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Comparative analysis of colchicine and bio-catharanthine as mutagenic agents for polyploid generation in wild passion fruit (*Passiflora foetida*)

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Abstract: Wild type Passiflora foetida or a bush passion fruit is unappealing plant with small fruit size (2-3 cm) to be cultivated as a fruit crop. Plant breeding by polyploidization may improve plant production including increase the fruit size. Many researchers have used colchicine as plant chromosome-modifying agent for polyploidization. However, this potent mutagen is very expensive in developing countries and toxic. Hence, an alternative mutagen needs to be considered, such as the natural bio-catharanthine. This study aims to evaluate the effectiveness of colchicine and bio-catharanthine mutagens in polyploidization of P. foetida plants. Experiment was arranged in split-plot design with three replications. Main plot was combination of the mutagens and concentrations, the subplot was soaking durations. Twenty-five seeds each were soaked for 1-, 2- and 3-days in distilled water as control, colchicine (0.05%, 0.1%, 0.15%) and bio-catharanthine (0.5%, 1% and 1.5%) solutions. Result showed, bio-catharanthine concentration of 1% at immersion durations of 3-days and 1.5% at 1-, 2- and 3-days could only produce 89, 56, 74, and 71% grade 1 mixoploid plants accordingly. On the other hand, colchicine succeeded in obtaining tetraploid plants at concentration of 0.15% with 3-days soaking time even though at lower result (5%). Lower colchicine concentrations with shorter or the same soaking period could only produce 5% grade 2 mixoploid plants. Colchicine with one-tenth lower concentration than bio-catharanthine concentration is still more efficient in inducing polyploidy. Further research by involving bio-catharanthine concentration and varying the soaking time must be pursued to obtain higher-level ploidy.

Keywords: Bio-catharanthine, colchicine, Passiflora foetida, polyploidy, tetraploid.

Introduction

Wild passion fruit (*Passiflora foetida*) is a wild plant classified as a weed, a fast-growing creeper that is potentially invasive, especially in areas where the climate is favorable for its growth. Wild Passion Fruit can cover native plants and disrupt local ecosystems so control is needed (Solomon et al., 2022). The stem is cylindrical and woody. The leaves are large and hairy, usually three- lobed with a shape that varies from ovate to angular. They are spirally arranged, with one leaf per node. The flowers are the most striking, with a white or light-yellow corolla and a pink spot in the center. The fruits are yellow when ripe and have a characteristic aroma (Patil Paikrao and Patil, 2013). Ecologically, this plant has roles, such as its seeds

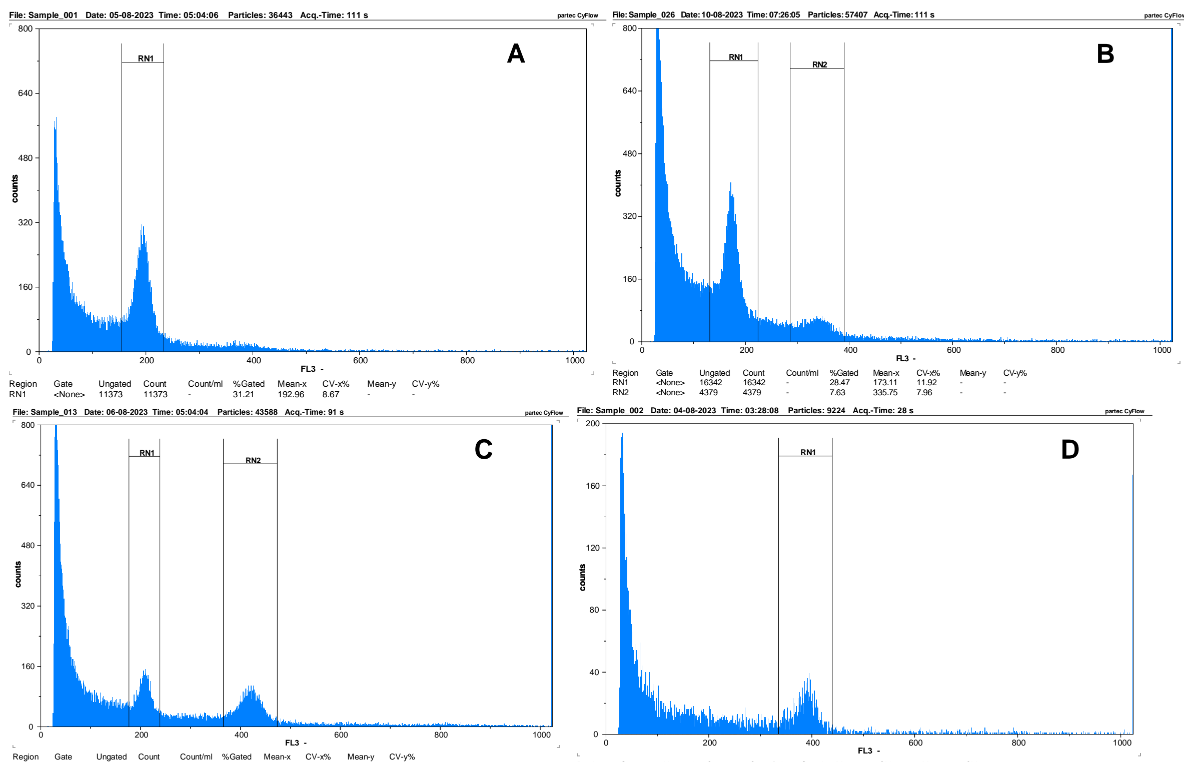

Fig. 1 The ploidy level of *P. foetida* plants based on flow cytometer. A (diploid) B (mixoploid grade 1) C (mixoploid grade 2) and D (tetraploid). Mixoploids have more than one peak on the histogram.

being a food source for a variety of birds and other wildlife. The plant's flowers often play host to various types of insects that help in the pollination of the plant (Solomon et al., 2022).

Passiflora foetida, commonly known in Indonesia as rotten passion flower, is a unique and versatile plant that has caught the attention of botanists, pharmacologists and herbal medicine enthusiasts around the world. In the field of health, this plant is widely used as an object of research because this plant is pharmacological in nature which has medical potentials (Chiavaroli et al., 2020). Many researchers have discovered the benefits of this plant. The fruits of *P. foetida* are rich in phenolic compounds that have antioxidant properties (Song et al., 2018), anti-inflammatory (Park et al., 2018), as an analgesic in traditional medicine (Asadujjaman et al., 2014).

In agriculture this plant has not attracted much attention from researchers and farmers. Unlike some other *Passiflora* species such as *Passiflora edulis* (passion fruit), *P. foetida* does not have significant commercial value as a horticultural crop or its edible fruit which is only small (2-3 cm) and unattractive to develop (Vijay et al., 2021). A step that can be taken to improve fruit quality such as fruit size is by polyploidization. The chemical mutagen often used in polyploidization is colchicine which aims to increase fruit size (Tammu et al., 2021; Sjahril et al., 2023).

Colchicine is a chemical compound that has been widely used in plant research to induce polyploidy, which is the presence of more than two sets of chromosomes in a cell or organism. Polyploidy has become an important method in the study of genetics and plant breeding as it can lead to the development of new genetic variations with the potential for trait improvement (Mo et al., 2020); Touchell et al., 2020).

Polyploidization using chemical mutagens for the development of wild and local plants has become a trend in recent years (Głowacka et al., 2010; Omezzine et al., 2012; Eng and Ho, 2019a; Le et al., 2020; Tammu et al., 2021). Polyploidization is seen as one of the most useful breeding methods to improve specific traits of plant varieties such as quality yield, or better environmental adaptation (Ruiz et al., 2020).

The success of colchicine-induced polyploidy can vary depending on species, cultivar, tissue type and culture conditions. Colchicine has been shown to be a highly effective mitotic agent for many plant species yet polyploidy induction is still highly variable (Touchell et al., 2020). The use of colchicine in polyploid research can pose several challenges, such as the production of mixoploids (organisms with both diploid and polyploid cells) and potential negative impacts on plant performance and fertility (Münzbergová, 2017; Eng et al., 2021). In addition, the consequences of colchicine use are still evident in second-generation plants, so at least third-generation polyploids should be considered in future comparisons (Münzbergová, 2017). Koutoulis et al. (2005) divided mixoploid plants into three classes, namely class 1 for mixoploid plants with diploid higher than tetraploid, class 2 for mixoploid plants where diploid and tetraploid are equal and class 3 for mixoploid plants with tetraploid higher than diploid

Bio-catharanthine is an alternative polyploidy agent from tamarind leaf extract that contains vincristine and vinblastine alkaloids, vindoline, and catharanthine which can inhibit cell division at the anaphase stage of mitosis (Rohmah et al., 2022). Bio-catharanthine can have a significant effect on plant morphology such as plant height, number of leaves, number of

Table 1. The ploidy level of *P. foetida* plants based on flow cytometer analysis.

Treatment	Region	Cell Count	Mean-x	CV-x%	Category	Percentage
Control	RN1	11,373	192.96	8.67	Diploid	100%
B1W1	RN1	9,147	211.10	6.40	Diploid	100%
B1W2	RN1	22,509	188.77	10.94	Diploid	100%
B1W3	RN1	3,770	185.94	8.12	Diploid	100%
B2W1	RN1	11,589	201.13	9.53	Diploid	100%
B2W2	RN1	19,479	212.60	8.93	Diploid	100%
B2W3	RN1	6,593	187.76	10.02	Diploid	11%
B2W3	RN1 RN2	15,394 2,204	186.71 374.01	9.88 6.20	Mixoploid Grade 1	89%
B3W1	RN1	1,778	194.12	7.52	Diploid	44%
B3W1	RN1 RN2	17,271 3,530	182.67 367.76	10.58 6.11	Mixoploid Grade 1	56%
B3W2	RN1	3,464	198.30	6.04	Diploid	26%
B3W2	RN1 RN2	18,265 2,934	196.24 392.39	10.76 6.16	Mixoploid Grade 1	74%
B3W3	RN1	5,177	198.60	5.27	Diploid	29%
B3W3	RN1 RN2	16,354 2,564	207.31 406.38	6.98 5.19	Mixoploid Grade 1	71%
C1W1	RN1	15,454	159.94	11.56	Diploid	100%
C1W2	RN1	13,993	162.04	11.70	Diploid	100%
C1W3	RN1	12,698	183.27	11.51	Diploid	100%
C2W1	RN1	22,441	171.16	11.71	Diploid	100%
C2W2	RN1	23,440	210.62	9.98	Diploid	33%
C2W2	RN1 RN2	23,327 3,105	177.13 357.00	10.87 6.23	Mixoploid Grade 1	67%
C2W3	RN1	13,620	177.58	9.50	Diploid	28%
C2W3	RN1 RN2	17,194 3,133	199.04 403.10	9.25 5.40	Mixoploid Grade 1	72%
C3W1	RN1	1,370	199.78	6.22	Diploid	43%
C3W1	RN1 RN2	20,988 2,602	188.06 376.74	10.10 5.78	Mixoploid Grade 1	57%
C3W2	RN1	16,686	201.65	8.43	Diploid	55%
C3W2	RN1 RN2	9,218 1,254	198.65 391.46	8.02 5.19	Mixoploid Grade 1	40%
C3W2	RN1 RN2	5,210 7,506	207.64 418.63	7.14 5.81	**Mixoploid Grade 2**	5%
C3W3	RN1	3,483	177.31	5.65	Diploid	45%
C3W3	RN1 RN2	7,455 1,183	210.28 414.58	6.05 4.48	Mixoploid Grade 1	50%
C3W3	RN1	1,448	386.71	5.76	**Tetraploid**	5%

Control (0% mutagen); B1 (0.5% bio-catharanthine); B2 (1% bio-catharanthine); B3 (1.5% bio-catharanthine); C1 (0.05% colchicine); C2 (0.1% colchicine); C3 (0.15% colchicine); W1 (1-day); W2 (2-day); W3 (3-day). Mean-X is the peak position on the histogram. If it has more than one Mean-X then the plant is mixoploid.

internodes, and number of roots per plant (Aziz et al., 2021), and able to produce polyploidy plants (Billa et al., 2022).

In this article, we tested the effectiveness of colchicine and bio-catharanthine mutagens in the polyploidization process in *P. foetida* plants. We will discuss how morphological and cytological changes are evidenced by the results of flow cytometry analysis based on the peak position of each leaf sample (Dirihan et al., 2013; Yan et al., 2016; Garavello et al., 2019).

Results

Effect of colchicine and bio-catharanthine on ploidy level of P. foetida

In this study, we obtained various ploidy levels based on the results of flow cytometry analysis. The mutagen colchicine still gave better results than bio-catharanthine as an alternative mutagen. A concentration of 0.15% colchicine was able to produce tetraploid plants as 5% and a concentration of 0.1% colchicine at a 3-days immersion period was able to produce grade 2 mixoploid plants as 5% (tetraploid and diploid cells were the same). Meanwhile, the 1% concentration of bio-catharanthine mutagen at a 3-days immersion period and a concentration of 1.5% at 1-, 2- and 3-days immersion periods were only able to produce grade 1 mixoploid plants (diploid cells were higher than tetraploid cells) (Table 1).

The position of the peak histogram of the visualization results of flow cytometry analysis on plant leaf cells can provide an overview of the differences between diploid plants and polyploid plants (mixoploid and tetraploid). The peak of diploid cells is only in the ±200 channel (Fig. 1A), the peak of mixoploid cells is in two channels ±200 and ±400 (Fig. 1B, C)

Table 2. The effects of colchicine and *bio-catharanthine* on plant height stem diameter number of shoots leaf length leaf width and leaf shape index of *P. foetida*

Mutagen concentration	Plant height (cm)	Stem diameter (mm)	number of shoots	Leaf length (cm)	Leaf width (cm)	leaf shape index
Control	185.51[a]	3.01[a]	2.94[a]	7.09[a]	4.23[a]	1.68[a]
B1	178.59[a]	3.07[a]	3.43[a]	6.98[a]	4.43[a]	1.58[a]
B2	192.51[a]	3.20[a]	3.26[a]	7.10[a]	4.25[a]	1.67[a]
B3	168.00[ab]	3.14[a]	2.93[a]	6.91[a]	4.31[a]	1.62[a]
C1	112.87[bc]	3.13[a]	3.95[a]	5.45[b]	4.44[a]	1.23[b]
C2	109.66[c]	3.00[a]	3.04[a]	5.19[b]	4.57[a]	1.14[b]
C3	103.77[c]	2.86[a]	3.17[a]	5.05[b]	4.29[a]	1.18[b]

Control (0% mutagen); B1 (0.5% bio-catharanthine); B2 (1% bio-catharanthine); B3 (1.5% bio-catharanthine); C1 (0.05% colchicine); C2 (0.1% colchicine); C3 (0.15% colchicine). The letters following the numbers within a column indicate a significant difference at $P < 0.05$ according to the LSD test (Least Significance Different). Plants were 5 weeks old.

Table 3. Results of correlation analysis between observation parameters

Parameters	Plant height	Stem diameter	number of shoots	Leaf length	Leaf width	Leaf shape index
Plant height	1.00					
Stem diameter	0.45[*]	1. 00				
number of shoots	-0.20[ns]	0.25[ns]	1. 00			
Leaf length	0.95[**]	0.48[*]	-0.17[ns]	1. 00		
Leaf width	-0.28[ns]	-0.08[ns]	0.38[ns]	-0.31[ns]	1. 00	
Leaf shape index	0.92[**]	0.45[*]	-0.24[ns]	0.97[*]	-0.52[*]	1. 00

Significant correlations are indicated by $**p < 0.01$ and $*p < 0.05$. * Significant ** highly significant [ns] non-significant.

and the peak of tetraploid cells is only in the ±400 channel on the horizontal axis (Fig. 1D).

Effect of colchicine and bio-catharanthine on morphological characters of P. foetida

The morphology of *P. foetida* plants induced by bio-catharanthine, as a whole, was not significantly different from the control plants. Plants induced with the mutagen colchicine were also not significantly different from the control plants in the parameters of stem diameter, number of shoots, and leaf width but showed differences in the parameters of plant height, leaf length, and leaf shape index. The flower and fruit morphology of mutant treatments was shown in Fig 2 and 3.

Correlation analysis between observation parameters

The results of correlation analysis on the observation parameters showed that plant height is significantly positively correlated to stem diameter (0.45), and highly significantly correlated to leaf length (0.95) and leaf shape index (0.92). Stem diameter was also significantly positively correlated to leaf length (0.48) and leaf shape index (0.45). Leaf shape index was significantly positively correlated to leaf length (0.97) and significantly negatively correlated to leaf width (-0.52). Positive correlation occurs when two variables move in the same direction or have a unidirectional relationship and vice versa with negative correlation, Negative correlation occurs when two variables move in the opposite direction or have an opposite relationship.

Discussion

Polyploid induction is one of the unconventional plant breeding strategies to see the impact of chromosome duplication in several plant species (Pelé et al., 2018; Corneillie et al., 2019; Doyle and Coate, 2019; Zhang et al.,2019; Heslop-Harrison et al.,2023). Flow Cytometry plays an important role in polyploidization related studies that can provide a distinction between standard induced polyploids or mixoploids of the same species with known ploidy levels. The use of FCM when compared to chromosome analysis has many advantages as it uses only a small amount of plant tissue such as leaves or other parts that cannot damage the plant itself. In addition, FCM does not rely on specific mitotic stages for analysis and determination of ploidy level, total nuclear DNA content (C-value) can also be estimated (Ho et al., 2024). Analysis of the relationship between morphological and cytological traits with flow cytometry analysis to detect the level of pluripotency in plants has been carried out in many studies (Eng and Ho, 2019b; Tammu et al., 2021; Tomaszewska et al., 2021; Sjahril et al., 2023). In various plant species, there is a direct relationship between plant ploidy level and different morphological characteristics such as plant height, stem diameter, and leaf shape. However, identification through morphological characters has some disadvantages such as environmental sensitivity and identification of mixoploid plants cannot be done. Flow cytometry is considered to be a more reliable, rapid, and simple method to analyze a large number of samples in a very short period of time (Sattler et al., 2016).

Analysis by flow cytometry can detect the level of pure diploid and tetraploid pluripotency by observing the peak position. This analysis can also detect mixoploids and the exact mixoploid grade can be determined because it analyzes a large population of cells. Other cytological analysis such as squash analysis is not effective in detecting mixoploids because only a few cells are observed so this analysis is also subjective (Eng et. Al., 2021). In some cases, polyploid plants can undergo a process called "reduction to diploid", where the extra set of

chromosomes is lost, resulting in a diploid plant (Corneillie et. al., 2019). Squash analysis is performed at the germination stage of the plant using the roots as the analytical material, which allows for changes after the plant has matured. Mature polyploid plants usually do not revert to diploid. Analysis using flow cytometry that uses leaves as analytical material is an advantage, not limited by plant age so that it can be done several times to ensure the consistency of ploidy levels. The use of colchicine as a chromosome-doubling mutagen in plants has proven successful in obtaining tetraploid plants (Samatadze et al., 2022; Taratima et al., 2023), however, the optimal procedure is specific to each species so that there is no standard concentration and duration of immersion in plants in general. Bio- catharanthine as a comparison mutagen in this study has also been shown to induce tetraploid plants in shallots (Billa et al., 2022), however, it has not been found in other plant species. Therefore, the optimal concentration of colchicine and bio-catharanthine solution, as well as the duration of soaking are very important for the success of polyploid induction.

Our results showed that colchicine was able to induce tetraploid *P. foetida* plants in seed soaking with a concentration of 0.15% colchicine for 3-days although the number of plants obtained was still very low (5% of the number of plant units per treatment). The concentration of 0.1% colchicine at soaking for 3-days was only able to induce grade 2 mixoploid plants (5% of the number of plant units per treatment), while the use of 1% bio-catharanthine concentration at a soaking period of 3-days and 1.5% concentration at soaking periods of 1-, 2-, and 3-days was only able to produce grade 1 mixoploid plants (Table 1, Fig. 1). In this study, the concentration of bio-catharanthine was ten times more concentrated than the concentration of colchicine, however, it was still unable to match the results obtained from polyploidy induction using colchicine.

Mixoploid plants still dominate in this study which consist of two kinds of chromosome pairs (2n 4n). In polyploidization mixoploid plants are undesirable because the unstable polyploid state often reverts entirely or partially to the diploid state after successive cell division cycles (Kolář et al., 2017; Esfahani et al., 2020). Diploid cells can replicate and divide faster than higher ploidy cells. Over time, the proportion of low ploidy cells increases, resulting in a loss of converted cells (Touchell et al., 2020). Morphologically, mixoploid plants often have no difference from diploid plants and is one of the disadvantages of the polyploidization method (Sjahril et al., 2023).

Ploidy levels and plant morphology are usually highly correlated. Polyploid plants may show increased or decreased plant height compared to their diploid relatives, depending on the species and environmental conditions (Chansler et al., 2016). In our study, it was shown that plant height with colchicine treatment resulted in shorter plants compared to control plants and bio-catharanthine treatment. There is no change in stem diameter in all treatments, this is thought to be due to the effectiveness of the mutagen given is still considered lacking, although managed to get tetraploid plants but still in very small numbers and mixoploid still dominate in each treatment. Polyploidization can cause changes in stem diameter, some polyploid plants show an increase in thickness compared to diploid plants (Pei et al., 2019), while mixoploid plants do not differ from diploid plants (Omezzine et al., 2012). Leaf length and leaf shape index of colchicine-treated leaves (0.05% 0.1% and 0.15%) were shorter than the control and bio-catharanthine mutagen treatments while there was no difference in leaf width parameters.

Materials and Methods

Plant material and seed preparation

Ripe fruits (yellow in color) were collected from *P. foetida* plants growing wild around Hasanuddin University campus. Seeds were separated and cleaned from the skin and slimy pulp then soaked in chlorine solution (2.62%) for ±3 minutes on clean seeds. Seed drying was carried out in an oven at 40 °C for 24 hours. The dried seeds were soaked in KNO_3 solution (0.4%) for 2-days to break the seed dormancy period. A total of 1800 seeds inducted by KNO_3 were treated with different colchicine concentration gradients and soaking durations.

Colchicine treatment and preliminary screening for putative tetraploids

This study was arranged by split plot and completely randomized design as environmental design with three replications. The main plot was the combination of mutagen kinds and concentrations (control, bio-catharanthine with 0.5% concentrations, bio-catharanthine with 1% concentrations, bio-catharanthine with 1.5% concentrations, colchicine with 0.5% concentrations, colchicine with 1% concentrations and colchicine with 1.5% concentrations) and the subplot was soaking durations (1-, 2-, and 3-days). First, we collected the seed and stored it. After all the components were ready, we started the treatments. Every 25 seeds were soaked for 1-, 2-, and 3-days at room temperature in distilled water as 0% mutagen control, colchicine concentrations of 0.05%, 0.1%, 0.15%, and bio-catharanthine concentrations of 0.5%, 1% and 1.5% dissolved in distilled water. After treatment, the seeds were washed with distilled water three times. The seeds were then transferred directly to seedling trays (hole size 6 cm x 6 cm) containing a mixture of soil and compost media (1:1 ratio). All treatments were repeated three times. In the first week, the germination rate was calculated and 100% germination was obtained in all treatments. Since a large population of seedlings (25 per treatment) was generated after seed treatment, we conducted an initial morphological screening on one-month-old seedlings to identify putative polyploid populations. Plants with unusual leaf morphology, i.e., dark green leaves, thicker leaves, coarser leaves, or leaves with abnormal margins were selected as putative polyploid plants, which were then tested by flow cytometry. For control plants (0%), seedlings with normal growth were selected.

Flow cytometric analysis

Approximately 0.5 x 0.5 cm area of fresh leaf tissue from each selected plant was minced with a razor blade in a dish containing 250 µl of Cystain Pi buffer extract for ±60 seconds. The extract was filtered on a sample filter tube to obtain approximately 0.2 µl of filtrate. A total of 800 µl of propidium iodide dye was added to the sample tube. The sample tube is placed under the needle sucker of the Partec Cy-Flow SpaceTM machine and pushed up manually so that the needle can draw up the filtrate into the system for analysis. The ploidy level

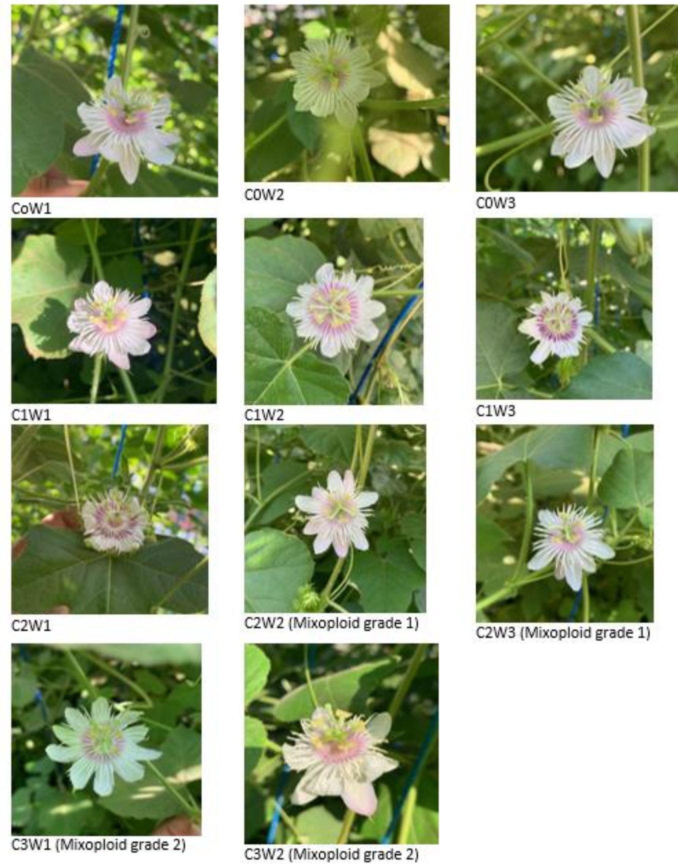

Fig. 2 The flower morphology of wild passion fruit mutant.

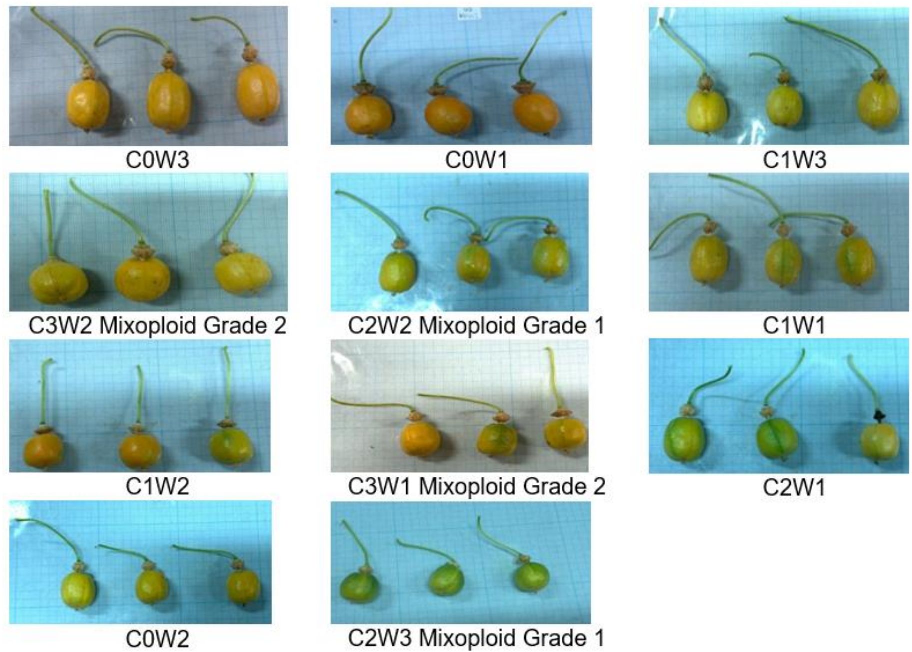

Fig. 3 The fruit morphology of wild passion fruit mutant.

reading can be obtained directly and displayed on the computer screen in the form of a histogram graph.

The peak histogram displays the number of cells counted and a comparison of the number of cells per peak can be seen if there is more than one peak (mixoploid). If the number of cells between peaks indicating diploid is greater than the number of cells in peaks indicating tetraploid is higher, it can be categorized as mixoploid grade 1 and vice versa for mixoploid

grade 3, while grade 2 has almost the same number of cells. The peak position can be seen from the number that appears on Mean-X and this is a marker to determine whether chromosome doubling occurs or not (same as the control).

Leaves from plants without mutagen treatment (0% concentration) were used as a control and the peak position was set so that it was in channel 200. The peak of the treatment leaf sample is compared with the peak of the control sample, if the peak of the treatment sample is in the channel 2 times that of the control, then it is counted as tetraploid (4n) and if there are two peaks that appear in the same channel as the control peak and twice the control peak then it is counted as mixoploid (2n, 4n). Mixoploid will be divided into three grades, namely grade 1 (diploid peak is higher than tetraploid peak), grade 2 (diploid and tetraploid peaks are equally high), and grade 3 (tetraploid peak is higher than diploid peak) (Koutoulis et al., 2005).

Flow cytometry analysis was performed on all plants per treatment per replicate. Six plant units per treatment were repeated three times, resulting in 18 plants for the same treatment. The percentage of ploidy level was obtained using the formula:

$$\frac{Number\ of\ plants\ per\ ploidy\ level\ per\ treatment}{Number\ of\ plants\ per\ treatment} \times 100\%$$

Morphological observations

Plant morphological traits including plant height, stem diameter, leaf width (w), leaf length (l), leaf shape index (l/w), and number of shoots were compared between control and treatment plants. Six plants per treatment per replicate were measured for height and stem diameter. Five fully formed leaves per plant per treatment per replicate were measured for leaf width and leaf length. Morphological data were analyzed using Split plot design and further tests using the Least Significant Difference Test (LSD) with 5% error level. In addition, the data obtained was also correlated with Pearson correlation at the 5% level.

Conclusion

The concentration of bio-catharanthine (0.5% - 1.5%) which is ten times more concentrated has not been able to match the results obtained from the use of colchicine (0.05% - 0.15%) as a chromosome multiplier agent in *P. foetida* plants. Tetraploid plants in very small numbers have been obtained in this study, namely at a concentration of 0.15% colchicine with seed soaking for 3-days, a concentration of 0.1% colchicine was able to induce grade 2 mixoploid plants in seed soaking for 3-days while in bio-catharanthine mutagens, grade 1 mixoploid plants were obtained. Morphological changes occurred in colchicine-induced plants, namely in the parameters of plant height, leaf length, and leaf shape index. Compared to the control, colchicine-induced plants at all concentrations produced shorter plants, shorter leaves and smaller leaf shapes compared to the control plants, while in the bio-catharanthine treatment there was no significant difference with the control plants. In this study, there were no dead plants due to the side effects of the two mutagens given, so it is recommended to increase the concentration and duration of immersion in each mutagen to get the limit of plant tolerance.

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