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RESEARCH ARTICLE

In vitro Polyploid Induction in Phalaenopsis Aphrodite by Pendimethalin

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Abstract: Phalaenopsis aphrodite is a type of orchid with high ornamental and economic value. However, its development for commercial needs in Indonesia is still hindered by the limited availability of varieties. Therefore, enhancing genetic diversity is necessary, one of which is through polyploid induction. Several previous studies reported that pendimethalin effectively duplicates the chromosome numbers of various plant species. The aim of this study was to induce polyploidy in Phalaenopsis aphrodite and to evaluate the effect of pendimethalin treatment at the early stages in vitro. This study was conducted from March to August 2024. The experimental design used was a Completely Randomized Design (CRD) with two factors, the concentration of pendimethalin and incubation time on the treatment media. A hundred of zygotic embryos were cultured on MS0 + pendimethalin at concentrations of 100, 300, and 1000 µM for 2, 4, and 6 days while the control was cultured on MS0 without pendimethalin. Polyploidy was confirmed through chromosome counting using the squash method. The results showed that treatment with 100 µM pendimethalin for 6 days resulted in 11.1% tetraploid Phalaenopsis aphrodite plants. Although tetraploid individuals were obtained, the induction efficiency was relatively low. In addition, the pendimethalin treatment was also associated with decline viability, reduced morphological parameters, and did not significantly affect the stomatal parameters. However, these findings serve as a preliminary data for further studies to optimize polyploid induction by pendimethalin treatment in Phalaenopsis aphrodite so it could be potentially applied to breeding programs and commercial production.

Keywords: Polyploid, Phalaenopsis Aphrodite, Pendimethalin

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Introduction

Phalaenopsis stands out as one the most-sought-after orchids in horticulture, primarily because of its appealing aesthetic qualities and extended blooming duration compared to other orchid varieties. The Phalaenopsis genus is estimated to comprise about 80 species distributed in tropical and subtropical regions and dominates approximately 79% of the total global orchid trade [1-3].

Phalaenopsis aphrodite is a species belonging to the Phalaenopsis genus originating from Taiwan [4]. Taxonomically, this orchid belongs to the kingdom Plantae, division Spermatophyta, subdivision Angiospermae, class Monocotyledonae, order Asparagales, family Orchidaceae, subfamily Epidendroideae, genus Phalaenopsis, and species Phalaenopsis aphrodite [5]. The flowers measure approximately 6-10 cm in length and are quite fragrant. The leaves reach up to 30 cm in length and 7 cm in width, with green adaxial and purplish abaxial. The inflorescences can reach 100 cm in length, bearing 20-30 flowers, with the flowering season begins around April [6, 7].

Naturally, the Phalaenopsis aphrodite flowers are naturally pure white and create an elegant appearance [8]. In addition to the floral attractiveness, the long blooming period makes this orchid highly desirable to consumers in various countries, such as the United States, Japan, and South Korea, as an ornamental plant [9]. Currently, Phalaenopsis aphrodite is imported mainly from Taiwan due to its superior quality [10, 11]. This orchid has a broad market potential and has the potential to become a locally grown commodity. However, its development in Indonesia is still hindered by the limited number of available varieties, necessitating efforts to increase genetic diversity to produce flower characteristics that align with consumer preferences.

Efforts to increase genetic diversity can be achieved through polyploid induction, the process of doubling the chromosome resulting in organisms with more than two sets of chromosomes [12, 13]. Through polyploid induction, new varieties of plants with improved traits and optimal productivity can be developed [14,15]. Polyploid induction is commonly performed through chemical agents, such as colchicine. However, colchicine is relatively expensive, making it less economically efficient [16].

Pendimethalin (N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine) is a dinitroaniline herbicide in the form of an orange-yellow solid that is easily dissolved in organic solvents. This compound is selective for controlling broadleaf weeds and grasses [17-19]. The mechanism of action of pendimethalin is similar to colchicine, as it binds to microtubule proteins and inhibits formations of spindle fibers, thereby disrupting chromosome movement during the metaphase stage [20, 21].

Several studies have used pendimethalin as an antimitotic agent to induce polyploidy in plants, such as Zea mays [14], Allium cepa L. var. aggregatum G. Don [15], Plumbago auriculata [13], Cerastigma wilmottianum Stapf [16], and Solanum melongena [22]. Pendimethalin is considered an alternative compound for polyploid induction because it is relatively less expensive than colchicine, making it more economically efficient [23, 24].

Previous studies have reported that pendimethalin effectively duplicates chromosomes in various plant species. However, polyploid induction using pendimethalin has not been reported in Phalaenopsis aphrodite. Therefore, this study aimed to induce polyploidy in Phalaenopsis aphrodite and to evaluate the effect of pendimethalin treatment at the early stages in vitro.

Materials and Methods

This study was conducted at the DNA Favorit Tissue Culture Laboratory in Bogor and the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Pakuan, Bogor, during the period of March to August 2024. The explants used in this study were zygotic embryos from Phalaenopsis aphrodite seed culture on MS0 media collected from the DNA Favorit Tissue Culture Laboratory, Bogor.

The experiment was conducted in Completely Randomized Design (CRD) with three replications and two factors: pendimethalin concentration and incubation time. The pendimethalin concentration factor consisted of three concentrations: 100, 300, and 1000 µM, and the incubation time factor consisted of three periods: 2, 4, and 6 days.

The pendimethalin stock solution was derived from the herbicide Prowl 330 EC, which contains 330 g/L of the active ingredient pendimethalin. The stock solution concentration was 10%. To prepare a 100 mL 10% stock solution, 30.3 mL of Prowl 330 EC herbicide was taken and dissolved in distilled water to reach a total volume of 100 mL. The pendimethalin solution for the treatment was prepared by diluting the stock solution to concentrations of 100, 300, and 1000 µM.

For polyploid induction, a hundred of Phalaenopsis aphrodite zygotic embryos were cultured on MS0 medium with pendimethalin at concentrations of 100, 300, and 1000 µM for 2, 4, and 6 days. As a control, the zygotic embryos were cultured on a medium without pendimethalin. Each treatment was composed of three replications. The cultures were incubated at 25-27°C in bright conditions.

Following treatment, the explants were transferred to MS medium containing 0.1 mg/L BAP for subculture. After an 8-week period, the plantlets were subsequently subcultured in ½ MS medium supplemented with 20 g/L sucrose and activated

charcoal. The parameters assessed in the study included explant viability, plant height, root number, leaf number, leaf length, leaf width, stomatal number, stomatal length, stomatal width, chloroplasts number, and chromosome number.

Explant viability was observed by counting the explants that grew at 8 weeks after treatment. The percentage of explant viability was calculated using the following formula:

$$Explant\ viability\ (\%) = \frac{Number\ of\ explants\ grown}{Number\ of\ explants\ planted}\ x\ 100\% \quad (1)$$

Morphology, anatomy, and chromosomes number were observed at 24 weeks after treatment. Due to the limited number of surviving plantlets, for chromosomes number observation, 3 plants of each treatment and control were conducted.

The number of stomata was observed by sampling stomata from the lower surface (abaxial) of the leaf. Samples were taken from the third leaf from the bottom, counting from the first fully opened leaf. A slide was performed by making a transverse incision on the surface of the leaf, then adding a drop of water, and covered with a coverslip. The stomatal number was determined by counting the stomata under the microscope in each field of view at 400× magnification. The field of view measured 0.25 mm², which was obtained from a camera connected to the microscope and calibrated with Image Raster software. Stomatal length was determined by measuring the length of the guard cells, while width was measured by the aperture of the guard cells. Stomatal measurements were performed on images acquired at 400× magnification using Image Raster software. Chloroplasts number were determined in each pair of guard cells under 400× magnification. For each sample, 15 randomly selected stomata from various fields of view were counted, and the mean of chloroplasts number per guard cell pair was calculated for each treatment.

Chromosome number were measured using the squash method. The root tips were cut at 8:00 a.m with a length of 1-2 cm, then immersed in 0.002 M hydroxyquinoline solution and stored for 24 hours in the refrigerator. The roots were washed three times with distilled water, followed by fixation in a 45% glacial acetic acid solution for 1 hour at room temperature. Post fixation, the roots hydrolyzed by immersing in a 1:3 solution of 1 N HCl and 45% glacial acetic acid for 30 minutes at 60°C. The samples were then stained by acetocarmine for 24 hours at room temperature. After staining, the tips of the root samples were cut to a length of 0.5 mm, then covered with coverslip, and gently tapped the coverslip with a rubberized pencil tip. The preparations were observed under a microscope at 1000× magnification.

Data on explant viability and chromosome number were presented descriptively as percentages. Data on plant height, root number, leaf number, leaf length, leaf width, stomatal number, stomatal length, stomatal width, chloroplasts number, and chromosome number were analyzed statistically. Non-viable explants were assigned as zero values and included in the statistical analysis. Prior to analysis, the data were transformed using square root transformation $\left(\sqrt{x+0.5}\right)$ to meet the assumptions of parametric tests. Data were analyzed using Analysis of Variance (ANOVA). If a significant effect was found, further comparisons were performed using DMRT (Duncan's Multiple Range Test) at $\alpha = 0.05$. All statistical analysis were conducted using SPSS version 16.0.

Results and Discussion

Explant Viability

The viability of the Phalaenopsis aphrodite explant was found to decrease following pendimethalin treatment (Table 1). Similar trends have been reported in other plant species, such as Plumbago auriculata and Solanum melongena, indicating that higher pendimethalin concentration induced the lower explant viability [13, 22]. Comparable responses have also been observed in colchicine-induced polyploidy, where viability of Brassolaeliocattleya declined after treatment [23].

The reduced viability of explants may be associated with the role of pendimethalin as a chemical mutagen. Mutagenic compounds have been demonstrated to inhibit cell division and disrupt plant tissue regeneration, thereby hindering explants to grow and develop [25]. Higher explant mortality was observed at 300 and 1000 µM. This may be due to accumulation of pendimethalin within cells. A similar response has been reported by [26 for other antimitotic agents such as colchicine, where more colchicine is absorbed into cells and diffuses to various parts of the cells as the concentration higher and prolonged exposure duration which then disrupts cellular mechanisms, resulting in higher plant mortality. Furthermore, chemical compound induction can also cause stress in plants, resulting in increased production of ROS (Reactive Oxygen Species). Excessive ROS has been demonstrated to cause damage to cellular components, disrupt metabolism, and ultimately lead to cell death [27, 28].

Table 1: Effect of pendimethalin treatment on explant viability at 8 weeks

Treatment		Number of Explants Planted	Viability (%)
Pendimethalin Concentration (µm)	Incubation Time (days)		
Control		100	80
100	2	100	7.50
	4	100	8.79
	6	100	6.67
300	2	100	6.29
	4	100	4.17
	6	100	0
1000	2	100	0
	4	100	0
	6	100	0

Plant Height

The highest mean in plant height parameter resulted from the control (Table 2). The treatments with 100 µM pendimethalin and incubation time for 2, 4, and 6 days showed mean decrease of plant height but did not differ significantly from the control. Meanwhile, the other treatments showed a significant mean decrease compared to the control.

Table 2: Effect of pendimethalin treatment on plant height at 24 weeks

Treatment		Plant Height (cm)
Pendimethalin Concentration (µM)	Incubation Time (days)	
Control		3.46d±0.39
100	2	2.90cd±0.36
	4	2.67bcd±0.29
	6	3.00cd±1.50
300	2	2.27bc±0.58
	4	1.83b±0.29
	6	0.71a±0.00
1000	2	0.71a±0.00
	4	0.71a±0.00
	6	0.71a±0.00

Data are presented as mean±standard deviation. Means followed by the same letter in the same column are not significantly different according to DMRT at α = 0.05. Data were transformed using square root transformation $(\sqrt{x + 0.5})$ prior to analysis

The pendimethalin treatment in this study affected plant height, with the treated plants exhibiting shorter compared to the control (Fig. 1). These responses are similar to the findings by [22], that shorter stature Solanum melongena occurred after the pendimethalin treatment. Comparable results have also been reported by [29], showing that colchicine treatment resulted in stunted growth in Dendrobium officinale.

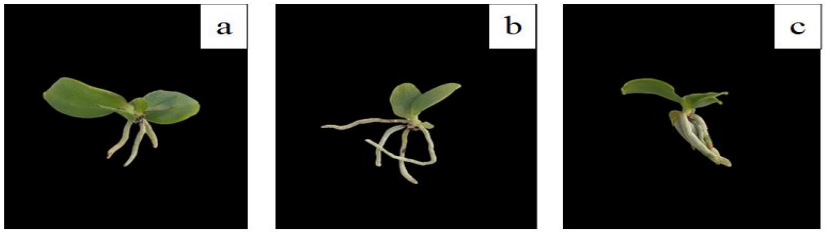

Fig. 1: Effect of pendimethalin treatment on plant height

Control (a); Pendimethalin concentration 100 µM, incubation time 2 days (b); Pendimethalin concentration 100 µM, incubation time 4 days (c); Pendimethalin concentration 100 µM, incubation time 6 days (d); Pendimethalin concentration 300 µM, incubation time 2 days (e); Pendimethalin concentration 300 µM, incubation time 4 days (f).

The decrease in the height of Phalaenopsis aphrodite plants in this study was associated by the effects of pendimethalin. Antimitotic compounds can inhibit cell elongation, thus affecting plant growth [30]. In addition, pendimethalin as a chemical mutagen may contribute to affecting growth hormones in plants, such as gibberellin (GA). A previous study by [31] reported that polyploid induction using EMS (Ethyl Methyl Sulfonate) reduced the gibberellin hormone in plants. The function of gibberellin includes stimulating cell division and elongation in the stem, so that the reduction of this hormone may affect plant height. [10] have also been reported that Zea mays treated with chemical mutagens exhibit shorter phenotype compared to the control plants due to lower gibberellin level.

Number of Roots

The control plants showed the highest mean on number of roots compared to the treated plants by pendimethalin (Table 3).

Table 3: Effect of pendimethalin treatment on number of roots at 24 weeks

Treatment		Number of Roots
Pendimethalin Concentration (µM)	**Incubation Time (days)**	
Control		4.22b±1.39
100	2	3.67b±0.58
	4	3.67b±0.58
	6	3.33b±0.58
300	2	3.33b±0.58
	4	3.00b±1.00
	6	0.71a±0.00
1000	2	0.71a±0.00
	4	0.71a±0.00
	6	0.71a±0.00

Data are presented as mean±standard deviation. Means followed by the same letter in the same column are not significantly different according to DMRT at α = 0.05. Data were transformed using square root transformation $\left(\sqrt{x+0.5}\right)$ prior to analysis

The decrease in number of roots in this study is consistent with findings by [32] that have observed a reduction in number of roots in Cymbidium aloifolium treated with colchicine. This may be attributed to chemical mutagens disrupting cell division in the root meristematic tissue, thereby inhibiting root growth [33]. Furthermore, pendimethalin as a mutagen may affecting the auxin hormone in plants. A previous study by [34], reported that colchicine induction can inhibit root formation and growth in plants due to changes in auxin levels. The functions of auxin include stimulating root formation and growth. When this hormone levels decrease, root formation and growth in plants can be disrupted. [35] have also been reported root growth in plants undergoing polyploid induction is inhibited attributed to lower auxin levels compared to diploid plants.

Number of Leaves

The mean number of leaves on control plants was 3.44. Most treatment results showed no significant differences compared to the control, except for the 300 µM pendimethalin treatment with a 6 days incubation time and all the 1000 µM pendimethalin treatment combinations (Table 4). Although there were no statistically significant differences in most treatments, the mean number of leaves tended to decrease compared to the control.

Table 4: Effect of pendimethalin treatment on number of leaves at 24 weeks

Treatment		Number of Leaves
Pendimethalin Concentration (µM)	Incubation Time (days)	
Control		3.44b±1.42
100	2	3.33b±0.58
	4	3.35b±0.58
	6	3.37b±0.58
300	2	3.17b±0.58
	4	3.07b±1.00
	6	0.71a±0.00
1000	2	0.71a±0.00
	4	0.71a±0.00
	6	0.71a±0.00

Data are presented as mean±standard deviation. Means followed by the same letter in the same column are not significantly different according to DMRT at α = 0.05. Data were transformed using square root transformation $(\sqrt{x+0.5})$ prior to analysis

The lower mean of leaves number is similar to report by [36], that found a reduction in the number of leaves of Dendrobium sp. compared to the control plants after treated with colchicine. The decrease may be associated with pendimethalin as a chemical mutagen, inhibits primordia formation in the apical meristem. Chemical mutagens, such as pendimethalin, can inhibit microtubule formation, preventing optimal cell division in plant meristems. Inhibited cell division slows the formation and development of leaf primordia, resulting in fewer leaves [37].

Leaf Length and Width

The mean of leaf length and width after treatment with pendimethalin was found to decrease compared to the control (Table 5).

Table 5: Effect of pendimethalin treatment on leaf length and width at 24 weeks

Treatment		Leaf Length (mm)	Leaf Width (mm)
Pendimethalin Concentration (µM)	Incubation Time (days)		
Control		22.89c±3.76	20.33c±2.74
100	2	15.56bc±3.56	11.03b±1.47
	4	17.22bc±3.86	10.95b±1.11
	6	21.67bc±14.11	11.08b±2.30
300	2	14.03bc±2.51	8.22b±3.34
	4	12.36b±8.13	7.03b±4.31
	6	0.71a±0.00	0.71a±0.00
1000	2	0.71a±0.00	0.71a±0.00
	4	0.71a±0.00	0.71a±0.00
	6	0.71a±0.00	0.71a±0.00

Data are presented as mean±standard deviation. Means followed by the same letter in the same column are not significantly different according to DMRT at α = 0.05. Data were transformed using square root transformation $(\sqrt{x+0.5})$ prior to analysis

This results consistent with findings by [15], that pendimethalin induction contributed to decrease in leaf length and width. [38] have also been observed the leaf length and width of Vanda orchid was decreased after the colchicine treatment. This may be associated with chemical mutagens can affect the physiological conditions of the plant, such as inhibited cell division and attributed the growth hormone imbalances, which resulting in stunted leaf growth [39]. Additionally, the reduction of leave size may be due to stress response in plants when exposed by chemical mutagens. According to [40], plants tend to produce smaller leaves in response to environmental conditions that are not ideal for their growth and development.

Number of Stomata

The mean of number of stomata per mm2 tend to decrease after the pendimethalin treatment (Table 6). However, the results did not significantly different compared to the control plants as shown in Fig. 2.

Table 6: Effect of pendimethalin treatment on number stomata at 24 weeks

Treatment		Number of Stomata per mm^2
Pendimethalin Concentration (µM)	Incubation Time (days)	
Control		24.44b±5.07
100	2	22.66b±2.31
	4	21.33b±2.31
	6	24.00b±4.00
300	2	21.33b±2.31
	4	22.67b±9.24
	6	0.71a±0.00
1000	2	0.71a±0.00
	4	0.71a±0.00
	6	0.71a±0.00

Data are presented as mean±standard deviation. Means followed by the same letter in the same column are not significantly different according to DMRT at α = 0.05. Data were transformed using square root transformation $(\sqrt{x + 0.5})$ prior to analysis

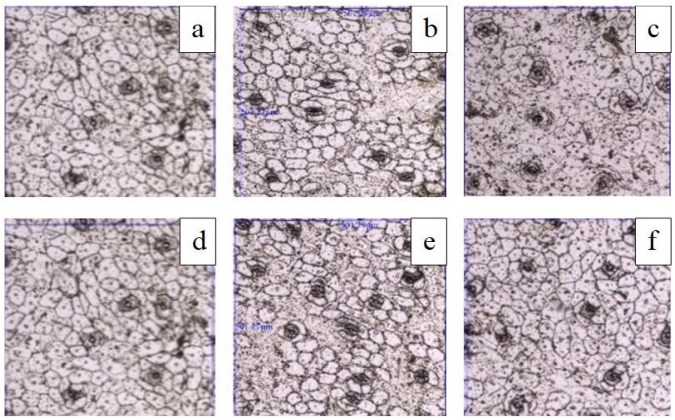

Fig. 2: Effect of pendimethalin treatment on stomatal number (400× magnification)

Control (a); Pendimethalin concentration 100 µM, incubation time 2 days (b); Pendimethalin concentration 100 µM, incubation time 4 days (c); Pendimethalin concentration 100 µM, incubation time 6 days (d); Pendimethalin concentration 300 µM, incubation time 2 days (e); Pendimethalin concentration 300 µM, incubation time 4 days (f).

This results align with study by [41], that polyploid induction did not affected the stomatal number significantly. No significant difference in number of stomata may be associated with genetic factors. [42] reported the stomatal count in plants is detemined by genetic traits passed down from the parent plants. In this study, the Phalaenopsis aphrodite plants originated from a single parental source, thus having uniform genetic characteristics. Genetic uniformity causes the plant's response to mutagen treatment to tend to be similar.

Stomatal Length and Width

The stomatal length and width on treatment of 100 µM pendimethalin for 2 and 4 days and 300 µM for 2 days shows an increase compared to control and other treatments (Fig. 3). However, the increasing in stomatal size did not statistically different (Table 7).

Table 7: Effect of pendimethalin treatment on stomatal length and width at 24 weeks

Treatment		Stomatal Length (µm)	Stomatal Width (µm)
Pendimethalin Concentration (µM)	Incubation Time (days)		
Control		41.27b±3.70	40.28c±5.35
100	2	40.24b±0.73	37.16b±1.53
	4	42.74b±1.61	41.75b±6.24
	6	44.33b±4.77	40.85b±0.35
300	2	43.57b±2.34	41.96b±2.41
	4	40.12b±0.77	37.02b±5.54
	6	0.71a±0.00	0.71a±0.00
1000	2	0.71a±0.00	0.71a±0.00
	4	0.71a±0.00	0.71a±0.00
	6	0.71a±0.00	0.71a±0.00

Data are presented as mean±standard deviation. Means followed by the same letter in the same column are not significantly different according to DMRT at α = 0.05. Data were transformed using square root transformation $(\sqrt{x + 0.5})$ prior to analysis

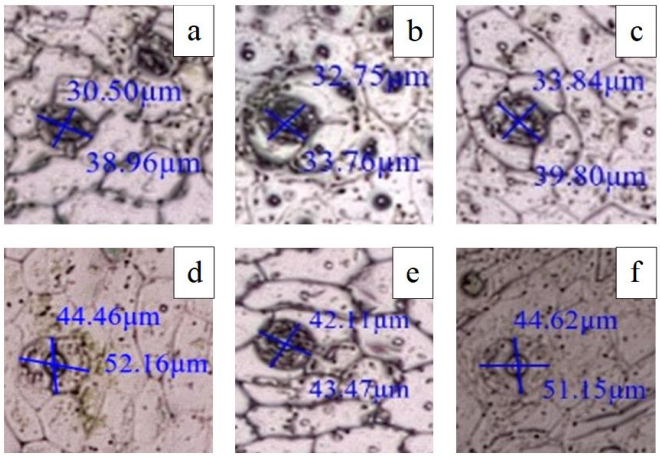

Fig. 3: Effect of pendimethalin treatment on stomatal length and width (400× magnification)

Control (a); Pendimethalin concentration 100 µM, incubation time 2 days (b); Pendimethalin concentration 100 µM, incubation time 4 days (c); Pendimethalin concentration 100 µM, incubation time 6 days (d); Pendimethalin concentration 300 µM, incubation time 2 days (e); Pendimethalin concentration 300 µM, incubation time 4 days (f).

A comparable result was observed by [43], that colchicine induction did not significantly affect the stomatal length and width in Glycine max.

Variation in stomatal size is inherited genetically. Previous study by [44] reported that genetic factors play a role in determining stomatal length and width. Additionally, the stomatal length and width in each plant are the result of adaptation to environmental conditions to support physiological processes, particularly metabolic needs. Stomata that are too large cause excessive transpiration rates, while stomata that are too small limit photosynthesis [45]. Therefore, drastic changes in stomatal size can affect plant physiological processes. Plants will tend to maintain their stomatal size within a specific range to ensure efficient physiological processes [46, 47].

Number of Chloroplasts

The treatment of 100 µM pendimethalin for 6 days shows the highest mean in number of chloroplasts (Table 8). The other treatments were also resulting the increase in the chloroplasts number. Although the number of chloroplasts increase, it did not significantly different compared with control and each treatment as shown in Fig. 4.

Table 8: Effect of pendimethalin treatment on number of chloroplasts at 24 weeks

Treatment		Number of Chloroplasts
Pendimethalin Concentration (µM)	Incubation Time (days)	
Control		49.11b±12.20
100	2	52.67b±2.52
	4	57.33b±8.33
	6	58.00b±2.65
300	2	54.67b±6.43
	4	55.33b±5.51
	6	0.71a±0.00
1000	2	0.71a±0.00
	4	0.71a±0.00
	6	0.71a±0.00

Data are presented as mean±standard deviation. Means followed by the same letter in the same column are not significantly different according to DMRT at α = 0.05. Data were transformed using square root transformation $(\sqrt{x + 0.5})$ prior to analysis

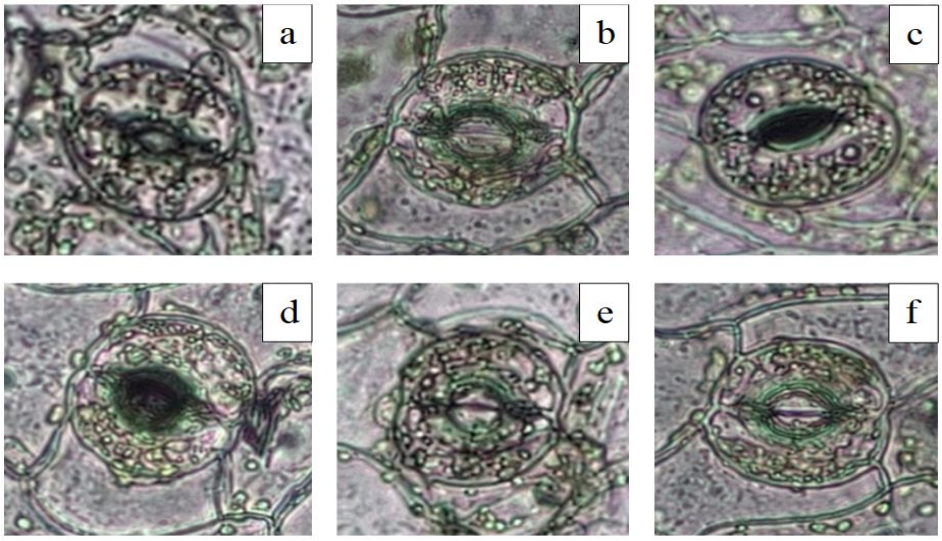

Fig. 4: Effect of pendimethalin treatment on chloroplasts number (400× magnification)

Control (a); Pendimethalin concentration 100 µM, incubation time 2 days (b); Pendimethalin concentration 100 µM, incubation time 4 days (c); Pendimethalin concentration 100 µM, incubation time 6 days (d); Pendimethalin concentration 300 µM, incubation time 2 days (e); Pendimethalin concentration 300 µM, incubation time 4 days (f).

This findings consistent with recent study by [48] that observed the chloroplasts number in Allium tuberosum did not exhibit significant differences. According to [49], chloroplast formation is determined by the genes in each plant. In several previous studies, polyploid induction resulted in plants with significantly increased chloroplast number compared to control plants [50, 51, 44]. However, significant changes in chloroplast count does not necessarily occur immediately, particularly during the initial stages of growth. Furthermore, environmental conditions also influence chloroplast count. In this study, explants were cultured in vitro, where nutrient supply was provided through the media and light intensity for the plants under controlled

conditions, thus their photosynthetic activity differed from that of plants grown in ex vitro environments. In general, plants in in vitro environments have low photosynthetic activity so that their chloroplast organelles have not developed optimally [52].

Number of Chromosome

The chromosome number was determined using the squash method. The results showed a difference in chromosome number between control and plants treated with pendimethalin (Fig. 5). The control Phalaenopsis aphrodite plants have a chromosome number of 2n = 38. This finding is consistent with [53], which reported that the chromosome number of Phalaenopsis orchids is 2n = 38. Thus, it is known that the diploid chromosome number of Phalaenopsis aphrodite is 2n = 38.

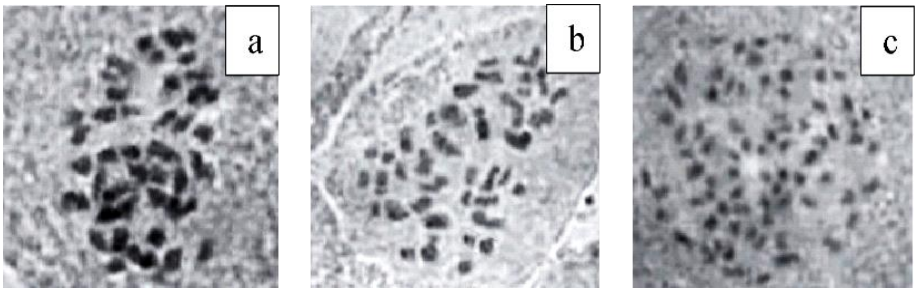

Fig. 5: Effect of pendimethalin treatment on number of chromosomes (1000× magnification)

Diploid (2n = 38) (a); Aneuploid (2n+2 = 40) (b); Tetraploid (4n = 76) (c).

In the treatment of 100 µM pendimethalin for 2 days, no increase in chromosome number was observed compared to the control. An increase in chromosome number was observed in the 100 µM pendimethalin for 4 days and the 300 µM pendimethalin for 2 and 4 days, ranging from 39 to 40. Although this increase occurred, the number did not reach a whole multiple of the diploid chromosome number. Therefore, it was classified as aneuploidy [54]. In the treatment with 100 µM of pendimethalin for 6 days, 2 samples were observed to have an increased chromosome number of 4n = 76. The increase was four times the diploid chromosome and it was classified as tetraploid plants [55]. Thus, the percentage of tetraploid plants in this study was 11.1%.

Pendimethalin is a chemical mutagen that can disrupt the mitotic division mechanism in cells, causing uneven chromosome distribution and leading to chromosome doubling. [22] induced Solanum melongena with 300 µM pendimethalin for 2 days and effectively doubled the number of chromosome by 75%. [13] reported that treatment of 800 µM of pendimethalin for 7 days produced 23.26% tetraploid Plumbago auriculata plants. [15] observed that pendimethalin treatment resulted in 45 tetraploid Allium cepa L. var. aggregatum G. Don plants. In our study, the induction rate was lower than previous studies. This findings may be attributed to different responses of each plant species to the same type of mutagen. Several factors that can affect the effectiveness in polyploid induction, including the plant species, type of explant, type and concentration of antimitotic agent, and exposure time [56, 57].

The obtained tetraploid plants in this study did not exhibit significant differences in phenotype such as higher plants and larger organ size compared to diploid. Previous studies have reported that polyploid plants typically exhibit an increase in cell and organ size. The tetraploid cells have twice the volume of their diploid cells. However, it does not necessarily lead to increase in organ size, as the rate of number cells division in polyploids can be reduced [58]. Recent study by [59] have observed that polyploid induction resulting in dwarfism in Allium sativum due to increasement in cell volume reduces cell division rate and slows its growth.

Polyploid induction have suggested to lead increase in stomatal length and width compared to diploid plants counterparts. However, in our findings, the phenotypic changes had not yet fully expressed. [60] reported that genetic factors play a role determining stomatal length and width. In genetically inherited characters, the changes are not always expressed directly. The ploidy instability may have occurred in our study since we were conducted in early developmental stage. A study by [61] have found that the tetraploid plants were stable after being maintained over multiple subcultures.

The squash technique is one of reliable methods for determine chromosome number and ploidy level directly [62]. This method is widely used and cost-efficient, but has limitations compared to the Flow Cytometry (FCM). However, the

FCM is less economically efficient. In addition, FCM is an indirect method to estimate ploidy level and it has to be supported by chromosome count [63].

Conclusion

In vitro polyploid induction using pendimethalin at various concentrations and incubation times affected the viability and growth of Phalaenopsis aphrodite at the early stages. Higher concentrations of pendimethalin and longer incubation times inhibit physiological processes, as well as the growth and development of plantlets. The ploidy was confirmed by chromosome counting with the squash method. Pendimethalin treatment at concentration 100 µM for 6 days resulted in 11.1% tetraploid plants. This result was relatively low compared to the previous studies. Additionally, no significant differences in stomatal parameters compared to diploid plants. This may be attributed to the instability of the ploidy as the plant is still at early stages of development. Therefore, these findings serve as a preliminary study on in vitro polyploid induction by pendimethalin in Phalaenopsis aphrodite. Further studies could examine different combinations of pendimethalin concentrations and incubation times in order to determine an effective treatment for inducing polyploidy in Phalaenopsis aphrodite, which then could be potentially applied to breeding programs and commercial production.

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Author's Contributions

Prasetyorini Djarot: Contributed to the conceptualization of the study, the development of the theoretical framework, provided general guidance on the methodology and analytical structure, and manuscript edited.
Deden Sukmadjaja: Contributed to data collection, data processing, validation of analytical results and ensured consistency between analytical results and research objectives.
Fadya Annisa Chaesarani: Contributed to data analysis, interpretation of results, preparation of the discussion section, and drafting of the manuscript.
Sukono: Provided academic guidance, scientific supervision, and comprehensive evaluation.

Ethics

This study is original and contains unpublished material. The corresponding author confirms that all other authors have read and approved the manuscript, and there are no ethical issues.

Conflict of Interest

The authors declare no conflicts of interest.

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