

# In Vitro Polyploidy Induction of Patchouli (*Pogostemon Cablin* Benth.) by Colchicine

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## Abstract

Patchouli (*Pogostemon cablin* Benth.;  $2n = 32$ ) is an aromatic herbaceous plant commonly cultivated for use in the the fragrance industry. As patchouli is propagated by cuttings polyploidization induction by colchicine treatment was conducted to obtain a new genotype with high patchouli alcohol content. This research aimed to increase patchouli genetic diversity with colchicine treatment by the formation of a polyploid plant. Axillary buds from single node cutting of aseptic plantlets were used as the explants. The experiment was arranged using a factorial completely randomized design with two factors, namely the concentration of colchicine and the immersion duration. Genetic diversity of patchouli was successfully enhanced by adjusting the colchicine concentration and immersion treatment. lethal concentration (LC) of 50% in patchouli was 0.132% and the LC 50% for soaking time was 60.16 hours. The number of chloroplasts, stomatal length and chromosome number increased with increasing ploidy, whereas stomatal density and the number of trichomes decreased. The chromosome number of 16 patchouli mutants in generation 4 was still unstable, and a chimera was found with mixoploidy between 20-80. A hierarchical dendrogram clustered 16 patchouli mutants into four different groups.

Keywords: colchicine, chromosome, patchouli, Sidi-kalang, trichome

## Introduction

Patchouli (*Pogostemon cablin* Benth.)  $2n = 2x = 32$  (Yan et al., 2016; All, 2019) is an aromatic annual herb plant that plays an important role in the fragrance industry (Ramya et al., 2013). Indonesia is one of the main patchouli producers, capable of supplying 90% of the world's patchouli oil requirement with an average annual patchouli oil production of 1100 tons per year while the total production of patchouli oil averages 1200 tons per year (Lawrence, 2008). An important

characteristic of high-value patchouli oil is the fixative nature (binding to other essential oils) which facilitates long-lasting effects. The major chemical compounds of patchouli oil are  $\alpha$ -patchoulene,  $\beta$ -patchoulene, patchouli alcohol, and  $\alpha$ -guaiene with patchouli alcohol (PA) as a determinant component of oil quality (Sundaresan et al., 2009). Currently no synthetic compounds have been identified that are capable substituting for patchouli alcohol (van Beek and Joulain, 2018). Patchouli is generally propagated by cuttings as it is genetically disinclined to flowering and hard to producing seeds (Li et al., 2011). However, the continuing use of cuttings for seedling production causes a decrease in yield and oil quality. Furthermore, this method of propagation makes it difficult for patchouli to form new varieties with high PA content. The yield and quality of oil produced nowadays remain low at 1.8-2% (Jos, 2004). This low yield is caused by several factors, including the low quality of plant genetics, cultivation technology, and post-harvest technology.

Mutation induction is one of the processes that can be carried out to achieve morphological improvement, enhance the content of secondary metabolites, and increase patchouli plant diversity. Through the polyploidization process, characteristics such as larger leaves in cassava (Zhou et al., 2017), larger shoot in gerbera (Gantait et al., 2011), and more flowers in carnation (Roychowdhury et al., 2011) can be obtained. Polyploid plants also increase the genetic diversity of patchouli. Colchicine is an alkaloid compound that can inhibit the formation of spindle threads in cell division, resulting in formation of polyploid plants (O.J. Eigsti, 1938). An increase in ploidy in plant cells is expected to increase the number of copies of genes responsible for the production of metabolite compounds. Polyploidy induction has been carried out on patchouli to obtain a new diversity on ploidy for producing patchouli with higher PA compounds.

This study aimed to increase the genetic diversity of patchouli (*Pogostemon cablin* Benth.) by the application of different colchicine concentrations over

varying soaking times in single-node cuttings culture *in vitro*. The expected result of genetic diversity was the formation of superior polyploid potential mutant genotypes with a high PA yield.

## Materials and Methods

The research was conducted from June 2017 to February 2019, at the Plant Tissue Culture Laboratory 2 and the Micro Technical Laboratory, Department of Agronomy and Horticulture, IPB University, Bogor Dramaga Campus of IPB University.

The plant material used for polyploidy induction was a single-node cutting of a Patchouli Sidikalang variety (collection, Dr. Ir. Ni Made Armini Wiendi, MS), cultivated for 8 weeks *in vitro* which was propagated on MS medium (Murashige and Skoog, 1962) + 4mg.L<sup>-1</sup> CaP + 30 g L<sup>-1</sup> sugar at pH 6.0. The experiments were arranged using a factorial completely randomized design of two factors excluding controls. The first factor was the concentration of colchicine and consisted of four levels (0.025%, 0.050%, 0.075%, and 0.1%), and the second factor was the soaking time, i.e. 24 and 48 hours. The experiment was replicated 40 times, with 3 explants for each replicate so that there were 960 observation units plus 40 control plants. Sub culturing was done four times at eight week intervals. Sub culturing between vegetative mutants (MV) was conducted by cutting axillary buds from the previous generation into a single-node cutting. The immersion for colchicine treatment was done by mixing the liquid media of MS + 4mg.L<sup>-1</sup> CaP + 30 g.L<sup>-1</sup> sugar at pH 6.0 with colchicine, while in the next subculture stage solid media with the composition of MS + 4mg.L<sup>-1</sup> CaP + 30 g.L<sup>-1</sup> sugar at pH 6.0. Explant were kept in 23°C growth room.

Measurements of the number of leaf and number of shoot were at the end of each generational period (8 week), before the next sub culture. Observations were made from the MV1 to MV4. The parameters observed included the number of leaves and the number of shoots. Histological analysis was carried out on the adaxial part of patchouli MV4 leaves. Leaves were taken from the 2<sup>nd</sup>-4<sup>th</sup> nodes from shoot tips of plantlets. Each treatment applied to 10 putative mutant plants, so there were 80 sample excluded control treatments. Observations were made with a magnification of 40x10 using a binocular microscope. Histology observation was done to determine stomatal density, stomatal length, the number of chloroplasts, and the number of trichomes. Stomatal length, chloroplasts, and trichomes were calculated from three replications with of each sample observed at 40x magnification. The stomatal density was calculated via the following formula:

$$\frac{\sum \text{Stomata}}{\text{area of microscope (0.1965 mm}^2\text{)}}$$

The cytological analysis, samples were taken from the root tip of 16 mutant numbers with two mutants per treatment. The analysis was conducted using the squashing method employed by (Rodiansah, 2007) with modifications. The root tip was sampled 08.00-09.30 and then cut and placed in 45% acetic acid solution for 15 minutes. Following this, the samples was immersed in a 1N HCl solution at 60°C for 3-5 minutes and left in Orcein dye 2% for 2-5 minutes. Chromosomes were observed under a microscope in the three fields of view of each sample with a magnification of 10 x 100. Stomatal density, the number of trichomes, the number of chloroplasts, and the number of chromosomes was calculated using ImageJ software. The diameter of the stomata was measured using Image Raster 2.1 software.

Morphological and histological data were analyzed using the ANOVA test, and where differences were observed a further DMRT test was performed using SAS 9.0 software. Lethal Concentration (LC) 50 data processed with probit analysis, and data variance is presented in scatterplots. Multivariate analysis data with dendrograms was processed with Minitab 16 software.

## Results and Discussion

### *The Survival Rate of Patchouli MV1 to MV4 Plantlets*

Patchouli single-node explants treated with colchicine displayed growth retardation, inhibited shoots formation, and increase mortality. Explant that survived and developed into plantlets showed the same survival rate observed as patchouli plantlet (Figure 1).

The death of explant was characterized by a change in color in the explant due to chlorophyll degradation. The highest mortality rate was observed in colchicine treatments with a concentration of 0.1% combined with a 24-hour soak duration and in treatment with a concentration 0.050% and 48- hour soak duration, leaving only a 5% of plantlets surviving at the end of the MV4 observation. This result is in line with previous work studying the effect of colchicine in stevia where treatment with a concentration of 0.08% and an immersion of 72 hours killed all explants in the treatment (Sinta, 2018). The death of the explants occurs be due to colchicine which is an inhibitor of the process of spindle thread formation in mitosis through inhibition of microtubule polymerization by binding to tubulin which may have disrupted the chromosome segregation in plants (Borisy and Taylor, 1967). Cells that have chromosome segregation inhibition will

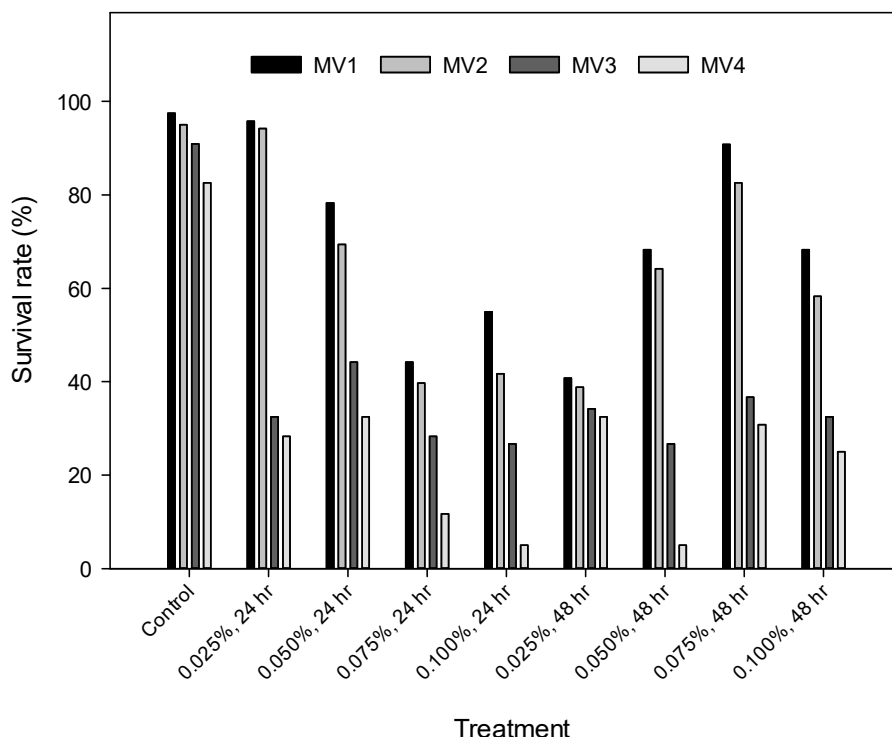


Figure 1. Survival rate of patchouli plantlets from MV1 to MV4 after colchicine treatment

attempt to find a new chromosome balance. Cells that succeed in forming a new rearrangement will survive, while cells that fail to form a new balance will experience death (Chaudhuri et al., 2000).

Lethal concentration (LC) 50 or 30 is the number of population died about 50% and 30% cause the chemical compound applied. According to Kangarasu et. al (2014), individuals who are around the LC50 value have the highest diversity of mutants. The analysis of Lethal concentration LC) 30% and LC 50% for the concentration of colchicine in patchouli amounted to 0.074% and 0.132%. This value indicates that at concentrations of 0.074% 30% of the population died and at a concentration of 0.132% 50% of the population died in MV1. LC 30% and LC 50% for patchouli immersion were 40.27 hours and 60.16 hours respectively. Hence, LC50 can be used as a basis for determining the concentration and the appropriate soaking time for the formation of mutant plants. Each species will have a different LC because their response to the colchicine concentration between species differ. For instance, LC 50% in stevia was shown to be 0.1% (Sinta, 2018) whereas in orchid plants, *Dendrobium lasianthera* it was 0.122% (Nugroho, 2015).

#### *In Vitro Morphological Evaluation, Number of Leaf and Shoot from Putative Mutants of Patchouli*

Table 2 shows the interactions between the concentration of colchicine and the immersion time that differed significantly for the leaf number variable

in the MV1, MV2 and MV3 generations did not display significant differences in the MV4 generation leaf number variable. Leaf growth was seen to be stunted in the MV1 generation and then began to stabilize in the MV2 to MV4 generation. Only the 0.025% concentration treatment with 48 hour soaking time duration did not appear to have inhibited leaf growth or any generation from MV1 to MV4. The control treatment had an higher average number of leaf than the plants treated with colchicine.

Previous research conducted by Nugroho (2015) on orchids showed a negative effect of colchicine treatment response on the number of leaves of the colchicine compared to the control treatments. Colchicine treatment was observed to inhibited the growth of the number of plant leaves at a higher immersion time of 72 hours (Nugroho, 2015).

Morphological observations were also carried out on the number of patchouli shoots in the MV1, MV2, MV3, and MV4 generations (Table 3). The interaction effect of concentration and immersion time did not significantly affect the number of shoots in the MV3 and MV4 generation. The interaction between the immersion period and the concentration showed no significant impact on the number of shoots and leaves of the MV4 generation indicating that the cell recovery process had proceeded so the cells could grow normally and display a similar a morphology of leaves and buds between treatment.

The number of shoots in the MV1 generation of

Table 2. Average number of leaf of MV1, MV2, MV3, and MV4 patchouli at eight weeks after colchicine immersion treatment

Generation	Concentration (%)	Immersion duration (hour)		Generation	Concentration (%)	Immersion duration (hour)	
		24	48			24	48
		Number of leaves				Number of leaves	
MV <sub>1</sub>	0.025	17.5±8.9b	24.5±15.4a	MV <sub>3</sub>	0.025	15.6±6.4e	23.5±3.8b
	0.050	10.3±9.4c	7.7 ±7.6cd		0.050	18.6±8.5cd	16.4±6.8de
	0.075	8.1±9.9cd	5.7 ±4.5de		0.075	18.6±7.9cd	26.2±9.4a
	0.100	10.3 ±11.5c	4.4e± 3.3e		0.100	16.7±7.1de	20.5±7.2c
	control	20.6 ±4.2			control	25.6±4.4	
MV <sub>2</sub>	0.025	17.9± 4.5ab	18.1± 5.9ab	MV <sub>4</sub>	0.025	26.7±5.7	27.3±6.8
	0.050	14.8 ±4.5c	18.6a ± 6.7a		0.050	28.9±6.3	26.85.9
	0.075	15.4±5.5bc	17.2± 5.5abc		0.075	22.2±5.4	30.8± 8.2
	0.100	19.1±6.1a	16.6± 6.7abc		0.100	28.9±11.9	26.7±13.3
	control	23.6±7.5			control	24.5±7.2	

Note: \*values followed by the same letters on the same row within the same MV show no significant difference based on DMRT test at α=5%

Table 3. Average number of patchouli shoots MV1, MV2, MV3 and MV4 after colchicine immersion treatment

Generation	Concentration (%)	Immersion duration (hour)		Generation	Concentration (%)	Immersion duration (hour)	
		24	48			24	48
		Number of shoot				Number of shoot	
MV1	0.025%	8.1±4.2a	8.6±4.9a	MV3	0.025	2.2±2.7	2.6±2.2
	0.050%	4.3±3.9b	2.9±2.9cd		0.050	2.2±2.4	2.7±2.2
	0.075%	2.8±3.2cde	1.8±1.8de		0.075	2.7±2.3	2.3±1.8
	0.100%	3.9±4.0bc	1.6±1.4e		0.100	2.6±2.4	2.3±2.2
	control	3.3±1.9			Control	2.7±0.9	
MV2	0.025%	1.7±1.3e	3.2±3.0b	MV4	0.025	2.6±1.2	2.4±2.6
	0.050%	3.0±3.0bc	3.1±2.8bc		0.050	2.4±2.9	1.9±2.1
	0.075%	2.0±1.6de	4.4±3.8a		0.075	2.8±2.9	4.0±2.8
	0.100%	3.0±2.9bc	2.3±1.6cd		0.100	1.8±2.2	2.7±3.6
	control	2.7±0.9			Control	3.4±1.9	

Note: \*Values followed by the same letters on the same row within the same MV show no significant difference based on DMRT test at α=5%

samples treated with a concentration of 0.025% appeared to be twice as higher when compared to the control plants. However, in other treatments the number of shoots tended to be lower or close to the control value. Sajjad et al. (2013) reported that, marigold which was treated with colchicine in vitro, at a concentration as low as 0.001% had shoots almost twice the number of controls. Whereas in other treatments colchicine inhibited growth terms of the in the number and length of shoots. Disruption in cell division may be due to colchicine, which prevents the formation of spindle threads, caused slower plant growth. The standard deviation values for all colchicine

treatments from MV1 to MV4 were higher compared to the controls. The higher standard deviation values indicate a greater deviation from average, and hence, a greater diversity of the individual in population treated with colchicine.

#### *Stomata, Chloroplasts, Trichomes, and Chromosomes Evaluation of Patchouli in MV4 Generation*

The observation of stomata and chloroplasts was the initial observation used as an indicator to detect polyploidy in plants. Hamill et al. (1992) revealed that tetraploid bananas have a stomata length longer than



its diploid relatives. The differences in the number of chloroplasts indicate that structural changes in the mitochondrial genome which are then expressed through chloroplasts number. An increase or decrease in the number of chloroplasts in individual plants indicates a change in the genetic traits of an that plant, which can be used as material for increasing genetic diversity (Perwati, 2012).

Interaction between concentration and time duration in variable number of chloroplast, number of stomata and number of trichome was significantly different while variable of stomatal density was not significantly different. Plants with treated colchicine display lower stomata density values, higher numbers of chloroplasts number and longer stomata diameters compared to control treatment plants (Table 4). Observation of stomata and chloroplasts number is the initial observation used as an indication to detect polyploidy in plants. The stomata density is closely related to the size of the stomata, with lower stomata density, smaller number of stomata. This indicates that the greater the size of the stomata the more chloroplasts produced (Padoan et al., 2013). Yan et al. (2016) reported that chloroplast number of octoploid plants was doubled that observed in tetraploids which demonstrate that the number of chloroplasts correlated positively with the level of plant ploidy.

Chloroplast comes from proto plastid which divides when the embryo develops. Like mitochondria, chloroplasts also have their own genome which can be used as a reference for determining ploidy (Kimball., 1994). Patchouli with colchicine treatment had an average number of trichomes lower than the control treatments. Patchouli oil is produced in

the trichomes, so that variation in these is expected to affect the production of patchouli oil (Croteau et al., 1987). The number of trichomes is also used as one of the histological variables with stomata and chloroplasts to determine the diversity of patchouli. The standard deviation of each histological variable was greater than in the control treatment. This shows the variety of mutants in individual treatments which indicates an increase in genetic diversity.

The distribution and variation of individual in the treatments are presented in Figure 2. Figure 2 describes the number of leaves, the number of shoots and the number of nodes. If increasing spread is observed in a diagram this can be interpreted as reflecting a higher level of diversity, while plots that are increasingly clumped suggest that the level of diversity is decreasing. The distribution of diversity was also analyzed for histological variables, specially the number of trichomes, stomata, and chloroplasts (Figure 2).

The number of trichomes, stomata density and the number of chloroplasts appeared to be more irregular throughout the populations when compared with morphological variables (number of shoots, leaves and nodes).

*In vitro* morphological variables, as indicated by number of leaf, shoots and node, did reveal any significant difference in diversity compared to histological variables (number of chloroplasts, stomata density, and number of trichomes. This likely due to the traits were not being fully expressed due to the environment, which is intentionally arranged uniformly for the *in vitro* culture. This is reflected in the

Table 4. Average number of chloroplasts, stomata length, stomatal density, and number of patchouli trichome generation MV4

Variable	Immersion duration (hour)	Cochicine concentration (%)			
		0.025	0.050	0.075	0.100
Number of chloroplast	24	23.2±6.1ab	20.3±6.6ab	24.6±10.5a	21.6±4.0ab
	48	20.2±4.2b	16.7±7.0c	22.1±6.5ab	22.3±6.9ab
	Control	16.0±0.6			
Stomata diameter	24	21.4±4.3	22.8±3.9	24.2±3.8	24.0±6.5
	48	21.5±6.6	20.1±3.8	21.7±6.5	22.5±4.3
	Control	20.6±0.7			
Stomatal density	24	138.9±35.8a	123.3±26.8a	87.8±34.5b	117.3±50.8a
	48	121.8±28.1a	131.1±46.7a	117.1±38.4a	96.3±41.4b
	Control	171.5± 9.8			
Number of trichomes	24	27.0±11.7a	21.7±10.7ab	22.9±10.6ab	26.9±5.8a
	48	26.6±11.9a	26.1± 9.5a	22.9±11.3ab	19.0±11.3b
	Control	39.5±5.31			

Note: \*Values followed by the same letters on the same row show no significant difference based on the DMRT test at α 5%

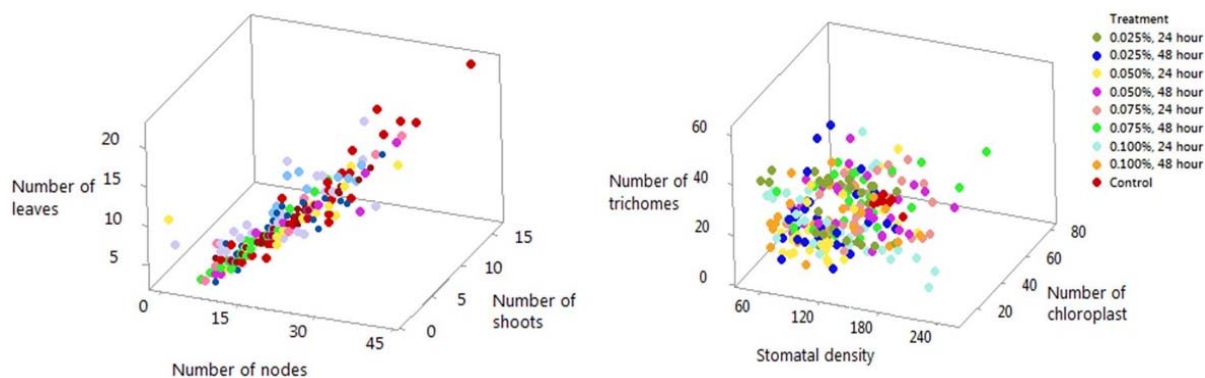


Figure 2. Distribution of MV4 mutant populations based on number of leaf, node, and shoot (left), stomatal density, number of trichomes, and number of chloroplast (right).

histological variables where there is diversity present in the population that is not morphologically visible. The characteristic of stomata density, the number of chloroplasts and the number of trichomes can be used as a more accurate reference to determine the differences in diversity between treatments.

The colchicine treatment induced an increase in the number of patchouli chromosomes in the MV4 generation which is reflected in some treatments having a higher number of chromosomes than the base chromosome with a range of chromosome numbers from 20-88 (Data not shown). The base number of patchouli chromosomes is  $2n = 32$  (Yan et al., 2016). From the observation of chromosomes, it was found that individuals with the number of chromosomes approaching control ( $2n$ ), experienced a reduction in the number of chromosomes (aneuploidy) or experienced a doubling of the number of chromosomes (polyploidy). This is in line with previous research (Sinta, 2018) in stevia that many aneuploid and mixoploid individual observed in the MV4 and MV5 generations where the maximum chromosome number obtained was 58 but this chromosome is in a state of mixoploid with a lower number of chromosomes which are 40, 42, 44, 36, 38 and 32. Plantlets treated with 0.100% concentration of colchicine and soaked for 48 hours with the number yielded the highest number of chromosomes (68-82),

which was twice as many as the number yielded in the control treatments (Figure 3).

The number of chromosomes seen in patchouli individuals mutated with colchicine varies and has not shown consistent results. This may mean that the individual mixoploids produced are still a chimeras. Similar research conducted by Sinta (2018), on stevia, also showed many mixoploid individuals in the MV5 generation. Another study on grape showed that mutant plants soaked in colchicine began to achieve stability in the 6-7<sup>th</sup> generation (Chang et al., 2014). Mixoploid patchouli from colchicine immersion need to be selected and sub cultured in the next generation. Sub-culturing was done to separate between chimeras and to produce a solid and stable patchouli mutant.

Analysis of 16 patchouli plants in term of the histology and cytology variables previously discussed showed that individuals with a higher number of chromosomes also had greater amounts of chloroplasts, longer stomata diameters, lower stomata densities and lower numbers of trichomes compared to the control plants (data not shown). Cluster analysis was performed on the histological and cytological variables of 16 MV4 generation mutants numbers coupled with two control plants (Figure 4).

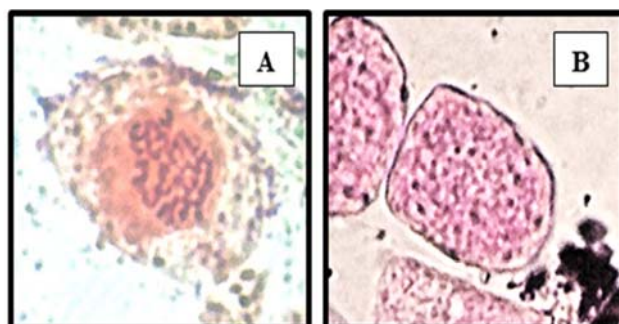


Figure 3. The number of patchouli control chromosomes and colchicine treatment; A = control chromosome and B = polyploid plant candidate chromosome treated with colchicine at 0.100% and soaking time of 48 hours

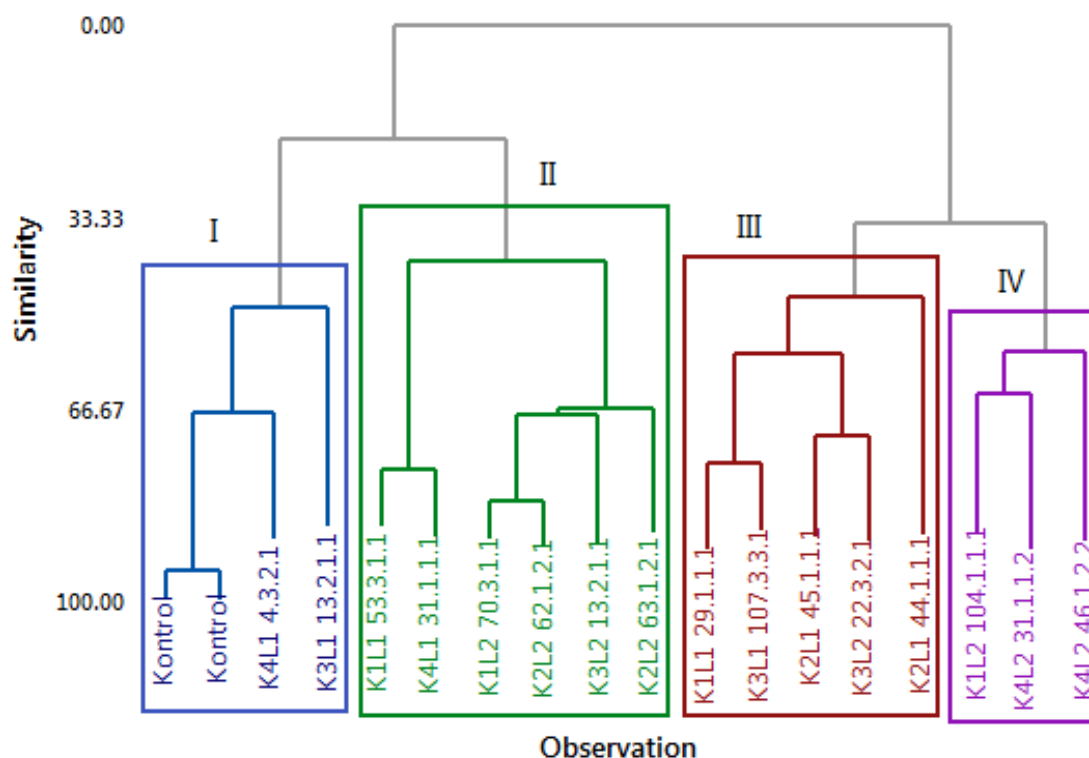


Figure 4. Analysis of 16 groups of MV4 patchouli mutant numbers based on the number of chloroplasts, stomatal density, stomata diameter, number of trichomes and number of chromosomes. K1L1 = 0.025%, 24 hours; K2L1 = 0.050%, 48 hours; K3L1 = 0.075%, 24 hours; K4L1 = 0.100%, 24 hours; K1L2 = 0.025%, 48 hours; K2L1 = 0.050%, 48 hours; K3L2 = 0.075%, 48 hours; K4L2 = 0.100%, 48 hours.

## Conclusion

Patchouli genetic diversity was successfully increased by colchicine and immersion duration treatment. LC 50% for the concentration of colchicine in patchouli is 0.132% and LC 50% for the length of soaking patchouli is 60.16 hours. The concentration of colchicine and soaking time increased the amount of chloroplast, stomata diameter, and a number of chromosomes and decreased stomata density and patchouli trichome. The chromosome of 16 patchouli mutants in the MV4 generation is not yet stable where there is still chimera with mixoploid individuals that have ploidy between 20 to 80. Recommendations treatment that has the highest level of diversity based on scatterplots are colchicine 0.075% with 48 hours soaking time. Hierarchical clustering/dendrogram clustering 16 numbers of patchouli mutants into four different groups.

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