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Research Article

Characterisation of Stomata in Mutant Butterfly Pea (*Clitoria ternatea* L.) Induced by Colchicine

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ABSTRACT

Butterfly pea (*Clitoria ternatea* L.) is a biopharmaceutical plant known for its bioactive compounds, particularly anthocyanins and antioxidants. Increases in stomatal size and density can enhance photosynthetic capacity by improving CO_2 uptake and gas exchange efficiency, which, in turn, stimulate the biosynthesis of antioxidant-related metabolites, such as anthocyanins. The study aims to increase the size of butterfly pea stomata by creating chromosome doubling through colchicine. Seeds of a deep blue single flower butterfly pea were soaked in colchicine for 24 and 48 hours at the concentrations of 0.5; 1.0; 1.5 mM, and a control (soaked in distilled water). The study collected data for chromosome analysis, stomata (density, width, and length), and chlorophyll content in butterfly pea leaves. The results revealed changes in chromosome number, stomatal density, and chlorophyll content, suggesting the occurrence of mixed polyploidy in the treated butterfly pea cells. Stomatal analysis showed that plants treated with 1.0 mM colchicine for 24 hours had a higher stomatal density on the abaxial leaf surface compared to the adaxial leaf surface. Although the width and length of stomata did not increase significantly compared to the control, there was a tendency for their size to increase, especially in butterfly pea leaves soaked in 1.0 mM colchicine for 24 hours. Chlorophyll content increased significantly in butterfly pea leaves in colchicine at a concentration of 1.5 mM for 48 hours. Colchicine-induced polyploidy and its effects on stomatal characteristics and chlorophyll content in butterfly pea.

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INTRODUCTION

Butterfly pea (*Clitoria ternatea* L.) grows well in tropical and subtropical regions and is drought-tolerant (Li et al. 2022). Butterfly pea plants exhibit a wide variety of flower characteristics. The number of petals ranges from one (single) to five (quintuple). The colour variations of butterfly pea corollas include pink, blue, dark blue, light blue, purple, and white. They are biopharmaceutical plants with significant potential in the health and industrial sectors (Fransiska et al. 2024). These plants are rich in bioactive compounds, such as anthocyanins, flavonoids, and alkaloids, which have antioxidant, anti-inflammatory, and neuroprotective properties and are a potential anticancer and antidiabetic agent (Alam et al. 2021). The flower extract has been widely used in traditional and modern medicine, including as a natural dye, pharmaceutical raw material, supplement, and cosmetic formulation (Thuy et al. 2021). As global demand for natural products increases, butterfly pea has promising prospect as a leading commodity in the biopharmaceutical and agribusiness industries.

Despite its numerous benefits, the development of butterfly pea as a biopharmaceutical crop still faces several challenges. One major issue is the low natural production of secondary metabolites, which can limit their effectiveness on an industrial scale. Besides, in tropical regions, butterfly pea is a wild ornamental plant that has not undergone systematic improvement. Consequently, no standard cultivars with high anthocyanin content have been developed. Furthermore, the growth and productivity of butterfly pea plants vary, particularly in relation to their adaptation to agroclimatic conditions. These factors limit the sustainable supply of high-quality raw materials for the pharmaceutical industries. Therefore, innovative and selective approaches are needed to increase bioactive metabolite content and optimal plant productivity (Abdolinejad et al. 2021; Zulchi et al. 2024).

One strategy that can be implemented to enhance butterfly pea's potential is genome manipulation, such as the induction of polyploidy or chromosome doubling (Zulchi et al. 2024). Polyploidy is an effective method for improving the quality and commercial value of ornamental plants (Jiang et al. 2022). Chromosome doubling in butterfly pea can be induced by colchicine, a chemical mutagen that induces polyploidy. Colchicine can inhibit the formation of spindle fibres during cell division, thus doubling the number of chromosomes in each cell (Hasanah et al. 2025). Colchicine is very effective in inducing polyploidy which results in increased production of secondary metabolites, flower size, colour intensity, and thickening leaves (Manzoor et al. 2019). For example, in *Celosia argentea*, polyploidy increases the production of alkaloids, phenols, and anthocyanin, which impart more intense colour and enhance its aesthetic value as an ornamental plant (Mostafa & Alhamd 2016).

The most important effect of polyploidy plants is an increase in cell size. Several studies have demonstrated polyploidy not only increases cell size such as flower diameter and leave thickness but also stomata and guard cells (Doyle et al. 2020; Catalano et al. 2021; Zhang et al. 2024). In some plants, stomatal length and stomatal frequency are rapid indirect methods to identify ploidy level and stomatal density has often been used as a morphological marker for identifying ploidy level (Beck et al. 2003; Yuan et al. 2009; Ye et al. 2010; Zhang et al. 2024). Stomata also play an important role in enhancing photosynthesis. Xiong et al. (2022) emphasizes the function of stomata in regulating photosynthetic induction and water use efficiency under fluctuating light, which will help improve crop photosynthetic efficiency.

The Agrotechnology Study Program, Faculty of Agriculture, UPN Veteran Jawa Timur, has ten potential accessions with varying flower diversity that can be used as genetic resources for mutation materials. The genetic diversity of butterfly pea flowers supports plant breeding processes through

mutation techniques, including chemical mutation with colchicine. Previous studies have reported that colchicine treatment can significantly alter stomatal density, size, and structural organisation as a consequence of chromosome doubling and cell enlargement. Polyploid plants induced by colchicine often exhibit reduced stomatal density but increased stomatal size compared to diploid controls, reflecting altered epidermal cell development. In addition, structural modifications such as changes in guard cell shape and stomatal aperture have been observed, which may influence transpiration rate and gas exchange efficiency. These stomatal alterations are frequently used as indirect indicators of successful polyploidization and have important implications for plant adaptation, water-use efficiency, and overall physiological performance (Hasanah et al. 2023). Therefore, this study focuses on the characterisation of stomata in butterfly pea mutants induced by colchicine.

MATERIALS AND METHODS

Plant Material and Research Design

Butterfly pea with its deep blue single flowers, was used as a source of genetic material in the Faculty of Agriculture collection garden at UPN Veteran Jawa Timur. Seeds from six-month-old butterfly pea plants were taken. The butterfly pea seeds were selected based on the percentage of germination.

This research used a completely randomised design consisted of two factors, namely colchicine concentration (0; 0.5; 1.0; and 1.5 mM) and soaking time (24 and 48 hours), resulting in eight treatment combinations. Each treatment consisted of five replications, so there were total of 60 experimental units.

Colchicine Treatment Application

The colchicine treatment began by dissolving colchicine powder in distilled water to obtain predetermined concentrations of 0; 0.5; 1.0; and 1.5 mM. Butterfly pea seeds were prepared by removing the pods and soaking 20 seeds in 50 mL of each colchicine solution and distilled water for control (0 mM). Seeds were soaked in a transparent, airtight plastic container (dimensions: 10 × 6 × 4 cm^3). The soaking duration consisted of 24 and 48 hours. After soaking, the seeds were rinsed with distilled water and air-dried at room temperature before planting.

Sowing and Planting of Butterfly pea

Butterfly pea seeds that have been soaked in the mutagen colchicine were sown in trays. Seeds were planted 0.5–1 cm below the soil surface and kept moist by regularly watering every morning and evening with a fine sprayer. Once the seedlings reached planting size, they were transplanted to the experimental field. The medium used in the field was a mixture of soil, rice husk charcoal, and goat manure in a 2:1:1 ratio to support optimal plant growth. Seedlings were planted at the specified spacing and then watered to field capacity. The plants were fertilised with a mixture of nitrogen, phosphate, and potassium, 3 g per plant every week until flowers appeared.

Analysis of Chromosome

Chromosomal analysis of butterfly pea plant was conducted to observe chromosomal changes due to mutations and polyploidy. Observations used the root staining method with acetoorcein with concentration 0; 0.5; 1.0; 1.5 mM to visualise chromosomes. Young roots were selected because their cells were actively dividing. They were then prepared using the squash preparation method, fixed in Carnoy's solution, and observed using an Olympus BX51 microscope. Chromosome size was measured using ImageJ or DP20 software,

while shape and structure were analysed using karyotyping. The results of this analysis helped to determine ploidy changes and the impact of colchicine treatment on the genetic structure of butterfly pea plant.

Analysis of Stomata

The length, width, and density of stomata were analysed using the Mold Method. Transparent nail polish was applied to the abaxial surface of the leaves, allowed to dry for 3-5 minutes, then affixed with clear tape and transferred to a glass slide. Observations were made using an Olympus BX51 binocular microscope (10 x 10 magnification), while stomatal density was calculated using a formula based on five different fields of view (4 x 10 magnification). Stomatal length and width were measured digitally using DP20 software, while the number of chloroplasts in guard cells was counted manually after observing the abaxial leaf layer sections.

Observation of Chlorophyll Content

Chlorophyll content was observed using a chlorophyll meter SPAD (*Soil Plant Analysis Development*) tool to measure leaf greenness related to chlorophyll content. Leaf samples were taken from the third to fifth leaves from the shoots of healthy plants. Each plant was measured at three different points: the leaf's base, middle, and tip. The measurement results were directly read on the SPAD screen in greenness index units. The measurement data were then recorded and averaged for each plant. Each treatment was conducted with three replications. All treatments were replicated according to the number of samples specified in the experimental design. The results of these leaf green observations are used as a physiological indicator of the butterfly pea plant's response to the treatment given.

Data Analysis

The data obtained were analysed using two ways analysis of variance (ANOVA) at a 5 % significance level, followed by the Tukey analysis to determine significant differences between treatments. All data processing was performed using the STAR (*Statistical Tools for Agriculture Research*) program.

RESULTS AND DISCUSSION

Chromosome Analysis

The chromosome analysis results show differences in the chromosome structure of butterfly pea roots after 24 hours of colchicine soaking. In the control treatment (0 mM), the chromosomes appeared regular with a normal number consistent with diploid conditions, indicating the absence of colchicine. Meanwhile, in the colchicine treatments of 0.5; 1.0; and 1.5 mM, the chromosomes appeared thicker and more compact, with differences in number and size, indicating chromosome duplication (Figure 1). In the control treatment (0 mM), chromosomes were clearly separated and evenly distributed, consistent with diploid cells. In contrast, colchicine-treated samples, particularly at 1.0 and 1.5 mM, exhibited thicker, more condensed chromosomes with increased chromosomal overlap and stacking, which are typical cytological indicators of polyploidy. This is consistent with the mechanism of action of colchicine, which inhibits spindle formation during cell division, resulting in chromosome failure and duplication. In 0.5 and 1.0 mM, cells with denser chromosomes were observed, while in 1.0 and 1.5 mM, chromosome stacking was more clearly observed, indicating the formation of polyploid cells marked with red arrows (Figure 1). This condition confirms that higher colchicine concentrations can increase the chance of polyploidy formation in butterfly pea root cells.

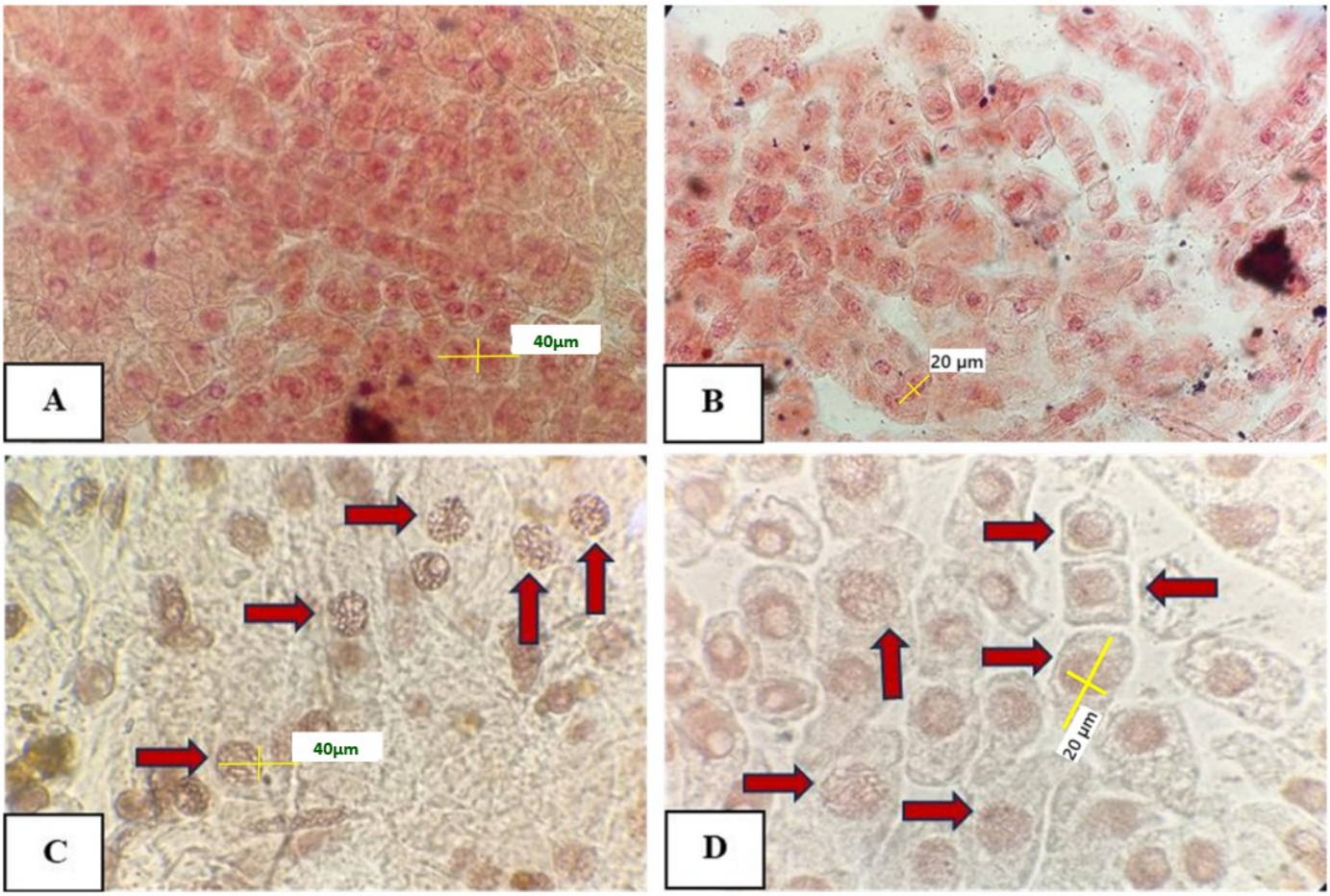


Figure 1. Chromosome structure of blue butterfly pea. (A). Control (0 mM) showing normal cell structure with nuclei that are uniformly distributed, (B). 0.5 mM concentration showed nuclei with early signs of chromatin condensation and irregular nuclear structure, (C). 1 mM, cells exhibited condensed and clustered chromosomes (indicated by red arrows), and (D). 1.5 mM treatment, enlarged cells with denser and thicker chromatin organization after 24 hours soaking in colchicine solution. Marked with red arrows are the polyploid cells.

The presence of polyploid cells is essential because it can increase genetic variation and has the potential to produce plants with superior agronomic characteristics, such as increased organ size, stress resistance, and higher accumulation of secondary metabolites. Refer to Manzoor et al. (2019) found that colchicine is a mitosis inhibitor widely used to induce polyploidization in plants by doubling the number of chromosomes in somatic cells. The mechanism of action of colchicine occurs during cell division, namely by inhibiting the formation of spindle threads so that chromosomes fail to segregate to the poles of the cell. This condition causes chromosomes that should be separated to remain in one cell nucleus, resulting in cells with a higher ploidy level.

The efficiency of chromosome doubling in colchicine-treated plants critically depended on both concentration and exposure time. Previous studies emphasised the importance of a precise balance between colchicine dose and treatment duration to induce polyploidy without causing excessive cytotoxicity (Manzoor et al. 2019). Low concentrations might have failed to disrupt spindle formation effectively, whereas excessively high doses might have inhibited cell division or caused cell death. In this study, the lower concentration (0.5 mM) appeared insufficient to induce stable chromosome doubling, whereas 1.0–1.5 mM colchicine promoted more frequent polyploid formation. This response was consistent with previous findings, which showed that moderate colchicine concentrations induced chromosome duplication by inhibiting microtubule polymerisation during metaphase, resulting in the retention of chromosomes within a single nucleus (Manzoor et al. 2019; Jiang et al. 2022).

Similar patterns reported in *Chrysanthemum* and *Celosia*, where increasing colchicine concentration enhanced polyploid induction efficiency until a toxicity threshold was reached (Manzoor et al. 2019; Jiang et al. 2022). Therefore, the observed response in butterfly pea confirmed that colchicine concentration played a decisive role in determining the success of chromosome doubling and the resulting morphological variation.

Stomatal Analysis

Stomatal Density

Observations of the stomatal density of upper and lower leaf surfaces of butterfly pea plant after 24 hours of soaking showed that colchicine significantly affected changes in stomatal density. Based on Figures 2 and 3, colchicine at a concentration of 1.0 mM resulted in the highest stomatal density in both the adaxial and abaxial leaf surfaces. In the adaxial leaf surface, the control treatment (0 mM) had the lowest stomatal density, while the 1.0 mM treatment produced the highest density, with approximately 31–32 stomata. These findings aligned with a report by Bian et al. (2025), which explained that poly-

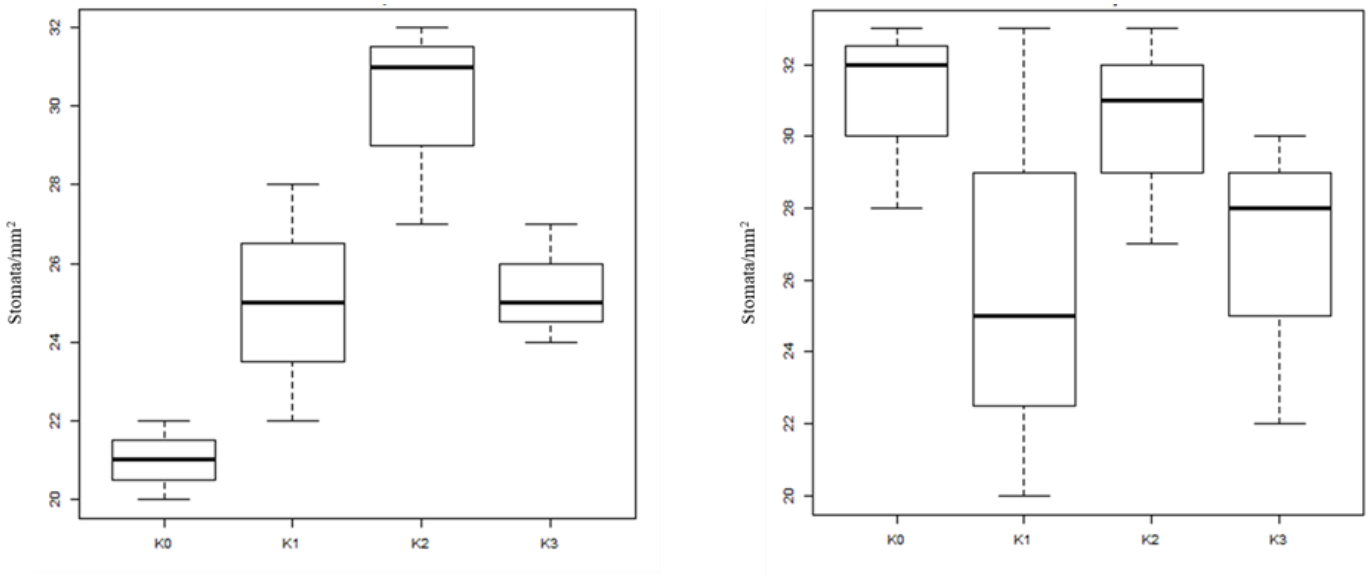


Figure 2. Stomatal density of adaxial leaf surface of butterfly pea at: A. 24 hours and B. 48 hours soaking in colchicine solution.

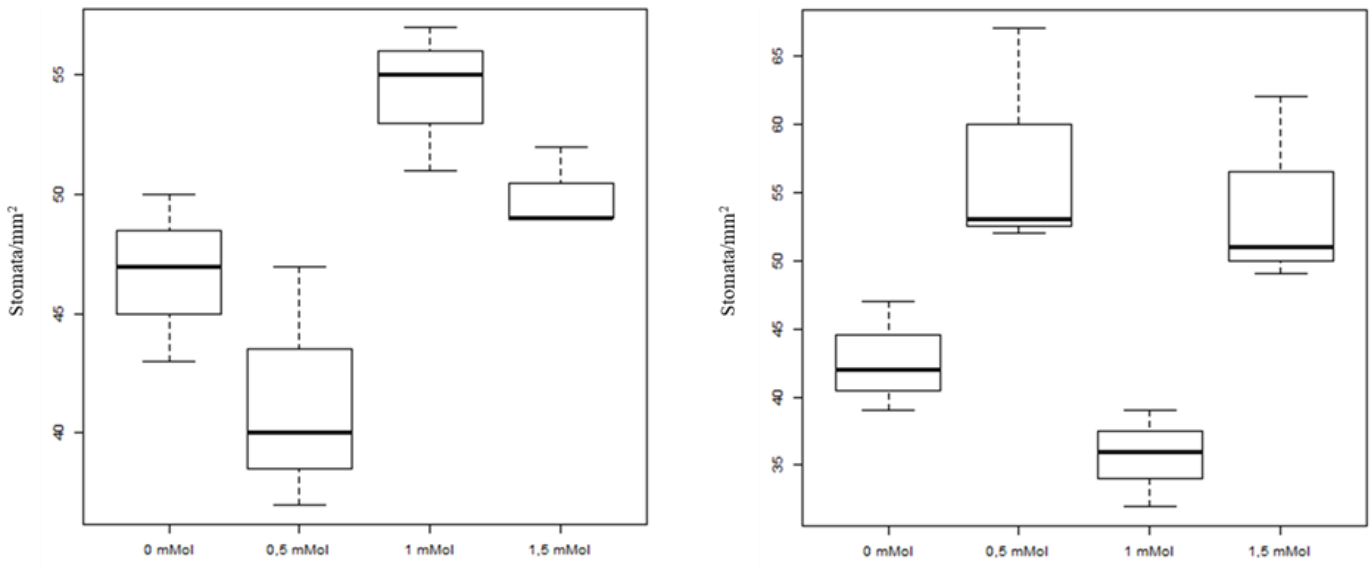


Figure 3. Stomatal density of abaxial leaf surface of butterfly pea at: A. 24 hours and B. 48 hours soaking in colchicine solution.

ploidy induction with colchicine could alter stomatal morphology, including its size and distribution, depending on the concentration and duration of treatment. Figure 4 showed that stomatal density on the abaxial leaf surface was higher than on the adaxial part. Increased stomatal density was associated with successful polyploidisation, which physiologically supported photosynthetic efficiency by increasing the surface area for gas exchange. Research by Abdolinejad et al. (2021) and Liu et al. (2021) also confirmed that colchicine effectively induced polyploidy in various plant species, in which physiological changes such as stomatal size and density were key indicators of successful polyploidisation. These results reinforced previous findings that appropriate colchicine concentrations, specifically 1.0 and 1.5 mM, played a crucial role in increasing stomatal density and could be used as a reference for developing cytogenetic techniques in butterfly pea plants.

Stomatal Length

The boxplot of stomatal length showed differences depending on the colchicine concentration and soaking duration. The length of stomata on the adaxial leaf surface of butterfly plants soaked in 24 hours at the concentrations of 0.5; 1.0; and 1.5 mM was higher than control (Figure 5). However, if the immersion was extended to 48 hours, the length of the stomata was shorter than the control. Stomatal length of abaxial leaf surface in all colchicine concentration for 24 hours was shorter than control. Meanwhile, the concentration colchicine of 1.0 mM at 48 hours was able to increase the stomatal length significantly compare to control (Figure 6). This indicates that concentration and soaking duration influence colchicine's effect on stomatal morphology. Similar results were found in studies on *Glycyrrhiza glabra* and *Carthamus tinctorius*, where colchicine treatment significantly increased stomatal length (e.g., from 85 nm in the control to 182 nm in the 0.1 % treatment) (Moghbel et al. 2015). Research conducted by Wu et al. (2022) on *Populus hopeiensis* showed that higher ploidy levels (octaploid versus tetraploid) resulted in longer and wider stomata, although with lower stomatal density per area. In butterfly pea plants treated with colchicine, increased stomatal length indicates the effect of polyploidisation, in which stomatal cells become larger when chromosomes double.

Table 1 showed an interaction between soaking time and colchicine

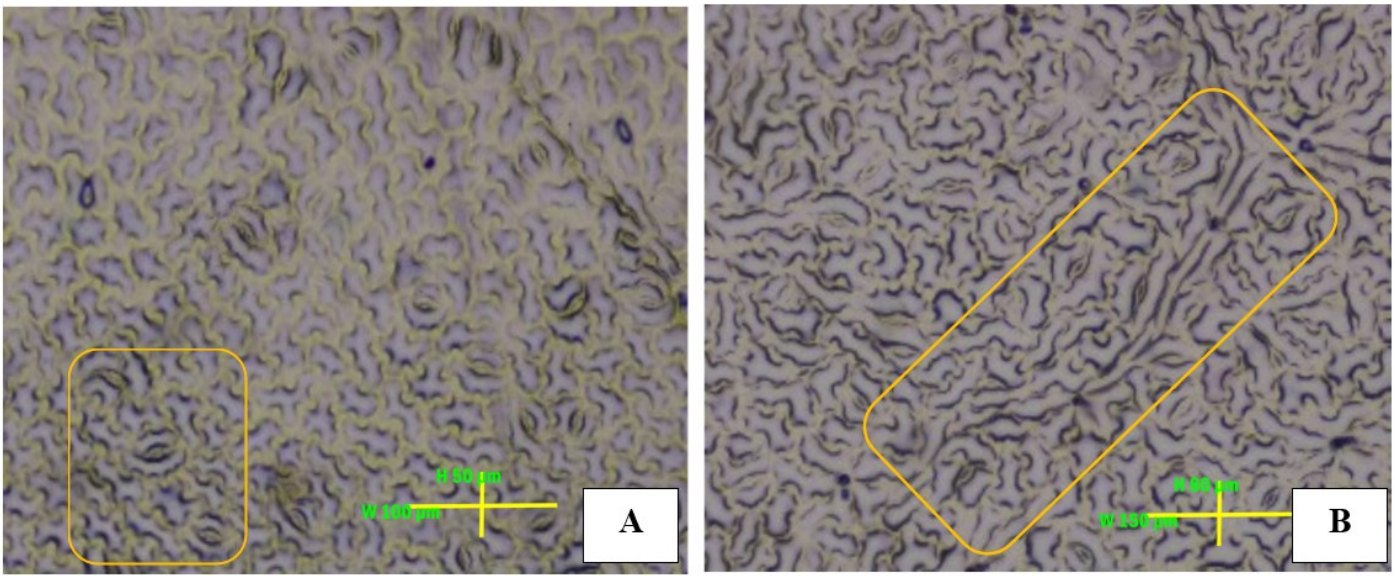


Figure 4. Stomatal density and epidermal cell pattern of butterfly pea leaf surface under different treatments. (A) Leaf epidermis showing higher stomatal density, illustrated with smaller epidermal cells and reduced spacing between stomata (highlighted in the marked square). (B) Leaf epidermis showing lower stomatal density, characterized by larger epidermal cells and wider stomatal spacing (highlighted in the marked square).

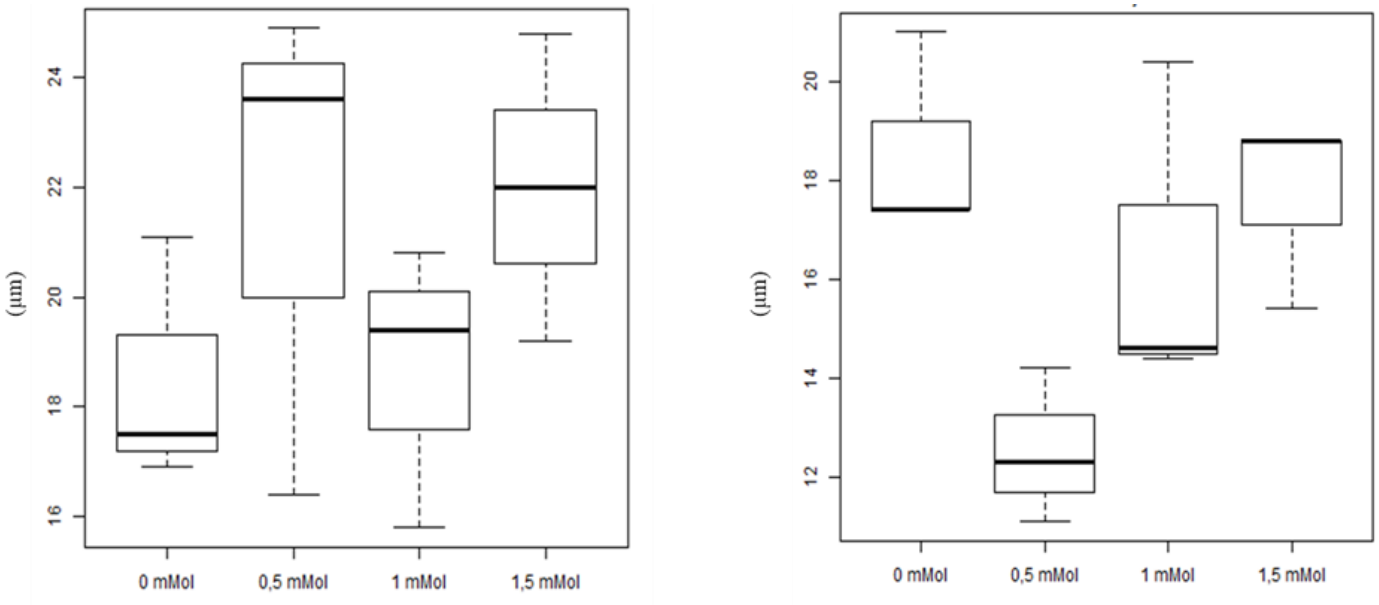


Figure 5. Stomatal length of adaxial leaf surface of butterfly pea at: A. 24 and B. 48 hours of colchicine soaking.

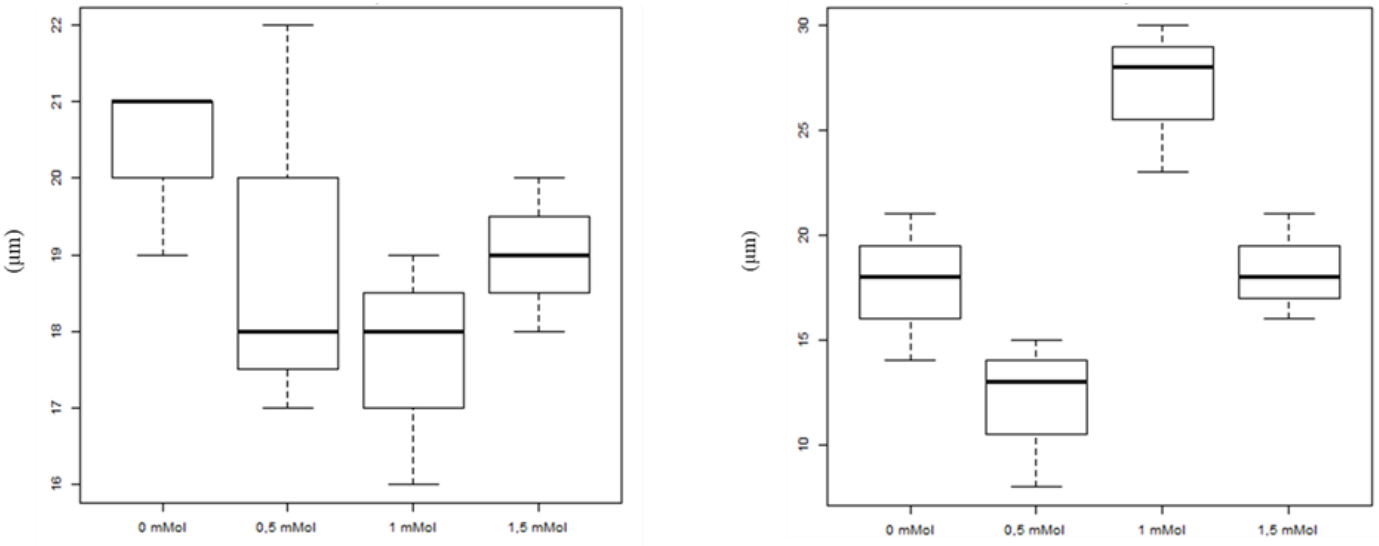


Figure 6. Stomatal length of abaxial leaf surface of butterfly pea at: A. 24 and B. 48 hours colchicine soaking.

concentration. The highest stomatal length on the abaxial leaf surface of butterfly pea was found at a concentration of 0.5 mM and 48 hours, namely 57.33 µm. This stomatal length was significantly different from concentration 0.5 mM in 24 hours soaking. However, the stomatal length was not statistically significantly different from the control. It seems that the role of colchicine is not very significant in increasing the length of the stomata of butterfly pea plants.

Table 1. Interaction of soaking time and colchicine concentration on the length of stomata on the abaxial of butterfly pea leaves.

Soaking time (Hours)	Concentration of colchicine (mM)			
	0	0.5	1	1.5
24	46.67[a]	41.33[b]	54.33[a]	50.00[a]
48	42.67[a]	57.33[a]	35.67[b]	54.00[a]

The interaction between colchicine concentration and soaking duration significantly influences stomatal morphology because both parameters determine the degree of chromosomal disruption during mitosis. Prolonged exposure allows colchicine to penetrate deeper into meristematic tissues,

increasing the probability of spindle inhibition and polyploid cell formation (Manzoor et al. 2019). In this study, variations in stomatal length across different treatment combinations indicate that polyploidisation occurred unevenly among cells, resulting in mixed ploidy levels. Similar interactions have been reported in *Carthamus tinctorius* and *Glycyrrhiza glabra*, where stomatal size varied significantly depending on both colchicine concentration and exposure time (Moghbel et al. 2015). Such interactions suggest that optimal polyploid induction is achieved not merely by increasing concentration, but by balancing exposure time to allow chromosomal duplication without inducing excessive cellular damage. These findings align with previous studies demonstrating that polyploid responses are highly dependent on treatment intensity and duration (Manzoor et al. 2019).

Stomatal Width

Based on the observations, the stomatal width of the adaxial and abaxial leaf surfaces of the butterfly pea plant showed variation influenced by colchicine concentration and soaking duration. At 24 hours, the 1.0 mM treatment tended to produce larger stomatal width than the control (0 mM), while at 48 hours, the pattern changed, with several treatments showing a decrease in median values and a narrowing of the data distribution (Figures 7 and 8).

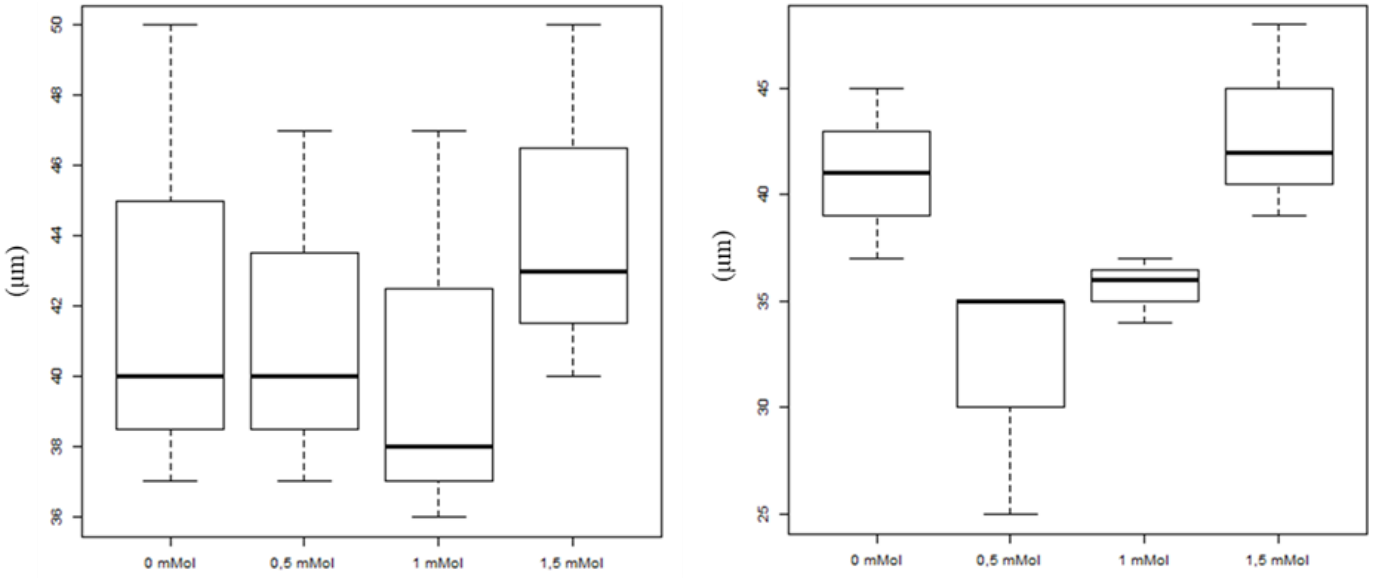


Figure 7. Stomatal width of adaxial leaf surface of butterfly pea at: A. 24 and B. 48 hours of colchicine soaking.

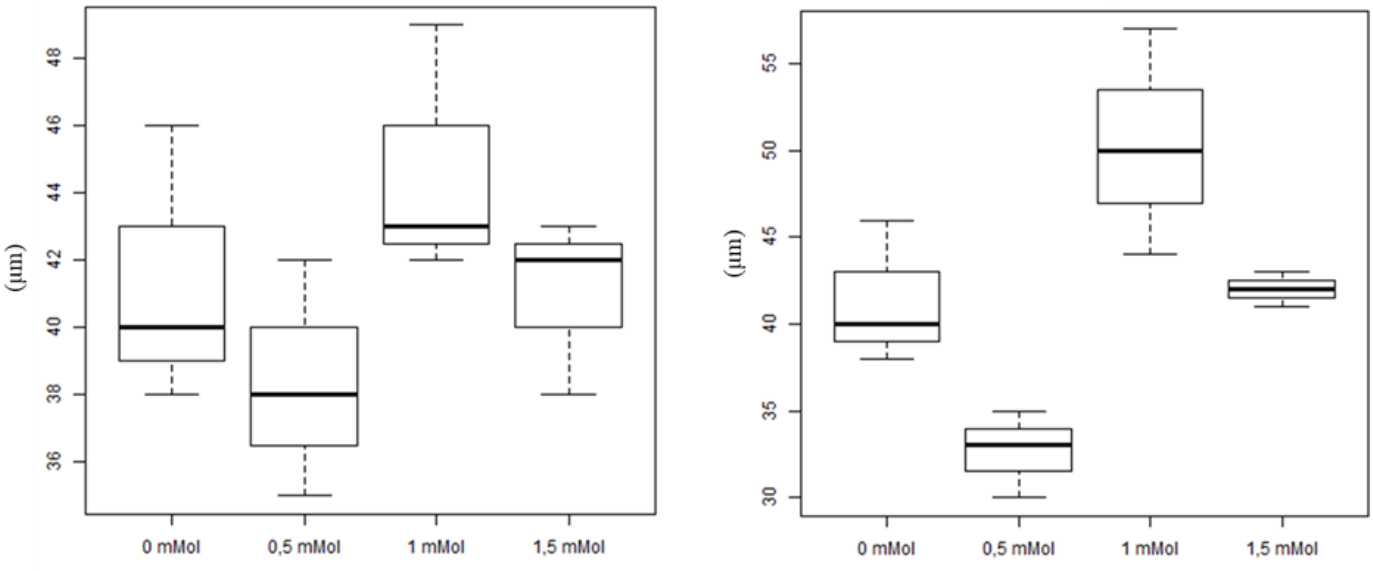


Figure 8. Stomatal width of abaxial leaf surface of butterfly pea at: A. 24 and B. 48 hours of colchicine soaking.

This indicates that colchicine can induce stomatal cell enlargement due to polyploidization, but this response is highly dependent on the dose and duration of treatment. According to Moghbel et al. (2015) in *Carthamus tinctorius* and *Glycyrrhiza glabra*, colchicine treatment increased stomatal dimensions (length and width) compared to the control, although their density decreased. Plants with higher ploidy levels (tetraploid–octaploid) exhibited larger stomata with significantly higher widths, although the number of stomata per unit area decreased. Although polyploid plants often exhibited reduced stomatal density due to increased cell size, the present study recorded an increase in stomatal number in some colchicine treatments. This phenomenon was also reported in other plant species, indicating that stomatal density responses to polyploidy were species-specific.

In some cases, polyploidisation stimulated the formation of additional epidermal cells or altered epidermal patterning, leading to increased stomatal frequency despite enlarged cell size (Wall et al. 2022). Such responses might have represented a physiological adjustment to maintain efficient gas exchange and photosynthetic capacity in leaves with altered cellular architecture. Additionally, changes in hormonal regulation and epidermal cell differentiation following chromosome doubling may have contributed to increased stomatal initiation. Therefore, the elevated stomatal density observed in butterfly pea may have reflected adaptive anatomical plasticity rather than a deviation from typical polyploid developmental patterns. (Wall et al. 2022). These data confirm that colchicine treatment of butterfly pea effectively increases stomatal size, particularly width, which may have implications for gas exchange and photosynthetic efficiency.

Chlorophyll Content

Chlorophyll content of butterfly pea after 24 hours of colchicine soaking showed that increasing the concentration of colchicine 0.5 to 1.0 mM tended to increase the chlorophyll content of butterfly pea plants (6.27-7.23) and decreased at a concentration of 1.5 mM (5.40). However, statistically, the chlorophyll content was not significantly different between the control and those given colchicine. After 48 hours, the chlorophyll content of butterfly pea plants showed a very significant increase at 1.5 mM colchicine, namely 8.50 (Table 2) and significantly different compared to the control (5.00). These results indicate that prolonged soaking duration combined with higher colchicine concentration had a pronounced effect on chlorophyll accumulation in butterfly pea leaves. Research by Rafique et al. (2018) showed that colchicine soaking treatment can increase chlorophyll content at low to medium doses due to the stimulation of photosynthetic activity and more optimal nutrient absorption. However, chlorophyll content tends to decrease at high doses and longer soaking times due to physiological stress, characterized by the accumulation of free radicals and disruption of green leaf pigment metabolism.

In this study, higher chlorophyll content observed at specific colchicine concentrations indicates improved photosynthetic potential. Similar results have been reported in polyploid plants where chlorophyll accumulation increased following chromosome duplication (Jiang et al. 2022). Furthermore, demonstrated that moderate chemical treatments can stimulate chlorophyll biosynthesis by improving metabolic efficiency and pigment stability, whereas excessive stress reduces chlorophyll content. Polyploid cells typically contain more chloroplasts per cell, which contributes directly to higher chlorophyll concentration and darker leaf coloration. Therefore, the increased chlorophyll levels observed in colchicine-treated butterfly pea are consistent with previous findings showing that polyploidisation enhances photosynthetic capacity through structural and physiological modifications (Rafique et al. 2018; Jiang et al. 2022).

CONCLUSIONS

Colchicine treatment was effective in increasing the chromosome number in butterfly pea (*Clitoria ternatea* L.) cells, indicating the induction of polyploidy. A colchicine concentration of 1.0 mM with a 24-hour soaking was identified as the optimum treatment for modifying stomatal characteristics, as it significantly influenced stomatal density as well as stomatal length and width in both adaxial and abaxial eaf surfaces. In contrast, a 1.5 mM colchicine treatment for 48 hours resulted in a significant increase in chlorophyll content, reaching approximately 1.5 times higher than the control, and can be considered the optimum condition for enhancing leaf physiological performance. The polyploid butterfly pea plants obtained in this study show potential as breeding materials for the development of plants with improved physiological traits. In addition, the observed improvements in stomatal characteristics and chlorophyll content suggest their potential use in secondary metabolite production, particularly through further breeding programs or plant cell culture approaches.

AUTHOR CONTRIBUTION

S. designed the research, supervised the entire process, and manuscript revision. A.S.H. collected and analysed the data, P.N.A. wrote the manuscript, and the article was published.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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