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Research Article

Performance of Putative Mutants and Genetic Parameters of *Plectranthus amboinicus* (L.) through Mutation Induction With Colchicin

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ABSTRACT

Genetic enhancement in vegetatively propagated crops can be done through mutation induction. Colchicine-induced mutation is one of the methods that can be employed to increase plant genetic diversity. This study aimed to determine the effect of colchicine on the performance and genetic parameters of MV3 generation of Plectranthus amboinicus (L.). This study was conducted at the Laboratory of Agricultural Seed Management, Plantation Research and Development Center, from June 2018 to June 2019. Nodes and shoots were used as explants. Mutation induction was performed using colchicine at concentrations of 0%, 0.02%, 0.04%, and 0.06%. Explant regeneration and subculture were done on MS0 medium. The number of plantlets yielded were 59 (0% concentration of colchicine), 60 (0.02%), 81 (0.04%), and 80 plantlets (0.06%), respectively. Results indicated that colchicine-induced mutation in an in vitro culture was able to generate high genetic diversity in both quantitative and qualitative characters of the plantlets. At the concentration of 0.04%, colchicine produced the highest frequency of putative mutants (28.4%). Genetic parameters in MV3 generation of P. amboinicus plantlets showed that five quantitative characters, i.e. plantlet height, number of leaves, number of shoots, leaf length, and number of roots had high heritability values at a concentration around the LC_{50} value (0.0275%).

1. Introduction

Plectranthus amboinicus (Lour.) Spreng, commonly known as torbangun in Indonesia (Wibisono et al. 2019), is a herbaceous plant, belonging to the Lamiaceae family, and can be found in Asia, Australia, and Tropical Africa (Hullatti and Bhattacharjee 2011). The leaves of this plant are widely used as traditional medicine because of its important medicinal and nutraceutical properties, such as anti-bacterial (Aguiar et al. 2015), anti-carcinogenic (Suresh et al. 2020), anti-streptococcal (Zhang et al. 2017), anti-inflammatory (Chen et al. 2014), anti-dandruff (Selvakumar et al. 2012), antioxidants and anti-bacterial (Bhatt and Negi

2012), anti-fungi (Brandao et al. 2013), anti-diabetic activities (Govindaraju and Arulselvi 2018), anti-rheumatic (Bhatt et al. 2013), anti-epileptic (Kumari et al. 2012), as well as anti-diarrhea (Shubha and Bhatt 2015). *P. amboinicus* can also be consumed to increase breast milk (Damanik et al. 2017).

In Indonesia, *P. amboinicus* regenerates vegetatively due to its tropical origin. This causes a disturbance in its physiological processes and resulted in plant unable to produce seeds (Saryoko et al. 2018). Vegetative propagation can lead to a lack of genetic diversity (Wang et al. 2021). Germplasm, biomass, and metabolite content in *P. amboinicus* need to be improved for future breeding

program purposes. Genetic enhancement in vegetatively-cultivated crops can be carried out through mutation induction. Mutation induction in *P. amboinicus* has been done using ethyl methanesulfonate (EMS) to evaluate plant growth and to obtain new variants (Sari et al. 2017). Interaction between EMS concentration and application method significantly affected plant height and number of leaves on *P. amboinicus*. Based on morphological changes, one putative mutant for *P. amboinicus* was produced from the study. Another study reported using gamma-ray irradiation to evaluate the compounds profile from the leaves of *P. amboinicus* mutant compared with its wild-type (Aisyah et al. 2020). From the hierarchical cluster analysis (HCA), they found that wild-type and mutant plantlets differed in their 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), stigmasterol, and hydroxymethylfurfural contents. In mutant plantlets, DDMP and stigmasterol content showed an increase compared with wild-type. On the other hand, hydroxymethylfurfural was not found in mutant plantlets.

Other than oryzalin and trifuralin, colchicine is one of antimitosis substances that can be utilized to produce polyploid plants (Dhooghe et al. 2009). Colchicine works by inhibiting the formation of spindle threads during cell division (Yan et al. 2016), leading to the formation of polyploidy (Kerdsuwan and Te-chato 2012). Polyploid plants generally have bigger leaves, stems, roots, and flowers, as well as higher secondary metabolites contents (Gaafar et al. 2017) than their diploid counterparts, a phenomenon called "gigas effects" (Manzoor et al. 2019). Application of mutation techniques in callus cells, followed by regeneration through organogenesis, is the method with the greatest chance of obtaining a lot of variants (Zhang et al. 2020).

Mutation inductions using colchicine have been frequently carried out in other plants, especially ornamental plants (Manzoor et al. 2019), *Rosa* spp. (Baig et al. 2016), *Rosa multiflora* Thunb. var. inermis and *Rosa roxburghii* F. Normalist (Feng et al. 2017), as well as papaya (El-Latif et al. 2018). However, in recent years, there has been no research focusing on colchicine-induced mutation in *P. amboinicus*. This study aimed to determine the effect of colchicine-induced mutation on the performance and genetic parameters of *P. amboinicus* (L.) MV3 (vegetative mutants 3) generation *in vitro*.

2. Material and Methods

Callus induction and regeneration of Plectranthus amboinicus

P. amboinicus plants were obtained from farmer's garden in Sentul, Bogor, West Java, Indonesia in June 2018. Samples were confirmed and deposited at Tropical Biopharmaca Research Center (Trop BRC), IPB University. Research was carried out in the Laboratory of Agricultural Seed Management, Plantation Research and Development Center, Cimanggu, Bogor from June 2018 to June 2019. Nodes and shoots were harvested and used as explants, cultured on a 0.4% (w/v) gellan gum-solidified Murashige and Skoog basal medium (MS) supplemented with 3% (w/v) sucrose and 0.5 ppm 2.4-D (2.4-*dichlorophenoxyacetic acid*) for four weeks until massive callus was formed. To stimulate the germination process, callus was then transferred to MS medium without any plant growth regulators added (MS0) for four weeks.

Polyploidy induction with colchicine treatment

Research was arranged in a completely randomized design with four treatments and three replications. Each replication included three bottles containing five calluses per treatment (the total callus population was 180 calluses). Four levels of colchicine concentration tested to induce polyploidy in *P. amboinicus* were 0%, 0.02%, 0.04%, and 0.06% with two hours of immersion time each. After immersion treatment, callus regeneration into plantlet was carried out by transferring callus to MS medium without any plant growth regulators added (MS0). LC_{50} was found at colchicine concentration of 0.0275%.

Subculture of M1 to MV3 mutant generation

After regeneration, plantlets (first generation mutants, MV1) were transferred to MS0 supplemented with 1.5 ppm 6-benzylaminopurine (BAP) and incubated for five weeks. The surviving plantlets producing new shoots were subcultured (into MV2 and MV3 generations) to MS medium without any plant growth regulators added (MS0). The number of MV1 plantlets treated with 0% colchicine was 21 plantlets. For colchicine concentration of 0.02%, 0.04%, and 0.06%, the number of plantlets was 19, 18, and 27 plantlets respectively. In MV2 generation, 0%, 0.02%, 0.04%, and 0.06% concentration produced 39, 32, 32, and 45 plantlets respectively. Meanwhile, in MV3 generation, treatment of 0% colchicine produced 59 plantlets, 0.02% produced 60 plantlets, 0.04%

produced 81 plantlets, and 0.06% produced 80 plantlets.

Data analysis

The percentage of normal and abnormal MV3 plantlets was analyzed using Microsoft Excel. Analysis of MV3 data (number of shoots, plantlet height, number of leaves, leaf length, number of roots) was performed using Boxplot Qualitative Test Minitab 17.0. Estimation of the value of genetic parameters, including phenotype and genotype variety, broad-sense heritability (Ritonga et al. 2018), as well as the percentage of putative mutant frequency were done using Microsoft Excel. Percentage value of putative mutants was obtained from the ratio of putative mutant frequency produced in each treatment to the total number of plantlets of the same treatment).

3. Results

Quantitative characters of MV3 mutant generation

Application of 0% colchicine yielded 91.5% normal MV3 plantlet (Figure 1). This number was the highest percentage observed from all treatments, while the lowest percentage was observed in 0.02% of colchicine concentration treatment. The diversity of quantitative characters could be seen from the data distribution and the extreme value of the boxplot analysis results (Figure 2). The observed number of shoots suggested a similar diversity, except for 0.02% and 0.04% colchicine concentration which showed several extreme values (Figure 2A). High variability in the 0.02% concentration was in accordance with the LC_{50} value, which was found at the concentration of 0.0275%. High variability in a character was usually observed at the concentration around LC_{50}.

Following the LC_{50} value, the highest variability in plantlet height was observed at the concentration of 0.02% (Figure 2B). However, extreme values were mostly found in plantlets from 0.04% concentration treatment. For number of leaves, the highest variability was found among plantlets from 0.04% colchicine concentration, but the number of extreme values was found at 0.02% the most (Figure 2C). Application of 0.06% colchicine affected plantlet leaf length, in which the highest variability and extreme values were both observed in this treatment (Figure 2D). These results suggested that higher concentration of colchicine affected leaf length more. For number of roots, the highest variability was found at the concentration of 0.02% colchicine, following the LC_{50} value.
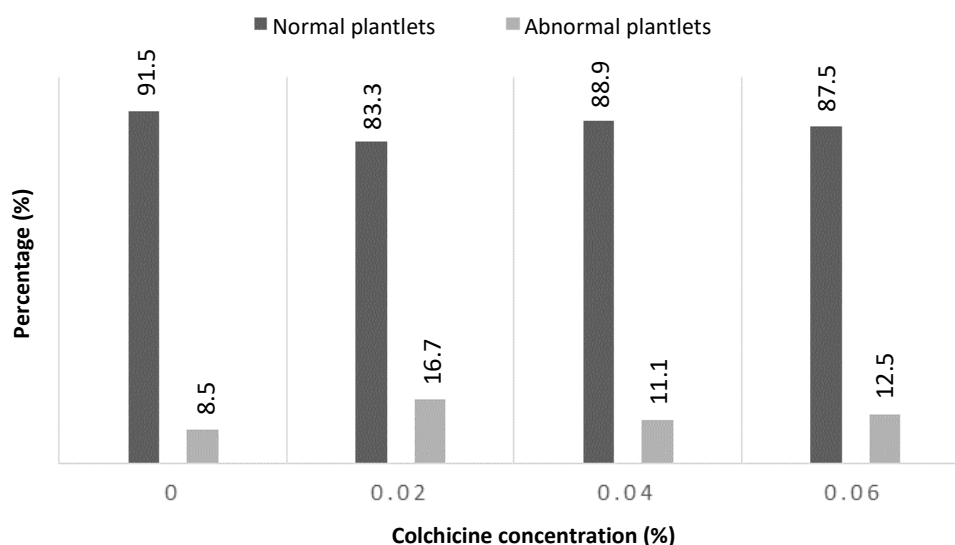

Figure 1 Percentage of normal and abnormal *Plectranthus amboinicus* plantlets in MV3 generation after five weeks.
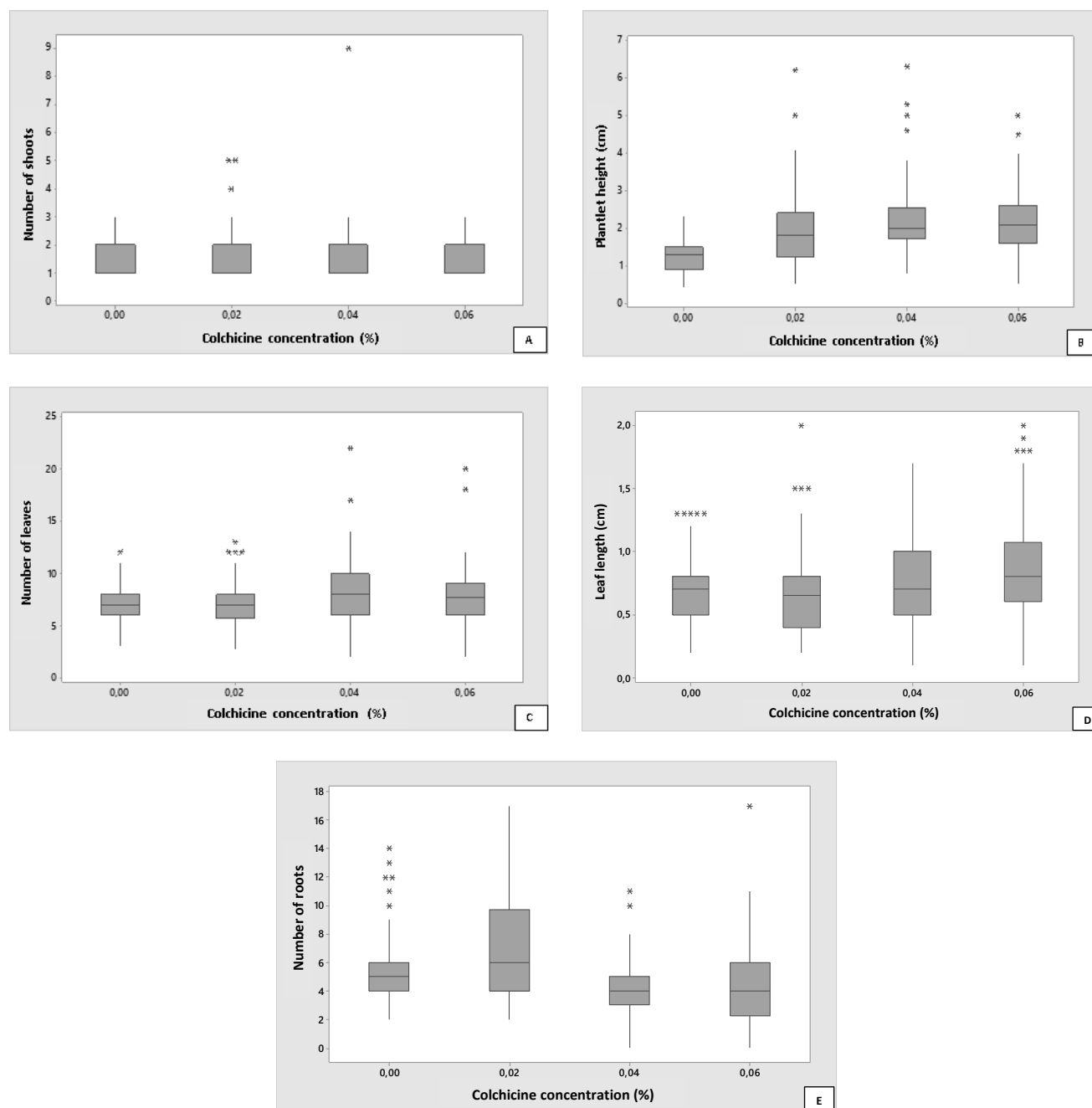
Figure 2 Boxplot analysis of *Plectranthus amboinicus* MV3 plantlets. A) number of shoots, B) plantlet height, C) number of leaves, D) leaf length, and E) number of roots.

Qualitative characters of MV3 mutant generation

Colchicine-induced mutation could also generate variability in plantlet leaf color, leaf and stem morphology (Figure 3). There were several variations in colchicine-induced leaf colors, i.e. yellowish-green (RHS 149A light yellow-green), dark green (RHS 144A dark green), and green variegata (RHS 145A yellow-green/RHS 141A dark green). Parental plants were all green (RHS 137 green). Leaves also became diversified morphologically, such as dwarf leaves, lanceolate-shaped leaves, flat-leaf edges, hairy leaf surfaces, and emarginate-shaped leaf tips. Changes in stem morphology were found in the number of stalks growing from one node. We found nodes with one, three, four, or five stalks simultaneously (Figure 3F–H).
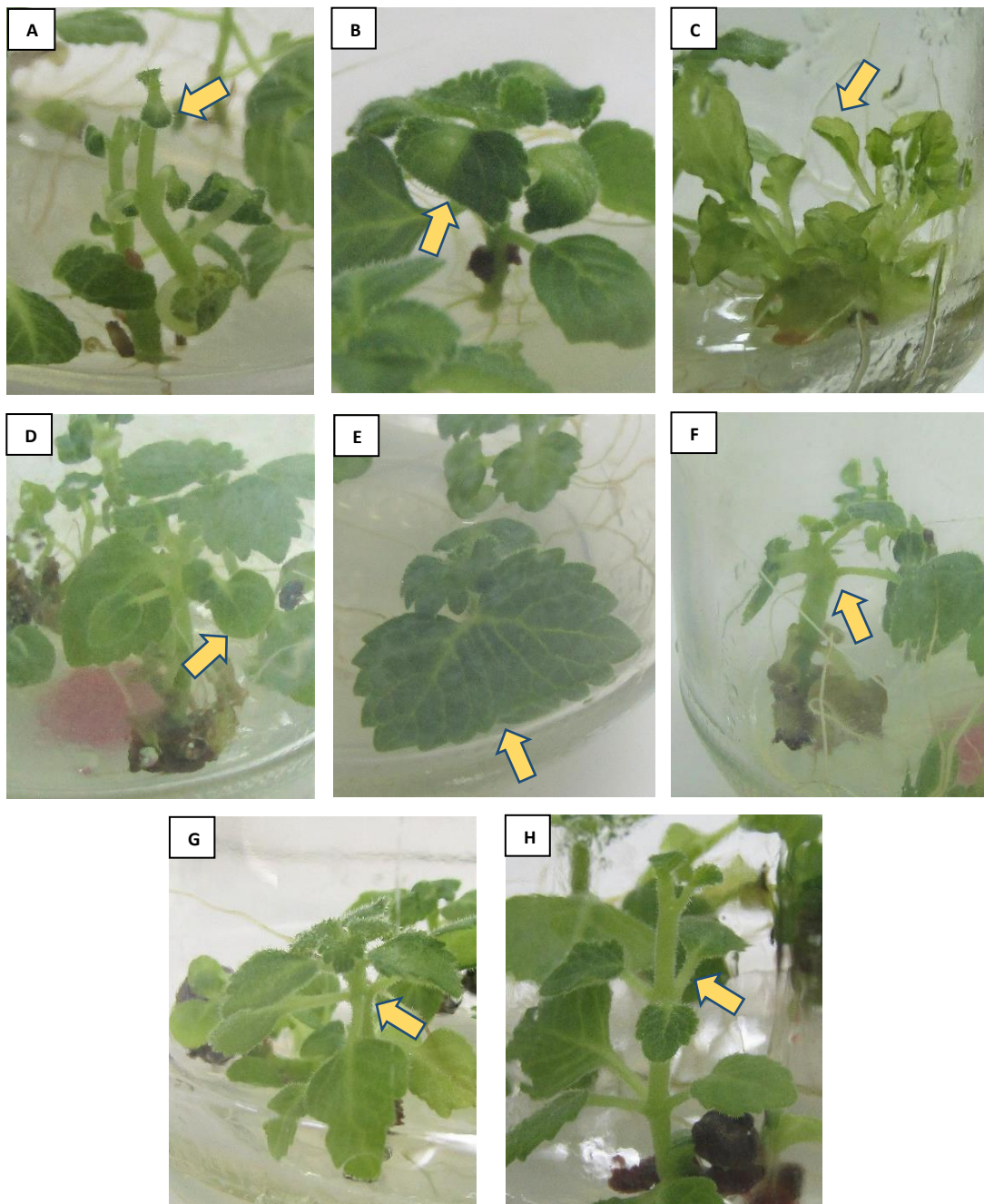
Figure 3 Variations in plantlets' qualitative characters after colchicine-induced mutation (five weeks post-subculture). A) dwarf leaf shape, B) green variegata leaf, C) yellowish-green and lanceolate-shaped leaf, D) flat-leaf edge, E) leaf tip with emarginate branching and dark green color, F) one node four stalks, G) one node three stalks, H) one node of three stalks, hairy stems, and leaves.

Distributions of the variation in plantlet qualitative characters were presented in Figure 4. Colchicine-induced mutation generated variations in leaf color compared with control treatment (0% concentration; Figure 4A), although these color changes did not differ strikingly from control leaves. Based on Figure 4A, leaves with yellowish-green color were mostly found among plantlets regenerated from 0.04% concentration treatment. Dark-green leaves were mostly found among 0.02% and 0.06% concentrations. At around LC_{50} value (0.0275%), dark green, yellowish-green, and variegata leaves were produced. On the other hand, callus immersion in 0.04% and 0.06% colchicine did not produce plantlets with green variegata leaves.
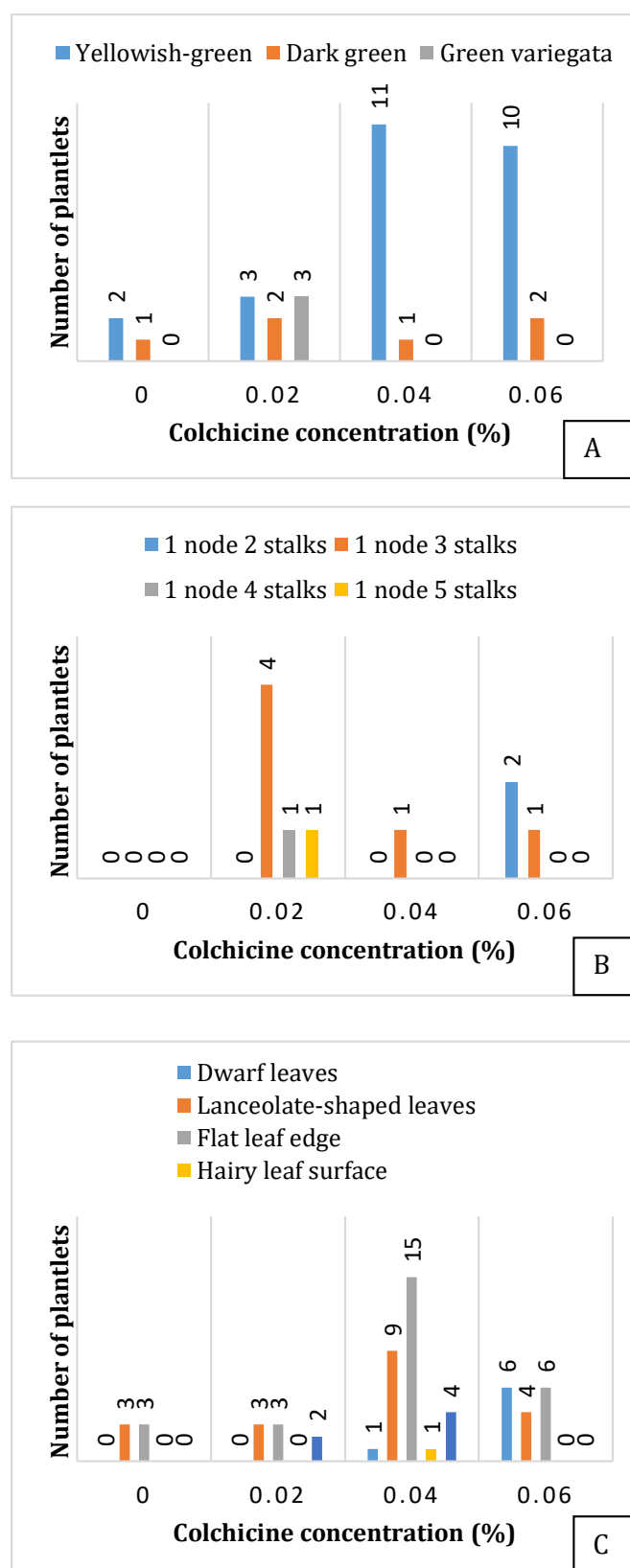
Figure 4 Variation in qualitative characters of MV3 generation through colchicine induction after 5 weeks post subculture. A) leaf color variations, B) stem morphology variations, C) leaf morphology variations.

Variation in stem morphology was observed from the number of stalks per node (Figure 4B). A higher number of stalks per node indicated the plantlet's potential to produce higher biomass compared with the control plantlet. Stem morphology variation was generated the highest around its LC_{50} value (0.02% colchicine concentration treatment), where we observed nodes with three, four, and five stalks. All control plantlets (treatment with 0% colchicine) had normal number of stalks per node, i.e. two stalks for each node. All plantlets from 0.04% concentration treatment had three stalks in their nodes. Meanwhile, some plantlets from 0.06% concentration treatment had one stalk in one node, and some others had three stalks instead. These results suggested that colchicine-induced mutation could be employed to increase plant biomass.

In Figure 4C, variation in leaf morphology was shown. The highest variation in leaf morphology was produced after treatment of 0.04% colchicine concentration. In this treatment, a variety of dwarf leaves, lanceolate leaf shape, flat-leaf edges, hairy leaf surfaces, and emarginate leaf tips could be observed. Hairy leaf surface only grew in plantlet from 0.04% concentration treatment. Figure 4C also showed that dwarf leaves grew as the colchicine concentration was increased.

Genetic parameters of MV3 mutant generation

Analysis results of MV3 generation genetic parameters showed that five quantitative characters of *P. amboinicus* generally had high heritability values (Table 1). MV3 generation also showed a high genetic variability. High heritability value were estimated at LC_{50} concentration (0.0275%) in three characters, i.e. number of shoots, plantlet height, and number of roots. Meanwhile, number of leaves and leaf length had moderate variances.

Heritability value from 0.04% concentration treatment was high for number of shoots, plantlet height, and number of leaves. The estimated value in leaf length was moderate, while for number of roots, it was low. As for 0.06% concentration treatment, the estimated value for plantlet height, number of leaves, and leaf length were high. The other two characters were low-valued.

The highest percentage of putative mutants was produced at a concentration of 0.04% colchicine (28.4%) and the second-highest was at the concentration of 0.02% (25%) (Figure 5). The percentages were obtained from the ratio of putative mutant frequency produced in each treatment to the total number of plantlets of the same treatment.

Table 1. Estimation of genetic parameter values of MV3 putative mutants

| Genetic Parameters	Colchicine Concentration (%)	σ^2E	σ^2P	σ^2G	h²bs (%)	Heritability Value
Number of shoots	0.02		0.99	0.64	64.34	High
	0.04	0.35	1.00	0.64	64.53	high
	0.06		0.39	0.03	8.22	low
Plantlet height	0.02		1.13	0.95	84.25	high
	0.04	0.18	0.90	0.73	80.29	high
	0.06		0.79	0.61	77.31	high
Number of leaves	0.02		4.94	1.41	28.57	moderate
	0.04	3.53	9.39	5.85	62.37	high
	0.06		8.30	4.77	57.47	high
Leaf length	0.02		0.15	0.06	43.46	moderate
	0.04	0.08	0.12	0.03	27.82	moderate
	0.06		0.20	0.11	56.72	high
Number of roots	0.02		16.50	9.41	57.03	high
	0.04	7.09	7.40	0.31	4.19	low
	0.06		7.23	0.14	1.97	low
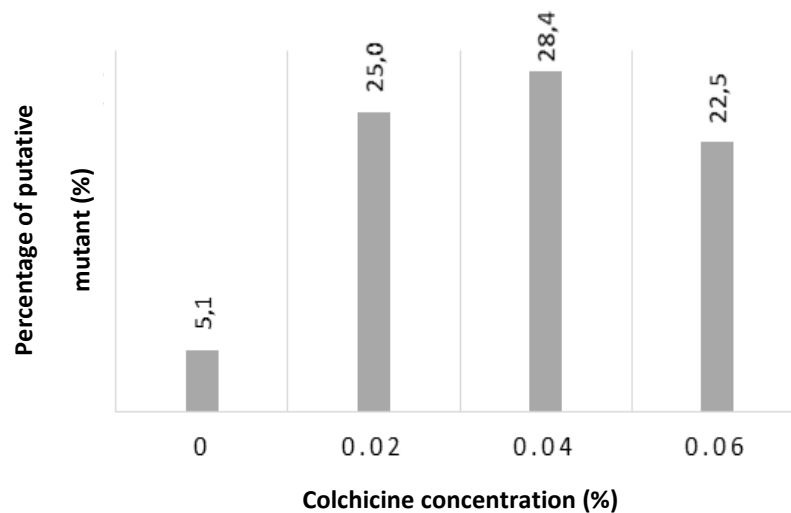
Figure 5. Percentage of putative mutants MV3 generation after colchicine-induced mutation

4. Discussion

Morphological characteristics of regenerant populations derived from colchicine-induced mutation have been studied in ornamental plants (Manzoor et al. 2019), such as plant height and stem diameter in *Rosa* spp. (Baig et al. 2016); number of shoots and number of leaves in *Rosa multiflora* Thunb. var. inermis and *Rosa roxburghii* f. *normalis* (Feng et al. 2017), as well as leaf color and abnormal leaves in papaya (El-Latif et al. 2018). Screening for putative colchicine-induced mutants using morphological markers is considered an indirect

assessment. The morphology of the mutants could be determined by comparing them with the control plants. The degree of morphological variations could reflect the genetic changes and indicate the effectiveness of colchicine induction treatment.

A boxplot could show the diversity of the data, and the whisker length showed the smallest and largest values (Krzywinski and Altman 2014). In MV3 generation, data of the five quantitative characters observed in all colchicine concentrations were not symmetrically distributed, as what could be seen from the box and whisker's length (Figure 2). This indicated that the treatments generated a high diversity in those characters. The observed diversity could serve as a reference for the next *P. amboinicus* plantlet breeding program and also became genetic material for mutant selection.

The number of normal plantlets displayed an increase in each generation, indicating that plantlet phenotype was becoming more stable. In MV3 generation, the percentage of normal plantlets from control treatment (0% colchicine) was 91.5% (Figure 1), which showed a 4.32% increase from the previous mutant generation. Normal plantlets regenerated from 0.02% colchicine treatment increased by up to 8.3% from its MV2 generation. Colchicine concentrations of 0.04% and 0.06% yielded 4.52% and 9.72% more normal plantlets in MV3 generation respectively, compared with their MV2 generation.

The number of abnormal-looking plantlets would decrease along with the increasing number of subcultures carried out. The phenomenon of normal and abnormal plantlets is due to the diplontic selection process, that is, competition between mutated and normal cells in cell dominance. Based on this, it is possible to observe chimeras that are mericlinal, periclinal, and sectoral in nature. Smaller explant size and medium without plant growth regulators would reduce the number of abnormal plantlets in the next generation (Nehra et al. 1992). Interestingly in MV3 generation, we still found 8.5% abnormal plantlets from control treatment (0% colchicine concentration). This could occur because abnormal conditions were caused by the use of plant growth regulators, the length of the incubation period of plantlets in the medium, and the frequency of subcultures. In control plantlets, abnormal conditions occurred probably due to the influence of plant growth regulators while they were still in callus phase. Plant growth regulators absorbed by callus cells could cause abnormality in the regenerating plants.

Plectranthus amboinicus plantlets' diversity in MV3 generation was observed in all five morphological characters (plantlet height, number of leaves, number of roots, leaf length, and number of shoots). The diversity of quantitative characters could be seen from the data distribution and the extreme values of the boxplot analysis (Figure 2). Extreme values observed in MV3 generation were larger than those in MV2 generation. This was due to changes in cell genetic material after colchicine induction treatment which led to cell rearrangement, where mutated cells must be able to adapt to their natural cell biology conditions with different chromosome numbers. This rearrangement process occurs along with the ongoing generation process. The increase in quantitative measurement is probably due to cells with a larger complement of chromosomes grow larger to maintain a constant ratio of cytoplasmic to nuclear volume and express more proteins (Abu-Qaoud and Shtaya 2014).

The qualitative characters showed that there were color variations due to colchicine induction. Leaf color is generally influenced by metabolite compounds from the flavonoid and carotenoid groups. Anthocyanins, one of the flavonoids, are responsible for producing several colors in plants, such as green, yellow, blue, purple, and red. Carotenoids scattered throughout the plant organs have an important role in photosynthesis (Dirar et al. 2019). Dark green, yellowish-green, and variegata leaf colors were found among MV3 plantlets (Figure 4A). This was due to the occurrence of chimeras, which are individuals composed of two or more different idiotypes in the apical meristem.

Leaf color is thought to be sectoral chimeras, where mutant cells develop vertically into tissue to form a sector. Hairy leaf surface appeared only at colchicine concentration of 0.04% (Figure 3). Hairy leaves could serve to reduce water evaporation, thus potentially making the plant to be resistant to drought stress. We also found leaf color changes in the control plants. We suspected that this was due to somaclonal variations that are common in tissue culture. Somaclonal variation is plant genetic or epigenetic variation that happens under *in vitro* conditions. Due to this, tissue culture techniques can be employed to produce plant variability without artificial mutation induction. However, mutation induction using colchicine increases plant variability.

It is important to identify cytochimeras (plants with different levels of ploidy in different types of tissues) when polyploidy is used for breeding purposes. Meristematic cells are usually divided into three histogenic layers (LI, LII, and LIII). The appearance of chimeras indicates that all cells presented in the histogenic layer (LI, LII, and LIII) of

the meristem tissue are not treated at the same time. Successful transformation of a diploid plant to a stable polyploid is dependent on a balance of colchicine concentration and exposure time, which doubles the genome content of the cells and ensures that the survival, growth, and development of induced plants are not severely affected (Montalban et al. 2011). Pigments in the cell vacuole generate color patterns depending on the ploidy level. During successive cell division, the mutated cells compete with normal surrounding cells for survival. If these mutated cells survive during diplotic selection, they are expressed in plants.

Heritability value has an important meaning in determining character selection. Heritability values are grouped into three categories, i.e. low, moderate, and high. Heritability is declared high if the heritability value is more than 50%, moderate if the value is 20-50%, and low if it is less than 20% (Ritonga et al. 2018). In this study, heritability values were found to be low, quite high, and high in MV3 generation. In general, four observed characters (plantlet height, number of leaves, roots, and shoots) had high heritability values for colchicine concentration of 0.02%, which was around the LC_{50} value (0.0275%). High heritability indicates a higher genetic influence than the environmental effect on the observed phenotype. Also, it was proved that high diversity and putative mutants could be obtained through colchicine-induced mutation by applying the correct concentration around LC_{50}.

As could be seen from Figure 5, the concentration around LC_{50} value (0.0275%) which is between the concentration of 0.02% and 0.04%, generated high percentages of putative mutants compared with other treatments. The highest percentage of putative mutants was produced at a concentration of 0.04% (28.4%), followed by the concentration of 0.02% (25%). Previous research results showed that colchicine induction through callus was most effective at LC_{50}. In *Tagates erecta*, mutation was induced through absorption of 0.0005% colchicine solution (LC_{50}) through roots or buds (Sadhukhan et al. 2014). In *Chrysanthemum carinatum*, a colchicine concentration of 0.2% (LC_{50}) was used for 3 days with a duration of 6 hours per day with a soaking method using wet cotton on the apical shoots (Kushwah et al. 2018).

5. Conclusion

Colchicine-induced mutation coupled with *in vitro* culture technique was able to produce high genetic variability both in quantitative and qualitative characters of the plantlets. The highest frequency of putative mutants resulted from colchicine induction was at a concentration of 0.04% (28.4%). Genetic parameters in the MV3 generation of *Plectranthus amboinicus* plantlets indicated that five quantitative characters observed in this study (plantlet height, leaf length, number of leaves, number of shoots, and number of roots) had high heritability values at a concentration around LC_{50} (0.0275).

6. Declaration of Conflicting Interests

The authors have declared no potential conflicts of interest concerning the study, authorship, and/or publication of this article.

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