The increase of steviol glycosides in stevia (*Stevia rebaudiana* Bertoni) through artificial polyploidy

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Abstract. *Stevia rebaudiana* is a plant that produces secondary metabolites with diterpene glycosides, commonly called steviol glycosides, the Steviol glycosides as a low-calorie sweetener. Rebaudioside A and stevioside are steviol glycosides that are abundant in stevia leaves. Apart from being a sweetener, stevioside in stevia leaves has the potential as an anticancer. The obstacle to developing Stevia in Indonesia is the availability of stevia seeds of low quality, so a strategy is needed to improve the plants and increase the availability of quality stevia seeds. Artificial polyploid is one strategy for plant improvement, including increasing the production of secondary metabolites. This study aims to increase the content of steviol glycosides through artificial polyploidy using colchicine in tissue culture. The treatment was immersed in the explants in 100 mg.L-1 colchicine for 0, 24, 48, 72, and 96 hours. The results showed that soaking for 24 hours produced 13.63% mixoploid explants. The mixoploid explants produced the highest levels of rebaudioside A at 4.286% and the highest stevioside at 13.047%.

1 Introduction

Stevia rebaudiana is a species of Stevia in the Asteraceae family and is commonly known as sugar leaf. Stevia originates from Brazil and Paraguay. The cultivation of Stevia is known as a natural sweetener in various countries worldwide, including East Asian countries, the United States, Canada, and Europe [1]. About 200 species belong to the Asteraceae family (Compositae), originating from Paraguay [2]. Among these species, the most widely planted is *Stevia rebaudiana*, which contains diterpenoid glycosides [3].

Stevioside is the main component of steviol glycosides in *Stevia rebaudiana* leaves (5 – 10% of the leaf dry weight), followed by reb A (2 – 4%), reb C (1-2%) and glucoside A (0.4 – 0.7 %). However, the composition can change according to the cultivar of the plant [4]. Steviosides are known to have a high level of sweetness compared to sucrose, which is around 200-300 times [4, 5]. Several studies have shown that, in addition to its sweetness, stevioside

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has benefits along with other steviol glycoside compounds, namely rebaudioside A, steviol, and iso steviol, as a therapy for various diseases, namely as anticancer, anti–diabetic, antihypertensive, anti–inflammatory and diuretic [5].

In Indonesia, the Stevia plant has not yet been widely developed. The obstacle to the development of Stevia in Indonesia is the availability of good-quality stevia seeds. Therefore, the development of Stevia in Indonesia needs to be strategically directed at improving plants and increasing the availability of good-quality stevia seeds.

Artificial polyploid is one strategy for plant improvement. Artificial polyploid is a technique used to artificially increase the number of chromosomes of a plant so that it becomes a polyploid plant. Artificial polyploidy, in general, increases the vigor of plant parts and is a method to increase the production potential of plants. Genomic multiplication can qualitatively improve secondary metabolites' chemical content profile [6]. Induction of polyploidy using antimitotic agents intended to increase plant biomass which can ultimately increase the content of secondary metabolites [7, 8]. Induction of polyploidy using 0.05% colchicine for 48 hours or 0.1% for 24 hours induced polyploidy in the germination of *Stevia rebaudiana* seeds [9]. Artemisia cina polyploid plant growth was induced using a combination of growth regulators 2,4–dichlorophenoxyaceticacid (2,4–D) 2 mg.L-1 and benzyl adenine 1mg. L-1 is higher than in diploid plants [10]. This study aims to determine the effect of artificial polyploidy using colchicine in tissue culture on leaf anatomy, ploidy level, and steviol glycoside content in *Stevia rebaudiana* Bertoni.

2 Materials and Methods

This research was conducted at the Tissue Culture Laboratory, Faculty of Agriculture and Business, Satya Wacana Christian University, Salatiga. Plant material was taken from the Center of Medicinal Plant Research Tawangmangu Central Java. Culture media was MS0 media with the addition of activated carbon charcoal, granulated sugar, and agar. HPLC Knauer GmBH Germany Model Smart Line Series with UV detector (Smart Line UV Detector 2500 A 5140) used Rebaudioside A and Stevioside as standard. Flow cytometry used BD Accuri™ C6 Plus with CyStain® PI from Sysmex was used for DNA relative content analysis. Colchicine C3915 from Sigma Aldrich was used for polyploidy induction in this research.

This study used a randomized block design with the following treatments:

Code of treatment	**Long Term Treatment of Induction with Colchicine**
P0	No immersing
P1	Immersing explants with 100 mg.L^{-1} colchicine for 24 hours
P2	Immersing explants with 100 mg.L^{-1} colchicine for 48 hours
P3	Immersing explants with 100 mg.L^{-1} colchicine for 72 hours
P4	Immersing explants with 100 mg.L^{-1} colchicine for 96 hours

2.1 Polyploidy Induction

Sterilized explants were put into Erlenmeyer tubes containing 100 mg.L^{-1} colchicine. The induction process runs for 24, 48, 72, and 96 hours with Erlenmeyer continuously shaken on a shaker. After the induction process, the explants are planted on MS0 media for the next

stage. The neutralization stage is carried out after the explants are induced polyploidy by moving the explants into MS0 media (without growth regulators) for 14 days. This neutralization stage aims to eliminate the effect of colchicine treatment. The next stage is the shoot propagation stage. The media used at this stage is MS0 media. At this stage, shoots appear in the leaf axils as peripheral buds and in the callus. This shoot propagation stage is for 14 days. The shoots produced at the propagation stage are then transferred to the root induction media. The media used was MS0 media plus activated carbon charcoal 0.1 gram.L-1. The root induction time is 7 – 28 days based on the appearance of the roots.

2.2 Observation of the number of viable explants after polyploid induction

Living explants were identified by their lack of necrosis, absence of contamination by microorganisms, and presence of budding points. Both live and dead explants were objectively observed, counted, and recorded on a daily basis.

2.3 Identify the ploidy level

Polyploidy is a condition in which a plant cell contains two or more complete sets of chromosomes. The ploidy level was determined using a flow cytometer, with three samples for every induction treatment. The analysis involved chopping a 0.5 cm2 leaf sample on a mini petri dish until it became smooth, then adding CyStain® PI Absolute Kit. The solution was then filtered with a microfilter, transferred to a 3.5 ml test tube (12 x 75mm), and mixed with dye + CyStain® PI Absolute Kit. The test was then conducted on the flow cytometer. The results of the flow cytometer are presented in the form of a diagram with units of total DNA content.

2.4 Stomata observation

After acclimatization, we observed the size and density of stomata on eight-week-old Stevia leaves. To observe the stomata, we selected leaves in the fourth position that were fully opened. We applied clear nail polish to the surface of the leaf epidermis and allowed it to dry. Then, we attached a piece of tape, slowly peeled off the nail polish, and placed it on a glass slide for preparation. The next step is to observe and measure the length, width, and density of stomata per unit area on leaves. This can be done using an Optilab USB camera device and the Image Raster program.

2.5 Chlorophyll content analysis

Chlorophyll content was analyzed on the third leaf of the plantlet using the Soil Plant Analysis Development (SPAD) chlorophyll meter, specifically the Konica Minolta SPAD-502 Plus.

2.6 Steviol glycoside content analysis

The analyzed Steviol glycosides were Rebaudioside A and Stevioside, which were determined using High-Performance Liquid Chromatography (HPLC). The analysis of Rebaudioside A and Stevioside involved preparing and determining a standard series of these compounds. Once the standard series was established, the levels of Rebaudioside A and Stevioside in the analyzed sample could be calculated and obtained using linear regression results.

2.7 Statistical analysis

The collected data was then analyzed. Analysis of descriptive data on the observed data of ploidy level, DNA content, size of stomata, density of stomata, chlorophyll content, and steviol glycoside content. The parametric data analysis using analysis of variance (ANOVA) at a 5% level was conducted to determine the number of explants alive after induction and Duncan at a 5% level was undertaken to determine the significance between the treatments. Analysis of data used Statistical Analysis Software (SAS) developed by the SAS Institute.

3 Results and discussion

3.1 Response of explants after induction with colchicine

Colchicine is a compound that has toxic properties and is a mutagenic agent for plants. Colchicine is an antimitotic agent that attaches to the tubulin dimers of plant cells, which causes depolymerization of the microtubules, thereby disrupting the cell cycle. It causes a decrease in the rate of cell division which causes growth inhibition. Colchicine not only has an effect on cell division but spreads throughout the cell, disrupts cellular mechanisms, and causes poisoning at high concentrations [11]. [12] stated that colchicine seems to affect the viscosity of the cytoplasm so that cells cannot function normally. The longer soaking time affected the live explants after induction. In the *Dioscorea zingiberensis* plant, the mortality rate of the explants reached 70% when treated with 0.3% colchicine for 12 hours of treatment and increased up to 81% for 60 hours of treatment [13].

Table 1 shows the highest percentage of live explants with an induction time of 0 hours. Furthermore, 24, 48, and 72 hours of induction time showed no significant difference with the 0-hour induction time treatment on the percentage of live explants. The 96-hour induction treatment showed that the percentage of living explants was significantly lower than the 0-hour induction treatment. Explants were indicated as growing or living if they did not experience necrosis, formed new shoots, and were not contaminated by mold, yeast, or bacteria.

Table 1. Percentage of living explants and number of shoots growing on day 28 after induction

Induction Time (Hour)	Living explants (%)	Number of shoots
0	100 ± 0.00 a	1,32 ± 0,10 b
24	88 ± 0,11 ab	1,48 ± 0,25 ab
48	76 ± 0,08 bc	1,52 ± 0,17 ab
72	84 ± 0,16 bc	1,92 ± 0,54 a
96	72 ± 0,10 c	1,48 ± 0,41 ab
CV (%)	13,128	22,11

The mean value followed by a different letter is significant (α=0.05) in the DMRT test

The number of shoots formed at the colchicine immersion time treatment was not significantly different from the 0-hour treatment except for the 72–hour treatment, which showed a significantly higher number of shoots than the 0–hour treatment. It was presumably the time of induction with colchicine at 24, 48, 72, and 96 hours that have not fully affected

toxic to stevia explants. [14] research reported that soaking *Impatients balsamina* sprouts in colchicine 0.01 % for 4, 6, 8, 12, and 24 hours had 20% – 40% more growing branches than control plants without immersion with colchicine Increased shoot growth on explants induced by the increased size of the transport bundle cells was caused by colchicine. [14] revealed that giving colchicine increased the size of the transport bundle cells so that the translocation process in plants runs more optimally. Colchicine-induced explants have adsorption capability nutrition in the media is better than explants without induction treatment with colchicine. Explants that absorb nutrients more efficiently can undergo morphogenesis, and organogenesis was more likely to result in increased shoot growth.

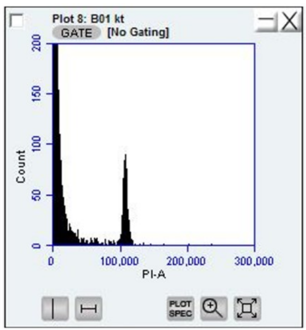
3.2 Effect of immersing time of colchicine on increasing ploidy levels of stevia explants

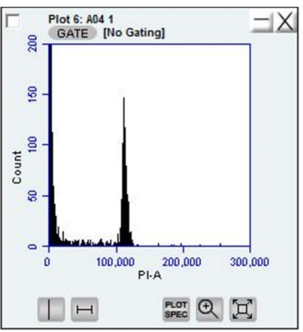
The analysis of plant ploidy level with a flow cytometer was presented in Table 2. Increasing the duration of soaking time did not affect the ploidy level of stevia explants. Alternation in ploidy level only occurred in the induction treatment for 24 hours. The 24–hour induction resulted in some explants with mixoploid 2n = 2x + 4x. The percentage of mixoploid explants from 24 hours of induction was 13.63%. The induction treatment of 48, 72, and 96 hours showed no alternation in the ploidy level, so it was still in diploid condition. During the induction period of 48, 72, and 96 hours, necrosis damaged the explant shoot, stimulating the growth of shoots only in the central region of the explants. The developing shoots were diploid because they did not undergo colchicine treatment.

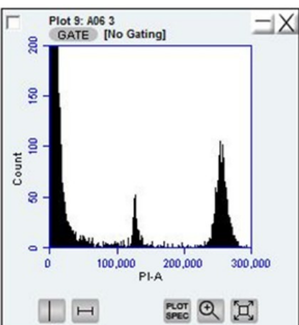
Table 2. The level of polyploidy of the explants among treatment

Induction Time (Hour)	Survive explant after 28 days (%)	Diploid Explant (%)	Polyploid Explant (%)	Ploidy level
0	100	100	0	-
24	88	86,37	13,63	mixoploid
48	76	100	0	-
72	84	100	0	-
96	72	100	0	

The results of the analysis of DNA content by flow cytometry was presented in Table 3. The samples with mixoploid were indicated by the presence of two peaks of DNA with a higher value of PI-A (Figure 1 c). The average peak yield in diploid explants resulting from tissue culture was 107.757. Then in mixoploid explants, the average first peak was 128.834, and the second peak was 256.726. The peak on mixoploid explants was approximately twice that of diploid plants, indicating a tetraploid second peak on mixoploid plants (Figure 1).







a.Control Plant (Diploidy) b. Diploidy Plants (2n=2x) c.Mixoploidy (2n=2x+4x)

Fig. 1. Identification of DNA content using a flow cytometer: a. DNA content in control plants; b. DNA content in a diploid plant (samples); c. DNA content of the mixed ploidy plants (sample)

Table 3. Results of DNA content analysis by flow cytometry

Ploidy level	Peak average (Calculated DNA content)	CV (%)
Diploid	107,757	3,79
Mixoploid	Peak 1= 128,834 Peak 2 = 256,726	Peak 1= 4,89 Peak 2 = 3,73

The alteration in ploidy level in stevia explants induced with colchicine for 24 hours was due to the activity of the compound. Colchicine inhibits the working system of microtubules, preventing proper formation or causing blockage of spindle threads. This failure to form spindle threads results in the failure of chromosomes to separate, leading to a lack of cell division. During the anaphase stage, the chromosomes fail to separate and remain in the same cytoplasm. However, each chromosome will separate from its centromere and enter the c-anaphase stage. This is followed by the formation of the cell nucleus wall. Because of this, the number of chromosomes produced doubled from 2x to 4x [15].

From the description above, the mixoploid condition is a condition in which one plant has two types of chromosome development. The results with two different peak points were tested using a flow cytometer. Mixoploid occurs due to uneven exposure to mutagen compounds in explant meristem cells. The mixoploid condition in stevia explants can be eliminated if continuous subculture is carried out so that the dominance of chromosome division will tend to be one of the ploidy levels.

The ploidy level is determined by calculating the DNA content, which can be read by the flow cytometer. The flow cytometer has been utilized in various tests to determine plant ploidy levels, as demonstrated in [16]. The flow cytometer was used to determine the ploidy level of guava plants by observing and calculating the peak value of DNA content. [17] emphasized that the flow cytometer is capable of quantifying the intensity of DNA luminescence in plant cell samples with minimal input for improved efficiency. In addition, [18] reported that the flow cytometer has a high level of accuracy and is capable of displaying data on mixoploid plants.

3.3 The size of stomata after treatment

The results of stomata measurements was presented in in Table 4. The mixoploid explants had stomatal length and width values greater than the diploid explants. Stomata in diploid plants had an average of length 2.10 ± 0.41 μm which was half of the stomata length of

mixoploid plants (4.13 ± 0.94 µm). The width of the stomata in diploid plants was an average of 2.04 ± 0.23 µm, while in mixoploid plants an average of 3.03 ± 0.92 µm. The increase in stomata size was caused by polyploid explant cells having larger size. Plants with a higher ploidy level generally have larger cell sizes than those with a diploid level as reported in another study [10]. Colchicine used in polyploid induction inhibited the formation of spindle threads causing a double number of chromosomes in one cell nucleus. Therefore, the cells size of polyploid plants become bigger than the size of diploid plant cells.

Table 4. The size and density of stomata

Ploidy Level	Length (µm)	Width (µm)	Density (100µm2)	Documentation
Diploid	2,10 ± 0,41	2,04 ± 0,23	1,92 ± 0,14	5,0 µm
Mixoploid	4,13 ± 0,94	3,03 ± 0,92	0,45 ± 0,07	5,0 µm

The stomatal density calculation of the stevia explant was presented in Table 4. The stomatal density in mixoploid explants was lowest, with an average of 0.45 ± 0.07 per 100 µm compared to diploid explants with an average of 1.92 ± 0.14 per 100 µm. The results of this study were related to the report from another study by [19] that the leaves of Passiflora edulis Sims tetraploid have a lower stomatal density than the diploid plants. Polyploid plants showed lower stomata density but larger stomata size than diploid plants. Stomata density in polyploid plants is affected by the larger size of the stomata and closer spacing between stomata [10]. Stomata density is the number of stomata in a unit area. The increasing number of stomata in a specific area unit indicates the higher value of stomata density, and vice versa. If in the same area unit but the number of stomata is small, it can be stated that the density was decreasing.

3.4 Chlorophyll content

The increase in chlorophyll content of mixoploid explants was presented in Table 5. The diploid explants had an average of 16.92 ± 0.98 SPAD units of chlorophyll, while the mixoploid explants had an average of 25.83 ± 1.19. Plants with more than 2x ploidy generally have different morphology and body anatomy from diploid plants [20]. An increase in the ploidy level from diploid to polyploidy causes an increase in the number of chromosomes to be more than in diploid plants. It caused the size of the cell and the cell nucleus to become more prominent.

Table 5. Chlorophyll content of stevia explants

Level of ploidy	Chlorophyll content (SPAD unit)
Diploid (2n = 2x)	16,92 ± 0,98
Mixoploid (2n = 2x + 4x)	25,83 ± 1,19

The chlorophyll in the leaves of polyploid plants is significantly higher than that of diploid plants, so the leaves of polyploid plants are darker green than those of diploid plants. Polyploid plants have more giant leaf cells and chlorophyll [21-24]. [25] revealed that polyploid Stevia has a more intense green leaf color, in which the green pigment on the leaves is chlorophyll. Mixoploid explants have more chromosomes than diploid explants, so more genes are involved in chlorophyll biosynthesis, which will increase the chlorophyll biosynthesis process. [26] stated that plants with a tetraploid ploidy level have a larger nucleus size; this allows the enzymes and proteins involved in the photosynthesis process to be produced in larger quantities. The increase in ploidy levels affects the level of sensitivity of plants in absorbing nutrients so that explants with mixoploid ploidy levels can absorb nutrients in the media more quickly and in large quantities.

3.5 Steviol glycoside levels

The steviol glycoside was higher in colchicine-induced explants, both diploid and mixoploid than in control explants without colchicine induction (Table 6). Steviol glycosides are secondary metabolites produced by the Stevia plant. One factor affecting the productivity of secondary metabolites is the plant's self-defense response to environmental stress [27]. Colchicine induction applied to stevia explants becomes a source of stress for Stevia explants. Colchicine, as a mutagen, can cause damage to explant cells so that explants respond to stress. Our data confirmed that the control treatment produced low Steviol glycosides. Table 6 shows that mixoploid explants had higher steviol glycoside levels (Rebaudiana-A 4.286% and Steviosida 13.047%) than diploid explants and control explants. The accumulation of secondary metabolites was higher in polyploid cells.

Table 6. Steviolglicoside content of Stevia explant

Ploidy Level	Time of induction (Hour)	Rebaudiosida-A content (%)	Stevioside content (%)
Control	0	0,806 ± 0,001	3,616 ± 0,126
Diploid	24	2,978 ± 0,078	9,125 ± 0,030
	48	2,864 ± 0,028	9,230 ± 0,058
	72	2,945 ± 0,182	8,916 ± 0,012
	96	2,578 ± 0,017	8,989 ± 0,041
Mixoploid	24	4,286 ± 0,537	13,047 ± 0,904

Stevia's biosynthesis of Steviol glycosides occurs via the methylerythritol 4-phosphate (MEP) pathway, which takes place in two organelles: the chloroplast and the endoplasmic reticulum. The chloroplast is responsible for the biosynthetic steps from pyruvate to kaurene, while the endoplasmic reticulum is responsible for the biosynthesis of kaurene to steviol. Steviol derivatives are produced in the cytosol. Mixoploid stevia explants exhibit larger cell sizes, as evidenced by the size of the stomata in mixoploid plants. This increase in cell size is likely due to a greater number of chromosomes and a higher number of genes contained within them. The number of genes is believed to contribute to the increase in enzyme activity in Steviol glycoside biosynthesis. It was in line with the results of research by [28], who stated that occasional mixoploidy plants showed increased stevioside content.

Mixoploid plants have larger chloroplasts, as evidenced by their higher total chlorophyll content compared to diploid plants. This increase in chloroplast size leads to an increase in

kaurene synthesis, which is vital for the formation of steviol. The amount of kaurene formed will determine the amount of steviol produced in the endoplasmic reticulum and will be passed on to the cytosol to form its derivatives, namely Rebaudiosida-A and Stevioside. A more significant number of genes have a role in activating enzymes and amino acids in the biosynthetic process, increasing the synthesis of steviol glycosides.

4 Conclusions

Artificial induction of polyploidy using 100 mg.L-1 colchicine for 24 hours of immersion resulted in a mixoploid ploidy level of 13.63%. Stomata size, chlorophyll content, and levels of steviol glycosides in mixoploid plants were higher than in diploid plants and control treatment. Steviol glycoside levels in mixoploid plants were Rebaudiana A 4.286% and Stevioside 13.047%.

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