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Assessment of Induced Polyploidy in Watermelon (*Citrullus lanatus* L.)

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Abstract

Watermelon (*Citrullus lanatus* L.), a diploid fruit crop with 2n=22 chromosomes, is valued for its sweetness and health benefits. Originating from South Africa, it belongs to the Cucurbitaceae family. Globally, annual production reaches about 117 million tons. Recently, seedless watermelons, derived from crossing tetraploid (4n=44) and diploid (2n=22) plants to produce triploid (3n=33) cultivars, have gained popularity over seeded types due to the inconvenience caused by seeds. In Pakistan, limited genetic studies on watermelon hinder breeding efforts. This research focused on developing methods to induce and identify polyploidy in watermelon using colchicine, an anti-mitotic agent, to produce tetraploids for seedless watermelon breeding. Two treatment methods were employed: seeds were soaked in colchicine solutions at 0.15% and 0.20% for 20 hours, and sprouts were soaked at the same concentrations for 30 minutes over two consecutive days. Results showed that colchicine treatments significantly affect plant growth. Treated plants had reduced length and leaf number but increased stem diameter. Leaves appeared darker, rougher, and thicker, with higher chlorophyll content correlating with the darker green color. The emergence of the first true leaf was delayed compared to controls. Pollen from treated plants had an increased diameter but decreased fertility. Additionally, stomatal length and width increased, while density decreased, indicating tetraploidy. An increase in the number of chloroplasts per guard cell was also observed. These morphological and anatomical changes confirm successful induction of polyploidy. Seed soaking with colchicine at 0.15% and 0.20% was particularly effective in developing tetraploid lines, which are valuable for seedless watermelon breeding programs.

Keywords: Watermelon, Polyploidy, Colchicine, Induction, Characterization

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Introduction

Watermelon (*Citrullus lanatus* L.), with a chromosome number of 2n=2x=22, belongs to the Cucurbitaceae family. It is an annual vine herb cultivated for its sweet fruit (1), and it is also known as the King fruit in summer. It originated in South Africa and is a commercially important fruit crop, similar to cucumbers, melons, and squashes (2). The ideal growing conditions for watermelons include sandy loam soils with good drainage (3) and a pH between 6.0 and 6.8 (4). Plants grow more slowly in heavy soils, and the overall quality of the fruit is usually less impressive.

In 2016, approximately 117 million tons of watermelon were produced globally, with China contributing 68% of the total. Over 1% of the world's total production came from Mexico, Iran, Uzbekistan, the United States, Turkey, Russia, Egypt, Brazil, Algeria, and Kazakhstan (5). Asia accounts for about 81 percent of global watermelon production (6). Across Pakistan, it is cultivated on 20,000 hectares, yielding a total output of 420,000 metric tons (7). Watermelon is widely used to treat health issues associated with aging, including heart disease, obesity, diabetes, ulcers, and some types of cancer (6). It is beneficial for your health because it contains important phytochemicals, including lycopene, citrulline, and other polyphenolic compounds with pharmacological effects (8). Watermelon is an important source of L-citrulline, a neutral α-amino acid that is the precursor of L-arginine, an essential amino acid necessary for protein synthesis (6). Studies have shown that eating watermelon can significantly lower blood pressure in overweight individuals, both at rest and under stress (9). The naturally occurring diploid watermelon, with two sets of chromosomes (2n=2X=22), is most common in nature, although autopolyploids can lead to

the development of triploid watermelons with three sets (3n=3X=33) and tetraploid watermelons with four sets (4n=4X=44) (1).

Nowadays, seedless watermelons are in high demand compared to seeded watermelons. Seedless watermelon is triploid, which is obtained by crossing tetraploid and diploid watermelons (10). Introducing new traits into plants through polyploidization is an important method used in breeding programs (11). The genetic makeup of seedless watermelons (triploid) consists of three sets of chromosomes (3n=33). These can be produced by crossing tetraploid watermelons with four sets of chromosomes (4n=44) with conventional diploid watermelons (which have seeds), which have two sets of chromosomes (2n=22). The concern is that tetraploid watermelons must be produced artificially. Producing tetraploids requires biotechnological methods. Many fruit plants utilize this technique for various purposes, e.g., mangosteen (12), durian (13), and watermelon.

Colchicine can be used to double the chromosome number (14), aiding in the production of tetraploid watermelons. Colchicine is widely used to induce polyploidy in plants. It prevents the formation of spindle fibers during cell division, leading to the formation of individual polyploids (15, 16). A higher concentration of colchicine results in a greater percentage of tetraploid cells; however, increasing the concentration also raises the risk of seed death during germination (17). This study aims to optimize colchicine concentration and application methods to induce polyploidy in watermelon and to investigate traits associated with change in ploidy levels.

Materials and Methods

The research was conducted at the University Research Farm (URF), Koont, PMAS-Arid Agriculture University, Rawalpindi, during 2022-2023. This area lies from Longitude 33.1166° N, Latitude 73.0111° E. The laboratory work was done in the Plant Breeding and Genetic Lab, Faculty of Agriculture, PMAS-Arid Agriculture University, Rawalpindi. Two watermelon verities Crimson Sweet and Augusta, were used and colchicine as an anti-mitotic agent to induce polyploidy

Experiment Layout

A randomized complete block design (RCBD) was employed to evaluate the effects of colchicine under two main experimental factors. The first factor was colchicine concentration, comprising 0.15% (C1), 0.20% (C2), and untreated control. The second factor was the method of colchicine application, in which seeds were soaked for 20 hours (A1), and sprouts were soaked for 30 minutes (A2). This factorial arrangement resulted in four distinct treatment combinations, each replicated three times, to ensure robust, reliable, and statistically sound data.

Seed Soaking in Colchicine

To prepare the seeds for colchicine treatment, they were first soaked in water for 24 hours. Afterwards, the seeds were placed in Petri dishes lined with cotton. Then, 20 mL of 0.15% or 0.20% colchicine solution was added to each Petri dish, each containing 17 watermelon seeds. The Petri dishes were covered and kept in a location protected from direct sunlight. The seeds were soaked for a total of 20 hours. The cotton layer was used to ensure that the seeds were partially submerged in the colchicine solution while maintaining full surface contact. Under these conditions, chromosome duplication was induced while the seeds still received sufficient oxygen.

Sowing of Soaked Seed

It took multiple washes to clean the seeds from each treatment in the Petri dishes. It was necessary to sow the clean seeds right away in trays already loaded with sterile coco peat, half an inch deep. The treated seeds were permitted to develop. The tag was added with complete information including concentration, time of treatment, and sowing date.

Sprout Soaking in Colchicine

The seed was planted in the seedling trays, and after 6-9 days when they sprouted and emerged enough to be treated, they were taken out carefully and soaked in a colchicine solution of 0.15% and 0.20% for 30 minutes. The tag was added with complete information, including concentration, treatment time, and sowing date.

Plantation

The colchicine-treated seeds with 2-4 leaves were then planted in the bed in the field area of the Plant Breeding and Genetics department at the university research farm, where plant-plant distance of 0.6m (2ft) and row-row distance of 2.4m (8ft) was maintained. Each bed and treatment was labeled and identified to facilitate observation.

Confirmation of Polyploids

For the confirmation of polyploids, the following morphological parameters were recorded: appearance of first leaf days after sowing, plant length (cm) after 55 days of germination, number of leaves per plant were counted, chlorophyll content using spade meter, stem diameter (cm) using digital calipar gauge, chloroplast number in guard cell using Jaskani et al.,(2005) approch (49), pollen fertility (%) and diameter (μm) using aceto-carmine staining technique, stomatal size (μm) and density (mm^2).

Statistical Analysis

Experimental data were analyzed using a t-test in Statistix 8.1.

Results

Chloroplast Number in Guard Cell

Statistical analysis shows a significant difference between the control and colchicine-treated plants C1A1 and C2A1, which effectively increase chloroplast numbers in guard cells of Crimson Sweet and Augusta. This suggests these treatment combinations promote higher chloroplast proliferation. The highly significant T-values (ranging from 33.46 to 41.22) indicate strong evidence that these differences are not due to chance. The lowest number of chloroplasts was observed in control plants of Crimson Sweet (5.667) and Augusta (5.9). However, the highest number of chloroplasts in Crimson Sweet (10.056 and 10.5) and Augusta (10.36 and 10.77) were found in plants germinated from seeds treated with colchicine concentrations (0.15% and 0.20%, respectively) in a single guard cell. Sprouts treated with colchicine at the indicated

concentrations show no difference; they have the same number of chloroplasts as the control. (Figure 1).

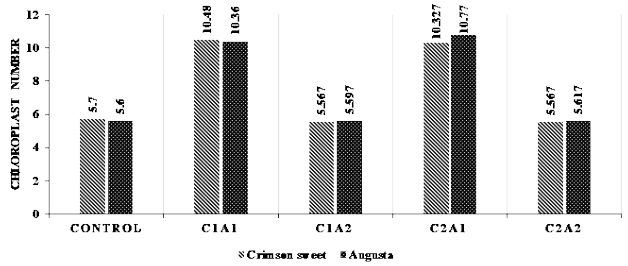

Figure 1: Impact of different concentration levels of colchicine and application method on chloroplast number in the guard cells of both varieties

Stomatal Size and Density

Statistical analysis showed significant differences in stomatal length, width, and density across various colchicine concentrations and control. The minimum stomatal length (16.91 µm) and width (10.67 µm), along with the maximum stomatal density (18.833 mm²), were recorded. At different colchicine concentrations (0.15% and 0.20%) during seed soaking, Crimson Sweet showed increases in stomatal length (24.37 and 25.52 µm, respectively) and width (19.16 and 17.57 µm, respectively). Meanwhile, Augusta exhibited increases in stomatal length (25.30 and 26.41 µm) and width (18.69 and 18.69 µm) at 0.15% and 0.20% colchicine, respectively (Figure 2). For stomatal length, treatments C1A1 and C2A1 in both varieties resulted in substantial increases, with t-values ranging from 88.11 to 139.92, indicating strong statistical significance and clear biological improvement compared to the control. A similar trend was observed for stomatal width, with t-values ranging from 40.20 to 89.60. Stomatal density significantly decreased in all plants treated with colchicine (both 0.15% and 0.20%), with values ranging from 12.563 to 10.75 mm². However, plants propagated from a sprout treated with colchicine (0.15% and 0.20%) showed no effect.

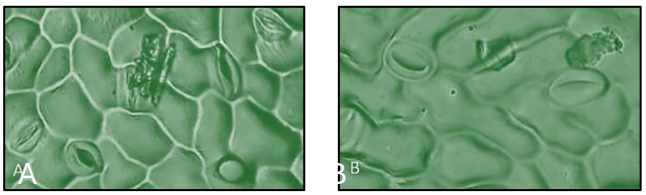

Figure 2: Difference in stomata size and density in (A) diploid and (B) tetraploid watermelon plants

Pollen Fertility and Diameter

The statistical study showed that seeds treated with 0.15% and 0.20% colchicine for 20 hours produced plants with larger pollen diameters. In Crimson Sweet, the pollen diameter increased to 5.51 µm at 0.15% and to 5.59 µm at 0.20% colchicine, respectively. However, the smallest pollen diameter (4.637 µm) was observed in the control treatment. The t-test analysis indicated that induced polyploidy treatments significantly affected pollen diameter in Crimson Sweet, with t-values ranging from 207.21 to 164.64. Plants treated with sprout soaking at 0.15% and 0.20% colchicine did not differ significantly in pollen grain diameter (Figure 3). Statistical analysis also revealed that pollen fertility (%) significantly decreased as colchicine concentration increased. In control plants, pollen fertility was highest at 82.908%, while it declined in all plants treated with colchicine at 0.1% and 0.20%, ranging from 81.957% to 76.653%.

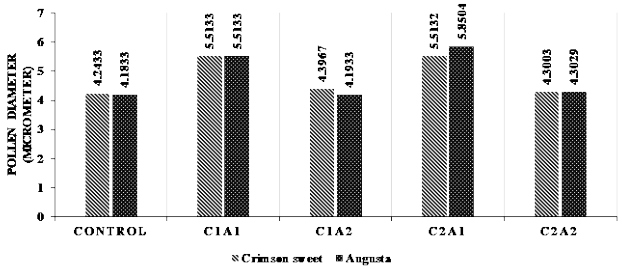

Figure 3: Impact of different concentration levels of colchicine and application method on Pollen diameter (µm) of both varieties

Appearance of First Leaf

There is a notable increase in the number of days until the first leaf appears in treated plants compared to the control. The delay in leaf emergence was the first clear indication that polyploidy had been induced by applying colchicine. Statistical analysis shows that, at various colchicine concentrations, the number of days needed for an effect to appear increased significantly (Table 1). Results indicated that the maximum number of days for the first leaf to emerge was recorded at 0.20% (12.083 days), while at 0.15% (11.389 days) for colchicine seed treatment in Crimson Sweet. In the control, the number of days is 6.5476. In Augusta, the first leaf appears almost the same as Crimson Sweet at 0.20% (11.913 days) and 0.15% (10.87 days).

Number of Leaves per Plant

The results show that control plants had the highest number of leaves (38.714 and 39.66 leaves). However, colchicine inhibits leaf production, decreasing the number of leaves in plants from seed treated with colchicine at 0.15% (26.889 and 30.84) and 0.20% for Crimson Sweet and Augusta, respectively (Table 1). The t-values (28.89–42.06) confirm that these differences are highly significant. There was no significant difference in leaf number between Crimson Sweet and Augusta plants treated with 0.15% and 0.20% colchicine during sprout soaking. These t-values confirm that all treatments significantly affected leaf production, with greater reduction observed under A1 treatments, indicating the sensitivity of vegetative growth to higher treatment levels.

Plant Length

In treated plants, vine length was approximately 27% shorter than in control plants. The maximum lengths recorded were 234.47 cm for Crimson Sweet and 286.31 cm for Augusta in the control group. In colchicine-treated plants, concentrations of 0.15% and 0.20% resulted in vine lengths of 170.17 cm and 167.67 cm in Crimson Sweet, and 209.67 cm and 191.33 cm in Augusta, respectively (Table 1).

Table 1: Impact of different concentration levels of colchicine and application method on morphological traits of both watermelon varieties

Treatment	First Leaf Appearance		Number of leaves		Plant length (cm)		Chlorophyll content		Stem diameter (cm)	
	crimson	Augusta	crimson	Augusta	crimson	Augusta	crimson	Augusta	crimson	Augusta
control	7.37	6.19	36.073	39.667	233.67	286.33	50.823	53.23	0.8467	0.95
C1A1	11.467	10.87	28.82	30.84	169.32	209.67	55.85	59.64	0.95	1.0733
C1A2	7.37	6.187	40.433	39.333	235.67	286.67	51.12	53.22	0.833	0.96
C2A1	12.127	11.913	26.74	29.763	166.93	191.33	55.85	61.113	1.0267	1.106
C2A2	7.41	6.17	38.433	40	234.67	284.67	50.937	53.203	0.8533	0.96

The very high t-values (ranging from 44.79 to 166.79) indicate that these differences are highly significant (α = 0.01). These observations were made 55 days after sowing, at which point control plants had nearly finished their vegetative growth. In contrast, plants from seeds treated with 0.1% or 0.20% colchicine remained in the vegetative stage and produced true leaves. Overall, the high magnitude of t-values across treatments confirms highly significant differences, showing that A1 treatments have a stronger inhibitory effect on plant elongation than A2 treatments.

Chlorophyll Content

The chlorophyll content in leaves varied significantly across different colchicine concentrations in plants grown from treated seeds and controls. The lowest chlorophyll levels were observed in the leaves of control plants of Crimson Sweet and Augusta (50.531 and 53.23). However, when seeds were treated with 0.15% and 0.20% colchicine, chlorophyll content increased in Crimson Sweet (55.411 and 55.958 mg/g FW, respectively) and Augusta (55.85 and 61.113 mg/g FW, respectively). There was no significant difference in plants with sprouts soaked in colchicine at these concentrations (Table 1). The very high t-values (110.68–176.33) indicate highly significant increases, especially under A1 treatments. The consistently high t-values show that all treatments significantly affected chlorophyll content, with A1 treatments producing the most substantial increase.

Stem Diameter

Analysis shows that seeds treated with colchicine concentrations (0.15% and 0.20%) significantly increase stem diameter. Low stem diameters of 0.84 and 0.95 cm were measured in control plants of Crimson Sweet and Augusta, respectively. Crimson Sweet's stem diameter increased to 0.956 and 1.045 cm when the seeds were treated with colchicine concentrations (0.15% and 0.20%, respectively). Similarly, Augusta's stem diameter increased to 1.0733 and 1.106 cm with colchicine concentrations (0.15% and 0.20%, respectively), indicating the plant is tetraploid. However, no significant difference was found between control plants and sprouts soaked in colchicine concentrations (0.15% and 0.20%) (Table 1).

Discussion

Based on the results, it was concluded that colchicine concentration was directly related to the number of days until the first leaf appeared. As the colchicine concentration increased, so did the time need for sprouting. Since colchicine-induced polyploidy disrupts normal cell division and growth processes, tetraploid plants took longer than diploid plants to develop their first leaves. Due to changes in genetic and cellular pathways caused by polyploidy, the timing of leaf emergence and overall plant development may vary. When *Hyoscyamus muticus* L was treated with colchicine, it was observed that the initial leaf of the tetraploid had a deformed appearance; previous studies clearly show that colchicine-induced tetraploids exhibit slower growth (18). Tetraploid plants grow slowly because the large number of chromosomes that must be synthesized during mitosis requires more energy and metabolites; consequently, cell division is reduced (19). The toxicity of colchicine was tested in Pelargonium seedlings during their early growth stages (20). Growth inhibition in colchicine-treated seedlings was also observed in balloon flower (21), tobacco (22), and London plane tree (23). The slower germination of colchicine-treated Trigonella seeds was due to reduced rates of meristematic cell division and differentiation (24). The reason pigeon peas take longer to germinate is that seeds treated with colchicine need time to develop a defense system against the toxic chemical (25).

Analysis of the effect of different colchicine concentrations on plant length showed that colchicine inhibits plant growth by reducing vine length at both concentrations in the seed soaking method. However, no significant difference was observed in the sprout soaking method for either variety (Crimson and Augusta). The reduction in growth indicates successful colchicine application to induce polyploidy. The decreased stem growth in tetraploids may be related to both low metabolite activity in these plants (26) and reduced cell division caused by a lack of growth hormones (27). The decrease in plant height in cotton and hairy vetch was due to a lower respiratory ratio, decreased metabolic enzyme activity, and limited auxin supply to cells under various colchicine treatments (28, 29). Colchicine induces polyploidy, but this process requires significant energy, which slows down cell division and limits the growth of balloon flower plants (21). The smaller distance between nodes was the reason for the lower plant height observed in Jimsonweed (30), azuki bean (31), purple coneflower (32), loquat (33), Lindernia sp. (34), and thyme (35), all of which are reported to experience a reduction in plant height due to colchicine.

Statistical analysis indicates that colchicine at various concentrations decreased leaf area, length, and width while increasing leaf thickness. Although colchicine-treated leaves were thick, shorter, and rough-surfaced, control plant leaves were broader, longer, and smoother. Previous studies have observed smaller, thicker leaves in colchicine-treated plants.

A reduction in cell division, which slows growth, or hormonal abnormalities may cause a drop in the number of leaves on plants treated with colchicine. Previous studies have shown that the number of leaves decreases in winged prickly ash (36), ornamental ginger (37), cowpea (38), balsam (39), and the orchid Vanda hybrid (40) when colchicine is used. Colchicine-treated watermelon, daylily, and alter Daeng orchid plants all exhibited shorter leaves (41-43). Treated plants display curled and wrinkled leaves. The development of leaf abnormalities in cowpea and melon under various colchicine treatments was also attributed to chromosome breakage, changes in ascorbic acid levels, decreased auxin supply, and changes in enzyme activity (44). Foliar abnormalities have also been observed in these species, as well as Japanese barberry, golden wattle, phlox, and alocasia (45-48).

According to the findings, colchicine treatments significantly increased chlorophyll synthesis, aligning with earlier observations of leaf color, which also indicated that plants treated with colchicine had dark green leaves the normal plant leaves. Tetraploid plants produce more chlorophyll and display darker leaves (49). Physiological disturbances and different cell division rates in colchicine-treated buds may be responsible for the color change in some mulberry leaves (50). The total chlorophyll content was higher, and the leaf color was deeper in tetraploids (29). Chromosomal doubling may increase cell size in most plant species, leading to broader, darker leaves with more chlorophyll (51). Tetraploid *Stevia rebaudiana* Bertoni plants had more glands, larger stomata, and a higher chlorophyll content index compared to diploid controls (52). Synthetic tetraploids outperformed their clonal diploid parents regarding net photosynthetic rate and chlorophyll content in leafy and fruit-producing mulberry (53).

Previous studies have shown that colchicine increases stem diameter. *Impatiens balsamina* L. was treated with a 0.01% colchicine concentration, which was found to increase the plant's diameter (54). Plants of *Cercis siliquastrum* have thicker, longer, and larger stems and roots. Colchicine solutions of 0.0%, 0.5%, 1.0%, 1.5%, and 2.0% were applied to seeds for 12, 24, and 48 hours, respectively (55). The effects of colchicine on the morphological characteristics of Artemisia plants and the induction of polyploidization were examined, revealing that the stem's diameter increased from 0.54 to 0.79 cm (56). The treatment group's plant height decreased by 35% compared to the control groups, while stem thickness increased, the number of generative shoots rose, and the length and width of leaves declined by 47%, 18%, 23%, and 22%, respectively.

The results show that colchicine increases the number of chloroplasts in guard cells. According to earlier research, guard cell chloroplast density, stomata frequency, and size are the main factors considered during the anatomical evaluation of cells (57). Compared to diploids, polyploids often have larger stomata in lower densities and more chloroplasts per guard cell (57, 58). The tetraploid *Dendranthema indicum* L. exhibited larger stomata, a greater number of them, and more guard cells per chloroplast than the controls (59). Tetraploid apples contain a much higher pigment content, especially chlorophyll a and b (60). The ploidy of regenerated plants has been estimated using somatic chromosome counts, but the number of chloroplasts can be counted more precisely in each guard cell pair of leaf epidermal cells (61). This method has been used to distinguish diploid from tetraploid melon regenerants. It has also been applied to distinguish tetraploid from diploid musk-melon plants that were grown after diploid seedlings were treated with colchicine (62), as well as in potato (63), and tomato (64-66). Chloroplast counting is an accurate indicator of polyploidy during plant development (49).

The enlargement of pollen grains under A1 treatments suggests successful induction of polyploidy, as increased nuclear content is commonly associated with larger reproductive cells. The diameter of pollen is directly related to the concentration of colchicine and increases as the concentration does. The induction of polyploidy, which increases the genomic size, may be responsible for this difference in pollen diameter. The cellular structure is altered by polyploidization to allow the expansion and development of the doubled genome. Larger pollen grains in polyploid plants treated with colchicine have also been observed in cotton (67), zoysia grass (68), physic nut (69), sunflower (70), and evergreen blueberry (71). The size of pollen particles can serve as a primary indicator for distinguishing plants with different ploidy levels. Plants of the night-blooming cactus that received a metaphase inhibitor had larger pollen grains (72). The uniform increase in stomatal size under A1 treatments across both Crimson Sweet and Augusta selections, supported by high t-values, confirms the successful and consistent induction of polyploidy. These findings suggest that stomatal measurements can be used as a quick and reliable screening tool for identifying polyploidy, while moderate treatments (A2) appear less effective in inducing such cytological changes. The 0.20% colchicine concentration was identified as the most effective for enlarging stomata. Polyploidy development is typically accompanied by changes in stomatal size. Stomatal size and density are indicators of polyploidy, making the identification of tetraploids a simple, effective, and non-destructive process. In contrast to tetraploids and triploids, diploid plants had 1.8 and 1.5 times more stomata than tetraploid or triploid plants (80). In patchouli plants treated with colchicine, a high ploidy level (tetraploid) was associated with increased guard cell length and decreased density (81). An increase in the size of polyploid cells may explain the maximum stomatal dimensions observed in colchicine-treated phlox plants (47). Because more genes are present, cells with greater genomic content enlarge to maintain the same cytoplasmic-to-nuclear volume ratio and to enhance protein expression. Jujube (82), citrus (83), poplar (84), purple coneflower (85), sundew (86), and field bean (87) have also been shown to develop the largest stomata with reduced stomatal density when treated with colchicine.

Conclusion

The experimental findings revealed successful induction of tetraploid plants using both colchicine concentrations

during seed treatment. Notably, a 0.20% colchicine concentration was the most effective in inducing tetraploids in Augusta plants, showing delayed first leaf emergence, darker thicker leaves with high chlorophyll content, shorter vines with larger diameter, and increased stomata size with more chloroplasts in guard cells, along with larger pollen but reduced fertility. In contrast, 0.15% colchicine showed the lowest tetraploid induction rate. The sprout soaking method was ineffective at both concentrations. Interestingly, the seed-soaking method produced significant numbers of tetraploid plants in both Crimson Sweet and Augusta, highlighting the importance of seed treatment for successful induction. In conclusion, the study emphasizes the crucial role of colchicine concentration and application method in inducing tetraploidy, with seed treatment showing promise for developing tetraploid plants in selected varieties. Further research is needed to understand the mechanisms and optimize the process for agricultural improvements.

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