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MORPHOLOGICAL CHARACTERS OF TRIPLOIDS AND TETRAPLOIDS PRODUCED BY COLCHICINE ON BUDS AND FLOWERS OF *Phalaenopsis amabilis*

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SUMMARY

Orchid breeders often used polyploidy to produce bigger and new flower characters using colchicine. Diploids, triploid and tetraploid progenies produced by cross and self-pollination of *Phalaenopsis amabilis* bud and flower using colchicine were being evaluated. This study was performed to answer the question of do morphological characters in acclimatization phase show polypoidy of *P. amabilis*. Sequential principle component analysis was performed to study morphological characters using plant diameter, leaf number, length and width from 4, 5, 6, 7, 8, and 9 months after acclimatization (MAA).The treatments given for polyploidization were: 50 ppm colchicine on young flower buds and then self-pollinated, 500, and 1000 ppm colchicine applied on buds and then flowers were self-pollinated and crossed with normal flower (control), and 50 and 500 ppm colchicine applied on self-pollinated and emasculated flowers for 3 and 5 days. Cluster analysis using R program resulted in 3 clusters with 45, 50, and 55% of Gower's dissimilarity on 9 MAA. Combination of morphological characters showed that the diploids distributed more evenly, but the triploids and tetraploids clustered together despite its treatments. Principal component analysis (PCA) and dendrograms were more similar at 8 and 9 MAA. The differences occurred caused by individual plant that was genetically different. This outcome showed that triploids and tetraploids of *P. amabilis* were more uniform than the diploids.

Key words: Acclimatization phase, diploid, moth orchid, PCA, polyploid, uniform

Key findings: Sequential principle component analysis of morphological characters using plant diameter, leaf number, length and width of diploids, triploids and tetraploids produced by applying colchicine on bud and flower of *P. amabilis* up to 9 months after acclimatization (MAA) showed that triploids and tetraploids were more uniform than diploids. The morphological characters were more synchronized with time. Leaf number was the dominant factor in the vegetative phase of *Phalaenopsis* toward the generative phase. The cumulative value of principal component analysis increased with time that showed more stable vegetative conditions. This study was conducted to evaluate plantlets morphological characters of *P. amabilis* with regards to colchicine treatments given at the bud and flower phase.

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INTRODUCTION

Phalaenopsis amabilis is one of Indonesian indigenous orchid species that often used as the source of *Phalaenopsis* hybrids found in the world (Christenson, 2001). In Indonesia, the hybrids found in the market were mostly imported. Hybrids in Indonesia were considered

small in size. The Orchid Mall (2013) stated that size of *Phalaenopsis* white hybrid that considered big is ≥ 13 cm. This condition had impelled Indonesia to find the hybrids from its own breeding, so it will be adapted to local condition. Polyploidization induction using colchicine has been proven to alter morphological changes in orchid (Griesbach, 1981; Sarathum *et al.* 2010; Atichart, 2013). This would be the basis for building new varieties in Indonesia, since it is required to ensure that constantly better and new varieties can be developed.

Morphological variability was observed in the *Phalaenopsis* spp .and also within hybrids that can be used as morphological characterization materials (Aziz and Sukma, 2015). Phenotypic difference after selfing, crossing between siblings and crossing can be observed with morphological marker that was influenced by environment. Morphological character usually is a qualitative character, such as the shape organs which is controlled by single gene on maize (Rieseberg, 1992).

Stock (2005) found that almost all of U.S. breeding has been with diploids, triploids, and the aneuploids that have resulted from breeding triploid reds to diploids and tetraploids. Aneuploids were also produced through attempts to increase flower size by breeding tetraploid reds to tetraploid pinks and stripes. Most attempts to increase size and flower count with diploid red breeding lines have resulted in the production of triploids. Unfortunately, triploid often could not produce seeds and the results of using 'anything that will breed', has produced a sea of aneuploids, which are then used in further breeding attempts. The outcome of this type of breeding is the well-known 'sterility barrier' so common in today's *Phalaenopsis* breeding.

Griesbach (1985) stated that most commercially valuable orchids are hybrids. In some instances, their hybridity can be quite complex involving up to 4 genera. Thus, both allo- and autopolyploidy could play a role in increasing fertility. Lu and Bridgen (1997) stated that sterile diploid hybrids revealed abnormal meiotic behaviors in *Alstro emeriaaurea* × *A. caryophyllae* and the aneuploid chromosome numbers, ranging from $2n = 1$ to $2n = 18$. The sterility of this hybrid is not caused by parental chromosome differences, but other complex fertility/sterility-regulating mechanisms are involved too. Further study on chromosome number is needed to anticipate the different number of ploidy found in the existing genotypes.

Multivariate analysis using principal component analysis (PCA) and cluster analysis shown to be useful in selecting genotypes for breeding program to achieve a plant breeder objective (Mohammadi and Prasanna, 2003; Niknejad *et al*., 2009). Ulaganathan and Nirmalakumari (2015) found that there is the need for breeders to exploit germplasm from distinct groupings produced by using principal component analysis and diverse clusters that showed intercrossing between genotypes to generate a broad spectrum of variability for effective selection for the development of high yielding cultivars on finger millet.

Sequential PCA can be used in determining correlation between sequential stages (Khademi *et al*., 2013). In this paper *P. Amabilis* progeny produced from previous research using colchicine application to increase the plant ploidy level. Diploids produced from all treatments (50 ppm colchicine on young flower bud and then selfed pollinated, 500, and 1000 ppm colchicine applied on the bud then the flower was selfed pollinated and crossed with normal flower (control), and 50 and 500 ppm colchicine applied on selfed pollinated and castrated flower for 3 and 5 days); triploids produced from bud treated with 500 and 1000 ppm colchicine and then crossed; tetraploids produced from 500 ppm colchicine applied on selfed pollinated and emasculated flower for 5 days. The plantlets further acclimatized and the vegetative variables were being observed to find the similarity between the different polyploidization sequentially from 4 up to 9 month after acclimatization (MAA).

MATERIALS AND METHODS

Diploids, triploids and tetraploids progeny produced with colchicine treatment acclimatized from in vitro culture (Table 1). Genetic materials used: No. 1-5 progeny from selfed pollinated flower then emasculated 50 ppm colchicine treated for 3 days, 6-8 progeny from selfed pollinated flower then castrated treated with 50

ppm colchicine for 5 days, 9-19 progeny from selfed pollinated flower then emasculated treated with 500 ppm colchicine for 5 days, 20-22 progeny from young flower bud treated with 50 ppm colchicine and then selfed, 23-28 progeny from young flower bud treated with 500 ppm colchicine and then selfed, 29-38 progeny from young flower bud treated with 500 ppm colchicine and then crossed, 39-42 progeny from young flower bud treated with 1000 ppm colchicine and then selfed, 43-49 progeny from young flower bud treated with 1000 ppm colchicine and then crossed. Morphological characters using leaf number, length and width, and plant diameter of diploids, triploids and tetraploids were observed from 4, 5, 6, 7, 8, and 9 month after acclimatization (MAA). Data were analyzed using sequential principle component analysis and cluster analysis using R program.

Table 1. *P. amabilis* progeny population produced from colchicine treatment on young flower bud and selfed pollinated flower used in the study.

No.	Ploidy level	Constituent accessions
1	Diploid	1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 15, 18, 20, 21, 22, 23, 24, 26, 27, 28, 30, 31,33, 36, 37, 38, 40, 41, 42, 43, 45, 47, 48, 49
2	Triploid	14, 19,29, 32, 34, 35, 44, 46
3	Tetraploid	10,13, 16, 17, 25, 39

Table 2. Clustering in *P. amabilis* progeny population produced from colchicine treatment on young flower bud and selfed pollinated flower.

Cluster	Number of genotypes	Constituent accessions
1	12	1, 49, 11, 38, 12, 46, 8, 20, 47, 45,28, 31
2	18	2, 9, 43, 13, 37, 15, 48, 16, 34, 27, 26, 42, 41, 6, 24, 18, 40, 21
3	19	3, 32, 30, 33, 14, 25, 10, 29, 36, 19, 17, 44, 22, 4, 23, 7, 5, 39, 35

Note: Numbers with black, green, and red color indicate diploid, triploid, and tetraploid plants, respectively.

RESULTS

Clustering with 100% diploids, triploids and tetraploids produced from colchicine treatments in the progeny population can be seen with the Gower's dissimilarity analysis (Table 2 and Figure 1). There was no pattern on number ploidy produced on specific treatment.Selfed pollinated flower then emasculated treated with 500 ppm colchicine for 5 days produced 18.18% triploids and 36.36% tetraploids. Young flower buds treated with 500 ppm colchicine and then selfed produced no triploids and 33.3% tetraploids. Young flower buds treated with 500 ppm colchicine and then crossed produced 40.0% triploids and no tetraploid. Young flower buds treated with 1000 ppm colchicine and then selfed produced no triploids and 25.0% tetraploid. Young flower buds treated with 1000 ppm colchicine and then crossed produced 28.6% triploids and no tetraploid (Table 2).

Cluster analysis were executed using R program resulted in 3 clusters with 45, 50, and 55% of Gower's dissimilarity on 9 MAA. Combination of morphological characters showed that the diploids distributed more evenly, but the triploids and tetraploids clustered together despite its treatments. Only progeny triploid no. 46 from young flower bud treated with 1000 ppm colchicine and then crossed with *P. amabilis* in cluster 1, others in cluster 2 and 3. Cluster 2 consisted of progeny triploid no. 34 (from young flower bud treated with 500 ppm colchicine and then crossed), and progeny tetraploids no. 13, 16

(from selfed pollinated flower then castrated treated with 500 ppm colchicine for 5 days), and 26 (from young flower bud treated with 500 ppm colchicine and then selfed). Cluster 3 consisted of progeny triploids no. 29, 32, 35 (from young flower bud treated with 500 ppm colchicine and then crossed), 14, 19 (from selfed pollinated flower then emasculated treated with 500 ppm colchicine for 5 days), and 44 (from young flower bud treated with 1000 ppm colchicine and then crossed), and progeny tetraploids no. 25 (from young flower bud treated with 500 ppm colchicine and then selfed), 10, 17 (from selfed pollinated flower then emasculated treated with 500 ppm colchicine for 5 days), and 39 (from young flower bud treated with 1000 ppm colchicine and then selfed).

Application of 500 ppm colchicine for 5 days on selfed pollinated flower then emasculated *P. amabilis* produced 22.2% triploids and tetraploids each in progeny population, whereas only 18.2% triploids and 9.1% tetraploid produced from the young flower bud treated with 1000 ppm colchicine. Selfed pollinated flower then castrated treated with colchicine produced 10.5% triploids, and 21.1% tetraploids, whereas young flower bud treated with colchicine produced 20.0% triploids and 10.0% tetraploids.

Data characters that can be described by 4 principal components for the whole data has a cumulative on 4, 5, 6, 7, 8, and 9 MAA were 65.2, 70.9, 65.9, 74.8, 76.8, and 82.1%, respectively for the first component (Table 3). Principal component analysis (PCA) and dendrograms were more synchronized on 8 and 9 MAA (Figure 2).

Table 3. Eigen analysis of the correlation matrix of leaf number, length, width and canopy diameter.

Time of observation	Principle component	Eigenvalue	Difference	Proportion	Cumulative
4 MAA	PC1	2.6076	1.5930	0.6520	0.6520
	PC2	1.0146	0.7514	0.2540	0.9060
	PC3	0.2632	0.1485	0.0660	0.9710
	PC4	0.1147	0.1147	0.0290	1.0000
5 MAA	PC1	2.8345	1.8282	0.7090	0.7090
	PC2	1.0063	0.9064	0.2520	0.9600
	PC3	0.0999	0.0406	0.0250	0.9850
	PC4	0.0593	0.0593	0.0150	1.0000
6 MAA	PC1	2.6366	1.7223	0.6590	0.6590
	PC2	0.9143	0.1993	0.2290	0.8880
	PC3	0.3575	0.2659	0.0890	0.9770
	PC4	0.0916	0.0916	0.0230	1.0000
7 MAA	PC1	2.9924	2.1617	0.7480	0.7480
	PC2	0.8307	0.6784	0.2080	0.9560
	PC3	0.1523	0.1276	0.0380	0.9940
	PC4	0.0247	0.0247	0.0060	1.0000
8 MAA	PC1	3.0704	2.4287	0.7680	0.7680
	PC2	0.6417	0.5522	0.1600	0.9280
	PC3	0.1984	0.1089	0.0500	0.9780
	PC4	0.0895	0.0895	0.0220	1.0000
9 MAA	PC1	3.2821	2.7335	0.8210	0.8210
	PC2	0.5486	0.4209	0.1370	0.9580
	PC3	0.1277	0.0861	0.0320	0.9900
	PC4	0.0416	0.0416	0.0100	1.0000

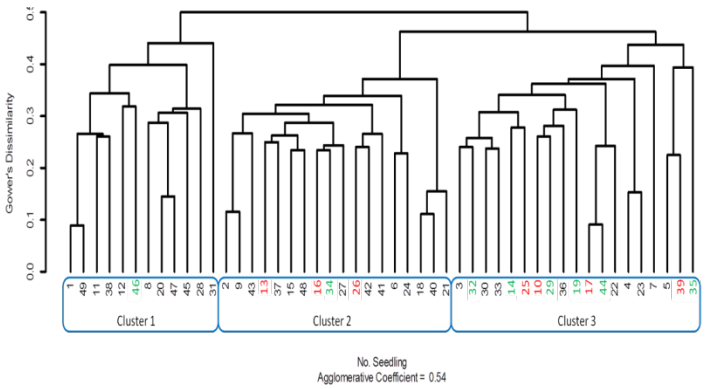

Figure 1. Dendrogram analysis of *Phalaenopsis amabilis* progeny population with different ploidy levels (diploid, triploid, and tetraploid) produced from colchicine treatment on young flower bud and selfed pollinated flower. Numbers with black, green, and red color indicate diploid, triploid, and tetraploid plants, respectively.

DISCUSSION

No pattern on ploidy number produced on specific treatment was observed (i.e. for the same treatment, for similarity between diploids, triploids, or tetraploids). This condition showed that every progeny is distinct and different with others. More polyploids produced if the organ treated with colchicine was young flower bud than selfed pollinated flower.

Leaf number as principal components for the whole data was cumulative on 4, 5, 6, 7, 8, and 9 MAA were 65.20, 70.90, 65.90, 74.80, 76.80, and 82.10%, respectively (Table 2). Leaf number was the dominant factor in vegetative phase of *Phalaenopsis* toward the generative phase. The cumulative value of principal component analysis increased with the observation time that showed more stable vegetative condition.

One *Phalaenopsis* pod consisted of thousands of seeds (Christenson, 2001). In polyploidy process with colchicine application, the number of ploidy produced took place by chance. Colchicine application produced tetraploids that made male and female diploid gametes. Selfing produced tetraploids progeny, whereas crossing produced triploids, pentaploids, etc. (Arditti, 1992). The differences in progeny produced caused by individual plant that was genetically different. The results from principal component analysis showed that triploids and tetraploids of *P. amabilis* progeny with colchicine application were more clustered together than the diploids that showed uniformity than the diploids (Figure 2) from 4 to 9 MAA. This founding shown that further explanation was needed for the mechanism processes involved in producing diploids, triploids, and tetraploids using young flower bud and selfed pollinated flower on *P. amabilis*.

CONCLUSIONS

Cluster analysis were executed using R program resulted in 3 clusters with 45, 50, and 55% of Gower's dissimilarity on 9 MAA. Combination of morphological characters showed that the diploids distributed more evenly, but the triploids and tetraploids clumped together despite its treatments. Principal component analysis (PCA) and dendrograms were more synchronized on 8 and 9 MAA. The differences occurred caused by individual plant that was genetically different. This outcome showed that triploids and tetraploids of *Phalaenopsis amabilis* were more uniform than diploids.

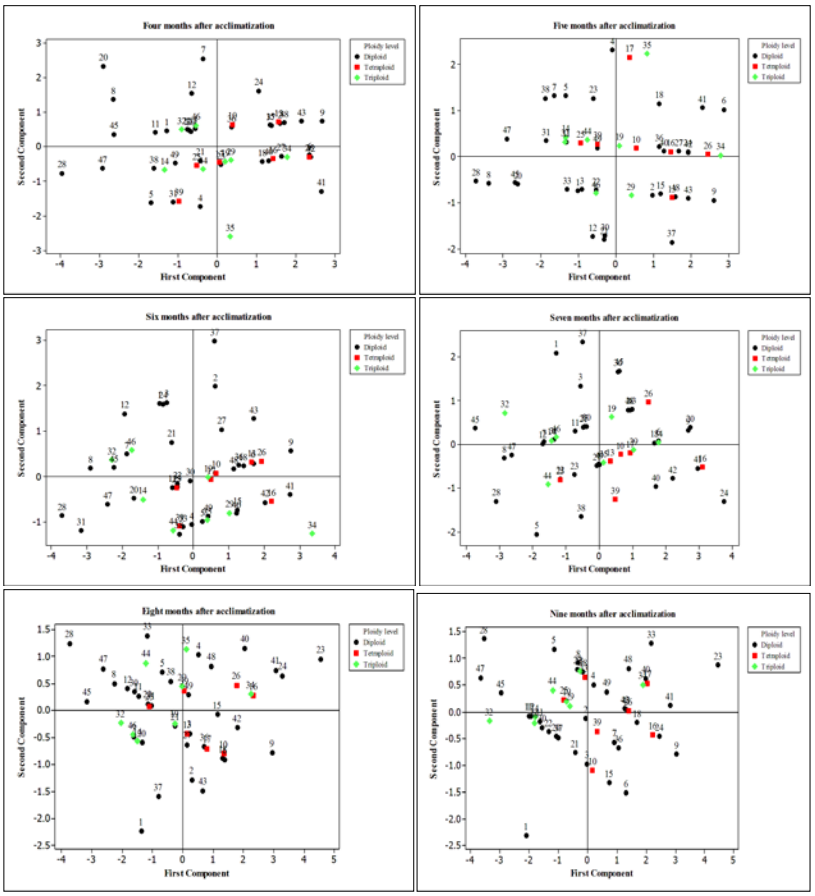

Figure 2. Principal component analysis of leaf number, length, and width, and canopy diameter of young flower bud and selfed pollinated flower colchicine-treated *P. amabilis* progeny population 4, 5, 6, 7, 8 and 9 MAA (black dot = diploids, green dot = triploids, red dot = tetraploids; No. 1-5 self-pollinated flower then emasculated treated with 50 ppm colchicine for 3 days, 6-8 selfed pollinated flower then castrated treated with 50 ppm colchicine for 5 days, 9-19 self-pollinated flower then emasculated treated with 500 ppm colchicine for 5 days, 20-22 young flower bud treated with 50 ppm colchicine and then selfed, 23-28 young flowerbud treated with 500 ppm colchicine and then selfed, 29-38 young flowerbud treated with 500 ppm colchicine and then crossed, 39-42 young flowerbud treated with 1000 ppm colchicine and then selfed, 43-49 young flower buds treated with 1000 ppm colchicine and then crossed).

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