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RESEARCH ARTICLE
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Determination of Polyploidy Induction Capacity of Toraja *Talas Bite* Taro (*Colocasia esculenta*) with Colchicine Mutagen

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ABSTRACT

Induction of *in vitro* polyploidy was carried out for plant breeding and development of local taro known as *talas bite* taro, (*Colocasia esculenta*) which is endemic to Toraja, South-Sulawesi, Indonesia, and is a neglected and underutilized crop but can be an alternative food source. This study aims to determine an efficient polyploidy induction method using *in vitro* trials on combinations of colchicine concentration and soaking time. The experiment was set using a completely randomized design (CRD), with colchicine and soaking time as treatments. Polyploidy of *talas bite* taro was induced by soaking the young shoots in 0.00, 0.05, 0.10, and 0.20% colchicine solutions with soaking time of 1-, 2-, and 3-days. Results showed that colchicine induction did not show significant differences in shoot emergence, and leaf emergence. However, there was a tendency to decrease in shoot and leaf emergence observed in plantlets treated with higher colchicine, indicating that some of the effects are prevalent. At a concentration of 0.20% and soaking time for 1- and 2 days the mortality rate was at $LC_{50,}$ producing mixoploid plantlets with morphological character and cytological analysis showing proof that the cells were mixoploid Type 2 (2n=2x, 4n=4x), signifying changes in the polyploidy level of the cells.

Keywords: Colchicine, *In vitro*, Polyploidy, *Talas bite*, Toraja local taro.

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INTRODUCTION

Taro is one of the minor root crops that is categorized as one of functional food and can be considered as an alternative staple food to non-rice food which also has a high carbohydrate and protein content (Azzahra et al., 2020) at least every 100g of taro corms contain 26.46g of carbohydrates and 1.50g of protein (Jyothi et al., 2019) and

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contains bioactive ingredients that are efficacious for health (Dagne & Mulualem, 2014). Apart from being used as food, the tubers, fronds, and leaves can be used as herbal medicine, as well as food wrappers. Leaves, tuber plant residue, and tuber skin can be used as animal feed in several regions or countries depending on their ethnicity or culture such as the ethnic of Toraja in South Sulawesi, Indonesia (Pasanda et al., 2022).

Taro is ecologically unique, this plant does not demand special growing conditions and can grow in extreme conditions (Andarini & Risliawati, 2018; Mukhopadhyay & Bhattacharjee 2016). In Indonesia, various types of taro grow in several regions but have not been well utilized, especially in Toraja (Maretta et al., 2021). The local taro commodity as a source of plant genetics from Toraja that has the potential to be improved is the *talas bite* taro. This *talas bite* taro (*Colocasia esculenta* var. Antiquorum) is localized to Toraja and is potentially an alternative food source for the community. Besides that, taro in general is always considered as potential food crop with high nutritional and pharmaceutical value (Aditika et al., 2021).

The corms of *talas bite* taro are bunch-shaped with white skin, flesh, and corms fibres. The size of the tuber is small with a smooth flesh texture, tender or soft and fluffy, with a savoury taste. In comparison, the *talas bite* taro is mostly looking alike the *satoimo* taro from Japan in the tuber and colour of petiole junction with leaf morphological and corm organoleptic character. The origin of the taro plant is still uncertain, but it is possible that a hybrid of *Colocasia esculenta* and wild taro was formed (Ahmed et al., 2020). *Talas bite* taro of Toraja could also be a product of natural hybrid with wild taro after introductions the Eddo type by colonial Japan or Chinese traders. However, the origin of *satoimo* is debatable and open further research (Maretta et al., 2020; Maretta, 2022). The relatively low production cost and wide adaptability make taro is considered a potential food in the future (Septianti & Sahardi, 2018), especially for the future transformation of food system (Cooper, 2023; Zhang, 2024).

Nevertheless, Taro in general has low productivity, especially for the *talas bite* taro. The low productivity is due to the small corm size and long harvesting period, thus reducing farmers' interest in cultivation making *talas bite* taro currently difficult to find. Therefore, much efforts are needed in cultivation and other agronomical aspects to increase the productivity and quality of *talas bite* taro. One of the attempts that can be made for better quality genetic traits is through plant breeding. Plant breeding causes the randomization of plant characteristics so that it can support plant improvement through selection methods giving rise to new populations with new genetic traits (Yulia et al., 2022; Anand et al., 2023). Plant breeding can be achieved by conventional cross-pollination, mutation induction or genetic transformation, protoplast fusion, or induction of polyploid plants with anti-mitotic compounds (Yemets & Blume, 2008; Martin et al., 2014). Improving the quality and quantity of these plants can be also attempted using the colchicine mutagen in mutation breeding techniques. This technique is carried out to doubling the plant chromosomes number from diploid to polyploid, thus producing superior plants with a larger morphological size than normal plants (Sinaga et al., 2014; Zhang & Cao, 2020).

Polyploid plants are plants that have three or more sets of chromosomes in their cells. These plants have more muscular characteristics, as well as larger roots, stems, leaves, flowers, and fruits (Sabana et al., 2022). The commonly used mutagen compound is the colchicine ($C_{22}H_{25}NO_6$) which is still relatively expensive. Colchicine is an alkaloid compound derived from the bulbs of *Colchicum autumnale* L. (Dhooghe et al., 2011; Gracheva et al., 2020).

Research on taro polyploid induction has been conducted on Redbud taro with 0.1% colchicine mutagen for 72 hours producing a polyploid percentage of 20.5% (Sen-Rong & Minghua, 2013), *kaliarung* taro with 0.2% colchicine mutagen for 72 hours producing a tetraploid induction efficiency value of 33.3% (Ermayanti et al., 2018) and peanut plants with 0.1% Bio-catharantin mutagen can double chromosomes into tetraploids (Muarifin & Daryono, 2015).

In addition to polyploid induction, in the cultivation of *talas bite* taro, it is also necessary to pay attention to seedling propagation techniques, one of which is the *in vitro* culture. *In vitro* culture technique can be used to obtain better and faster propagules in larger quantities and relatively shorter times and does not depend on the season compared to the conventional propagation method. *In vitro* culture is a method of propagating seeds from plant parts *in vitro* in the form of cells, tissues, or organs under aseptic conditions, such as in *talas bite* taro that has been done by Pasanda et al. (2022), and tissue culture propagation for bananas where uses buds as explants in the formation of banana seedlings (Sabana et al., 2022). While the induction of *in vitro* polyploidy in *talas bite* taro has still little information available.

Polyploidy induction is expected to be a breakthrough in obtaining superior cultivars in *talas bite* taro. Using the *ex-vitro* method in the induction of polyploidy in this variety will require a lot of colchicine mutagen. Hence in this study, we propose to use *in vitro* as our method to obtain an efficient polyploidy induction protocol with a combination of colchicine concentration and its soaking time.

MATERIALS & METHODS

Place and Time of Research

The research was conducted at the Laboratory of Plant Bioscience and Reproduction Biotechnology, Department of Agronomy, Faculty of Agriculture, Hasanuddin University, Makassar. It was conducted from July to November 2023, when shoots from the *talas bite* taro plant were abundant. The shoots will be used as explants during *in vitro* experiments.

Materials

The materials used for explants in this study were *talas bite* taro shoots obtained from North Toraja Regency. Other materials used were colchicine as the chemical mutagen,

Murashige and Skoog (MS) media for the culture, detergent, alcohol 70% and 96%, aluminium foil, scalpel, bactericide (Agrept), fungicide (Dithane-45), and distilled water.

The tools used were plant culture bottles, Erlenmeyer, measuring cylinder, pH meters, autoclaves (Raypa®), ovens (Memmert), a laminar airflow cabinet (LAFC) (Telstar AH-100), Petri dishes, an automatic gas Bunsen burner, tweezers, and scissors. The ploidy level was measured using Flow Cytometry equipment (Partec Cy-Flow Space™).

Methods

Preparing Planting Material

Tubers obtained from the field were sown at evenly spacing rows on sterile husk charcoal planting media, covered thinly with sterile compost and was kept moist during germination. After the shoot tips emerged from the corms, surface sterilization was carried out by washing the corms with running water, followed by sterilization in a LAFC. Shoot tip explants were then soaked in a mixture of 2g L^{-1} bactericide (Agrept) and 2g L^{-1} fungicide (Dithane-45) for 30 minutes. Explants were then washed three times with distilled water and further sterilized by immersion in 50% Bayclin (v/v) and rinsing three times again with sterile distilled water. Petiole and tuber parts damaged by the sterilization treatment were peeled off and discarded. The clean shoots were then planted *in vitro* on half-strength MS media without sucrose in culture vessels.

Colchicine Induction

In vitro shoots that had been obtained free of bacterial and fungal contamination 2 weeks after initiation were cleaned, cut to a size of ± 1 cm at the base with 2 cm tall shoots. Sterile shoots were then treated with colchicine solution of 0.05, 0.1, and 0.2% and distilled water as a control with a soaking time of 1-, 2-, and 3-days, respectively. After the mutation treatment of colchicine, the explants were rinsed thoroughly three times with distilled water and air dried on sterile tissue paper in an opened Petri dish for five minutes insight a running LAFC. Then the explants were planted in the media, tightly covered and covered with plastic wrap.

There were 12 treatment combinations of colchicine combination and soaking time. Each treatment combination was repeated three times, so there were 36 experimental units. Each experimental unit consisted of 3 plants in each in culture bottles.

Observation of Ploidy Level with Flow Cytometry

Ploidy characterization were carried out on young leaf. The resulting increase of plant ploidy levels were identified using the Partec® Cyflow™ Space ploidy analyser equipment. Place about 0.5 cm^2 of fresh leaf sample into a plastic Petri dish and add 250µL of nucleus extraction buffer. Shred the sample using a sharp razor blade for 60s. Add the remaining nucleus extraction buffer and incubate for 30s to 5min at room temperature. Filter the sample using CellTrics™ 30µm into a sample tube. Then allowed to stand for 1 minute. Add 800µL of staining buffer. Analysis begins after a short incubation of 30-60s. The sample tube is placed on the Partec® Cy-flow™ Space suction syringe for further automated analysis. Cell nuclei contained in the sample will be analyzed and identified in the flow cytometer through the laser beams captured by the detector, and the detection results will be displayed in the form of digital data. The results analysis is displayed as a graph that can be observed on the computer screen (Sjahril et al., 2021; Novitasari, 2023).

Parameter Observation

Observations were made every day until 8 weeks after planting (WAP). Parameters measured and observed were percentage explant mortality, shoot emergence rate, number of roots, leaf emergence rate and number of leaves.

The percentage of explant mortality and the percentage of survival rate were calculated by the formula:

$$\text{Percentage mortality rate} = \frac{x}{n} \times 100\%$$

Remarks: x=explant died

n=number of explants planted

The emergence rate of shoots, roots and leaves was observed daily in each culture bottle by counting how many shoots, roots and leaves began to appear or grow. The number of leaves and roots was observed daily by counting the number of fully formed roots and leaves.

Data Analysis

The qualitative observation data were analyzed descriptively, which was preceded by calculating the percentage value of each observation variable. The quantitative observation data obtained were analyzed using factorial ANOVA with the completely randomized design (CRD) as environmental design. If significantly different, further analysis was carried out with the least significant difference test (LSD) (α=0.05).

RESULTS

Mortality Rate (%)

Soaking method was determined as the polyploidization procedure in this study. Observation was made to determine the mortality rate of *talas bite* taro at more than LC_{50} of the population due to colchicine treatment and soaking duration. The results provide information on the effective dose that can cause 50% mortality in a population treated with colchicine. The results of mortality rate observations can be seen in Fig. 1.

Explant Growth

Based on the results of variance analysis of the speed of forming buds and the speed of forming leaves showed that the interaction, colchicine treatment and soaking time had no significant effect. The fastest shoot formation speed was found in the treatment of 0.00% colchicine concentration with 1-day soaking time (2.19-days) while the longest shoot formation speed was found in the treatment of 0.20% colchicine concentration with 2-days soaking time (2.77-days). Furthermore, the fastest leaf formation speed was found in the treatment of 0.00% colchicine concentration with a soaking time of 3-days, while the longest leaf formation speed was found in the treatment of 0.10% colchicine concentration with a soaking time of 2-days (3.77-days). The average speed of forming buds and speed of forming leaves can be seen in Table 1.

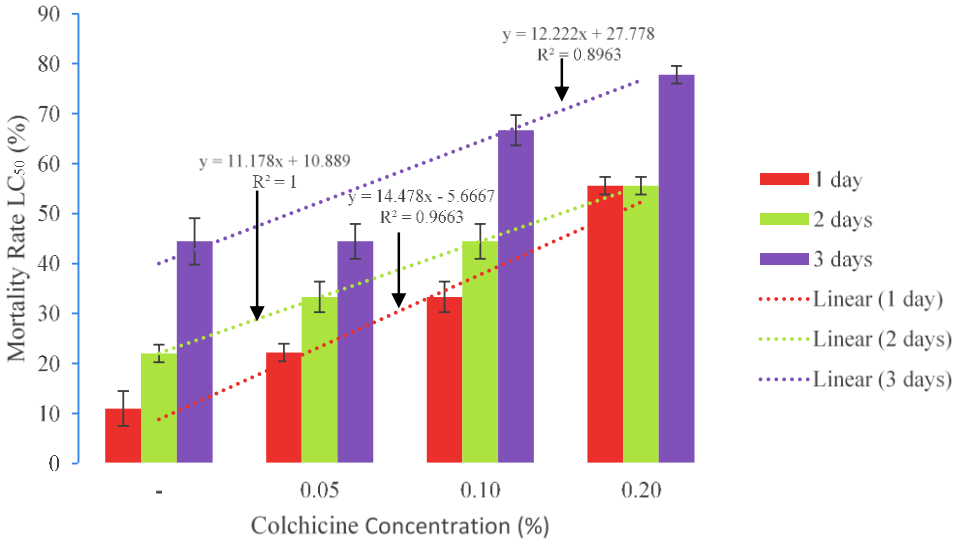

Fig. 1: Mortality rate of *talas bite* taro due to colchicine concentration treatment and soaking duration

Table 1: Average growth of shoot emergence and root emergence of *talas bite* taro on treatment of colchicine and soaking time 4 weeks after treatment.

Treatment		Observation	
Colchicine (%)	Soaking time (Day)	Shoot emergence (Day)	Leaf emergence (Day)
c0: 0.00	h1	2.19	2.96
	h2	2.41	3.18
	h3	2.28	2.86
c1: 0.05	h1	2.47	3.22
	h2	2.43	3.22
	h3	2.51	3.21
c2: 0.10	h1	2.45	3.38
	h2	2.66	3.77
	h3	2.40	3.43
c3: 0.20	h1	2.61	3.64
	h2	2.77	3.32
	h3	2.42	3.41
Significance		Ns	ns

Remarks: Colchicine concentration (c0=0.00%, c1=0.05%, c2=0.10%, c3=0.20%) soaking time (h1=1-day, h2=2-days, h3=3-days). ns=not significantly.

Based on visual observations made on the *talas bite* taro planlets, it can be seen that there is swelling and height differences that occur in the *talas bite* taro stems treated with colchicine. The longer plant soaking time in colchicine, the slower it grows in height (short plants). Visual observations of *talas bite* taro plants after colchicine treatments and the length of soaking time can be seen in Fig. 2.

Ploidy Level Analysis by Flow Cytometry

Based on the results in Fig. 3 and Table 2 which shows flow cytometry of 4 samples: 1 control sample and 3 treatment samples, it shows that colchicine treatment 0.05% and 0.10% with soaking time 1- and 2-days can cause ploidy effects on *talas bite* taro explants. Plants without colchicine treatment (control) were plants that were detected to be diploid (2n) with a histogram peak at channel 200. On the other hand, plants treated with 0.05%–0.20% colchicine and soaked for 1- to 3-days showed mixoploid characteristics (2n–4n). This can be seen from the histogram peaks in channels 200 and 400. These mixoploids are included in the Type 1 mixoploid group, namely plants whose diploid cells are higher than tetraploid cells. In other words, if the number of diploid and tetraploid cells almost the same or equal the ploidy level is mixoploid Type 2 (Koutoulis et al., 2005; Novitasari, 2023).

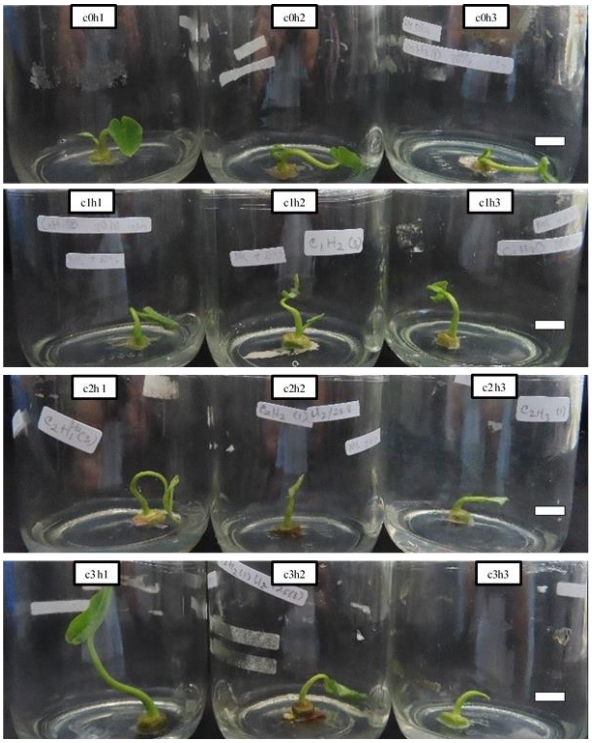

Fig. 2: The growth of *talas bite* taro with the treatment of colchicine concentration and soaking time. C=Concentration of colchicine (c0=0.00%, c1=0.05%, c2=0.10%, c3=0.20%). H=soaking time (h1=1-day, h2=2-days, h3=3-days). c0=control sample (diploid, 2n=2x); c1h1 sample (mixoploid, 2n=2x, 4n=4x); c2h2 sample (mixoploid Type 1, 2n=2x, 4n=4x) and c3h1 sample (mixoploid Type 2, 2n=2x, 4n=4x). White bar scale=1 cm.

DISCUSSION

Polyploidy induction research was conducted on taro plants using colchicine mutagens with concentrations of 0.00%, 0.05%, 0.10%, and 0.20% with soaking times of 1-, 2- and 3-days by *in vitro* method to see the interaction

Table 2: Result of ploidy analysis with flow cytometry in *talas bite* taro

Treatment	Chromosome detected	Mean x	CV-x%	Category
c0h1	2n	196.52	7.97	Diploid
c1h1	2n–4n	163.60 + 202.18	32.97 + 14.98	Mixoploid
c2h2	2n–4n	206.06 + 418.14	26.87 + 3.25	Mixoploid
c3h1	2n–4n	200.84 + 398.27	16.52 + 8.29	Mixoploid

Remarks: c0h1=0.00% colchicine concentration with soaking time 1-day, c1h1=0.05% colchicine concentration with soaking time 1-day, c2h2=0.10% colchicine concentration with soaking time 2-days and c3h1=0.20% colchicine concentration with soaking time 1-day.

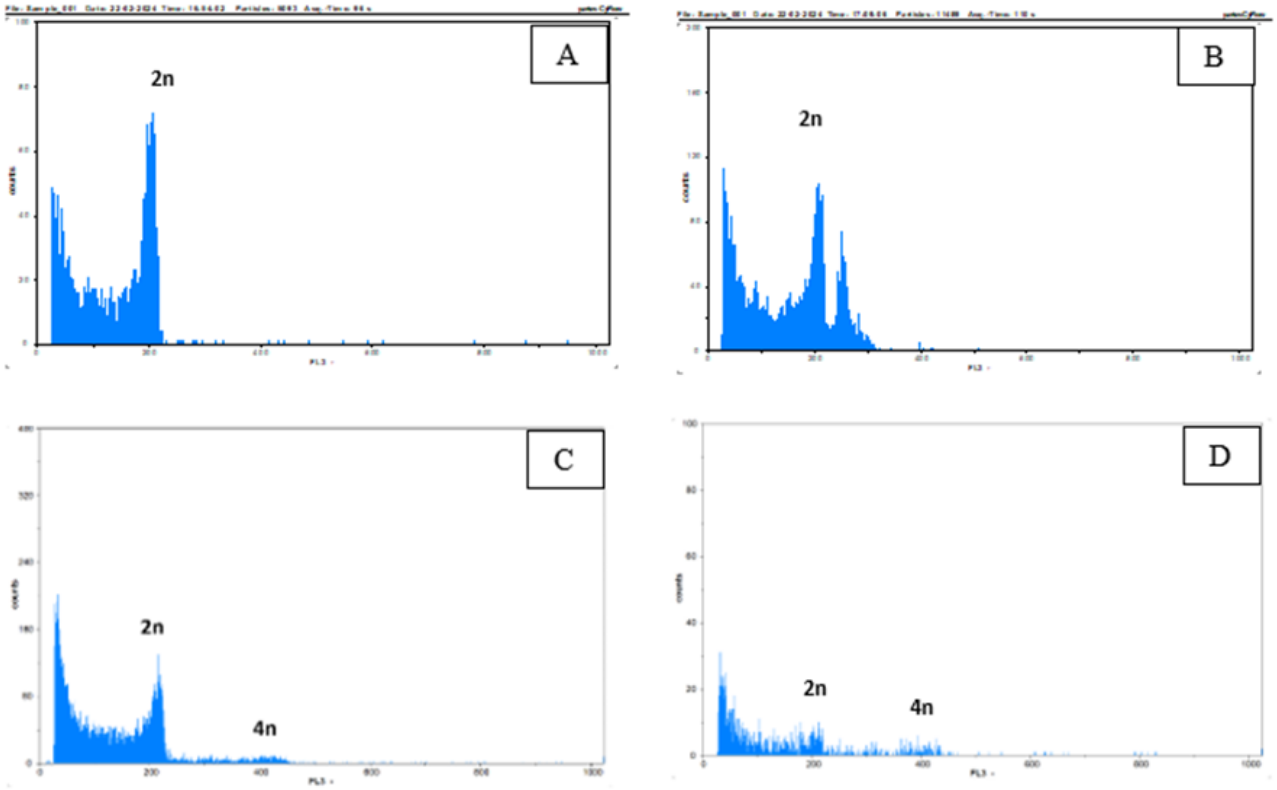

Fig. 3: Histogram of flow cytometry analysis from 3 samples. A=control sample, diploid (2n=2x), B=c1h1 sample, mixoploid (2n=2x, 4n=4x), C=c2h2 sample, mixoploid Type 1 (2n=2x, 4n=4x) and D=c3h1 sample, mixoploid Type 2 (2n=2x, 4n=4x).

between colchicine concentration and soaking times that are effective in producing polyploid plants. The treatment of 0.20% concentration with 3-days soaking time (c3h3) showed the highest mortality rate (78%) followed by 67% in the treatment of 0.10% colchicine concentration with 3-days soaking time (c2h3). The lowest mortality rate was found in the treatment of 0.00% colchicine concentration with 1-day soaking time (control) (11%) showing that soaking the explant in water alone could cause death.

Mortality rates above 50% (LC_{50}) began to occur at a concentration of 0.10% with a 3-days soaking period (c2h3) to a concentration of 0.20% with a 1- to 3-days (c3h1 and c3h2) soaking period. The higher concentration of colchicine used tended to cause greater mortality (Fig. 1). Giving excessive treatment or not in accordance with the ability of the plant will be fatal and can even kill the plant (Arindyaswari et al., 2021). The high mortality rate that occurs is thought to be due to the toxic effects of the use of colchicine mutagens by affecting plant cell growth to be abnormal and causing poisoning and even death in plants (Limera et al., 2016; Kharde et al., 2017). The target gene, the dose of mutagen used, and the sensitivity of the target organism are some of the factors that affect the occurrence of mutations in plants (Khursheed et al. 2019). Determining the lethal dose value and exposure soaking time to antimitotic colchicine compound at (LC_{50}) is important because it can be one way to determine the optimal mutagen dose frequency by reducing the level of damage. In this study it was observed at colchicine concentration of 0.20% with soaking time exposure of 1-day (c3h1).

The results of the variance of the observation parameters of *talas bite* taro growth showed no significant effect on the shoot emergence and leaf emergence parameters treated with colchicine concentration and soaking time. When viewed from the observation of shoot emergence and leaf emergence, the treatment without colchicine concentration (c0) produced the fastest shoot emergence and leaf emergence compared to other treatments. The treatment of colchicine concentration between 0.05%–0.20% produced days to shoot emergence and leaf emergence tended to be longer. One of the characteristics of colchicine-induced plants is experiencing slow growth changes from normal plants. Using colchicine solution at certain critical concentrations will block the microtubule arrangement of the spindle fibers. This will cause mitosis to become irregular (Girsang et al., 2021; Ermayanti et al., 2018). Based on visual observations of *talas bite* taro growth (Fig. 2), it is clear that the longer the colchicine soaking exposure time given caused the plant

growth rate to be longer (especially the colchicine concentration treatment of 0.10% and 0.20%), this is in line with the research of Wiendra et al. (2011) which states that the higher concentration of the treatment used tends to inhibit the rate of growth and shoot formation in *Impatiens balsamina* L. plants using the colchicine mutagen.

The ploidy level was detected by performing flow cytometry analysis using a flow cytometer (Partec® Cy-flow™). The level of plant ploidy was determined by observing the data in the form of graphs or histograms displayed on the computer screen obtained from the signal of the detector on the flow cytometer. According to Fomicheva & Domblides (2023), flow cytometry analysis is influenced by numerous factors including lysis buffer, buffer supplement, nuclei isolation, tissue origin, cytometer setup, acquisition settings, and data processing.

The results of flow cytometry analysis conducted on *talas bite* taro with colchicine concentration treatment and soaking time *in vitro* resulted in plants that were detected diploid (colchicine concentration 0.00% or without colchicine treatment) and mixoploid (colchicine concentration 0.05–0.20%) can be seen in Fig. 3 and Table 2. Based on these data, *talas bite* taro induced with colchicine concentration and soaking time can produce polyploid plants although only mixoploid.

Mixoploid plants are plants that have diploid and tetraploid cells, it happens that not all cells in the plant tissue are exposed to mutagens (Poerba et al., 2017). The use of colchicine solution concentration and the length of treatment time do not reach the right condition, so tetraploid plants cannot be obtained. Conversely, if the concentration is too high or the treatment time is too long, then colchicine will show a negative effect: the appearance of the plant becomes unsightly, many cells are damaged, or even cause the death of the plant (Manzoor et al., 2018; Novitasari et al., 2023). Therefore, it is necessary to find the right concentration, effective and efficient application/ soaking time for *talas bite* taro. Changes that occur in plants due to the use of colchicine can vary. Because, there are plants that experience mutations in almost all parts, from the growing point to the generative organs, while some only experience mutations in some parts. Therefore, it is possible that the colchicine given to each plant does not affect all plant cells but only some cells (Manzoor et al., 2019). A compound's toxicity effect may have an impact on plant sample survival. Colchicine is a very harmful substance that only slightly promotes growth. Colchicine should be applied sparingly and for longer periods of time in order to minimize harmful effects and boost the ratio of polyploid formation (Manzoor et al., 2019). However, high concentrations with increased exposure time still cause a high percentage of plant death (Mo et al., 2020). It is recommended that further research be carried out on colchicine induction in *talas bite* taro using different explant sources and further analysis by Karp method (1991) should be carried out to count the number of chromosomes and karyotyping to obtain more accurate data.

Conclusion

The research has succeeded in obtaining polyploid plants even though only mixoploid Type 2 (2n=2x, 4n=4x) with a tendency of tetraploid was detected. The treatment of 0.10% colchicine concentration with a soaking time of 2-days is the optimum treatment with a death rate around LC_{50} and also the CV-x% was the lowest in 2n-4n is recommended for further laboratory research and field trial.

Conflict of Interest

The authors declare there is no conflict of interest

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Authors' Contributions

RS was the lead researcher, conception, and design of the study. MFA provided advice and analysis and/or interpretation of data in the research. MR, ES, and YL provided advice and direction in the research. AT and R assisted in laboratory activities. AAP and WS provided samples of "talas bite" from the field. AA and N helped in the analysis of data and drafting of the manuscript. ISP and KK helped in improving the writing of the article.

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