Morphological characterization of Colchicine-induced Mutants in Stevia rebaudiana

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Abstract. Stevia rebaudiana is a sweet herb that belongs to the family Asteraceae. Stevioside, a natural sweetener derived from Stevia leaves are 100-300 times sweeter than sucrose and contain a complex mixture of sweet diterpene glycosides. The mutagenic effect of colchicine to improve leaf and steviol glycosides yield in stevia was investigated. Stevia plant were treated with five different colchicine concentrations (0.5%, 1.00%, 1.50%, 2.0%, 2.5% and untreated plantlet (0.0%) act as control) for 48h. The plants were arranged in a completely randomized block design. Treated plants were characterized based on morphological traits such as plant height, leaf length and leaf thickness. The results obtained revealed significant difference (p≤0.05) in the morphological traits of the mutants when compared with the control. Overall, 2.0% colchicine concentration resulted in higher average plant height by 36.0% ± 0.6 leaf length increments by 43.0% ± 0.7 and the leaf thickness increment by 5.0% ± 0.1. The improvement of the mutants’ traits is concentration dependent and increases with increment in colchicine concentration. Thus, we therefore suggested that 2.0% concentration should be employed in improving stevia growth and yield related traits.

Keywords: Colchicine Concentrations; Leaf Thickness; Steviol Glycosides Yield; Stevia rebaudiana

1. Introduction
Stevia rebaudiana Bertoni is a native herbaceous perennial plant found in highlands of Paraguay that belongs to the family of Asteraceae [1]. Stevia leaves are made up of various sweet tasting secondary metabolites or glycosides namely stevioside (5–10%), rebaudioside-A (2–4%), rebaudioside-B, C, D, M and dulcoside, which are high potency sweeteners with comparable sweetness of around 300–400 times sweeter than common sugar or sucrose. Rebaudioside-A is the highly prized glycoside as it is the best tasting with no leafy or metallic aftertaste and sweeter in comparison to other glycosides present [2]. Stevia is sourced as a natural noncalorie sweetener and an alternative to the synthetic sweeteners...
One method known in improving secondary metabolite contents in plants is by using chemical mutation. Applying polyploidy agents or chemicals has been known to be used as a mutating mechanism in the breeding of polyploid plants. Polyploid plants experience increments in organs sizes as example in the leaf thickness and width, plant growth, lengths and changes in secondary metabolites yields especially in medicinal plants [4]. Polyploid plants may occur naturally due to some cytological mechanisms, or by applying the plants with anti-mitotic agents such as colchicine, oryzalin and trifuralin. Colchicine is a natural product extracted from the bulbs and seeds of the plant known as meadow crocus or Colchicum autumnale L. [5, 6]. The way it works is by preventing microtubules formation during cells mitosis and this then resulted in polyploidy in the plants. These artificial polyploidy effects by colchicine may induce some changes in morphological, physiological, cytological and even in the expression of gene levels with number of studies reported successful artificial polyploidy induction in different plants [7, 8]. There have been numerous research studies conducted on polyploidy in ornamental plants in order to improve the size of the flowers, create sturdier stems, improving the hardness and robustness of the plants and intensification of colours of the plant [9]. A research on the plant Lolium multiflorum where the treated seeds with 0.2 and 0.4% colchicine solution resulted in the significant effects on certain agronomic traits and allele frequency in the plants [10]. Another method in introducing colchicine were by the dipping of leaf petiole of Torenia fournieri with 0.01% colchicine solution resulted in the significant effects on morphological traits [11]. In other studies, the yield potential, height of the plant, fresh weight and number of leaves for potato plant that were applied with colchicine during the sprouting buds’ stage, showed improvement in all the parameters mentioned [12]. Plant mutation through colchicine has showed a remarkable positive result and are able to greatly improve the yield of the plant secondary metabolite. Therefore, present study was carried out to investigate the mutagenic effect of colchicine on agromorphological traits in stevia.

2. Materials and methods

2.1. Preparation of plant material Stevia rebaudiana Bertoni

Cuttings of Stevia rebaudiana plants from the assession Jalan Kebun were obtained from the greenhouse of Universiti Teknologi-MARA Cawangan Puncak Alam. The methods of obtaining the plant samples were as following: 1) Microcuttings of stevia were obtained from mother plant. 2) The microcutting were cut slanted to increase surface area and dipped inside a rooting hormone powder. 3) The dipped microcuttings were then placed inside a mist-box setup to allow for optimal root growth under normal sunlight exposure. 4) After 2 weeks of root development the plantlets were taken out of mist-box and placed into polybags filled with mixed soil ready for colchicine treatment. The methods are slight modification from previous studies [13].

2.2. Chemical mutation of Stevia rebaudiana Bertoni using colchicine

Plantlets of Stevia rebaudiana were left to grow for one month. Pure colchicine powder was obtained from Shaanxi Huike Botanical Development Co. Ltd. After one month the shoot tip (ST) of the plants were cut. Each plant was cut at three different shoot tip point. Sterile cotton balls of 1cm by 1cm in size were placed on the pre-cut ST of the plant. Colchicine powder was diluted using room temperature distilled water to five different concentrations of 0.50%, 1.00%, 1.50%, 2.00% and 2.50% with distilled water was act as control at 0%. Using a dropper three drops of each colchicine concentrations were dropped onto the cotton plug to soak in colchicine. The cotton plugs were again soaked with three drops of colchicine after 24 hours and were exposed to colchicine for a total of 48 hours. After 48 hours the cotton plug was taken off to allow for the new growth of the shoot tip. The method was performed following [14] with slight modification.

2.3. Stomatal observation

The cytological features of the plantlets were evaluated and compared with each other. Stomatal density measurement was used for identification of variation in plantlets. Two well expanded leaves from each the untreated and treated plantlets were selected at random. The bottom part of the leaves was covered with a thin layer of clear nail polish and allowed to dry. After drying the polish, it was removed carefully
using forceps. It was placed on a glass slide and observed through the light microscope. The stomata were then photographed. The calculation of stomata density was done using the formula (Stomatal density = number of stomata in entire field of view (FOV) / area (mm²)) [15].

2.4. Morphological study and data collection
Two months after treatment, the treated plant shoot tip was examined morphologically and also screened for any ploidy changes. Data were recorded on the shape of leaf, length of leaf, thickness of leaf, and plant height.

2.5. Statistical Analysis
The obtained data were statistically analysed using one-way ANOVA and subsequent comparison of means was performed using the post-hoc Tukey test at 95% confidence interval. Statistical analysis was carried out using computer software (SPSS for Windows, ver. 22.0).

3. Results and discussion
The specific function of the colchicine is to disrupt the formation of microtubules in mitosis in order to induce polyploid cells [16]. The use of colchicine can increase the number of chromosomes, often disturbs the mitosis process of the plant resulting in higher ploidy and multiple stages of cell division at one time.

3.1 Effect of colchicine on the leave shape and morphology

![Figure 1 Comparison between control (a) and colchicine-induced stevia plant (b)](image)

Figure 1 Comparison between control (a) and colchicine-induced stevia plant (b)
Table 1. Leaf shapes and characteristics based on different treatments

| Accessions | Plant type | Leaf Shape | Tip  | Base  | Venation | Margin   |
|------------|------------|------------|------|-------|----------|----------|
| Control    | Loose      | Lanceloate | Obtuse| Cuneate| Reticulate| Incised  |
| 0.50%      | Loose      | Obovate    | Acute| Cuneate| Reticulate| Incised  |
| 1.00%      | Loose      | Obovate    | Acute| Cuneate| Reticulate| Incised/Dentate |
| 1.50%      | Loose      | Obovate    | Acute| Cuneate| Reticulate| Incised/Dentate |
| 2.00%      | Loose      | Obovate    | Acute| Cuneate| Reticulate| Incised   |
| 2.50%      | Loose      | Obovate    | Acute| Cuneate| Reticulate| Incised   |

Colchicine-induced stevia plant demonstrated bigger size of leaves as compared to the control plant (Fig 1a and 1b). In plants, colchicine often induces “giga” effects meaning to enhance the sizes of various plant organs mainly the leaves and stems. This confirmed the result showed in this study. The leaves of stevia are the most important organ of the plant as it contains the highest content of the steviol glycosides (SG) in general. Other parts of the plants such as the plant stem do contain steviol glycosides although in a lower concentration. Hence the leaves in stevia have the highest economic value. The introduction of colchicine onto the plant showed physical changes in terms of the shapes of the leaves and the size of the stem. The leaves of the untreated stevia plant have a slim, oblong and elongated shape as compared to the shape of the treated stevia leaves that developed a rounder and wider shape of the leaves (Table 1). The shapes of the leaves showed that the treated leaves with acute and rounder leave positively increase the yield of secondary metabolite stevioside. This is parallel with the report made by previous studies [17].
The untreated plant presented leaves (Figure 2b) that have a more serrated margin while the treated group with colchicine showed margin in leaves that are mixed either serrated or dentate margin (Figure 2a and c). This indicates some alteration in the gene of the plant resulting in the mixed phenotype shown by the shapes and margin of the leaves. A study reported [18] on two strains of stevia that differ morphologically where the two strains (strain A with obtuse and strain B with acute leaf tip, with the plant type of the former being compact and the latter being loose) and strain A showed higher stevioside value but lower rebaudioside-A content as compared to strain B. This showed that there is correlation between the morphology of stevia with the steviol glycoside contents and yield. However further confirmation through molecular work will be needed to indicate the changes occurring in the genetic makeup of the plant.

3.2 Effect of colchicine on the plant height, leaf length, leaf thickness and mean stomatal density.

Table 2. Analysis of plant height, leaf length, leaf thickness and mean stomatal density in response to colchicine treatment in stevia

| Accessions | Plant height (cm) | Leaf length (µm) | Leaf thickness (µm) | Stomatal Density (µm²) |
|------------|------------------|------------------|--------------------|----------------------|
| Control    | 40.21±0.1a       | 2.69±0.2a        | 163.90±1.5a        | 83±5.7ab             |
| 0.50%      | 46.44±0.2b       | 3.36±0.1b        | 173.90±1.5bcdef    | 93±5.7ba             |
| 1.00%      | 65.32±0.2c       | 5.33±0.6ce       | 171.70±1.9bc       | 137±5.7cdf           |
| 1.50%      | 75.36±0.1d       | 6.39±0.1d        | 176.60±2.4d        | 151±5.7cdef          |
| 2.00%      | 86.39±0.1e       | 6.88±0.1ec       | 181.00±2.2e        | 142±5.7cdef          |
| 2.50%      | 76.50±0.2f       | 6.06±0.1f        | 174.60±2.6d        | 125±5.7cdef          |

Note: Different alphabet denotes significant differences at p ≤ 0.05

The analysis of variance showed that colchicine levels significantly affected the leaf morphology in terms of the mean plant height, leaf length, leaf thickness and the stomata density. The entire treatment group showed significant increase of plant height in comparison to the control. The highest plant height (86.39±0.1), the leaf length (6.88±0.1) and leaf thickness (181.00±2.2) were found at plant treated with 2.00% of colchicine concentration. The leaf length demonstrated steady increment as the concentration of colchicine increased. However, there is non-significant difference between the treatments with 1.00% and 2.00% colchicine concentration. This suggested that the usage of 1.00% colchicine produces comparable leaf length against 2.00% of colchicine concentration. The leaf length is a vital morphology criterion to be considered as stevia plant most prized organ is the leaf part because it contains the most content of steviol glycoside and steviosides. The leaf thickness showed an increase of 5.00% from the control plant. There was a significant difference between the control treatment with all the other colchicine induced group except for 0.5%. However, 1.0%, 1.5%, 2.0% and 2.5% colchicine treatments showed no significance difference for the leaf thickness.
The identification of variation in plants can be determined by the stomatal morphology [19]. The environment and abiotic factors i.e. temperature and water content does not affect the stomata density. Hence, it is a reliable method to determine variation between plantlets [20]. Analysis of variance showed the highest mean for density of stomata at 1.50% of colchicine treatment. The increment in comparison with control was recorded by 29.0%±0.1. There is a significant difference between control and the treatment group except for the 0.50% treatment of colchicine. The result showed an increase of around 59 stomata higher when treated with colchicine. The size of treated stomata also showed bigger size and girth in comparison to the control. This improvement in stomata size and density indicate the increment in steviol glycosides content as reported by the previous study [1].

4. Conclusion
The induction of colchicine in stevia demonstrated the effectiveness of colchicine as an inducer and mutating agent. The different plantlets features obtained in this experiment generated immense variability for morphological characteristics. Based on the morphology parameters studied, the best treatment to produce better plant height, increment in leaf length and thickness is by using the 2.00% concentration of colchicine. Meanwhile the application of 1.50% of colchicine showed the highest value for stomata density. These plantlets need to be multiplied and evaluated further for glycoside profile and analyzing the genotypes through molecular work for supporting evidence of mutation. Breeding of the mutant stevia will generate new variations with respect to biomass and steviol glycoside content. This will greatly benefit stevia improvement as a low-calorie sweetener and future cash crops.

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