on the stomata observations, the GMP3 variety has Gramineae stomata type, which shows the length of the axis of the neighbouring cell is parallel to the stomata axis (Figure 1). The type of stomata of the GMP3 mutants obtained in each 0.1% and 0.2% colchicine treatment did not show changes in the shape of the stomata. This indicates that colchicine does not affect the stomata's shape. According to Moghbei et al. (2015), the type of olive leaf stomata obtained on each treatment, which is submersion and drops at various concentrations (0.25%, 0.5%, 0.75%, and 1%), did not change the shape of the stomata. This is because colchicine does not affect all parts of the cell, just a few cells or random cell mutations. According to Syukur et al. (2015), colchicine does not affect all cells or cause random cell mutations. Cells that are actively dividing are sensitive to colchicine, whereas differentiated cells are less sensitive to this mutagen. According to Moghbei et al. (2015), the sensitivity of each plant species to colchicine application will be different even from the existing plant part.

Based on the data results (Table 1), the width of the stomata aperture in the GMP3 mutant has a value range of 1.06-2.06, an average of 1.67 with a standard deviation of 0.38. Several types of GMP3 mutants had smaller stomata aperture (1.06-2.06 µm) compared to controls (2.54 µm). This is because the concentration of colchicine given to these mutants is too high, so that colchicine will inhibit the process of enlargement or opening of stomata.

In terms of stomatal length, the GMP3 mutant has a range of 17.33

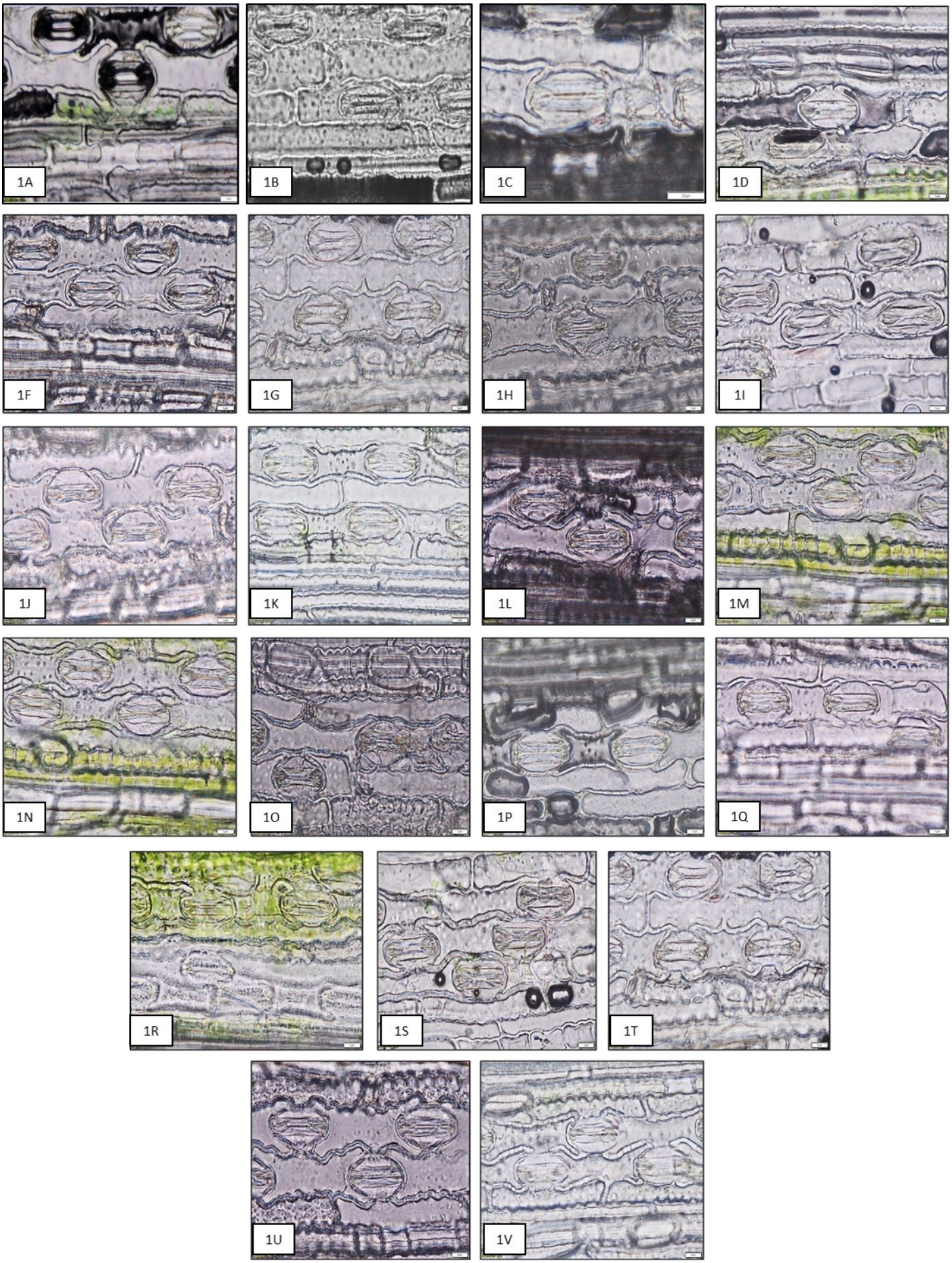

Figure 1. Structure of stomata in GMP3 variety and 21 GMP3 mutant varieties.
Note**:** 1A:GMP3, 1B: Mutan1, 1C: Mutan2, 1D: Mutan3, 1E: Mutan4, 1F: Mutan5,1G: Mutan7, 1H: Mutan8, 1I: Mutan9, 1J: Mutan10, 1K: Mutan11, 1L: Mutan12, 1M: Mutan13, 1N: Mutan15, 1O: Mutan16, 1P: Mutan17, 1Q: Mutan18,1R: Mutan19, 1S: Mutan21, 1T: Mutan22, 1U: Mutan23, 1V: Mutan24. Bar = 1 µm.

Table 1. The average value of stomata characters in the GMP3 variety and 21 GMP3 mutant varieties.

No.	Variety Name	Stomata Aperture Width (µm)	Stomata Length (µm)	Stomata Width (µm)	Number of Stomata	Stomata Density (mm)	Stomata Index (%)
6.	GMP3	2.54	19.20	11.71	77	392	0.32
1.	M / C 0.1/2H/01	1.30	18.72	9.12	95	484	0.41
2.	M / C 0.1/3H/15	1.10	19.39	11.62	87	443	0.39
3.	M / C 0.1/3H/16	2.06	19.78	10.13	74	377	0.30
4.	M / C 0.1/3H/02	2.21	17.90	9.07	97	494	0.38
5.	M / C 0.1/3H/10	1.63	18.43	10.80	77	392	0.32
7.	M / C 0.1/3H/04	1.06	18.72	12.82	79	402	0.34
8.	M / C 0.1/3H/07	2.06	19.73	9.74	89	453	0.41
9.	M / C 0.1/3H/06	1.73	17.33	10.75	59	300	0.30
10.	M / C 0.1/3H/03	1.97	18.96	11.86	72	366	0.34
11.	M / C 0.1/3H/14	2.26	18.34	8.59	91	463	0.38
12.	M / C 0.1/3H/02	1.15	21.02	10.61	93	473	0.40
13.	M / C 0.1/3H/08	1.44	18.58	10.03	84	428	0.41
15.	M / C 0.1/3H/05	1.54	18.38	10.27	88	448	0.42
16.	M / C 0.1/3H/01	1.44	18.38	11.52	84	428	0.39
17.	M / C 0.1/1H/01	1.44	20.74	10.75	82	417	0.39
18.	M / C 0.1/2H/01	2.21	18.43	9.65	97	494	0.45
19.	M / C 0.2/1H/04	1.44	18.58	10.05	95	484	0.41
21.	M / C 0.1/3H/09	1.87	18.86	10.46	98	499	0.42
22.	M / C 0.2/3H/10	1.63	18.43	10.80	80	407	0.44
23.	M / C 0.1/3H/12	1.44	21.31	9.00	73	371	0.30
24.	M / C 0.1/3H/11	2.11	17.9	8.54	89	453	0.43
	Average	1.67	18.94	10.29	85	432	0.38
	Std. Dev.	0.38	1.03	1.10	10.11	51.66	0.04

Note: M= Mutant, C = Colchicine, 0.1-0.2%: = Colchicine concentration, H = Day

-21.31, an average of 18.94 with a standard deviation of 1.03, while the GMP3 mutant stomata aperture width has a range of 8.54-12.82, an average of 10.29 with a standard deviation of 1.10. Meanwhile, the stomata's size of the GMP3 mutant variety increased in length and stomata aperture width compared to the GMP3 variety (Table 1). This suggests that colchicine affects the character of stomata's length and width. Although in this study colchicine affected the length and width of stomata, the induced colchicine at a concentration of 0.1-0.2% could not yet be indicated as a superior variety of sugarcane because each character measured was not more than 1.25 times. This is in accordance with what has found by Miguel and Leonhardt (2011) that polyploid orchidaceae plants are those that have stomata that are wider and longer than control plants by at least 1.25 times the length of control plants. Polyploidy plants with stomatal character can be characterized by the size of their cells getting bigger and visible in the epidermal cells, and cell nucleus (Suryo 2009).

The number of stomatal characters based on the data (Table 1) has a value range of 59-98, an average of 85 with a standard deviation of 10.11, while the density of stomata in the GMP3 mutant has a value range of 300-499, an average of 432 with a standard deviation of 51.66. Some GMP3 mutants have more stomata numbers than GMP3 variety, while some mutants also have higher stomata density than GMP3 variety (Table 1). It can be said that colchicine can induce polyploidy formation in GMP3 variety. According to Gantait et al. (2011) reported the stomata and epidermal cells of *Gerbera jamesonii* Bolus cv. are greater in size than those of diploid plants, their stomatal density is higher than that of diploid plants. The value of olive leaf stomata density obtained in

the colchicine treatment has increased stomata density (Rohmah et al. 2017). Stomata density in plants is closely related to plant resistance to drought stress, while stomata size and stomata density are closely related to plant resistance to water stress (Yanny et al. 2022). Based on this research, it shows that the colchicine-induced mutant causes drought resistance by having high stomata density.

The GMP3 mutant stomatal index characters have a value range of 0.30-0.45, an average of 0.38 with a standard deviation of 0.04. Some GMP3 mutants had a higher stomatal index than the control (Table 1) and colchicine at a concentration of 0.1-0.2% caused the stomatal index to increase from 0.30-0.45%. This is because the colchicine-induced mutant causes an increase in the size of the stomata. As found by Moghbei et al. (2015) that the stomata index on olive leaves treated with 0.2-0.5% colchicine increased the size of the stomata index from 0.21-0.24%. An increase in stomata size indicates an increase in stomata frequency in plant mutations. The number of stomata affects the density of stomata. Stomata density is high if the number of stomata increases. According to Asif and Khalil (2019), there is harmony between stomata size and stomata frequency, the larger the size of the stomata, the higher the stomata frequency. It was found that a dose of 75 Gray was given to the mutant plantlets, and the resulting stomata frequencies ranged from 54.47 to 77.4. Hanafy and Akladious (2018) obtained the stomata frequency range of *Trigonella foenumgraecum* to be 93.23-159.39 for the mutated plants.

Colchicine induction in GMP3 mutant variety showed larger stomata size, increased number of stomata, and high stomata index (Table 1). Sattler et al. (2016) stated that colchicine-induced maize experienced an increase in stomatal density, length, and width. This shows that colchicine can be used as a reference for sugarcane breeding because sugarcane plants with larger stomata can increase the rate of photosynthesis where the effect of this rate of photosynthesis is able to increase the growth and productivity of sugarcane plants (Prabowo et al. 2022).

Phenetic analysis

The similarity index of 21 GMP3 mutant varieties and GMP3 variety ranged from 0.20-1.00 (Table 2). The higher similarity index indicates the results of the phenetic analysis between samples are getting closer, while the lower similarity index indicates the results of the phenetic analysis are getting farther away (Hamidah et al. 2016).

Analysis of kinship relationships aims to group between plant populations based on the same character or characteristics to determine whether they are closely related or distantly related. The coefficient of similarity can be used to determine how distant or close a group is related. Genetic distance and kinship, as indicated by the coefficient of similarity, are correlated. The closer the kinship between people, the lesser the genetic distance, and the greater the similarity coefficient value (Purnomo et al. 2020; Mahfut et al. 2021).

The results show that M9, M5, M24, M18, M11, M4, M17, M2, M22, M21, M19, M15, M13, M12, M10, and M3 are the only ones to possess the similarity index of 1.00. This similarity between GMP3 mutations and GMP3 variety explains their close association. The characteristics of stomata length, stomata aperture width, and stomata index were found to be similar. According to Purnomo et al. (2012), the greater the similarity index indicates no difference between the objects being compared. This indicates that some mutants of the GMP3 variety are closely related.

Table 2. Similarity index on GMP3 varieties and 21 mutants of GMP3 varieties.

	GMP3	M1	M2	M3	M4	M5	M7	M8	M9	M10	M11	M12	M13	M15	M16	M17	M18	M19	M21	M22	M23	M24
GMP3	1.00																					
M1	0.16	1.00																				
M2	0.33	0.40	1.00																			
M3	0.75	0.00	0.40	1.00																		
M4	0.16	0.50	0.40	0.20	1.00																	
M5	0.25	0.00	0.25	0.33	0.00	1.00																
M7	0.20	0.25	0.50	0.25	0.25	0.50	1.00															
M8	0.50	0.60	0.50	0.33	0.60	0.00	0.16	1.00														
M9	0.25	0.00	0.25	0.33	0.00	1.00	0.50	0.00	1.00													
M10	0.75	0.00	0.40	1.00	0.20	0.33	0.25	0.33	0.33	1.00												
M11	0.16	0.50	0.40	0.20	1.00	0.00	0.25	0.60	0.00	0.20	1.00											
M12	0.33	0.75	0.60	0.16	0.40	0.25	0.50	0.50	0.25	0.16	0.40	1.00										
M13	0.33	0.75	0.60	0.16	0.40	0.25	0.50	0.50	0.25	0.16	0.40	1.00	1.00									
M15	0.33	0.75	0.60	0.16	0.40	0.25	0.50	0.50	0.25	0.16	0.40	1.00	1.00	1.00								
M16	0.16	0.50	0.75	0.20	0.50	0.33	0.66	0.33	0.33	0.20	0.50	0.75	0.75	0.75	1.00							
M17	0.33	0.40	1.00	0.40	0.40	0.25	0.50	0.50	0.25	0.40	0.40	0.60	0.60	0.60	0.75	1.00						
M18	0.33	0.75	0.33	0.16	0.75	0.00	0.20	0.80	0.00	0.16	0.75	0.60	0.60	0.60	0.40	0.33	1.00					
M19	0.33	0.75	0.60	0.16	0.40	0.25	0.50	0.50	0.25	0.16	0.40	1.00	1.00	1.00	0.75	0.60	0.60	1.00				
M21	0.33	0.75	0.60	0.16	0.40	0.25	0.50	0.50	0.25	0.16	0.40	1.00	1.00	1.00	0.75	0.60	0.60	1.00	1.00			
M22	0.33	0.75	0.60	0.16	0.40	0.25	0.50	0.50	0.25	0.16	0.40	1.00	1.00	1.00	0.75	0.60	0.60	1.00	1.00	1.00		
M23	0.25	0.00	0.25	0.33	0.00	0.00	0.00	0.20	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	1.00	
M24	0.33	0.75	0.33	0.16	0.75	0.00	0.20	0.80	0.00	0.16	0.75	0.60	0.60	0.60	0.40	0.33	1.00	0.60	0.60	0.60	0.00	1.00

There are two main categories into which the GMP3 mutant accessions can be divided: cluster A and cluster B (Figure 2). The 18 accessions in Cluster A, which has a similarity index of 0.25, are M7, M9, M5, M24, M18, M8, M11, M4, M16, M17, M2, M22, M21, M19, M15, M13, M13, and M1. The similarity of this kinship relationship is based on the similarity of the characters of the width of the stomata opening and the length of the stomata. Sub Cluster I of Cluster A is made up of M7, M9, and M5, whereas Sub Cluster II is made up of M24, M18, M8, M11, M4, M16, M17, M2, M22, M21, M19, M15, M13, M13, and M12. Cluster B has a similarity value of 0.30. The closeness of this kinship relationship is based on the similarity of the characteristics of the width of the stomata opening, the number of stomata, and the density of stomata which consists of 4 accessions, including GMP3, M23, M10, and M3. Two subclusters of Cluster B exist: Sub-Cluster I, which only contains M23, and Sub-Cluster II, which contains GMP3, M10, and M3.

The dendrogram's clustering pattern reveals distinct distinctions between GMP3 and its variant mutations. In this case, the GMP3 variety as a commercial variety, showed a clear separation from the mutant group of the GMP3 variety. At the similarity index of 0.25 (Figure 2), the two clusters formed. Clusters A and B are grouped with a similarity coefficient of 0.20. The two clusters are similar in that they have high stomatal density and high stomatal index. The distinctive features in cluster A were the length and width of the stomata, which are both large and narrow stomata opening. While in cluster B the length and width of the stomata are small, and the number of stomata is large. The clustering pattern based on the dendrogram shows the grouping of GMP3 mutants based on the similarity of stomata characters. Many comparable GMP3 mutants will have a higher similarity value, which is why they are grouped together in the same cluster or subcluster. Because of variations in stomata characteristics, the findings of the cluster analysis revealed that the mutants of the GMP3 variety did not cluster in a single cluster.

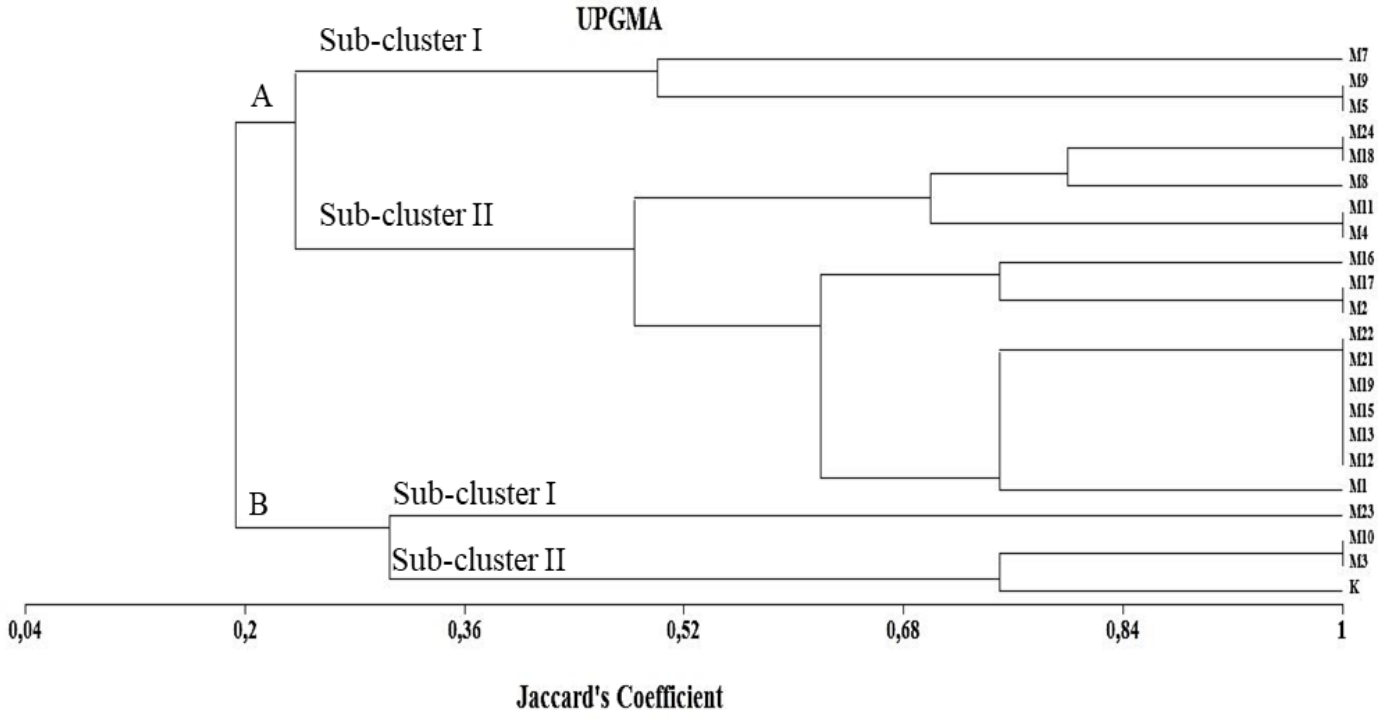

Figure 2. Dendrogram of phenetic relationship of 21 GMP3 mutants and GMP3 variety using Jaccard's coefficient.

Principal Component Analysis (PCA)

The grouping of GMP3 varieties and GMP3 mutant varieties are divided into two clusters (Figure 3), Cluster I consisting of GMP3, M7, M9, M10, M17, and M23 based on the characters of stomata length and stomata aperture width. Cluster II consists of M2, M8, M11, M16, M22, and M24 which are grouped based on the character of the number of stomata, stomata index, and stomatal density. According to Sari et al. (2016), the length and direction of the arrows indicated the stomata characters that most influence the grouping. Arrows pointing to a particular group indicate the most influential stomata character. The length of the arrow is directly proportional to the character of the stomata. The characters that play a role in the grouping of GMP3 mutant accessions are presented in Table 3.

PCA is used to indicate the distance between groups in the specimen under study. According to Adebisi et al. (2013) and Hamidah et al. (2016) PCA is also used to determine the character of stomata that plays a dominant role in group formation.

There are two characters are playing an important role in grouping GMP3 mutant varieties (Table 3). These two characters, namely stomata aperture width (LBS) and stomata cell length (PS) were simultaneously able to separate all samples on PC1 and PC2. In the main component analysis, there is an eigenvalue that shows the percentage value of the contribution of each grouping (Sultan et al. 2010). Eigenvalue of >0.02 indicates the most important character in cluster grouping (Stevens & Tello 2014). The size of the eigenvalues shows the influence of each character, which can be seen from the short length of the projection formed (Sari et al. 2016). The six stomata characters were varied by 40.54 percent with the help of the total variation on axis 1, which had an eigenvalue of 2.43, and by 19.02 percent with the help of the total variation on axis II, which had an eigenvalue of 1.14.

Based on the findings of this study, the characteristics that influence sample separation between cluster analysis and principal component analysis share commonalities aside from the cluster grouping pattern (I and II). Jalil et al. (2020) stated that there is congruence between the

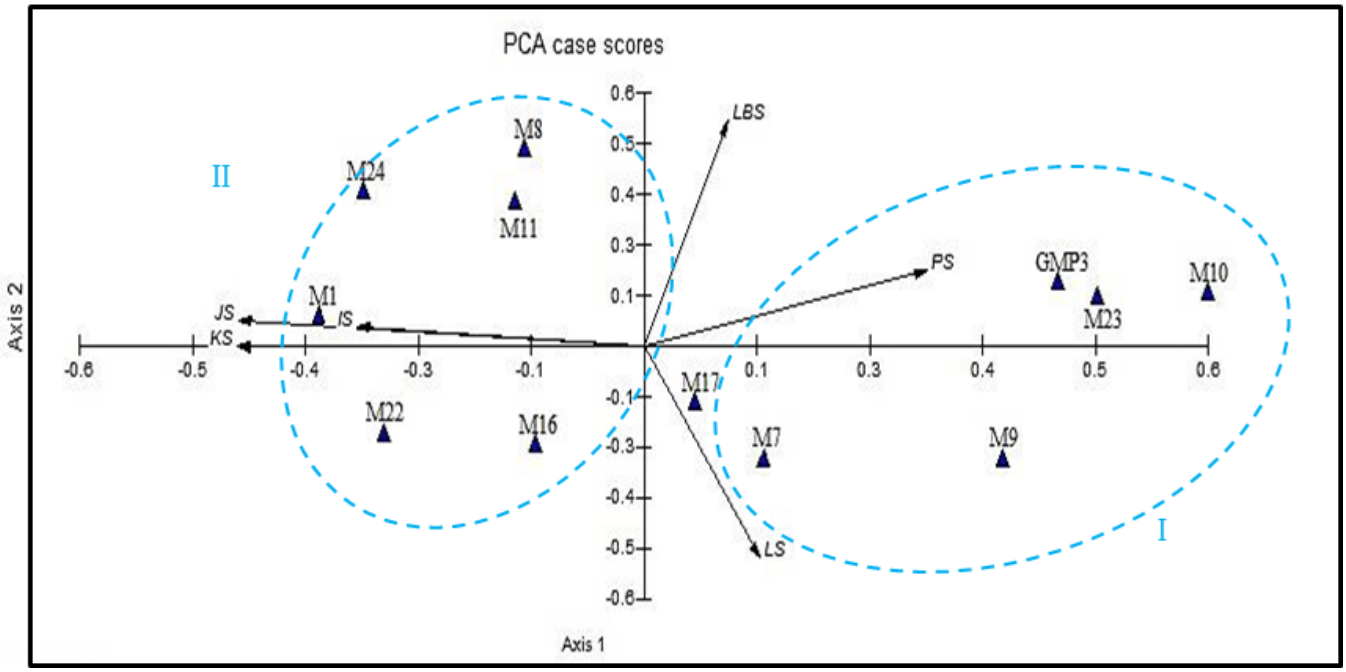

Figure 3. Principal Component Analysis on 21 mutants GMP3 varieties and varieties of GMP3 based on the characters of the stomata.

results of cluster analysis and principal component analysis. Both analyses can separate a number of botanically similar species and are widely used techniques for discovering cluster structures in numeric taxonomy.

Table 3. Characters that play a role in grouping GMP3 mutant varieties.

Character Code	Character Name	PC1	PC2
LBS	Stomata Aperture Width	0.117	0.706
PS	Stomata Cell Length	0.395	0.235
LS	Guard Cell Width	0.162	-0.661
JS	Number of Stomata	-0.567	0.081
KS	Stomata Density	-0.568	-0.003
IS	Stomata Index	-0.401	0.061
	Eigenvalues	2,433	1,141
	Percentage (%)	40,544	19.023
	Cum. Percentage (%)	40,544	19.023

CONCLUSION

In conclusion, the current study's findings revealed that 21 mutants of the GMP3 variety possessed a range of anatomical characteristics, including greater stomata size, smaller stomatal opening width, high stomatal density, a large number of stomata, and a high stomata index.

AUTHORS CONTRIBUTION

M. data validation, reviewing and final manuscript preparation. P.K. set up the experiment, carried out the entire experimental work, analyzed and interpretation of data, writing-editing. A.S, S.W, and E.S. guide in setting up the experiment and manuscript preparation. All authors have read and approved the manuscript for publication.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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