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Stevia Local Tawangmangu Generation M1 Result of Oryzalin Treatment

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Abstract. *Stevia rebaudiana Bert. (Stevia) is used in the commercial and health industries because of its steviol glycosides (stevioside, rebaudioside A, rebaudioside C) and flavonoids. Polyploidy induction of stevia plants using oryzalin was held to increase the diversity and produce superior varieties. This research aims to obtain stevia diversity with different characteristics from its diploid. The Tawangmangu variety of stevia seedlings was experimentally designed using a completely random design. Nine combinations of oryzalin concentrations (1.5, 2.5, and 3.5µM) and immersion times (4, 6, and 8 hours) treatments were applied to 15 stevia seedlings each. Stevia without treatment was used as a control. Treatments were applied directly to the shoots on the second internode from the tip. Observations on target shoots included the number of survivors, morphological variables (height, number of internodes, internode length, leaf size, leaf thickness, stem diameter, and leaf color), stomata, and plant ploidy level. Data analysis on the number of live plants used frequency and descriptively. Morphological data consisting of plant height, number of shoots, number of internodes, internode length, leaf length, leaf width, leaf thickness, and stem diameter were analyzed using boxplot graphs and descriptive to describe the diversity of M1 stevia treatment results. Leaf color and stomata were analyzed descriptively. The results showed that up to 3.5µM concentrations of oryzalin and 6 hours of immersion time were safe to use as a mutation agent with above 67% survival rate. Various oryzalin treatments of Tawangmangu stevia varieties yielded polyploidy morphological growth indications in height, number of internodes, internode length, stem diameter, leaf size, leaf thickness, leaf color, stomata, and stem diameter. In addition, there are growth variations such as chimeras, rosettes, and leaf splitting. However, further flow cytometry tests showed that oryzalin concentration and immersion duration directly on the vegetative material did not produce polyploid stevia individuals.*

Keywords: *antimitotic, concentration, immersion time, microtubulin inhibitor, polyploidization.*

Citation
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INTRODUCTION

Stevia (*Stevia rebaudiana* Bert.) is a natural sweetener plant used as a sugar substitute in commercial and healthcare industries that is widely used for illness therapy because it has anti-cancer, anti-hypersensitive, anti-hyperglycemic, and anti-microbial activities. Steviol glycosides content in stevioside, rebaudioside A, and rebaudioside C forms of stevia has a sweetness 200 to 300 times higher than cane sugar. Stevia also contains total flavonoids with high antioxidant activity (Amarakoon, 2021; Moongngarm et al., 2022; Peteliuk et al., 2021).

Stevia grows optimally in semi-humid areas with an average temperature of 6°C and rainfall of 1500-1800 mm/year. In a suitable environment, the flowering age of stevia occurs about 50 to 100 days after planting. Stevia is harvested at the flowering phase when its steviol glycol content reaches peak levels. Stevia could be propagated using seeds, vegetatively, and tissue culture, but propagation by seeds has some major constraints, namely low production, viability, and germination of stevia seeds. Vegetative propagation with stem cuttings is also not easy because the emergence and growth of shoots are influenced by several factors, such as the part of the plant used, the length of the cuttings, the number of internodes of the cuttings and the time of making the cuttings (season). The most widely practiced stevia propagation is through tissue cultures (Gunasena et al., 2021). The increasing demand for stevia should be balanced with improvements in planting materials and cultivation techniques so that stevia yields an increase (Amarakoon, 2021). awangmangu local variety of stevia has stevioside levels of 11.38-16.09% and Rebaudioside A 1.37-1.85%. The stevioside content of local varieties is within the range of stevioside stevia from Paraguay, which is 5.1-21%, while the rebaudioside A content of local varieties is lower than the range of rebaudioside A stevia from Paraguay (0-12%) (Penner et al., 2004). Efforts are needed to improve the quality of stevia.

Planting material improvements could be done using the superior seeds obtained from the results of crosses, induction of mutations, and assembly of the new high-yielding varieties due to high biomass yields and levels of secondary metabolite compounds. Crossbreeding of stevia is difficult due to stevia's self-incompatibility, leading to a low probability of successful self-pollination. In addition, obtaining viable seeds is challenging due to low germination capacity (Sinta et al., 2018). Plant breeding with polyploidization, irradiation, or genome editing is one of many solutions to increase plant diversity and seed availability in plants that are difficult to develop conventionally. Several plant mutation induction (polyploidization) could produce polyploid plants with higher growth and secondary metabolite compound content (Adabiyah et al., 2019; Gantait & Mukherjee, 2021).

Polyploid plants experience chromosome number changes from their origin due to mutation induction. There are two kinds of mutation induction agents (mutagens), namely physical mutagens (gamma rays, x-rays, beta rays, and UV rays) and chemical mutagens (EMS, DES, colchicine, and oryzalin) (Ermayanti et al., 2018; Lestari, 2021). Research related to mutation induction effect on the content of plant secondary metabolite compounds was carried out at *Dendrobium officinale* (Pham et al., 2019), *Solanum bulbocastanum* (Caruso et al., 2013), *Physalis peruviana* L. (Çömlekçioğlu & Özden, 2020), *Melissa officinalis* (Talei & Fotokian, 2020), *Capsicum frutescens* (Pliankong et al., 2017), *Artemisia cina* (Kasmiyati et al., 2020) and *Cnidium officinale* (Kim et al., 2021). Oryzalin is a safer mutation agent because it does not inhibit the vegetative growth of plants (Surya et al., 2016). Mutation induction using oryzalin in local Tawangmangu stevia plants is expected to increase the diversity of stevia plants with properties different from those of diploid stevia plants.

MATERIALS AND METHODS

This study used one-month-old Tawangmangu local variety of stevia seedlings with a height of about 10 cm. The experimental research used a completely randomized design (CRD) with two factors, namely oryzalin concentration (1.5, 2.5, and 3.5 µM) and immersion time (4, 6, and 8 hours (h)). This study used nine combination treatments and the untreated stevia plants as control. Each treatment consisted of 15 individuals. Treatments were applied on the second internode from the top, and the observations were performed on the shoots that grew from the internode. Research conducted in Innovation Agency in Tawangmangu from January to April 2023.

Morphological observations were made once a week on each stevia from 14 days after planting (dap) to 60 dap. The observations were performed on live and healthy plants (not attacked by pests and diseases). The observation variables included the number of living plants, plant height, number of internodes, internode length, leaf length, leaf width, leaf thickness, stem diameter, and leaf color. The leaf color was observed using a Colorimeter (Research Lab Tools) Smartphone-based app (Arunachalam et al., 2022). Stomatal observations were performed using an Olympus CX22LED microscope with a built-in camera after stevia was three weeks old. Stomatal preparation was done by taking one leaf (third leaf from the tip or shoot of the plant) from each treatment at 09.00 am. The leaf surface was smeared with clear nail polish and allowed to dry. The dried polish was attached to a clear tape and peeled off. The specimen on the tape was attached to the glass object and then observed (Rohmah, 2019; Sinta et al., 2018). The number of live plant data was analyzed using frequency and descriptive analysis. Plant morphological data such as plant height, number of shoots, number of internodes, internode length, leaf length, leaf width, leaf thickness, and stem diameter were analyzed using boxplots and descriptive graphs. Data on leaf color and stomata were analyzed descriptively. The analysis was carried out to see the diversity of stevia plants due to the application of oryzalin treatment.

RESULTS AND DISCUSSION

Percentage of Survive Plants in Various Oryzalin Treatments

Differences in oryzalin concentration and immersion time used as a treatment for stevia mutation induction resulted in different numbers of surviving stevia. About 66.43% of the entire 135 treated stevia individuals survived. The highest survival was obtained in the treatment combination of 1.5µM-4 hours and 2.5µM-4 hours (14 individuals each). The least survival was obtained in the combination of 3.5µM-8h (7 individuals). The survival of treated stevia tended to be lower than the control (without treatment). The tendency to decrease stevia survival occurred as the concentration of oryzalin as a mutation agent and the length of immersion of stevia buds increased (Table 1).

The treatment combination of oryzalin concentration and immersion time used in this study is safe enough to be used on stevia plants with the number of deaths below 50%. Large concentrations of oryzalin as the agent used, immersion time, and the interaction between concentration and immersion time cause a decrease in the ability of plants to survive (Erboğa et al., 2021; Ridwan & Witjaksono, 2020; Wang et al., 2020). The type, part, and size of the treated plant affect the survival ability of the plant (de Carvalho et al., 2016). Zahumenická et al. (2018) stated that high concentrations of antimitotic agents and longer application times caused a decrease in the viability rate of Anemone sylvestris plants. However, the use of oryzalin was safer than colchicine.

Table 1. Percentage of live stevia in the combined treatment of oryzalin concentration and soaking time after 60 days after planting (dap)

Treatment	Initial Amount	Death	Living Percentage(%)
Control (no treatment)	15	0	100
1.5µM-4hours	15	1	93
1.5µM-6hours	15	5	67
1.5µM-8hours	15	7	53
2.5µM-4hours	15	1	93
2.5µM-6hours	15	5	67
2.5µM-8hours	15	4	73
3.5µM-4hours	15	6	60
3.5µM-6hours	15	5	67
3.5µM-8hours	15	8	47

Morphology of stevia plants

The treatment of oryzalin concentration and immersion time influenced all growth organs of the local variety of stevia plants, including plant height, number of internodes, internode length, and stem diameter (Figure 1), as well as leaf length, leaf width, and leaf thickness (Figure 2). Almost all treated stevia plants had lower height, number of internodes, internode length, and stem diameter than untreated stevia (control).

Plant height in the 2.5µM-8h treatment has homogeneous data compared to other treatments and has an outlier with a value lower than the control median (20 cm), which is 18.5 cm (sample 11). The treatment of 3.5µM-6h has extreme data, which is 30.80 cm (sample 1) higher than the control median (20 cm). The internode of control is higher than the treated stevia. The lowest internodes mean value was obtained in the 1.5µM-6h treatment (4 internodes), while the lowest median was obtained in the 1.5µM-6h and 3.5µM-8h treatments (3 internodes). The data distribution of the 3.5µM-6h treatment was smaller than that of other treatments. The highest data distribution was found in the 3.5µM-8h treatment. The 2.5 µM-6h treatment had 1 outlier whose value was higher than the median of the control (9 internodes), which was 11 internodes (sample 1).

The data distribution of internode length of control stevia was the smallest compared to the treated stevia. Stevia treated with 1.5µM-8h and 3.5µM-8h had the same median value, which was higher than the control, which was 2.2 cm and 2.0 cm, respectively. The lowest median was found in the treatment of 2.4µM-8h, which was 1.1 cm. The treatment of 1.5µM-4h had one extreme data, which was 8 cm (sample 11). The treatment of 2.5µM-8h had one outlier data, which was 5 cm (sample 8), higher than the median of control stevia. Treatments of 2.5µM-6h and 2.5µM-8h had data distribution below the median of the control (Figure 1). The stem diameter of the treated stevia tended to be smaller than the control stevia. Outlier data above the median was found in the treatment of 1.5µM-4h sample 5 (0.19 cm), treatment of 2.5µM-4h sample (0.22 cm), and treatment of 3.5µM-4h sample 1 (0.24 cm), while in extreme data above, the median was found in the treatment of 2.5µM-8h sample 5 (0.3 cm), treatment of 3.5µM-6h sample 1 (0.2 cm) and treatment of 3.5µM-8h sample 3

(0.15 cm) (Figure 1).

As a mitotic division inhibitor, Oryzalin was applied to actively dividing plant parts (seeds, stem internodes, shoots, and callus), causing α-tubulin instability. Oryzalin binds to plant tubulin at concentrations below 500 nM. The inhibition that occurs in α-tubulin causes structural changes at the endoplasmic reticulum (ER) and golgi apparatus of plants, leading to plant morphological changes (Handayani et al., 2017; Langhans et al., 2009; Morejohn et al., 1987; Touchell et al., 2020). The combination of oryzalin concentration and immersion duration inhibits the vegetative growth rate of plants and can cause plant growth to become stunted. High concentrations of Oryzalin are toxic to plants and cause disruption of plant growth (de Carvalho et al., 2016; Handayani et al., 2023; Silalahi et al., 2020).

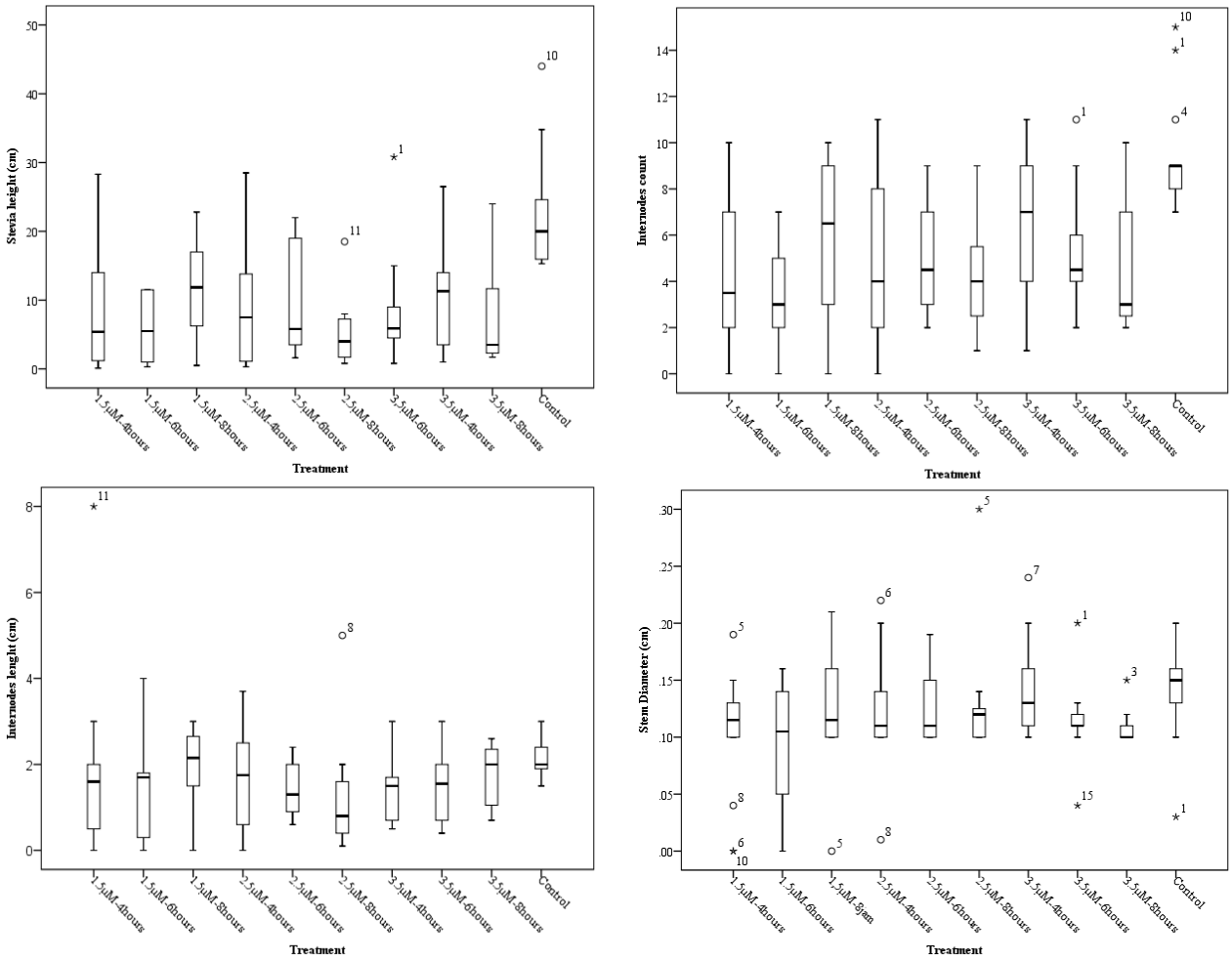

Figure 1. The effect of the combination of oryzalin concentration and immersion time on plant height, number of stevia internodes, internode length, and stem diameter at 60 days after plant.

Jurnal Biodjati 9(1): 102-116, May 2024

JURNAL BIODJATI

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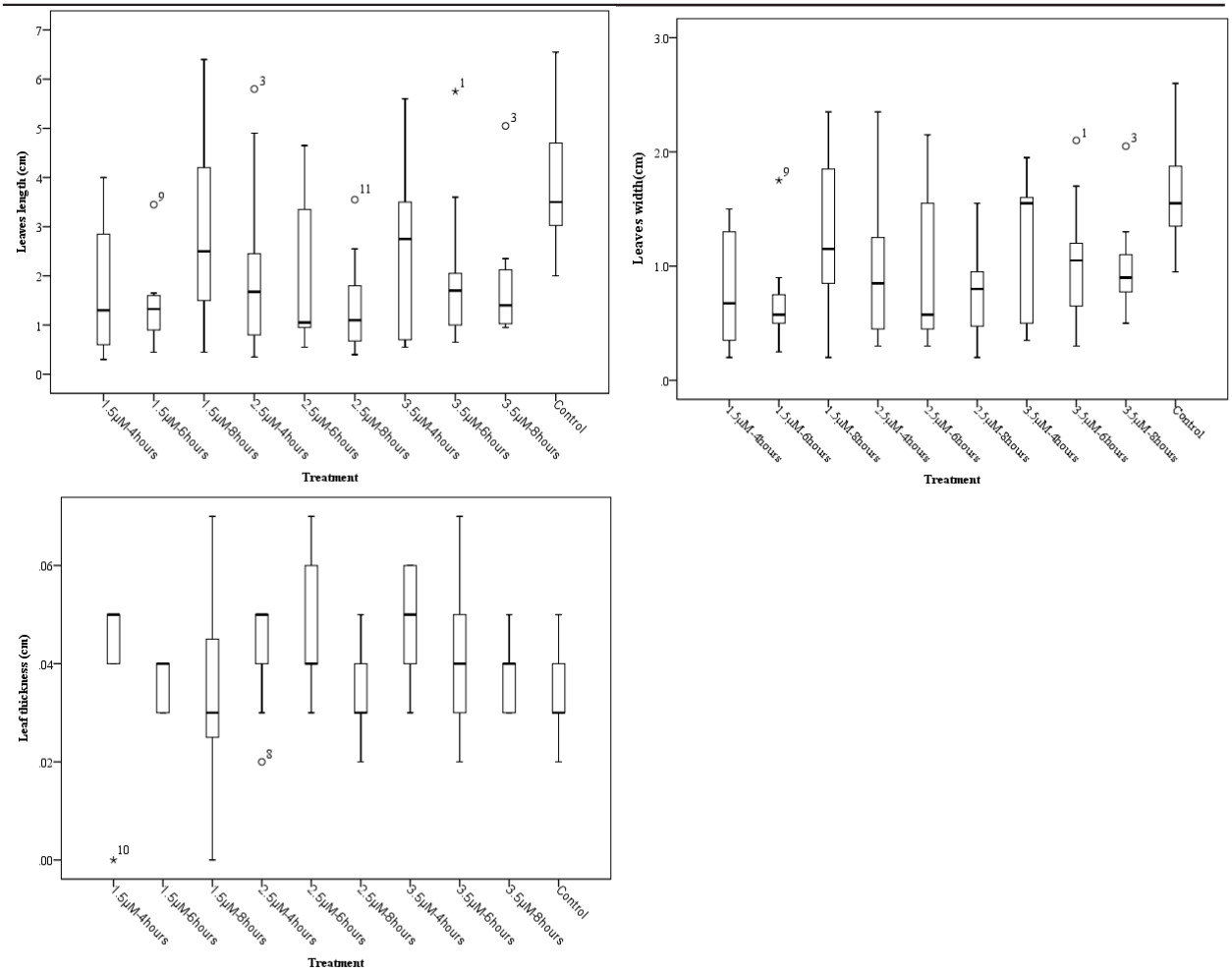
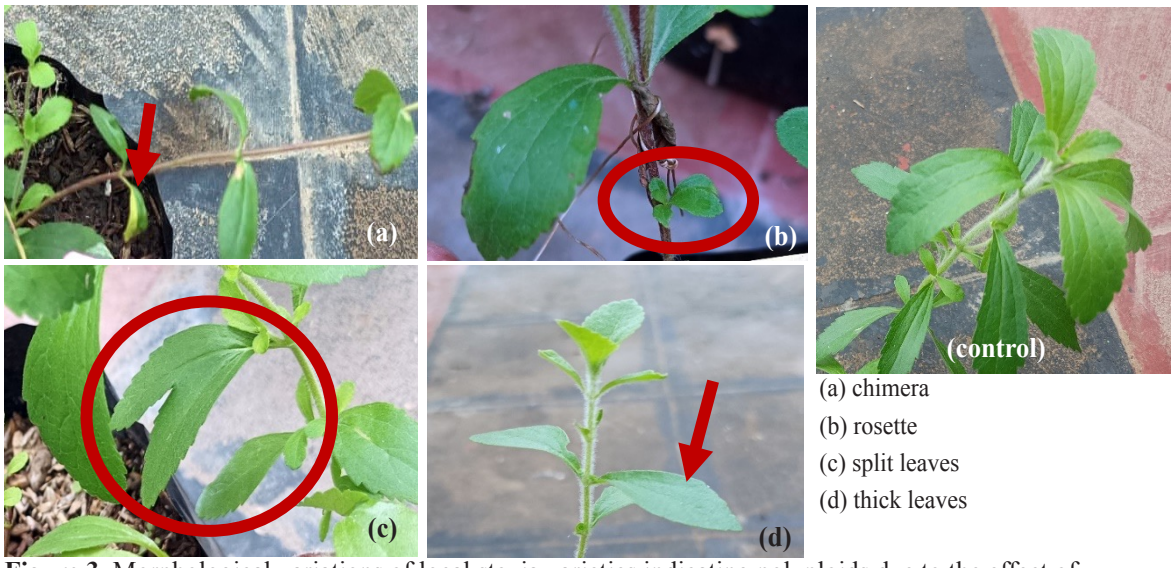


Figure 2. Effect of oryzalin concentration and immersion time on leaf length, leaf width, and leaf thickness of stevia at 60 days after plant



(a) chimera
(b) rosette
(c) split leaves
(d) thick leaves

Figure 3. Morphological variations of local stevia varieties indicating polyploids due to the effect of concentration and immersion duration of oryzalin

The height, internode number, internode length, and stem diameter growth of the treated stevia tended to be lower than the control plants. Rahman et al. (2017), in their research, state that the use of oryzalin produces tetraploid Artemisia annua with lower height and stem growth than diploid, while colchicine causes a decrease in tetraploid height and stem growth compared to diploid. Wen et al. (2022) stated that dwarfing growth is one of the most visible phenotypic expressions in tetraploid garlic compared to diploid. However, some treatment combinations cause higher growths than control plants, especially in stem diameter parameters (Deans et al., 2021; Friska & Daryono, 2017). Yadav et al. (2013) stated that autotetraploid stevia experienced a decrease in leaf internode length. The morphology of stevia plant growth in the parameters of plant height, number of internodes, internode length, and stem diameter shows an indication of polyploidization.

Most treated stevia leaf length data was smaller than the control (Figure 2). Outliers data were obtained in the 1.5µM-6h treatment sample 9 (3.5 cm), 2.5µM-4h treatment sample 3 (5.8 cm), and 3.5µM-8h treatment sample 3 (5.1 cm). The outlier found in the 1.5µM-6h treatment has a value below the median of the control. In addition to the outlier value, one extreme data was obtained in 3.5µM-6h treatment individual 1 with a leaf length of 5.8cm. The leaf width of the 2.5µM-8h and 3.5µM-6h treatments had normal distribution, the 3.5µM-4h treatment had negative distribution, and the other treatments had positive distribution. Outlier data were obtained in the 2.5µM-8h treatment in samples 14 (leaf width 1.6 cm) and 11 (leaf width 1.6 cm), 3.5µM-6h treatment in sample 1 (leaf width 2.1 cm) and 3.5µM-8h treatment in sample 3 (leaf width 2.1 cm). Extreme data were obtained in the 1.5µM-6h treatment in sample 9 (leaf width 1.8 cm). Figure 2 also shows that the leaf thickness of treatments 1.5µM-8h, 2.5µM-6h, and 3.5µM-6h had positive distribution. The treatments of 1.5µM-4h, 1.5µM-6h, 2.5µM-4h, 3.5µM-4h, and 3.5µM-8h had negative distribution. Normal distribution was obtained in the 2.5µM-8h treatment. Most treatments produced higher leaf thickness than control stevia with extreme data and outliers smaller than the control median. Morphological variations of stevia plants also showed a significant difference in leaf thickness.

Some treated stevia plants showed indications of polyploidy, as stated by Yadav et al. (2013), Zhang et al. (2020), and Zahumenická et al. (2018) that tetraploid plants have higher leaf length, width, and thickness than their diploid counterparts. The application of oryzalin to watermelon conducted by Bae (2020) resulted in changes in leaves to be smaller, thicker, and wrinkled on the leaves, small, thick, and wrinkled in tetraploid watermelon. The increase in leaf thickness is due to the thickening of the mesophyll layer (a layer that fills almost half of the total leaf thickness) (Zeng et al., 2019).

Stevia stomatal

The oryzalin treatment also affected stevia stomata (Table 2). Almost all concentrations of oryzalin at three immersion durations affected stevia's size in terms of length and diameter. The oryzalin treatment also affected the epidermal cells of the stomata, and there was a tendency to thicken the epidermal cells of the stomata of the treated stevia leaves compared to the control stevia leaves. Larger stomatal size in polyploid plants with lower stomatal density had been reported by Zhou et al. (2017) on cassava plants. Tang et al. (2010) stated that stomatal observation is one of the rapid and

effective methods for estimating tetraploid plants before transplantation to the field.

Stevia plants that were oryzalin-induced showed differences in morphological characteristics from control plants. One of the morphological characteristics seen is the difference in the size of the induced stevia stomata with the control, and some treatments show differences in stomatal density. It is in line with the results of research (Rahmi et al., 2019; Ridwan et al., 2018; Ridwan & Witjaksono, 2020; Wang et al., 2020). As the microtubules destabilizer (microtubules inhibitor), Oryzalin disrupts and inhibits the function of microtubules, consequently inhibiting plant growth. Microtubules are fundamental components that support the diverse morphology and dynamics of various cell types, cell cycles, and developmental stages of plants and animals. Most microtubule inhibitors produce cytotoxicity in plant and animal cells. Oryzalin causes aberrant growth with milder effects than higher microtubule depolymerizing agents and also induces anisotropic expansion of epidermal cells (Ishida et al., 2021; Roll-Mecak, 2020). Stomata are one of the plant characters affected by chromosome manipulation and the polyploidization process, where changes in stomatal size and density due to polyploidization can affect the photosynthetic process of plants (Handayani et al., 2023).

Erboğa et al. (2021) stated that oryzalin usage increased the length and width of the leaves stomatal and decreased the stomatal density. The chromosome number increased due to polyploidization increases in the stomatal size, which caused decreases in the stomatal density. Tetraploid plants have an increase in the number of chloroplasts in stomatal guard cells twice from diploid. Thus, stomatal density and size can serve as an index to distinguish tetraploid L. ruthenicum from its diploid because the size and density of stomata, as well as the frequency of chloroplast content of polyploidized plants, are inversely proportional to their ploidy level (Kara & Doğan, 2022; Rao et al., 2019). In this study, the treatment of oryzalin concentration and immersion time caused increases in the stomatal size but did not affect the stomatal density of stevia leaves. There was no increase in the size of stomatal guard cells and the size of non-stomatal epidermal cells (Figure 4). It agrees with Šmarda et al. (2023) that the stomatal density of the polyploidization effect is not always lower, although most are lower than the diploid.

Table 2. Effect of concentration and duration of oryzalin immersion on stomata of some morphologically polyploid stevia plants

Treatment	Stomata length	Stomatal diameter	Notes
Control	48.55	38.85	Normal
1.5µM-4hours (5)	51.99	42.73	no epidermal thickening occurs
1.5µM-6hours (13)	48.55	38.85	epidermal thickening occurs, cells are damaged
1.5µM-8hours (14)	61.10	38.91	epidermal thickening occurs
2.5µM-4hours (3)	55.98	42.39	epidermal thickening occurs, cells are damaged
2.5µM-6hours (3)	64.43	50.57	epidermal thickening occurs
2.5µM-8hours (15)	52.18	42.04	epidermal thickening occurs
3.5µM-4hours (12)	61.57	49.10	epidermal thickening occurs
3.5µM-6hours (15)	55.67	40.78	no epidermal thickening occurs
3.5µM-8hours (5)	50.09	40.80	epidermal thickening occurs

Leaf colors

The various concentrations of oryzalin with three different immersion times influenced the color of stevia leaves (Table 3). The control stevia had a dark sea green leaf color, while the treated stevia had 14 different leaf colors from the control leaf. The three most common stevia leaf colors were dark sea green (29 individuals), asparagus green (19 individuals), and smoke green (16 individuals). It is presumed that the color difference in stevia treatment results was affected by pigments or compounds contained in stevia leaves. One of the organs that affected the green color of the leaves was the stomata, which contain green pigments (chlorophyll). Zeng et al. (2019) stated that the doubling chromosomes due to polyploidization causes modification of vegetative characteristics such as a decrease in plant height, thicker leaves with a darker green color, and an increase in root diameter accompanied by root number and length decrease. The dark green color of leaves treated with mutation agents can be used as an indicator that the polyploidization process occurs in plants due to the presence of higher chlorophyll content (Bharati et al., 2023; Handayani et al., 2023; Yadav et al., 2013). The results of this study indicate that some plants that have been treated with oryzalin concentration and immersion duration have darker leaf color compared to stevia leaves without treatment (control).

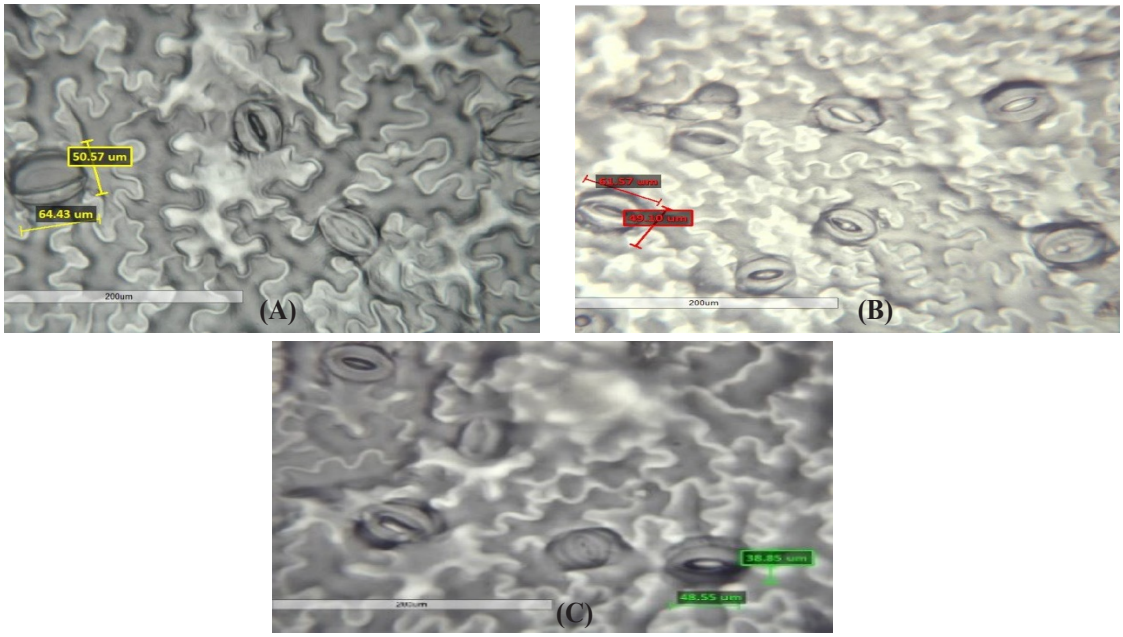


Figure 4. Effect of concentration and duration of oryzalin immersion on stevia stomata indicated as polyploid based on morphology. (A) 2.5µM-6hours (3); (B) 3.5µM-6hours (12); (C) Control or stomata of stevia leaves with out treatment. Total magnification 400x. Bar scale = 200 µm(micrometer). µM = micromolar.

Table 3. Color variation of stevia after treatment of oryzalin concentration and immersion time

Colors	Treatment								
	Control	1.5µM -4h	1.5µM -6h	1.5µM -8h	2.5µM -4h	2.5µM -6h	2.5µM -8h	3.5µM -4h	3.5µM -6h	3.5µM -8h
Dark sea green	15		3		4	5	5	5	4	3
Asparagus green		2	4	1	1	4	2	2	2	1
Medium sea green			3	1				1		
Green smoke		6	1	2	2		1	1	1	2
Light green		3	1		1	1			1	1
Sea green					2	1				
Dark olive green		1					3	2		
Bitter green		1								
Cactus green			1	1	2	1			2	
Verdun green							1		1	
Pale green			1			1				1
Olive drab					1	1				
Sirroco green				1						
Yellow-green								1		
Yellowish brown								1		

Notes: µM = microMolar; h = hours

Ploidy level

Based on the treated stevia plant morphology that showed different growth indications from the control plants, stevia in the 3.5µM-6h treatment with sample number 7 was further tested by flow cytometry. The results of flow cytometry showed that the tested stevia plants did not show polyploidy. The results of the flow cytometry test of diploid plants (control) and treated plants display a peak level at a height of 100 (Figure 5). Morphological changes in plants indicate polyploids can occur due to genetic changes due to the induction of mutation agents or the influence of physiological, biochemical, and transcriptomic changes (Ruiz et al., 2020).

Mutation induction is an attempt to change the morphology and traits of plants due to genetic changes. Successful induction requires a synergistic pairing of the penetration efficiency of microtubulin inhibitors (antimitotic agents) and plant meristematic tissues. In addition, success depends on the length of exposure, the concentration of antimitotic agent used, and in in-vitro applications depends on the interaction between basal media and growth regulators (Mullins et al., 2021; Touchell et al., 2020). The success of mutation induction is influenced by determining the duration and dose of exposure to mutagen compounds that inhibit the formation of microtubule compounds. The optimal immersion time in oryzalin solution differs with different concentrations but is around one day for *Lilium rosthornii* with a concentration of 0.01% (34.6 µM) (Wang et al., 2020). In addition, de Carvalho et al. (2016) also stated that handling techniques and the condition of the plants at the time of treatment application also affect the sensitivity of the plants to the given oryzalin because it becomes a bridge to facilitate the penetration of the antimitotic agent into the plant tissue.

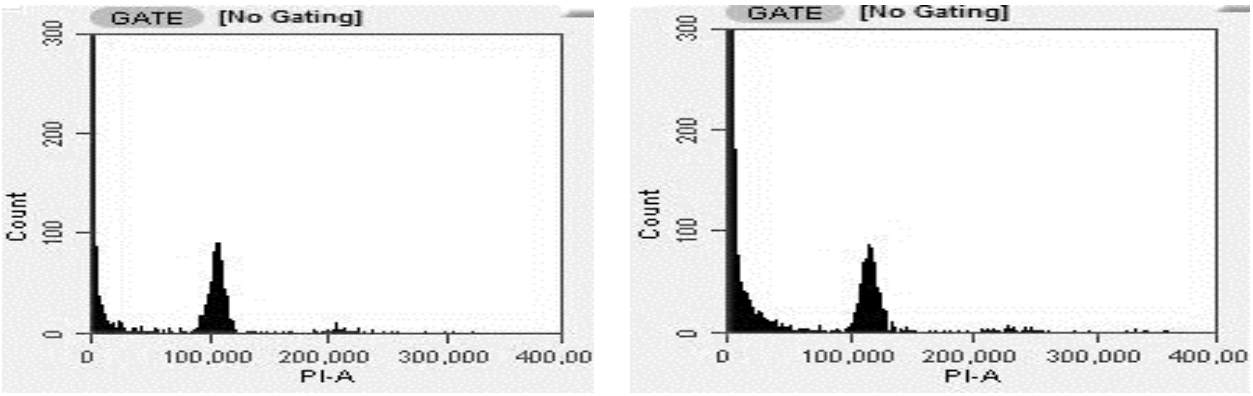


Figure 5. Results of flow cytometric analysis of diploid stevia plants (control) (a) and selected treated stevia plants (2.5µM-6hours sample number 3) (b)

CONCLUSION

Oryzalin concentration up to 3.5µM in 6 hours of immersion time was safe to induce mutation because it produced 67% live stevia. The oryzalin combination treatment applied to the stevia variety Tawangmangu generated morphological growth that indicates polyploidized including height, number of internodes, internode length, stem diameter, leaf size, leaf thickness, leaf color, stomata, and stem diameter. In addition, there were variations in growth, such as chimeras, rosette growth, and leaf splitting. However, further testing using flow cytometry showed that the treatment of concentration and duration of oryzalin immersion directly on vegetative material did not produce polyploid stevia individuals. Further research on the higher oryzalin concentration, longer immersion time, and stevia meristematic parts treated to obtain tetraploid stevia.

AUTHOR CONTRIBUTION

D.S. designed, conducted, collected research data, analyzed, interpreted data, drafted, and finalized the manuscript; P. and S.A. provided guidance, advice, input and supervision during the research process and manuscript drafting.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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