

## **Polyploidy Induction of Moth Orchid (*Phalaenopsis amabilis* (L.) Blume) by Colchicine Treatment on Pollinated Flowers**

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### **ABSTRACTS**

*The effectiveness of colchicine treatment at inducing polyploidy in *Phalaenopsis amabilis* ( $2n=2x=38$ ) was investigated. Colchicine was applied on *P. amabilis* pollinated flowers and the development of the pods (fruit) was examined. Colchicine concentration in the experiment were 0, 50, 500, 1000, and 2000 mg L<sup>-1</sup>, with three and five days duration. The result showed that higher colchicine concentration decreased pod length and pod number. Visual screening of the seedlings obtained from colchicines treatments were classified into normal seedlings (NS), i.e. those having similar morphology to control, and putative polyploidy mutant seedlings (PMS). All colchicine-treated PMS were significantly different compared to control in the basal organ of the protocorm (BOP) length and width, leaf length, and root length and diameter. The PMS chromosome number analysis revealed that 50 mg L<sup>-1</sup> for three or five days colchicine application and 500 mg L<sup>-1</sup> for five days colchicine application produced 60 and 100% tetraploid mutant seedlings, respectively.*

**Keywords:** chromosome number, colchicines concentration, pollinated flower, polyploidization

### **INTRODUCTION**

*Phalaenopsis amabilis*, one of the species under *Phalaenopsis* genus, is important in floriculture because of their wide adaptability in various environmental conditions. This species can be found widespread in rain forest of Indonesia to the southern part of Philippines, and east to New Guinea and Queensland, Australia (Christenson, 2001). *Phalaenopsis amabilis* from South Cianjur, West Java, grows on lowland and has potentials to be developed into new commercial varieties. This *P. amabilis* ( $2n=2x=38$ ) flowers are lack of ornamental qualities and improvement in their flower characters, especially in the flower form and size could increase the ornamental values.

Flower characters of *Phalaenopsis* are generally one of important aspects that acquire a tremendous attention from orchid breeders and growers. Those of undesired

flower characters could possibly be improved by polyploidization. According to Griesbach (1981; 1985), perfection of flower in the white *Phalaenopsis* types, have probably been achieved by polyploidy. In the recent time, a polyploidy program had developed by Chen *et al.*, (2011b) in Taiwan, to convert over 20 diploid *Phalaenopsis* species to tetraploid, in order to fill up the needs of the industry. Polyploidy also very important and have been improved various crops characteristics: watermelon (Jaskani *et al.*, (2005), *Phlox* (Zhang *et al.*, (2008), tomatillo (Torres *et al.*, (2011)), etc. Polyploidy can increase genetic variability and improve flower characteristics, therefore floriculture is probably the most benefited from this technique (Griesbach, 1985). Those of several previous studies have been showed valuable role of polyploidy in orchids and various crops improvement.

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Polyploidy induction by using anti-mitotic agent is very common technique in polyploidization, one of them that very often used in plant is colchicine (Eigsti and Dustin, 1957). *Phaenopsis* Golden Sands is one of several orchid varieties that has been obtained by colchicine treatment (Griesbach, 1985). Despite colchicine has been widely used for orchid or various crops polyploidization, an appropriate polyploidy induction protocol with high yielding of desired polyploidy mutant plants is still limited. Effectiveness of polyploidy induction is determined by, among others, plant material used in the treatment. Protocols for polyploidy induction using colchicine have been established on various plant materials. The most often technique and plant material used in orchid polyploidy inductions were protocorm or seedling immersion in aqueous colchicine solution as performed by Griesbach (1981), Chaicharoen and Saejew (1981), Sarathum *et al.*, (2010), Kerdsuwan and Te-chato (2012), and Atichart (2013). However, these *in vitro* approaches are often laborious and resulted in high percentage of contamination by microorganisms, and also can produced unwanted chimeric plant. In another study, Nakasone (1960) used different orchid plant materials, such as flower spike, seeds, seedling, and cutting, but very few polyploidy plants produced.

Selecting the right plant material is a main key in orchid polyploidization to increase the rate of effectiveness and eliminate chimeric result. In this study, the plant materials used were relatively different with the published studies, that is using pollinated flower for colchicine treatment. This study was conducted to establish a novel and more effective technique in obtaining polyploidy mutant plant of *P. amabilis*. This technique offers several benefits as flower is a reproductive organ which can be easily manipulated by self or cross pollination to generate genetic variation (segregation), and produced progeny with larger quantity of polyploidy mutant seedlings.

## MATERIALS AND METHODS

### *Plant material and colchicine treatment*

The plant materials used were blooming *P. amabilis* from South Cianjur, West Java, Indonesia. Blooming plants were placed in shaded house and were self pollinated manually. Three days after self pollination, the flowers were castrated to discard their sepals, petals, and labellum. The ovary and stigma of castrated flowers were covered with absorbent cotton, and applied with colchicine solution using small brush until saturated for production of polyploidy seeds/progenies. Colchicine concentrations used in the experiment were 0, 50, 500, 1000, and 2000 mg L<sup>-1</sup>. Treated colchicine flowers were covered with aluminium foil to prevent evaporation for the duration of three or five days and were nested under each levels of concentration. Colchicine treatments were applied to 1-2 flowers per plant and repeated four times, so there were 40 plants in the experiment.

### *Pod harvesting and seed sowing*

All of mature pods of colchicine-treated and controls were harvested at 24 weeks after pollination (WAP). The number of harvested pod was counted from each levels of colchicine treatment, and the pod number percentage was calculated by using formula:

$$\% \text{ Pod number} = \frac{\text{Pod number}}{\text{Colchicine treated flower number}} \times 100\%$$

The pod length (the pod with pedicel) was recorded using calipers. All of harvested pods then were rinsed with tap water and the surface was sterilized by dipping into 70% (v/v) ethanol for 5 mins followed by passing briefly through the flame for three times. The pods were placed in a sterile petri dish and were cut vertically into two parts. The seeds were cultured on Knudson C medium supplemented with 2% (w/v) sugar, 6% (v/v) coconut water, 6% (w/v) pineapple and banana, 0.7% (w/v) agar and 0.2% (w/v) activated charcoal. The cultured

seeds were incubated at 23 °C under continuous illumination (1000 Lux). The cultured seeds were observed at 8 weeks after sowing (WAS) to evaluate the pod number with germinable seeds and the range of protocorm number per pod. In order to observe the quantity of protocorm per pod, the range of protocorm number per pod was classified into three groups, which were high (protocorm>250), medium (250>protocorm>50), and low (protocorm<50).

### ***Seedling subculture***

Colchicine treatment on pollinated flowers which produced progeny of more than 200 seedlings were subcultured on fresh Knudson C medium, those were 0 mg L<sup>-1</sup> (control), 50 mg L<sup>-1</sup> for three and five days, and 500 mg L<sup>-1</sup> for five days. The seedlings from each colchicine concentration were selected randomly at 15 weeks old after sowing and were subcultured to fresh Knudson C medium with five seedlings per flask. The experiment were arranged in Completely Randomized Design (CRD) with progenies from each colchicines-treated pollinated flowers as treatment, and consisted of five independent replications with five culture flasks per replication. During the growing period of 16 weeks after subculture (WAS), seedlings were observed every two weeks for new leaves and roots. Visual screening, morphological, and anatomical characteristics analysis were assessed at 16 WAS. Screening was conducted visually by comparing its morphological characters similarity and dissimilarity of the progeny seedlings (produced from colchicine-treated pollinated flower) from the diploid control seedling, and then classified them into two group, namely: normal seedling (NS) (similar to control) and the putative polyploidy seedling (PMS) (different to control). Both of seedling group (NS and PMS) then were analyzed morphologically (measuring the length and width of basal organ of the protocorm (BOP) and leaf, and length and diameter of root) and anatomically (length and width of stomatal) by using calipers and light microscope (40x magnification), respectively.

### ***Cytological analysis of chromosome number***

Both seedling group (NS and PMS) were examined by chromosome number counting to identified polyploidy. Chromosome preparation was determined according to Griesbach (1981) with some modifications. The length of actively growing root tips harvested was about 1 cm. They were then incubated for five hours in 50 mg L<sup>-1</sup> colchicine solution at 5-6 °C for metaphase arrested. Root tips were rinsed with distilled water and transferred to fixative and hydrolyzed solution of HCl 1 N : glacial acetic acid (3:1, v/v) for 3-4 hours in room temperature. Root tips were stained with 45% (v/v) aceto-orcein solution for 1-2 hours and transferred on a glass slide then squashed. Root tips samples were taken from five seedlings of each colchicine treatment and its chromosome number were observed under light microscope at 100x magnification with immersion oil.

### ***Statistical analysis***

Data were analyzed by using SAS 9.0. Significant differences of pod number percentage and leaf and root number between treatments were further analyzed using Duncan's Multiple Range Tests (DMRT), comparison of the characters of the putative polyploidy mutant seedlings and the normal seedlings were analyzed using orthogonal contrast at P< 0.05.

## **RESULTS AND DISCUSSION**

### ***Fruit (pod) forming and in vitro seed germination***

The stigma of pollinated flower of *P. amabilis* began to close three days after pollination. The pollinated flowers were able to develop into pod on both control and colchicine treatments. Several pollinated flowers treated with colchicine were not developed and dropped prematurely before they reached harvest time (24 weeks after pollination/WAP).

Higher colchicine concentrations resulted in shorter pods at 16 WAP (Table 01). Several colchicine-treated pods were dry and dead, especially those treated with 1000 and 2000 mg L<sup>-1</sup> colchicine for both three and five days. Dead pods can be marked by their color which gradually turned to be yellowish green and finally brown. Colchicine effect that hampered the pod development and even dead may caused by toxicity of colchicine that achieved at high concentration (1000 and 2000 mg L<sup>-1</sup>). Nakasone (1960) reported that colchicine treatment in orchid spike caused bud dropped at concentration of 1000 to 10000 mg L<sup>-1</sup>. Wu *et al.*, (2007) also reported that colchicine treatment to young flower bud of *Lilium* at 2000 mg L<sup>-1</sup> resulted in percentage of death up to 81.3 and 47.0% in Con Amore and Acapulco varieties, respectively. Toxic effect of colchicine on different plant material was reported by Kun-Hua *et al.*, (2011) on *Zingiber* shoot bud *in vitro*, its dead percentage increased significantly at higher colchicine concentration from 10.0% (1000 mg L<sup>-1</sup>) to 83.3% (4000 mg L<sup>-1</sup>).

Exposure duration to colchicine treatment, showed that no significance difference between three and five days on pod number percentage produced over all colchicine concentrations. The pod number percentage was significantly affected by colchicine concentration (Figure 01). The pod number percentage decreased at higher colchicine concentration. This result was different to Ghani *et al.*, (2014), survival of *Mentha* rizhome and tip meristem were affected by both colchicine soaking time and concentration.

All of control pods (eleven pods) produced seeds that could germinate and developed into protocorm on Knudson C medium. In contrast, only nine out of 25 pods (36%) of the colchicine-treated had germinated. Colchicine treatments

which produced pods with germinable seeds were 50 mg L<sup>-1</sup> for three and five days, 500 mg L<sup>-1</sup> for five days, and 1000 mg L<sup>-1</sup> for three days. Treatment of pollinated flowers with higher colchicine concentrations decreased pod number with germinable seeds. Moreover, several pods of colchicine treatment produced very few of non germinable seeds, such as 500 mg L<sup>-1</sup> for three days, 1000 mg L<sup>-1</sup> for five days, and 2000 mg L<sup>-1</sup> for three and five days. Those conditions indicated that colchicine (especially in high concentration) had affected to the post-pollination process and then it may had impacted to the fertilization directly or indirectly.

The range of protocorm number per pod varied between control and colchicine concentrations (Table 01). Colchicine treatment of 500 mg L<sup>-1</sup> for five days and 1000 mg L<sup>-1</sup> for three days which produced shorter pods than those of controls, had the range of protocorm number per pod at medium (250>protocorm>50) and low (protocorm<50), respectively. Those results showed that higher colchicine concentrations were not only affected on the appearance of the pod, but also its inside where embryo formation and development occurred. It may caused embryo unformed or non-viable embryo formed that impacted to the protocorm number per pod, since the control germination was good/high (protocorm>250). Colchicine treatment of 50 mg L<sup>-1</sup> for three and five days produced pods with length and protocorm number per pod relatively equal to control. Nakasone (1960) reported that treatment of *Vanda* and *Dendrobium* seeds with colchicine of 1000 up to 10000 mg L<sup>-1</sup> for more than four days caused interference on their germination. Similar results were also reported in non-orchid plants, in *Cyclamen* seeds by Takamura and Miyajima (1996) and *Platanus acerifolia* seeds by Liu *et al.*, (2007).

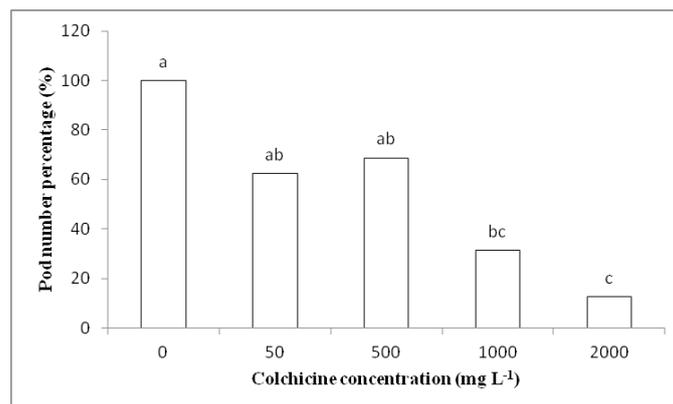
**Table 01: Pod development characteristics and protocorm produced at different colchicine concentration levels and durations of application**

Colchicine (mg L <sup>-1</sup> )	Duration	Colchicine-treated pollinated flowers number	Pod number <sup>a</sup>	Pod length mean (cm)	Pod number with germinable seeds <sup>a</sup>	Range of protocorm number per pod
0	3 days	4	4/4 (100.00)	5.12	4 (100.00)	High (protocorm>250)
0	5 days	7	7/7 (100.00)	4.92	7 (100.00)	High (protocorm>250)
50	3 days	5	3/5 (60.00)	5.26	3 (100.00)	High (protocorm>250)
50	5 days	7	4/7 (57.14)	4.52	3 (75.00)	High (protocorm>250)
500	3 days	7	5/7 (71.42)	3.52	0 (0.00)	None
500	5 days	7	6/7 (85.71)	3.68	2 (33.33)	Medium (250> protocorm >50)
1000	3 days	7	4/7 (57.14)	2.80	1 (25.00)	Low (protocorm<50)
1000	5 days	7	2/7 (28.57)	2.60	0 (0.00)	None
2000	3 days	5	1/5 (20.00)	2.00	0 (0.00)	None
2000	5 days	4	0/4 (0.00)	0.00	0 (0.00)	None

Pod length was observed about 16 weeks after pollination (WAP).

Protocorm observation was performed at 8 weeks after sowing (WAS).

<sup>a</sup>=value in the brackets showed percentage (%).



**Figure 01: Effect of colchicine concentrations on pod number percentage of *P. amabilis*. Different letters indicate significant difference at P< 0.05 according to Duncan's Multiple Range Test (DMRT)**

### ***Seedling growth and development***

In general, the seedlings from all colchicine treatments and control showed good growth. New leaves and roots emerged from the seedlings during incubation period up to 16 WAS. However, there was one seedling that showed abnormal morphology which found in colchicine treatment of 500 mg L<sup>-1</sup> for five days. The abnormal seedling had no leaves until the end of observation (16 WAS) and appeared as rooted protocorm. Rahayu (2014) also found abnormal seedlings of *P. amabilis*

and *P. amboinensis* which generated from protocorms treated by immersion in colchicine solution, which showed shorter and thicker stem and leaf than its diploid control seedling. Those abnormal seedlings may suspected as an octoploid seedling. As described by Chaicharoen and Saejew (1981) that found the auto-octoploid exhibited abnormalities in both growth and appearance, it grew very slowly and produced only few roots and had thick and wrinkled leaf.

There were significant differences in the leaf and root number of seedlings amongst all colchicine concentration and control during incubation period (Table 02). The seedlings obtained from colchicine treatment of 500 mg L<sup>-1</sup> for five days, grew slowly compared to others colchicine treatments including control, with leaf and root number were 0.83 and 0.68 times lower than those of control seedlings respectively, at the end of observation (16 WAS). The variation in the performances of seedlings treated with different colchicine concentrations could be considered as an early indication that colchicine treatment on pollinated flowers of *P. amabilis* was effective in inducing polyploidy mutant seedlings. In general, the polyploidy mutant seedlings obtained by colchicine treatments had slower growth characteristic compared to diploid control seedling (wild type). As observed by Griesbach (1981) that found diploid *Phalaenopsis* plant grew faster than colchicine-treated tetraploid. Kerdsuwan and Te-chato (2012) also observed similar growth characteristic in *Rhynchosyilis* tetraploid, root number and length were lower than control, but there was an opposite result in leaf number where tetraploid was higher.

**Visual screening of the putative polyploidy mutant seedling, cytological identification of the polyploidy mutant seedling, and the percentage of polyploidy mutant seedling**

Screening of the putative polyploidy mutant seedling conducted visually by comparing

the diploid control seedling and the seedling obtained from colchicine treatments at 16 WAS. There were two types of seedlings as result of screening: normal seedling (NS) and the putative polyploidy mutant seedling (PMS). The NS had morphological characteristics relatively similar to the diploid control seedlings, whereas the PMS were different to diploid control seedlings. Screening of seedlings obtained from colchicine treatment of 50 mg L<sup>-1</sup> for three days, 50 mg L<sup>-1</sup> for five days, and 500 mg L<sup>-1</sup> for five days, resulted the percentage of PMS were 27.35, 39.20, and 92.80%, respectively.

In this study, there was a character observed that had never been studied in identification of putative polyploidy mutant seedling of *P. amabilis* before, i.e. was basal organ of the protocorm (BOP) on seedling (Figure 02). There were significant differences based on orthogonal contrast test for several characters observed, such as BOP length and width, leaf length, and root length and diameter, between diploid control seedlings and seedlings obtained from all of colchicine treatments (Table 3). Moreover, comparison of those characters observed between the NS and the PMS of all colchicine treatment resulted in significant difference for almost all of characters observed except for leaf width. The PMS of all colchicine treatment had 1.5 times length and width of BOP than the NS. Similar results were recorded from colchicines concentration of 50 mg L<sup>-1</sup> for three and five days, and 500 mg L<sup>-1</sup> for five days.

**Table 02: Leaf and root number mean of seedlings obtained from colchicine treatments of pollinated flowers and control at 4 to 16 weeks after subcultured (WAS)**

Colchicine	4 WAS		8 WAS		12 WAS		16 WAS	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	1.44a	0.90a	1.96	1.04ab	2.47a	1.49a	3.12a	2.06a
50 mg L <sup>-1</sup> (3 days)	1.17b	0.85a	1.79	0.97bc	2.53a	1.48a	3.05ab	1.96ab
50 mg L <sup>-1</sup> (5 days)	1.46a	0.80ab	1.97	1.05a	2.43a	1.34a	2.83bc	1.78b
500 mg L <sup>-1</sup> (5 days)	1.41a	0.68b	1.79	0.94c	2.21b	1.16b	2.60c	1.42c

Means followed by different letter within the same columns indicate significant difference at P< 0.05 according to Duncan's Multiple Range Test (DMRT).

Sample size of each colchicine treatment and control were 125 seedlings, except in 50 mg L<sup>-1</sup> colchicine (3 days) with 117 seedlings at 8-16 weeks after subcultured (WAS).



**Figure 02: Basal organ of the protocorm (BOP) sizes on seedlings at 500 mg L<sup>-1</sup> colchicine (5 days colchicine application). The putative polyploidy mutant seedling (left) and the normal seedling (right)**

However, the morphologicals changed of the PMS were not always higher than both control and the NS as observed in leaf and root length of seedling (Table 03). Alteration of colchicine-treated polyploidy was varied or species dependent and it may was not always appeared in gigantic form, especially at *in vitro* condition. Similarly with Chen *et al.*, (2009) that observed leaf length of tetraploid *P. hieroglyphica* was significantly lower than its diploid. However, Heping *et al.*, (2008), found that significant different in width and length of *Dioscorea* leaf with higher values of tetraploid than their diploid were only at *in vivo* condition (no significant different at *in vitro* condition). The most common characteristic changed of polyploidy was gigantic form or darken leaf color. Chaicharoen and Saejew (1981) found some characteristics changed of tetraploid *Dendrobium* which were greener and thicker leaf than their diploid. Those leaf characters changed were also found by Omidbaigi *et al.*, (2010) in basil (non-orchid). Jaskani *et al.*, (2005) observed gigantic form of tetraploid watermelon leaf with higher leaf area index than diploid counterparts. Torres *et al.*, (2011) discovered tomatillo polyploid showed higher plant height and fruit weight and diameter. Zhang *et al.*, (2008) found tetraploid *Phlox* flower with higher size but lower leaf length.

Anatomical observation revealed that the PMS of all colchicine treatment had both length and width of abaxial stomatal significantly larger (1.12 and 1.17 times, respectively) than the

NS. Similar results also occurred in colchicine concentration of 500 mg L<sup>-1</sup> for five days (Figure 03). These results were in accordance with some orchid studies of tetraploid seedlings which were obtained by colchicine treatments, by Chen *et al.*, (2009), Kerdsuwan and Te-chato (2012), and Rahayu (2014), as well as in non-orchid plants such as in *Anthurium* by Chen *et al.*, (2011a) and in *Citrus nobilis* by Yulianti *et al.*, (2014). Stomatal size was often used as indirect polyploidy identification as performed by Miguel and Leonhardt (2011) that used stomatal length comparison to identify polyploidy in some orchids.

The chromosome number of the NS and PMS of each colchicine concentration were examined using a light microscope to confirm ploidy levels. The result showed that the NS of each colchicine concentration were 100% diploid ( $2n=2x=38$ ). All of colchicine concentration of 50 mg L<sup>-1</sup> for three and five days, and 500 mg L<sup>-1</sup> for five days were effective in obtaining polyploidy mutant seedlings. Colchicine at 50 mg L<sup>-1</sup> for three and five days had percentage of polyploidy mutant seedling 60% of the overall PMS examined. The highest percentage of polyploidy mutant seedling obtained from colchicine concentration of 500 mg L<sup>-1</sup> for five days, with all of PMS examined were identified as polyploidy mutant seedlings (100%) (Table 04). The chromosome number of diploid control and tetraploid mutant seedling of *P. amabilis* were presented in Figure 4.

**Table 03: Comparison of some characters of seedlings obtained from colchicine treatments of pollinated flowers and control at 16 weeks after subcultured (WAS)**

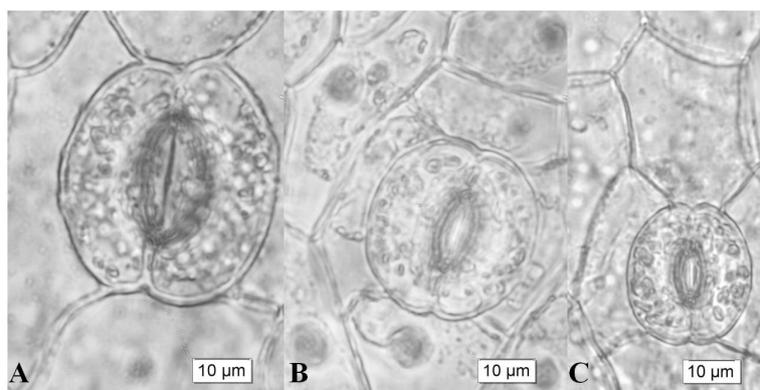
	BOP <sup>a</sup>		Leaf <sup>b</sup>		Root <sup>a</sup>		Stomatal <sup>b</sup>	
	Length (mm)	Width (mm)	Length (cm)	Width (cm)	Length (cm)	Diameter (mm)	Length (µm)	Width (µm)
Control	2.19	1.73	3.32	0.7	4.27	1.49	29.04	29.47
All colchicine treatment	3.26	2.26	2.48	0.68	3.5	1.79	30.54	28.48
Contrast test	*	*	*	ns	ns	*	ns	ns
Control	2.19	1.73	3.32	0.7	4.27	1.49	29.04	29.47
PMS (all colchicine treatment)	3.91	2.66	2.29	0.67	2.96	1.87	32.24	30.77
Contrast test	*	*	*	ns	*	*	ns	ns
NS (all colchicine treatment)	2.61	1.86	2.67	0.68	4.05	1.72	28.83	26.19
PMS (all colchicine treatment)	3.91	2.66	2.29	0.67	2.96	1.87	32.24	30.77
Contrast test	*	*	*	ns	*	*	*	*
NS (50 mg L <sup>-1</sup> 3 days)	2.49	1.79	2.88	0.74	4.71	1.73	28.83	26.24
PMS (50 mg L <sup>-1</sup> 3 days)	3.61	2.4	2.31	0.63	3.15	1.73	29.07	29.71
Contrast test	*	*	*	*	*	ns	ns	ns
NS (50 mg L <sup>-1</sup> 5 days)	2.41	1.78	2.97	0.66	4.19	1.56	27.98	24.53
PMS (50 mg L <sup>-1</sup> 5 days)	3.28	2.31	2.59	0.6	3.34	1.45	31.56	28.9
Contrast test	*	*	ns	ns	ns	ns	ns	*
NS (500 mg L <sup>-1</sup> 5 days)	2.94	2.02	2.15	0.66	3.25	1.86	29.68	27.79
PMS (500 mg L <sup>-1</sup> 5 days)	4.83	3.27	1.97	0.79	2.39	2.44	36.1	33.69
Contrast test	*	*	ns	*	ns	*	*	*

NS = the normal seedling, PMS = the putative polyploidy mutant seedling.

No significant different (ns) and significantly different (\*) according to contrast test at P< 0.05.

<sup>a</sup> Five seedling per colchicine treatment and control samples with five replications.

<sup>b</sup> Three different spot view per leaf per colchicine treatment and control samples with five replications. Stomatal were observed using light microscope with 100x magnification.

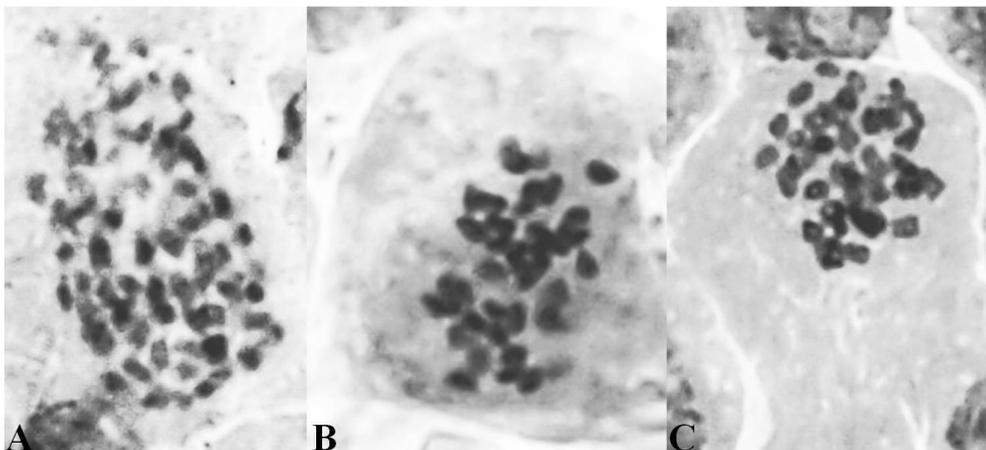


**Figure 03: Stomatal sizes of abaxial leaf at 500 mg L<sup>-1</sup> colchicine (5 days colchicine application), a. the putative polyploidy mutant seedling, b. the normal seedling, and c. control seedling**

**Table 04: Effectiveness of colchicine treatments on pollinated flower of *P. amabilis* at inducing poliploidy**

Colchicine	Seedling type	Number of seedling examined	Number of diploid seedling	Number of polyploidy seedling	Percentage of polyploidy (%)
Control	NS	5	5	0	0
50 mg L <sup>-1</sup> (3 days)	NS	5	5	0	0
	PMS	5	2	3	60
50 mg L <sup>-1</sup> (5 days)	NS	5	5	0	0
	PMS	5	2	3	60
500 mg L <sup>-1</sup> (5 days)	NS	5	5	0	0
	PMS	5	0	5	100

NS = the normal seedling, PMS = the putative polyploidy mutant seedling  
Counting of chromosome number was performed after 16 weeks after subcultured (WAS)



**Figure 04: Somatic chromosomes from root tip seedlings of *P. amabilis*: a. polyploidy mutant seedling ( $2n=4x=76$ ) and b. normal seedling ( $2n=2x=38$ ) from colchicine treatment at  $500 \text{ mg L}^{-1}$  (5 days colchicine application), and c. control seedling ( $2n=2x=38$ ) (100x magnification)**

The cytological examinations proved that colchicine treatment on pollinated flowers of *P. amabilis* was effective in producing progeny of polyploidy mutant seedlings. Another research that used orchid generative organ also was conducted by Nakasone (1960) which used young flower spike of *Vanda* as a material for colchicine treatment, but no polyploidy seedling produced in the study. In non-orchid plants, Wu *et al.*, (2007) reported the polyploidy induction in *Lilium* obtained 18% triploid mutant progeny by crossing colchicine-treated flowers with diploid control flowers. Other orchid studies with

colchicine treatment on protocorm reported by Chaicharoen and Saejew (1981) and Griesbach (1981), also resulted in approximately 50% tetraploid mutants. Sarathum *et al.*, (2010) and Kerdsuwan and Te-chato (2012) reported the percentage of tetraploid mutant orchid were 43 and 60%, respectively. Polyploidy induction in non-orchid plant resulted in 50% polyploidy mutant in *Platycodon grandiflorus* (Wu *et al.*, 2011). Chimeric result of polyploidy induction also has been reported by Petersen *et al.*, (2003) that found high percentage of ploidy chimeras in colchicine-treated planlet of *Miscanthus*.

## CONCLUSIONS

Colchicine treatments on pollinated flowers of *P. amabilis* were effective in obtaining a high number of polyploidy mutant seedlings (progenies). The length of pod and percentage of pod number were inhibited by colchicine treatment at higher concentration. Visual screening of seedlings (especially for basal organ of the protocorm character) obtained from colchicine treatments was effective in sorting polyploidy mutant seedlings from its diploid seedlings. Colchicine at 500 mg L<sup>-1</sup> for

five days was the most effective treatment for polyploidy induction, this treatment resulted 100% of putative polyploidy mutant seedlings were tetraploid.

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