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Original article

Induction of Polyploidy in Stevia Plants (*Stevia rebaudiana* Bertoni) Using Colchicine

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Abstract

The primary objective of this study was to establish a protocol for induction of polyploidy in *S. rebaudiana* var. Levent 93. As a plant material in vitro propagated seedlings of stevia variety Levent 93 were used. For induction of polyploidy, shoot tips with 3-4 leaves of seedlings were immersed in 0.5% concentration of colchicine solution for 1, 2 and 4 hours. Then, shoot tips treated with colchicine solution and distilled water were taken into rooting medium. Plants that developed successfully in the rooting medium were transferred to pots and acclimatized to the external environment. Flow cytometry ploidy analysis was performed on plants treated with colchicine solution after approximately 8 months. According to the ploidy analysis results, it was determined that 17 plants were diploid and 3 plants were tetraploid among 20 plants that survived after treatment with colchicine. All tetraploid plants were observed in 2 hours treatments. As a result of the study, the viability rate, plant height, number of leaves, number of branches, chlorophyll amount, ploidy levels and core DNA contents of stevia plants treated with colchicine were determined and diploid and tetraploid plants were compared. When diploid plants and tetraploid plants are compared, it is concluded that tetraploid plants have smaller averages in terms of plant height average, while they have larger averages in terms of leaf number average and number of branches.

Keywords: *Stevia Rebaudiana*, Polyploidy, Tetraploid, Colchicine, Flow Cytometry.

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INTRODUCTION

Stevia rebaudiana is a diploid (2n) plant with chromosome number of 22. It is a perennial herbaceous plant belonging to the Asteraceae family, found in the mountainous lands of Paraguay (Yadav et al. 2011; Azizan et al. 2020). *Stevia rebaudiana* is grown as the number one herb in many countries, including Japan, China, Korea, Indonesia, Tanzania, Mexico, the United States, and Canada (Madan et al. 2010; Zhang et al. 2018). The main producers of stevia are Japan, China, Taiwan, Thailand, Korea, Brazil, Malaysia and Paraguay. Currently, stevia is consumed in Japan, Brazil, Korea, Israel, the United States, Argentina, China, Canada, Paraguay, and Indonesia (Crammer and Ikan 1986; Singh and Rao 2005; Yadav et al. 2011) and no adverse effects have been reported from its use to date (Kinghorn and Soejarto 1985; Brandle and Rosa 1992; Yadav et al. 2011). Stevia was used for the first time in Japan for the food and pharmaceutical industry and its use is increasing day by day (Uskutoğlu et al. 2019). It is also used as a natural sweetener in many countries such as North and South America, Southern Europe, Korea, Thailand, China, India and Bangladesh. China is the largest stevia producer in the world, and the United States and North America are the largest stevia consumers (Al-Taweel et al. 2021). This plant, originating from Paraguay and Southwest Brazil, was first introduced in Turkey by being grown in Akdeniz University in Antalya in 2013 (Turgut et al. 2013). Stevia is a natural sweetener due to the steviol glycosides it contains. Stevia leaves are composed of glycosides or secondary metabolites called stevioside (5-10%), rebaudioside-A (2-4%), rebaudioside-B, C, D, M and dulkoside, which have various sweetness properties. The most important organs of the plant are the leaves, as they have the highest steviol glycoside content. Other parts contain lower concentrations of steviol glycosides (Azizan et al. 2020). The average stevioside ratio in stevia varies between 4-12%, and the rebaudioside-A ratio varies between 2-4% (Kaplan and Turgut 2019), and the amount of steviol glycosides contained in the plant determines its quality. These are high-potency sweeteners with approximately 300-400 times sweetness compared to ordinary sugar or sucrose and have zero calories (Azizan et al. 2020). For this reason, stevia is used as a natural sweetener in many countries. It is used safely by diabetics as it does not increase the sugar level in the blood. It is preferred in diet products due to its zero-calorie feature.

There are currently 5 Stevia varieties (Levent 93, Turgut 82, Güney 04, Turgut and Tutuncu) registered in Turkey and included in the National Variety List. Among them, Levent 93 variety stands out in terms of both leaf yield and leaf quality. Levent 93 variety has a dry leaf yield of 4.5-5.0 kg/ha from the second year, and the total steviol glycoside (TSG) rate in dry leaves is 20%, the stevioside (Stv) rate is 9-10% and the rebaudioside A (Reb A) rate is 8-10%. However, it is always possible to improve leaf yield and quality of stevia by using different breeding methods.

To improve steviol glycosides yields in *S. rebaudiana*, polyploidy breeding may be a promising approach. Polyploidy has been used to develop new crop cultivars; including wheat, cotton, potato and sugarcane (Zhang et al. 2018). Polyploidy is defined as the presence of more than two sets of

chromosomes in somatic cells (Şehirali and Özgen 2013). Polyploidy can occur naturally in plants, or it can be obtained by disrupting mitosis by using antimitotic agents. Colchicine, oryzalin and trifluralin are commonly used mitotic agents to obtain polyploidy plants (Goluch et al. 2021). In addition to these, chemicals such as chloral hydrate, ether, chloroform and phenyl urethane and heat shocks are also used in polyploidy plant breeding (Şehirali and Özgen 2013). Among these chemicals, the most commonly used chemical is colchicine.

Colchicine is a natural alkaloid obtained from the *Colchicum automnale*. Its chemical formula is $C_{22}H_{25}NO_6$. Colchicine can be applied to seeds, roots and shoots. The amount and duration of colchicine may vary depending on the type of plant species and plant tissue to be applied. In addition, there are different application methods such as dripping colchicine on plant tissues, contacting the plant with cotton or dipping in colchicine. Colchicine prevents the formation of spindle fibers in cell division, preventing the chromosomes from accumulating in the midplane during metaphase, and thus normal anaphase and telophase phases do not occur. Thus, polyploidy cells with twice the normal number of chromosomes are formed (Şehirali and Özgen 2013). Polyploidy applications are a valuable breeding method used to increase transition time in long-term breeding programs. Polyploidy is an important plant breeding tool that increases genetic diversity. By changing the chromosome groups and the number of genes in a cell, it can change plant characteristics in desired or undesired directions (Madani et al. 2021).

Although polyploidy studies have been done on stevia before, there are no studies on our domestic stevia variety Levent 93. For this reason, Levent 93 variety, which is superior in terms of yield and quality, was used in this study. The aim of this research is to double the chromosome number and obtain polyploidy plants by applying colchicine to Levent 93 stevia cultivar. As a result, it is aimed to compare polyploidy plants and diploid plants obtained.

MATERIALS and METHODS

The study was carried out in the Faculty of Agriculture in Akdeniz University and in Güney Agripark R&D Center (Antalya) in 2021 and 2022. As a plant material *in vitro* propagated seedlings of *Stevia rebaudiana* Levent 93 variety were used. On the other hand, colchicine ($C_{22}H_{25}NO_6$) was utilized as mutagen agent. For induction of polyploidy, shoot tips with 3-4 leaves of seedlings (Fig.1a) were immersed in 0.5% concentration of colchicine solution for 1, 2 and 4 hours (Fig.1b). After purchasing colchicine with a purity of more than 99%, it was kept closed and protected from light at +4 °C.

a **b**

Figure 1. Shoot tips: a= shoot tips taken from seedlings b= immersion of shoot tips in colchicine solution

Polyploidy Experiment

The study was started by preparing the mutagen colchicine to be applied to the plants. 0.5% colchicine solution, which was decided in line with the studies researched and examined, was prepared one day before the application. The prepared solution was taken into a suitable bottle and wrapped with aluminum foil to protect it from light. Then stored at +4 °C until mutagen application. In the experiment, mutations were targeted in plants by treating stevia shoot tips with 0.5% concentration of colchicine for 1 hour, 2 hours and 4 hours. One sample was taken from each plant, which was approximately 3-4 months old. Shoot tips with 3-4 leaves of Levent 93 seedlings were dipped into colchicine solution in the morning. According to the researches, because colchicine interacts with light, the treatment of plants with colchicine solution was done in the dark condition. After immersion, the shoot tips were removed from the colchicine solution at the end of 1 hour, 2 hours and 4 hours by keeping the time. Then it was rinsed thoroughly with distilled water. In the study, the control samples were immersed in distilled water. Then, shoot tips treated with colchicine solution and distilled water were planted in trays with 50% peat and 50% perlite mixture and taken into rooting medium (11.01.2022). After about 2 months, the cuttings were taken from the top of the rooted plantlets, transferred to trays with a mixture of 50% peat and 50% perlite. They placed in a rooting medium containing 80% humidity, 22ºC temperature, 12 hours day length and 48 µm light intensity, and the plants were expected to develop in a controlled manner. The plants that developed successfully in the rooting medium were transferred to the pots (9 x 8 cm) and they were accustomed to the external environment.

Ploidy Analysis

Ploidy analysis was performed on plants treated with colchicine solution. For this purpose, flow cytometry analysis was done in the Department of Field Crops of Agriculture Faculty in Namık Kemal University. In flow cytometry analysis, 20-30 mg of disease-free, clean and fresh leaf tissues obtained

from young and healthy plants were used. Ploidy analysis of stevia leaf samples was performed by accepting barley leaves as standard due to their similar DNA content. Twenty milligrams of stevia leaves and 20 milligrams of barley leaves were cut into a sterile container and then 400 µl of the disintegrating buffer solution was added and the leaf samples were cut with a razor blade. It was passed through a filter and taken into a tube. After that, 1400 µl of DAPI dye solution was placed on the tube and kept in the dark until flow cytometry analysis. After all leaf samples were prepared in this way, the tubes were read in the flow cytometry device, respectively, and the ploidy levels were recorded.

RESULTS and DISCUSSION

As stated before, shoot tips of stevia seedlings were treated with 0.5% concentration of colchicine for 1 hour, 2 hours and 4 hours. Then, treated shoot tips were rooted successfully in the mixture of 50% peat and 50% perlite. After 2 months, cuttings were taken from the shoot tips of the live plants and planted in the same mixture for rooting. The number of plants that survived this period is given in the table 1.

Table 1. Survival rates in plants grown from shoot tips treated with colchicine

Treatment Time (Hour)	Total Number of Plants	Number of Live Plants	Live Plant Ratio (%)
Control	45	45	100
1 Hour Treatment	45	18	40.0
2 Hours Treatment	45	12	26.6
4 Hours Treatment	45	6	13.3

Table 2. Survival rates of plants developing from shoot tips of living plants

Treatment Time (Hour)	Total Number of Plants	Number of Live Plants	Live Plant Ratio (%)
Control	45	45	100
1 Hour Treatment	18	10	55.5
2 Hours Treatment	12	7	58.3
4 Hours Treatment	6	3	50.0

When Tables 1 and 2 are examined, the highest viability rate was found to be 100% in control plants, and the lowest viability rate was 13.3% and 50% in 4 hours applications of colchicine solution. It was observed that the viability rate of the plants decreased as the treatment time of the shoot tips in the colchicine solution increased. The reason for this can be explained by the fact that colchicine is a very toxic chemical (Elçi and Sancak 2013) and the lethal effect of excessive colchicine concentration (Şehirali and Özgen 2013).

Table 3. Minimum, maximum and average values of plant height, number of leaves, number of branches and chlorophyll content of mature plants

Observed Features	Measured Values	Control	1 Hour Treatment	2 Hours Treatment	4 Hours Treatment
Plant Height (Cm)	Minimum	45.1	33.0	20.0	25.6
	Maximum	75.0	63.9	35.4	42.5
	Average	62.4	46.2	26.8	32.8
Number of Leaves (Number)	Minimum	16.0	17.0	20.0	24.0
	Maximum	55.0	60.0	47.0	31.0
	Average	35.4	35.5	30.1	27.3
Number of Branches (Number)	Minimum	1.00	3.00	2.00	2.00
	Maximum	5.00	6.00	11.0	5.00
	Average	3.00	4.20	6.20	3.30
Chlorophyll Content (Spad)	Minimum	18.2	10.2	9.70	13.0
	Maximum	26.9	23.9	26.2	14.0
	Average	23.9	17.7	14.9	13.6

In this study, the responses of control, 1 hour, 2 hours and 4 hours applications to plant polyploidization in colchicine solutions at 0.5% concentration were investigated. The main aim of the study is to obtain tetraploid stevia plants by doubling the diploid chromosome number. As a result of the study, the viability rate, plant height, number of leaves, number of branches, amount of chlorophyll, ploidy levels and core DNA contents of stevia plants treated with colchicine were determined (Table 3).

Colchicine showed a negative effect on the viability rate of plants. In the study, stevia shoot tips were treated with colchicine solutions at different times (1 hour, 2 hours and 4 hours), and 81 plants out of 180 plants in the first stage and 65 plants out of 81 plants in the second stage were able to survive. Among the plants treated with colchicine, the plants that were most adversely affected in terms of viability were the plants exposed to 4 hours of application, and the plants that were least affected were the plants that were exposed to the 1-hour application.

When the stevia seedlings were examined in terms of average plant height, it was determined that the plants exposed to the 2 hours application were shorter, while the control plants were taller. When diploid plants and tetraploid plants were compared in terms of plant height average, it was observed that diploid plants had a higher average height than tetraploid plants. Similarly, in studies conducted on stevia, it has been reported that colchicine applications caused a significant decrease in plant height (Yadav et al. (2013); Talei et al. (2020)). In other studies, on stevia, Oliveira et al. (2004) found that plant height ranged from 27.8 cm to 96.8 cm. In our study, plant height ranged from 20.0 cm to 75.0 cm. These results are similar to those found by Oliveira et al. (2004), but the minimum and maximum values were different. It is believed that this is due to differences in growing conditions. Rameshsingh et al. (2015) found the highest plant height as 83.44 cm in triploid plants and the lowest plant height as

71.55 cm in tetraploid plants. They found the plant height as 78.88 cm in diploid plants. We did not encounter any triploid plants in our study. We observed the lowest plant height in tetraploid plants as 22.5 cm and in diploid plants as 20.0 cm. this difference may be due to the different number of plants tested, different stages of plant growth at the time of observations, and differences in growing conditions. When applying colchicine at a concentration of 0.05%, Talei et al. (2020) and Grad and Gomaa (2020) found the highest plant height as 135,44 cm and 134.33 cm, respectively, while in our study with the same dose, we found 63.9 cm. The differences in these results are though to be due to the different stages of plant growth at the time of observations and the differences in care procedures such as fertilization. Azizan et al. (2020) reported that the highest average plant height was at 2% colchicine concentration, while it was the lowest in the control group. These result do not match with the findings Yadav et al. (2013), Talei et al. (2020) and our study. The different result obtained from these studies on the same plant species are thought to be due to the difference in methods used by Azizan et al. (2020), Yadav et al. (2013), and our study. Additionally, applying different chemicals to plants at different stages of growth can also yield different results.

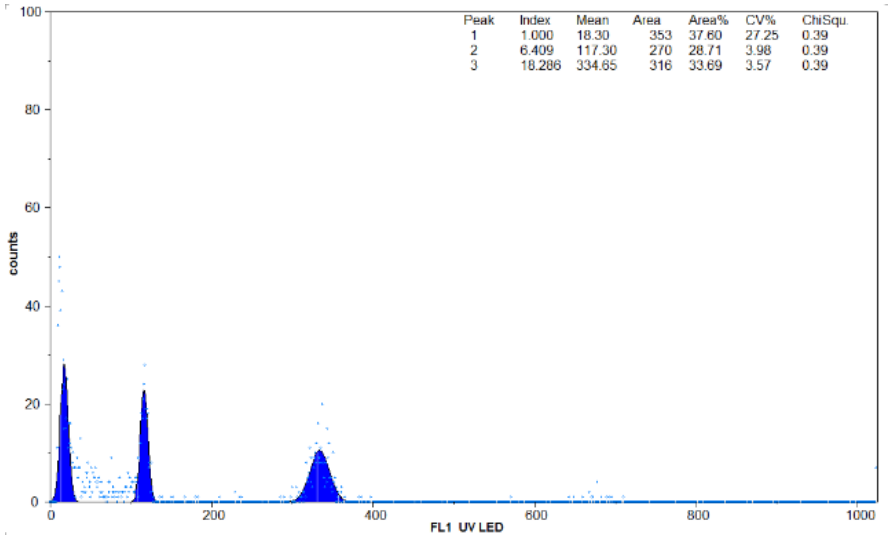
When examined in terms of average leaf number, it was determined that plants exposed to 4 hours of treatment have fewer leaves, while plants exposed to 1 hour of treatment have more leaves. When the leaf number averages of diploid and tetraploid plants were compared, it was observed that diploid plants have a higher average leaf number than tetraploid plants. When other studies on Stevia were examined, Grad and Gomaa (2020) found the highest average leaf number in 0.1% concentration colchicine applications as 825.67 and the lowest average leaf number in 0.2% concentration colchicine applications as 177.67. It is estimated that the main reason for the emergence of similar results in these studies is the application method of colchicine. It is thought that since plants are treated more with colchicine through the dipping method than the cotton-mediated contact method, it may be more effective at a lower dose. In the study conducted by Grad and Gomaa (2020), different concentrations including the concentration used in the current study, which were control, 0.025%, 0.050%, 0.100%, and 0.200%, were applied, and the lowest average leaf number was found in the case of exposure to the highest colchicine concentration. This result is consistent with the current study. In general, it can be said that the exposure time to colchicine affects the average leaf number.

Compared in terms of average number of branches, control plants were found to have fewer branches, while plants treated for 2 hours had more branches. It was observed that tetraploid plants had a higher average number of branches than diploid plants. Rameshsingh et al. (2015) reported that the highest average number of branches was 13.5 and the lowest was 9.11. In the current study, the highest average number of branches was determined to be 6.2 and the lowest was 3.0. The reason for the different results may be due to the measurements taken and the fact that the plants were in pots and in an early growth stage, while Rameshsingh et al. (2015) evaluated the field performance of plants, which

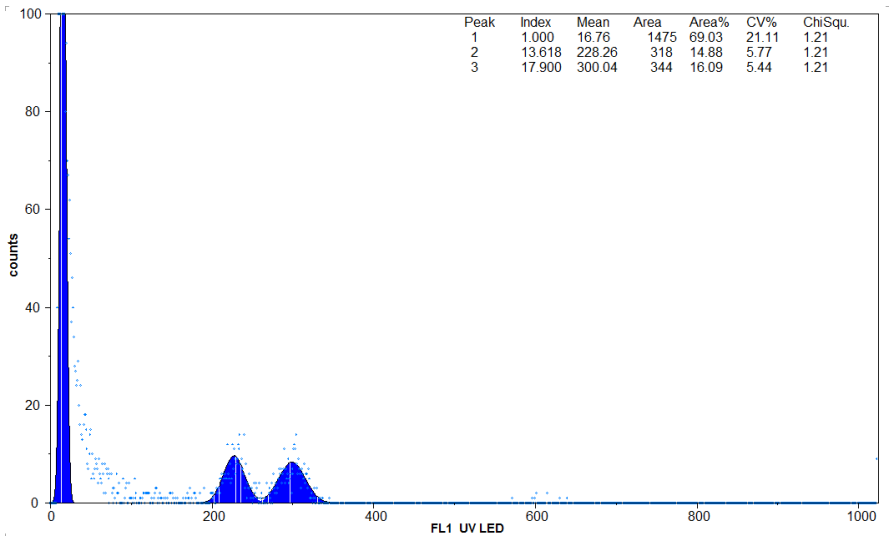
was assumed to be in a later growth stage. Talei et al. (2020) found the highest number of branches at a 0.05% concentration of colchicine application. They reported that concentrations of 0.10% and 0.20% reduced the number of branches. These results indicate that the 0.05% colchicine dose increases the average number of plant branches and higher doses have a reverse effect. It is important to determine the appropriate dose for the average number of branches. In addition, it was observed in our study that the application at different durations also had an effect on the average number of plant branches. Similarly, in a study by Grad and Gomaa (2020), the highest average number of branches was found to be 21.33 in colchicine applications at 0.05% concentration. They determined the lowest average number of branches to be 3.33 in colchicine applications at 0.2% concentration. These results are consistent with the current study and may provide a useful reference for those evaluating the effect of colchicine doses and durations on the average number of plant branches.

A portable chlorophyll measuring device was used to measure the chlorophyll content of the plants. Measurements were made from 3 different leaves randomly selected from each plant, and their averages were recorded. The maximum amount of chlorophyll was 26.9 spad in control plants, and the minimum amount of chlorophyll was 9.7 spad in 2 hours colchicine applications. It was observed that the mean chlorophyll content of the plants decreased as the duration of the shoot tips in the colchicine solution increased. Yadav et al. (2013) reported that the amount of chlorophyll increased significantly in tetraploid plants. Zhang et al. (2018) reported that the chlorophyll content was 29.00 in diploid plants, and 41.03 in tetraploid plants.

Ploidy analysis was performed on plants treated with colchicine solution after about 8 months. For this purpose, flow cytometry device was used. According to the ploidy analysis results, it was determined that 17 plants were diploid and 3 plants were tetraploid among 20 plants that survived after treatment with colchicine. All tetraploid plants were observed in 2 hours treatments. A computer image of the results of flow cytometry analysis in diploid and tetraploid plants is given (Fig 2). When other studies on stevia were examined, Yadav et al. (2013), Rameshsingh et al. (2015), Mahdi et al. (2018), Xiang et al. (2019), Talei et al. (2020) obtained polyploid plants in their studies. Also, Zhang et al. (2018), Ghonema et al. (2015) while obtaining tetraploid plants at 0.05% colchicine dose, Talei et al. (2020) could not obtain tetraploid plants at 0.05% colchicine concentration.



a



b

Figure 2. Display of flow cytometry analysis results in computer environment: a= image of a diploid plant b=image of a tetraploid plant

The core DNA content is called the total amount of DNA present in the cell and is measured as the C value. (Şahin 2019). In this study, DNA content of stevia plants was calculated by means of flow cytometry and is given in Table 4. It has been reported that there is a close relationship between the core DNA content and the ploidy level, and the DNA content increases as the ploidy level increases (Tuna et al. 2004; Şahin 2019). Indeed, our study supports these results. In a study on stevia, Xiang et al. (2019) reported that the cytological analysis of tetraploid stevia plants showed that the chromosome number was doubled and that the flow cytometry analysis showed that the DNA content was doubled. Examining

other studies, Chen et al. (2011) reported that according to the results of flow cytometry analysis in Anthurium andraeanum plants, tetraploid plants with twice the DNA content of diploid plants were obtained. Tavan et al. (2015) determined the DNA contents and ploidy levels of Thymus persicus plants by means of flow cytometry. They confirmed the results of flow cytometry with chromosome counting studies.

Table 4. Stevia-Barley Fluorescence Intensities and DNA Contents

Treatment Time (Hour)	Stevia Fluorescence Intensity	Barley Fluorescence Intensity	Barley Core DNA Contents	Stevia Core DNA Contents
Control	116.11	299.06	10.65	4.13
1 Hour	113.33	308.24	10.65	3.92
1 Hour	118.30	293.95	10.65	4.29
1 Hour	109.71	280.20	10.65	4.17
1 Hour	115.13	259.74	10.65	4.72
1 Hour	117.00	276.22	10.65	4.51
1 Hour	116.26	329.18	10.65	3.76
1 Hour	109.35	271.45	10.65	4.29
1 Hour	120.64	281.53	10.65	4.56
1 Hour	112.37	265.72	10.65	4.50
1 Hour	106.99	265.97	10.65	4.28
2 Hours	112.12	281.54	10.65	4.24
2 Hours	102.84	244.85	10.65	4.47
2 Hours	106.55	279.79	10.65	4.06
2 Hours	117.30	334.65	10.65	3.73
2 Hours	208.71	278.77	10.65	7.97
2 Hours	213.50	262.19	10.65	8.67
2 Hours	228.26	300.04	10.65	8.10
4 Hours	112.62	280.52	10.65	4.28
4 Hours	114.61	297.13	10.65	4.11
4 Hours	108.11	269.13	10.65	4.28

In this study, tetraploid stevia plants unfortunately could not survive. As a matter of fact, Şehirali and Özgen (2013) supported our results by stating that the development of polyploid plants is quite weak compared to diploid plants, their osmotic pressure is low and they need more time for cell divisions. At the same time, they added that in addition to the problems of polyploid plants, they are sensitive to nitrogen and phosphorus fertilizers, their growth rate is slow and infertility may occur. As a matter of fact, the polyploid plants obtained in this study were in a very weak condition compared to other plants and showed very little development and lost their vitality after a while. In order to protect and reproduce polyploid plants, shoots developed from polyploid plants were sensitively approached, and even

explants taken from some polyploid plants were taken into tissue culture but still could not maintain their viability.

Conclusion

According to the results of this study, colchicine provides chromosome folding in stevia plant. In order to obtain polyploidy in stevia, immersing the shoot tips in 0.5% colchicine solution for 2 hours gives successful results. However, since polyploid plants could not maintain their viability, they could not be compared with diploid plants in terms of steviol glycoside amounts. This study will be useful in determining the appropriate dose and time for obtaining polyploid plants in other studies to be carried out on stevia, and it is predicted that it will be possible to compare with diploid plants by obtaining more polyploid plants.

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Conflicts of interest

There is no conflict of interest between the authors of the article.

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