Research Article

In Vitro Polyploidization and Growth Identification of Chrysanthemum Cultivars

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Abstract | Improving genetic of ornamental varieties can be created via artificial polyploid induction. In the present study, in vitro technique was used to examine the effects of Bio-Catharanthine extracted from periwinkle plants in induction and growth of the tetraploid Chrysanthemum plants. The four-week-old in vitro grown nodal segments were treated with various concentrations of Bio-catharanthine (0.05%, 0.1%, 0.15%, 0.2% w/v) for 8 h. Treated explants were cultured on Murashige and Skoog (MS) medium and the survived treated explants were sub-cultured to fresh MS medium for further growth and evaluation. The Ploidy level of plantlets was confirmed by flowcytometry and the induced tetraploids were cultured on multiple shoot proliferation and root induction media. In total, 6 tetraploids (16.66%), 8 triploids (22.22%) and 3 mixoploids (8.33%) were recovered from the tested Pinka Pinky plantlets. 2 tetraploids (33.33 %) and 2 triploids (33.33 %) were obtained from the surviving plants of Lolipop. No significant differences were observed in shoot, internode, or leaf numbers between diploid and tetraploid plantlets, but tetraploids produced significantly more roots. Stomatal measurements showed larger guard-cell length and width, larger stomatal aperture, and lower stomatal frequency in tetraploids compared with diploids.

Received | August 12, 2025; **Accepted** | December 04, 2025; **Published** | August 22, 2026
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Citation | Inderiati, S., M. Farid, Feranita and K. Mantja. 2026. In vitro polyploidization and growth identification of chrysanthemum cultivars. *Sarhad Journal of Agriculture,* 42(4): 1433-1443.
DOI | https://dx.doi.org/10.17582/journal.sja/2026/42.4.1433.1443
Keywords | Bio-catharanthine, Ornamental, Flowcytometry, Tetraploid

Introduction

Chrysanthemum species are primarily farmed for cut flowers, potted and bedding plants. Chrysanthemum's popularity as a great cut flower reaffirms it's a second most popular of cut flowers worldwide after rose (Boukhebti *et al.*, 2020). It is known for its beautiful flowers, vibrant color, and diverse floral types and shapes. With the rapid transition of the global floriculture business, markets and consumers are demanding new varieties with increased ornamental value and ready-to-use products. Cut flower quality is determined quantitatively through stalk length, number of leaves, weight and flower size, in addition to qualitative characteristics such as flower color, fragrance, physical damage, disease, and freshness (Roh *et al.*, 2017). Since chrysanthemums represent the top

ornamental crop worldwide, introducing novelties with varied ornamental attributes into the market is necessary to sustain global competitiveness in the most dynamic sector of floriculture. In addition to the floral color, the ornamental traits of chrysanthemums include flower type, shape, floral scent, flowering time, vase life, and biotic and abiotic stress resistance (Azadi *et al*., 2016; Mekapogu *et al*., 2022).

Historically, new chrysanthemum cultivars have been developed through conventional breeding methods. However, these methods can be imprecise and require several generations before the desired traits are obtained and a stable strain is produced. One strategy to accelerate breeding development is a chromosome doubling event called polyploidization. Polyploidization is common in the plant kingdom and has been associated with increased genetic diversity in some plant lineages (zhang *et al*., 2019; Wang *et al*., 2024). Desirable consequences of polyploidy for plant breeding include the buffering of deleterious mutations, increased heterozygosity and hybrid vigor (Sattler *et al*., 2016). Consequently, polyploids often have phenotypic traits that are distinct from diploids, including larger leaves, flowers and often exhibit a faster growth rate (Zhang *et al*., 2023; Wang *et al*., 2025). Inducing tetraploid can enhance genetic variation, which is useful for breeding programs aimed at developing new varieties or hybrids with specific traits (Madani *et al*., 2021; Nourozi *et al*., 2025). Additionally, tetraploid plants may improve adaptability to environmental conditions, including tolerance to abiotic stress factors like drought (Xu *et al*., 2019; Tossi *et al*., 2022; Li *et al*., 2024) and resistance to biotic stress factors such as pests and diseases (Li *et al*., 2019; Li *et al*., 2024; Koziara-Ciupa & Trojak-Goluch. 2025).

Polyploidy can be induced through application of antimitotic agents and polyploidization of chrysanthemum has been successfully through in vitro mutagenesis using mutagen of colchicine (Kushwah *et al*., 2018; Kushwah *et al*., 2018). The current study employed in vitro mutagenesis with Bio-catharanthine derived periwinkle plant (*Catharanthus roseous* L.*)* to attempt whole-genome duplication in chrysanthemum. Unlike previous study that confirmed of in vivo bio-catharanthine induction, the current report is concerned with the comprehensive study on artificial in vitro polyploidy as a method of induction. Because in vivo variation induction has various limitations, in vitro variation induction may be a preferable option for competent variation introduction, handling a large number of plant materials, and extremely quick variant multiplication (Li *et al*., 2019; Sharma *et al*., 2024).

In vitro technique was used in this study to examine the effects of bio-catharanthine in induction and growth of the tetraploid chrysanthemum plants. Polyploid plants were confirmed through flowcytometry and in vitro growth and stomatal morphology of polyploidy plants were compared. These newly induced polyploid plants could be useful for advance quality genetic of chrysanthemum to provide better clones for enhancing ornamental value and environmental persistence.

Materials and Methods

Polyploidy induction with Bio-catharanthine

The experiment was conducted in the laboratory of tissue culture of Faculty of Agriculture of Hasanuddin University, Makassar from July to December 2024. The polyploidy inducer used was bio-catharanthine, a commercial product from the research group of the Faculty of Biology at Universitas Gadjah Mada, Indonesia. Polyploidy induction of chrysanthemum was performed using nodal segments from in vitro chrysanthemum cv. Pinka Pinky and cv. Lolipop. The both cultivars chosen for their commercial value, fast growing cultivars that is easy to handle in vitro and have been shown to respond well to polyploidy induction. Each explant consisting of two internodes without leaf. The nodal stems were immersed in a liquid of bio-catharanthine at concentrations of 0.05%, 0.1%, 0.15% and 0.2% (w/v) and shaken in a shaker (150 rpm) for 8 hours. The concentrations and duration were selected from a dose-response trend line that optimized tetraploid induction in the chrysanthemum using colchicine (Ridwan *et al*., 2024).

The crude extract of roseus plant (bio-catharanthine) was diluted with double-sterilized water to make up the liquid concentrations. Each treatment consisted of 5 replications and each replication comprised 3 nodal. The treated nodal were rinsed three times with sterile water before planting in solid modified Murashige and Skoog (MS) medium with a pH of 5.7–5.8. The cultures were incubated in a room with a temperature 25°C and light intensity provided by two fluorescent lamps for 16 h per day. Survival

rate and regeneration were observed after 10 days of bio-catharanthine treatment. The brown and black explants were considered as dead and removed from the culture. Healthy explants cultured in new MS medium and allowed to regeneration and normal growth for ploidy determination.

Ploidy analysis based on flowcytometry

The ploidy level of in-vitro induced chrysanthemum was analyzed using flow cytometer. The upper fully opened and green leaves of 1.5 months of induced plants and controls were collected and washed with running water and dried with tissue paper. The leaves measuring 0.5–1.0 cm^2 are finely chopped and immersed in 0.5 mL Nuclease Extraction buffer (Sysmex). After 2 minutes incubated at room temperature, the mixture was sieved using a cell Trics™ 50 µm filter, followed by the addition of 750–1000 µL staining solution which contain 2 µL Staining buffer, 12 µL Propidium Iodide and 6 µL RNAse A (Sysmex 05-05022). After 1 minute incubated, the nuclear suspensions were analysed using a Partec CyFlowSpace flow cytometer using 'Cystain TM PI absolute P' reagent kit (Sysmex 05-05022). The results of the flow cytometry analysis were displayed on a computer screen in the form of graphs using the Flow Max software version 2.81.

Leaf materials from untreated (control) plantlets of Pinka Pinky and Lolipop cultivars were used as diploid standards for each cultivar tested. Reading for diploid control plants were positioned at a channel of about 200; therefore, tetraploid plant will be indicated with readings at a position of around 400.

The identification of polyploidy

In vitro growth of diploid and tetraploid chrysanthemum

The diploid and induced tetraploids confirmed through flow cytometry analysis were cultured on multiple shoot proliferation and root induction media subsequently. The in vitro performance of tetraploids over control diploids was studied on the basis of shoot, leaves, stem, node growth, rooting time and number of roots. The in vitro culture bottles were set according to a complete randomized design on the shelves of an incubation room and each explant/plant was considered as an experimental unit. The in vitro growth experiments were restated thrice using six explants per replication and observation was carried out daily.

Stomatal analysis

The cytological features of diploid and tetraploid plantlets were evaluated and compared with each other. Stomata guard cell length and stomatal density measurement used for identification of polyploidy plantlets. For this purpose, fresh leaves of diploid and tetraploid regenerated plants of the two chrysanthemum cultivars at the same stage were placed on slides to observe their lower epidermal cells. This part of the leaves was covered with a thin layer of clear nail polish and allowed to dry. After drying the polish, it was removed carefully with glass glue then placed on a glass slide. The stomatal density, stomatal length and width of the diploid and tetraploid leaves were observed and measured by optical microscopy. The number of stomata per field of view (234 µm x 295 µm) under the 40x objective was used to calculate the density of stomata in six different leaf samples of each cultivar tested. To determine the stomatal length and width, six randomly selected stomata from each view were measured.

The data were assessed using ANOVA with the Tukey HSD test and the results were expressed as mean ± standard error of mean.

Results and Discussion

Survival of bio-catharanthine treated plants

The survival plant was evaluated after ten days of incubation of treated explants on MS medium. The survival rate of explants in both cultivars used decreased with increasing bio-catharanthine concentration (Table 1). However, the local cultivar of Pinka Pinky showed higher subsistence as compared to an introduction cultivar of Lolipop, as no lolipop explant survived when treated with the highest concentration of the mutagen (0.2%). This result is different from previous polyploidization research which showed that bio-catharanthine did not lower germination rate of black rice (Kurniawan *et al.*, 2023; Setyati *et al.*, 2024) and orange watermelon (Setiyobudi *et al.*, 2024). However, none of the studies reported polyploidy in the bio-catharanthine treated plants. This comparison shows that sensitivity to mitotic agents could differ between plant species and cultivars. Bio-catharanthine treated shoots along with the controls were maintained in a sustainable manner with regular subcultures at an interval of 30 days.

Table 1: *Influence of various concentrations on induction of polyploid plantlets from the in vitro cultures of Chrysanthemum*

Cultivar	Conc. (%)	No. of treated explant	Survival plant (%)	No. of plant tested	Tetraploid	Ploidy (%) Triploid	Mixoploid	Diploid
Pinka pinky lolipop	0.05 0.1 0.1.5 0.2 0.05 0.1 0.15 0.2	15 15 15 15 15 15 15 15	13 (86.7) 11 (73.3) 9 (60) 9 (60) 11 (73.3) 9 (60) 6 (40) 0	999 99960	1(11.1)1 (11.1) 2(22.2) 2(22.2) 002 (33.3)0	2 (22.2)2 (22.2)2 (22.2)01 (11.1)2 (33.3)0	1 (11.1)1 (11.1)01 (11.1)0000	5 (55.5)5 (55.5)5 (55.5)4 (44.4)9 (100)8 (88.9)2 (33.3)0

Table 2: *Effect of ploidy level on in vitro growth of chrysanthemum (± SE)*

Cultivars	No. of Shoots	Shoot induction time (day)	No.of Leaves	Root induction time (day)	No. of roots	No. of internodes
Pinka pinky *diploid tetraploid* lolipop *diploid tetraploid*	1.33 ± 0.071.50 ± 0.13 1.55 ± 0.141.33 ± 0.08	4.50 ± 0.00a 5.83 ± 0.07b 6.91 ± 0.09 9.55 ± 0.13	12.83 ± 0.5013.50 ± 0.07 15.00 ± 1.32 17.17 ± 0.65	5.83 ± 0.07a 7.83 ± 0.07b 11.17 ± 0.22 12.17 ± 0.74	14.83 ± 0.07b 16.20 ± 0.04a 8.35 ± 0.21b13 ± 0.35a	12.33 ± 0.54 12.67 ± 0.07 10.33 ± 0.74 14.33 ± 0.79

Means ± standard errors followed by different letters in each column are significantly different at 5 % probability level.

The polyploidy induction rate was calculated as the percentage of polyploids among the tested plants. All concentrations used induced polyploids in Pinka Pinky cultivar with the highest tetraploid induction rate was at 0.15 % and 0.2 %; each concentration yielded 2 tetraploids out of 9 plantlets (22.22 %). In total, 6 tetraploids (16.66%), 8 triploids (22.22%) and 3 mixoploids (8.33%) were recovered from the tested Pinka Pinky plantlets. When Lolipop explants were treated with 0.15 % bio-catharanthine for 8 h, 2 tetraploids (33.33 %) and 2 triploids (33.33 %) were obtained from the surviving plants (Table 2).

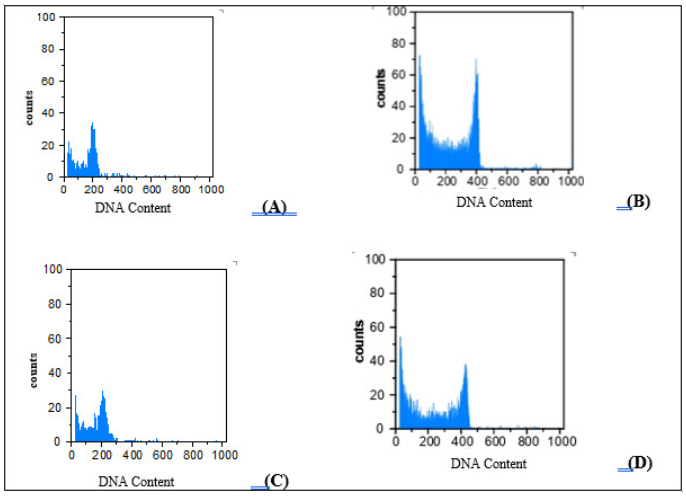

Figure 1: *Analysis of ploidy by flow cytometry. (A) Flow cytometric histograms of the nuclear DNA content in diploid (2x) and (B) tetraploid(4x) leaf samples for Pinka Pinky plants, respectively. (C) Flow cytometric histograms of the nuclear DNA content in diploid (2x) and (D) tetraploid(4x) leaf samples for Lolipop plants.*

Analysis by flowcytometry

In the current experiment, the ploidy levels of the plantlets from bio-catharanthine-treated, as well as control plantlets were determined with flowcytometry analysis and the DNA content of diploid and tetraploid plants are shown in Figure 1. The differences between flow cytometer results of diploid and tetraploid plants can be observed from the peaks of a histogram. The histograms from flowcytometry analysis reveal two kinds of peaks of the nuclear DNA content corresponding to 2x (diploid) and 4x (tetraploid) respectively. In proportion to the square root of ploidy, each peak had around twice the fluorescence intensity. These results confirm the ploidy level of untreated chrysanthemums (control plants) as diploid and bio-catharanthine treated plants as tetraploid.

The control samples of diploid chrysanthemum set up at channel 200 for flow cytometry analysis, therefore, samples that recorded in channel 400 indi-cated as tetraploid, and samples that had two chan-nels, signalled as mixoploid. The analysis showed that the channel or peak of the graph signifying relative DNA content of Pinka Pinky cultivar was at 204.23 for the diploid control and peaked at 411.73 for the tetraploid, both with CV values of 4.80 % and 3.71 % respectively (Figure 1). The peak of the diploid (control plant) and tetraploid Lolipop samples were at channel 207.15 and 422.10 with CV values of 5.61 % and 4.66 %, respectively. The flowcytometry analysis method was initially used to determine ploidy level and screen for mutant plants, followed by

in vitro growth characteristic and stomatal analysis. Flowcytometry is a simple and effective tool for ploidy analysis, capable of quickly and accurately identifying the ploidy of mutant plants without limitations on tissue or cell stage (Sattler *et al*., 2016). Plant tissue such as leaves, stems, roots, flowers, peels, and seeds can be used for flowcytometry identification, and the required samples are minimal (Wu *et al*., 2023).

Bio-catharanthine can doubled the chromosome number of peanut (Muarifin *et al*., 2021) and shallot plants (Billa *et al*., 2022). In current study, higher concentration of the mutagen resulted in polyploid in both cultivars while the lowest concentration did not alter the DNA content of Lolipop. On the other hand, the concentration of 0.1% and 0.15% led to genotype mutations and produced polyploid plants in Pinka Pinky cultivar. Previous study reported in vivo mutation of *Passiflora foetida* after treated with 1 to 1.5% Bio-catharanthine (n = 25) for 24 hours only formed mixoploids (Kasim *et al*., 2024). Because the effective method varies by species, there is no general concentration of mutagen or length of treatment in plants. The duration of soaking treatment might be sufficient for the inhibition of spindle structure in this in vitro mutagenic of chrysanthemum. Though, the higher tetraploid might be observed with the flowcytometry analysis through a longer duration of bio-catharanthine treatment.

Previous investigations revealed that the treatment time of the polyploidization agent affects the degree of ploidy produced. Soaking the bulbs of shallots (n = 75) with bio-catharanthine 0.2% for 12 hours resulted in the chromosomes 3n (triploid) and the bulb treated with bio-catharantine 0.4% (12 hours) increases the ploidy level to 4n (tetraploid) chromosomes (Billa *et al*., 2022). The peanut seeds (n = 40) treated with 0.15% catharanthine content solution for 12 hours did not induce polyploidy. However, the increase in exposure time to 24 hours, induced polyploidy both tetraploid and octoploid plants (Muarifin *et al*., 2021). The induction with bio-catharanthine for 48 hours soaking treatment led to two mitotic cycles which facilitate a higher possibility of the mitotic destruction (Kurniawan *et al*., 2023). Therefore, the effect of induction on each tissue can also be different if the combination of solution concentration and soaking time is not appropriate (Billa *et al*., 2022). Indeed, the success of polyploidization induction is not only influenced by the concentration of the mutagen but also the duration of immersion.

Bio-catharanthine as periwinkle leaves extract contains vincristine and vinblastine, which can act as anti-mitotic compounds on the chromosomes of plants (Rohmah *et al*., 2022). Vincristine and vinblastine function to suppress microtubule activity through the β-tubulin side binding mechanism (Mohammed, 2016). The inhibition of spindle formation by the catharanthine produces an unbalanced chromosome number of two daughter cells, resulting in a polyploid cell (Skubnik *et al*. 2020). Vincristine and vinblastine work as anti-mitotic agents by hindering microtubule polymerization, comparable in effect to colchicine; however, because their dose-response and toxicity profiles differ, the dosage must to be carefully optimized.

The identification of polyploidy

The in vitro growth of diploid and tetraploid chrysanthemum

Polyploid identification is an important step in polyploid induction since polyploids differ from diploids in morphology, anatomy, and physiology. In this study, leaf stomata and in-vitro plant growth were used to identify polyploids and to evaluate the influence of mutagenic agent on growth of induced tetraploid plants.

The in vitro growth of chrysanthemum tetraploid and diploid (control plants) on the MS medium were evaluated after they have been transferred from the regeneration medium. No significant difference was found between the tetraploid and diploid plantlets in terms of the mean number of shoots, internodes and leaves of both cultivars. However, the number of roots of the tetraploid Pinka Pinky and Lolipop was significantly different from the mean number of roots of their diploid counterparts. From the in vitro growth parameters recorded, polyploidization resulted in the slower growth of shoots and roots of chrysanthemum cv. Pinka Pinky, and the shoot and root induction time of Lolipop controls were slightly faster than their tetraploid counterparts (Table 2).

These phenotypic data (Table 2, Figure 2, Figure 3) are similar to earlier bio-catharanthine studies in peanut (Muarifin *et al*., 2021) and red spinach (Shafura *et al*., 2022), which reported that tetraploid plants grew better than diploids. Higher ploidy is often linked to larger cell size, resulting in thicker leaves and larger

stomata. Consequently, several studies have shown that tetraploids produce greater biomass (fresh and dry weight) and develop more extensive root systems (Huang *et al*., 2022).

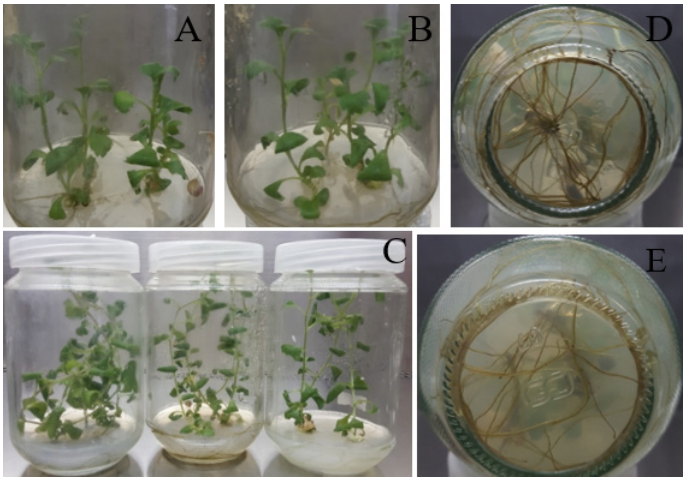

Figure 2: *Morphological observation of diploid and tetraploid Chrysanthemum cv. Lolipop. Shoot regeneration of tetraploids (A) and Diploids (B). (C) Shoot tip growth of tetraploids (left), diploids (middle and right). Rooting of tetraploid (D) and diploid (E).*

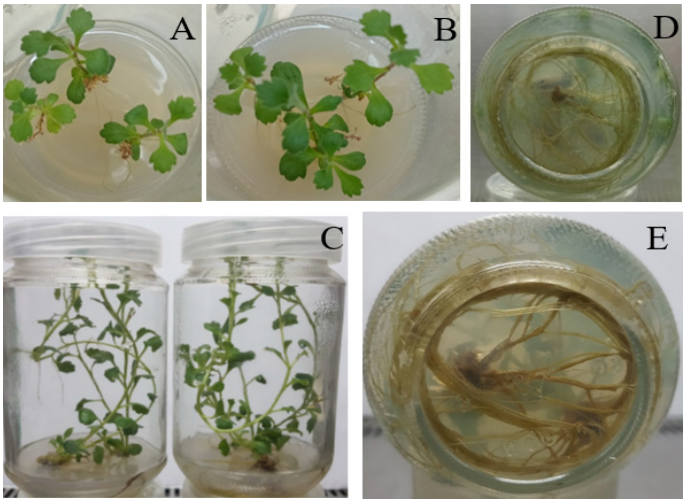

Figure 3: *Morphological observation of diploid and tetraploid Chrysanthemum cv. Pinka Pinky. Shoot regeneration of tetraploids (A) and diploids (B). (C) Shoot tip growth of diploids (left) and tetraploids (right). Rooting of diploid (D) and tetraploid (E).*

Although bio-catharanthine is an uncommon mutagenic agent, several studies have described the impact of this ethanolic extract of the periwinkle plant on plant morphology. In Pamelo (*Rutaceae* sp.) bio-catharanthine treatment altered several morphological traits of plant height, number of leaves, number of roots, and number of nodes (Aziz *et al*., 2021). Previous investigation in *Zephyranthes rosea* reported morphological changes caused by bio-catharanthine application such as number of leaves and leaf surface area, root biomass, stomatal size and density, as well as flowering time (Wardana *et al*., 2019). Bio-catharanthine also affected the chlorophyll content of red spinach (Shafura *et al*., 2022) and significant differences found in the size, thick and color characteristics of bio-catharanthine treated and untreated leaves of watermelon (Setyobudi *et al*., 2024). These studies underline the feasibility of bio-catharanthine as a mutagenic agents like colchicine for inducing polyploidy in crops.

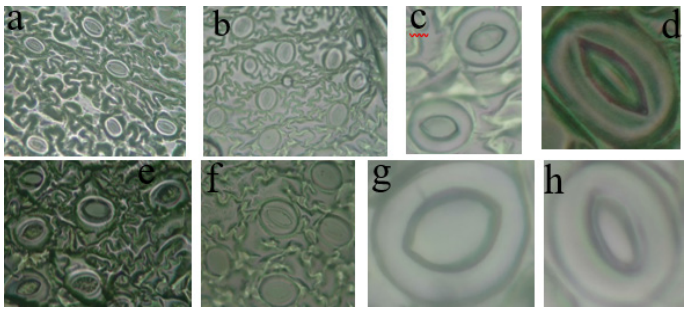

Figure 4: *Comparison of the stomata and guard cells between diploid and tetraploid plants. Tetraploid plant (a,c) and diploid plant (b,d) of Pinka Pinky. Tetraploid plant (e,g) and diploid plant (f,h) of Lolipop. a,b,e,f, 10x magnification and c,d,g,h 40x magnification.*

Stomata analysis

Polyploidization led to broaden plant cells and organs. In this study, both cultivars showed different size of stomata based on their ploidy levels. The individual stomata became longer, the guard cells increased in size, and the density of stomata per unit area decreased in tetraploid plants (Table 3. & Figure 4). The length and width of stomata in tetraploids Pinka Pinky were 27.15 and 19.73 µm respectively, while in diploids were 22.60 and 14.22. Tetraploid Lolipop had wider (23.56) and longer (34.51) stomata as compare to diploid. The average number of stomata per field (under a microscope) on tetraploids Pinka Pinky and Lolipop was 5.80 and 6.00 respectively, less than one-half of that of diploids. Bio-catharanthine also enlarge the stomatal size and decreased density of black rice (Kurniawan *et al*., 2023). The enhanced of stomatal size due to increased chloroplasts in guard cells, leading to a larger stomatal size (Jeloudar *et al*., 2019; Yao *et al*., 2023).

The tetraploids plantlets had larger stomata compared

Table 3: *Comparison of morphological traits of stomata (mean ± SE) in diploid and tetraploid plantlets in chrysanthemum*

Chrysanthemum cultivars	Stomata density (no./microscopic field)	Stomata length (µm)	Stomata width (µm)
Pinka pinka tetraploid diploid *lolipop* tetraploid diploid	5.80 ± 0.2a (*n* = 6)9.40± 0.5b (*n* = 6) 6.00± 0.3a (*n* = 6) 13.60 ± 0.7b(*n* = 6)	27.15 ± 0.3a (*n* = 36) 22.06 ± 0.5b (*n* = 36) 34.51 ± 0.6a (*n* = 36) 26.30 ± 3.1b (*n* = 36)	19.73 ± 0.5a (*n* = 36) 14.22 ± 0.4b (*n* = 36) 23.56 ± 0.2a (*n* = 36) 15.24 ± 1.9b (*n* = 36)

Means ± standard errors followed by different letters in each column are significantly different at 5 % probability level.

to diploids and the increased stomata size results in decreased stomata frequency. A pattern also documented in several other plants such as *Cannabis sativa* (Parsons *et al.*, 2019), *Stevia rebaudiana* (Zhang *et al.*, 2016), and *Tectona grandis* (Windarsih *et al.*, 2024). Mutagen exposure in plants can increase cell size, including stomatal guard cells. This is principally due to the mutagen's effect on the cytoskeleton, specifically microtubules, which are essential for the organization and growth of cells and tissues. The disruption of microtubules can alter the development of tissues, including the epidermis and impact the size of surrounding cells near stomata (Li *et al.*, 2022).

A negative relation was observed between the stomatal density and ploidy level. The results of stomatal density showed that tetraploid plantlets significantly had lower stomatal density than diploid plantlets. It has been suggested that the lower stomata frequency in tetraploids was due to the larger stomata and epidermal cell size, as well as reduced stomata differentiation. Stomatal size and density have an inverse relationship, as larger stomatal size results in decreased stomatal density (Tossi *et al.*, 2022). Stomatal density was affected by size, where the length and width of the stomata can be used as an indicator of changes in polyploidy (Fu *et al.*, 2019; Windarsih *et al.*, 2024). Stomatal size measurement has become a quick method for detecting polyploidy in numerous plants (Bhattarai *et al.*, 2021; Eng *et al.*, 2021; Kurniawan *et al.*, 2023; Windarsih *et al.*, 2024).

The absence of pronounced growth differences in this study might be due to the in-vitro growth condition. Under the standardized in vitro condition used, biomass accumulation including shoot, leaf, and internode numbers did not differ significantly between diploid and tetraploid plantlets, indicating that the controlled environment masked ploidy-related growth advantages. Tetraploids may exhibit superior performance only at elevated light intensities; under the moderate light, the two ploidy levels displayed comparable growth (Wang *et al.*, 2025). A study on *Melia volkensii* plant showed no significant difference in leaf growth between tetraploid and diploid plants in vitro; in contrast, a significant increase in leaf growth was observed in potted plants. This variation in leaf development could be due to species and growing conditions (Dushimimana *et al.*, 2023).

The significance of increased stomatal size and reduced stomatal density in chrysanthemum remains to be determined. However, studies in other plants have demonstrated that altered stomatal morphology changes the balance between water loss and CO_2 uptake, which can affect photosynthesis, stress coping mechanisms, and disease susceptibility (Bertolino *et al.* 2019; Emily *et al.*, 2020; Hou *et al.*, 2024). Furthermore, the internal environmental adaptation of tetraploid *Lycium ruthenicum* differed significantly from that of diploids under normal growth conditions. The tetraploids showed superior drought resistance, as large amounts of ABA accumulated in them and strongly induced the expression of osmotic-protective proteins, increasing overall drought tolerance (Rao *et al.*, 2020).

An enlargement of stomata in polyploids had a better stomata conductivity to support the bigger size of stomatal cells and pores in relation to water and CO_2 efficiency (Dunn *et al.*, 2019; Smarda *et al.*, 2023). Low stomatal conductance can reduce transpiration and prevent excessive water loss due to transpiration, thus greatly increasing the survival rate of transplants (Fu *et al.*, 2019). Therefore, it is interesting to further investigate the growth and development of the in vitro induced tetraploid chrysanthemum after being successfully acclimatized. Hence, the phenotypic characteristics become useful for screening tetraploids in chrysanthemum. The tetraploid plantlets obtained from this study were sub cultured several times and prepared for soil adaptation before evaluating their ornamental value and the benefit of altered stomatal morphology.

Conclusions and Recommendations

The current study sets the foundation for producing polyploid Chrysanthemum morifolium using the uncommon antimitotic agent bio-catharanthine. From the Pinka Pinky plantlets we obtained six tetraploids, eight triploids and three mixoploids, while two tetraploids and two triploids were recovered from the Lolipop plantlets. Varying the bio-catharanthine concentration altered stomatal size and density and affected plantlet growth. For future work, we recommend increasing the bio-catharanthine concentration and varying immersion times to raise ploidy rates.

Acknowledgments

The authors are thankful to the Agrotechnology Study Program, Faculty of Agriculture, Hasanuddin University, Makassar for extending research facilities to carry out the laboratory works and flowcytometry analysis.

Novelty Statement

The present study employed plant natural mutagen derived to attempt whole-genome duplication in chrysanthemum, marking one the first report of its kind, and demonstrating significant potential for both research and commercial applications. This approach has shortened in vitro mutagenesis more affordable, sufficient and reliable.

Author's Contribution

Sitti Inderiati: Developed the initial experiment proposal and worked with method development, developed bio-catharanthine treatment, conducted flow cytometry analysis and carried out the laboratory work, data collecting and analysis.
Muhammad Farid BDR: Developed the initial experiment proposal and worked with method development,assisted with writing and editing.
Feranita: Optimized tissue culture methods for Chrysanthemum, assisted with writing and editing.
Katriani Mantja: Optimized tissue culture methods for Chrysanthemum.
Sitti Inderiati: Developed bio-catharanthine treatment, conducted flow cytometry analysis and carried out the laboratory work, data collecting and analysis, assisted with writing and editing.

Generative AI or AI assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest
The authors declare that there is no conflict of interest.

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