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# Morphological and Anatomical Comparison between Tetraploid *Stevia rebaudiana* (Bertoni) Bertoni and its Parental Diploid

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**ABSTRACT**

***Stevia rebaudiana* (Bertoni) Bertoni is a perennial herbaceous plant that produces natural low glycemic-index sweeteners alternative to sugar cane. The genetic improvement of Stevia needs to be investigated to increase its productivity. Although this plant has a narrow genetic diversity, genetic improvement through polyploidization may succeed. Meanwhile, genotypic characters of tetraploid Stevia were previously reported. This study then observed the phenotypic characters of the tetraploid plants, which aimed to evaluate the morphological and anatomical characteristics of tetraploid Stevia clones compared to its wild-type parental diploid plant. Three tetraploids and one diploid clone were grown in the field for 12 weeks. The results of qualitative descriptive characters showed that the tetraploid clones B60.3H8, P1T22 and P3T5 had habitus type, shoot bud shape, leaf margin, leaf venation, shape, and type of stem, type of flower, and seed similar to the diploid parental clone. However, the quantitative characters showed that the tetraploid clones had leaf size, stem diameter, root size, length of the single flower, length of the flower stalk, and length of fruit which were more extensive than those of the parental diploid clone. The leaf color of tetraploids was a darker green, and the range of initial flowering times was earlier. This finding gives more understanding of the phenotypic and anatomical characteristics of Stevia tetraploids compared to their parental diploid plant.**

## 1. Introduction

Stevia, *Stevia rebaudiana* (Bertoni) Bertoni, is a herbaceous plant native to Paraguay (Soejarto 2001). This plant has high commercial value because it contains a natural sweetener used as an alternative source of cane sugar (Singh *et al.* 2019). The sweetness source is steviol glycosides, which comprise glycosides stevioside (4-13%) and rebaudioside-A (2-4%), mainly found in its leaves (Brandle *et al.* 1998). The glycosides are also beneficial for diabetic patients because it has zero glycemic indexes and can control glycemic and lipid profiles in type 2 diabetic patients (Ajami *et al.* 2020; Gardana *et al.* 2003). Steviol glycosides are 70-400 times sweeter than sugarcane (Chughtai *et al.* 2020; Perez *et al.* 2016), but Stevia still has a bitter aftertaste. This bitter aftertaste compound in Stevia leaves is caused by its high stevioside content.

Furthermore, Stevia cultivation in Indonesia is still constrained by several factors, such as low production and limited areas of cultivation which usually require 700 to 1,500 meters above sea level (m asl), a temperature of 20–24°C, and an average rainfall of at least 1,400 mm/year (Djajadi 2014). The regional climate adaptation and Stevia's agronomical properties are limited by its narrow genetic diversity (Carvalho *et al.* 2011; Yadav *et al.* 2011). Therefore, increasing its genetic variation is necessary to facilitate the selection to obtain superior genotypes.

Polyploidization can be an option to widen the genetic diversity of the crop. Tetraploidy in Stevia generated doubled chromosome numbers (2n) = 4x = 44 (Brandle and Telmer 2007; Yadav *et al.* 2011). Polyploidization may affect some plant traits. In *Dianthus broteri*, diploidization affected physiological behavior in transpiration, photosynthetic, and growth

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rate (Dominguez-Delgado *et al.* 2021). Meanwhile, phenotypic traits analysis helps to characterize the proteome profile between diploid and tetraploid *Paulownia australis* (Wang *et al.* 2017).

Before, we reported genotypic tetraploidization of Stevia with greater Stevioside and Rebaudioside-A content (Adabiyah *et al.* 2019). However, the phenotypic characterization of the tetraploid Stevia genotypes has not been performed. Therefore, our research evaluated the morphological and anatomical characteristics of tetraploid *Stevia rebaudiana* compared to its parental diploid. These characteristics are beneficial to complement the genetic properties of the tetraploid Stevia clone we previously obtained with its structural properties.

## 2. Materials and Methods

### 2.1. Planting Materials

The starting materials for this research were *in vitro* plantlets of *Stevia rebaudiana* from the collection of Plant Cell and Tissue Culture Laboratory-Research Center for Genetic Engineering BRIN, Cibinong, West Java, Indonesia. We used one diploid Stevia as control, and three tetraploid clones, namely B60.3H8, P1T22, and P3T5. Clone B60.3H8 was obtained by oryzalin treatment. Meanwhile, clones P1T22 and P3T5 were obtained by 0.1% colchicine treatment. The plantlets were acclimatized and reproduced by cutting for 11 weeks. Then the plants were planted in an experimental field in Cibodas Botanical Garden-BRIN, Cianjur, West Java, Indonesia (1,275-1,425 m asl). Thirty plants were prepared for each clone to undergo further observations.

### 2.2. Plant Morphological Characterization

The morphological characteristics were observed on the plant habitus, shoot bud, leaf, root, flower, fruit, and the seed of plants aged 8-12 Weeks After Planting (WAP). 5-10 plant samples represented each clone for this determination. The morphological characteristics were described by referring to the Plant Descriptors for Cultivated and Wild Sunflower from the International Board for Plant Genetic Resources (1985), the Plant Identification Terminology (Harris and Harris 2001), as well as Flora of Great Britain and Ireland Vol. 4 Campanulaceae-Asteraceae (Sell and Murrell 2006).

Leaf area in a plant was measured using the gravimetric method (Sitompul and Guritno 1995) by comparing the weight of the leaf replica to the weight of a determined square millimeter of the replica material. The leaf area was estimated by using the equation:

$$LD = \frac{Wr}{Wt} \times LK$$

LD = Leaf area ($mm^2$)
Wr = Paper weight of leaf replica (g)
Wt = Weight of leaf replica in square shape (g)
LK = Area of leaf replica in square shape ($mm^2$)

Leaf color was recorded according to the Munsell Plant Tissue Color Chart (1977). The leaves were taken from three-leaf positions, i.e., the upper, middle, and lower stems. The positioning of the leaves was based on the stem nodes, from bottom to top. The top position was taken from the stem nodes 9-10, the middle position was from the stem nodes 6-7, and the lower position was from the stem nodes 3-4. Flower characteristics were observed on five plants per clone, taken throughout the development of flowers, from bud to maximum blooms. Fruit characteristics were observed at the time of harvest 50-60 days after planting. Morphological observations of the flower and fruit were done using Olympus CX21FS1 binocular microscope (Japan) and Optilab Advance plus digital microscope camera (Miconos, Indonesia). The flower was taken randomly from the main shoot or the branch.

### 2.3. Plant Anatomy Analysis

The histological observations of plants were carried out by making a transverse section of leaves, stems, and roots using the semi-permanent Wholemount method (Sass 1951). Incisions were made using samples of plant parts aged 8-10 WAP. For anatomical analysis, the leaf and stem from the middle main stem on the 6-7$^{th}$ nodes were isolated, and young fibrous roots 1.5 mm in diameter were taken.

Histological observations were carried out using an EZ4HD Stereo microscope with Leica Application Suite (LAS) software and a Dino-Lite Digital Microscope (AM7115MTF, Taiwan) with 4 × 10 times magnifications. The leaf tissue's thickness and the roots and stems' diameter were measured using the Image Raster Application.

### 2.4. Ploidy Level Confirmation

Ploidy level was reconfirmed on young leaves using a flow cytometer (BD Accuri+, USA). The leaves of Stevia diploid plants were used as the standard. Leaf pieces (0.5 × 0.5 cm) were placed on a petri dish. Then they were dropped with 1.5 ml of UV-Ploidy cysteine buffer, then chopped finely with a razor blade. The chopped leaves were filtered through a 30 m millipore sieve. The filtrate was put in a cuvette

tube for analysis. The analysis was carried out at a wavelength of 400 nm and a speed of 1,000 nuclei per sec (Ermayanti *et al.* 2013).

### 2.5. Data Analysis

Qualitative data were analyzed descriptively, while quantitative data was processed through analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) using IBM SPSS Statistics version 21.0 with a 95% confidence level. In addition, Principal Component Analysis (PCA) was also done according to Statistical Software for Excel (XLSTAT) 21 Program. Principal Component Analysis was carried out separately according to morphometric and anatomy measurement data.

## 3. Results

### 3.1. Plant Morphological Characteristics

#### 3.1.1. Habitus and Shoot Bud

Eight weeks after planting, the habitus of parental diploid and tetraploid Stevia clones was characterized as an herb (perennial herbaceous), having short-sized with less than 1 m in height, and many branches on the main stem as well as heavy leaf canopy and wet stems. Diploid and tetraploid clones had the same type and shape of buds, namely *gemma terminalis* (Figure 1). Furthermore, the terminal leaf buds were classified as naked because they had no protective leaves.

#### 3.1.2. Leaf Morphology

Polyploidy did not affect the plant leaf morphology either. Diploid and all tetraploid clones had the same type of leaf arrangement, i.e., a single leaf on a stalk attached directly to the stem and arranged in cross pairs (*folium oposita*) along the main stem. The leaf is classified as a spatulate resembling a spoon or spatula, with leaf strands shrinking, tapering from the base, and rounding off the edges with the top of the pointed leaf (*acutus*). The leaf has a ratio of length and width of 3:1. In addition, the leaf was thin and had a soft fleshy leaf structure (*interveium*).

The diploid and tetraploid clones had the same leaf shape and type (Figure 2). The leaf margin of the diploid (Figure 2A) and the tetraploid (Figures 2B-D) had a notched-like jig-saw margin with teeth pointing toward the apex (*serrated leaves*).

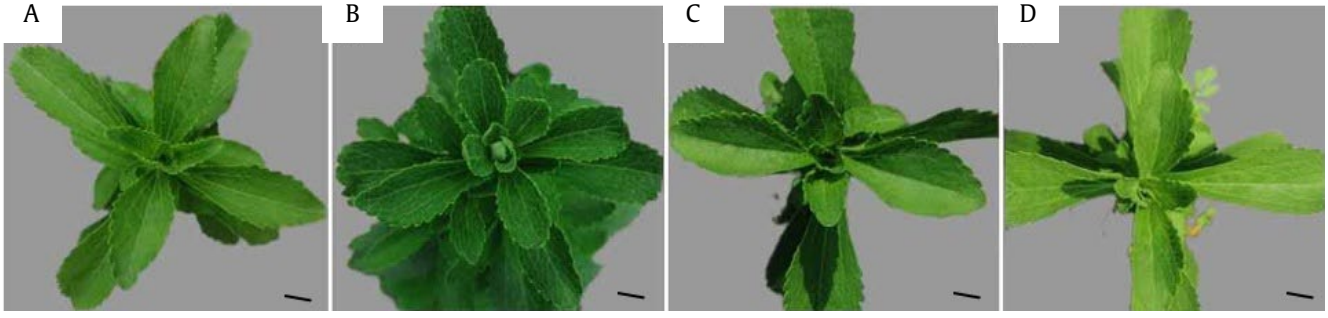



Figure 1. *Gemma terminalis* type of shoot bud of *S. rebaudiana*. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 1 cm

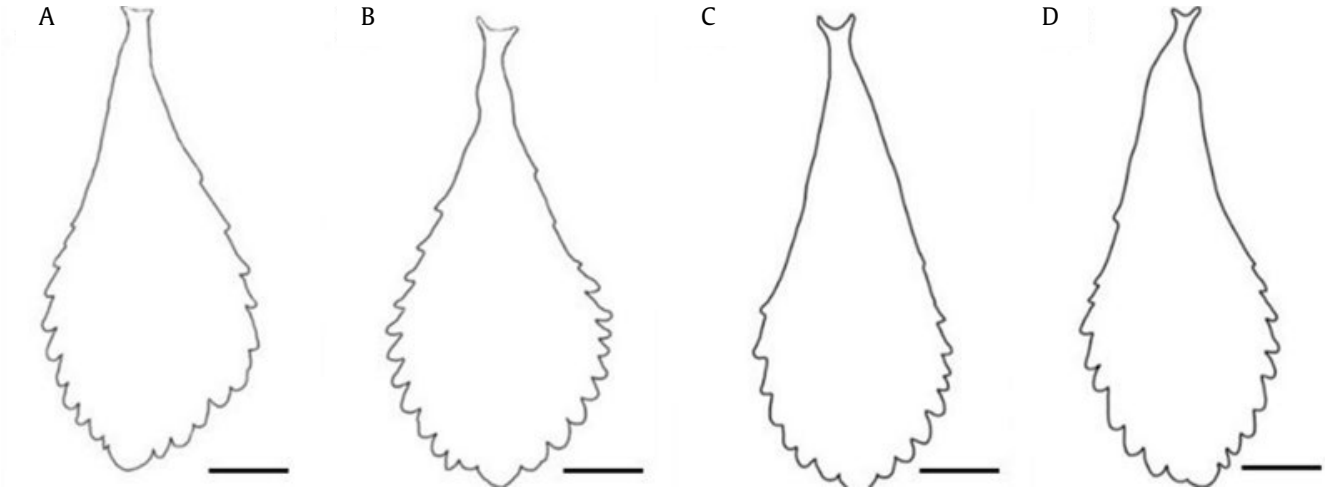



Figure 2. Leaf margin of *S. rebaudiana*. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 1 cm

Table 1 shows that the tetraploid clones' leaf character (length, width, area) is bigger and significantly different from the parental diploid clone. The leaves of the tetraploid P3T5 clone had the largest size. In addition, the leaf color of tetraploids indicated a color number of 7.5 compared to 5 for the diploid clone. It means that the tetraploids had darker green leaves than the diploids. Meanwhile, the diploid clone is brighter (3/2 level) than the tetraploid clones (3/4 level).

### 3.1.3. Stem Morphology

Diploid clone and all tetraploid clones had the same stem type and shape, i.e., rod type (herbaceous) (Figures 3A-D). The stem of the diploid and the tetraploid clones had a rounded shape (*terres*) with a rough surface with the presence of trichomes. Polyploidization did not affect the color of the stem. Both diploid and tetraploid clones had the same color, yellowish-green. The stem diameter of the tetraploid plants was more significant than the diploid plants (Table 2).

### 3.1.4. Root Morphology

The results show that roots of diploid (Figure 4A) and tetraploid clones (Figures 4B-D) had the fibrous root type (*radix adventicia*). The whole root emerged from the stem base. Tetraploid B60.3H8 had the longest root, while P1T22 and P3T5 had the shortest roots compared to the parental diploid clone. The most oversized root diameter was found in P1T22. The roots of the parental diploid were thin and long (Table 3).

Table 2. Stem diameter of *S. rebaudiana*

| Clone | Stem diameter (mm) |
|---|---|
| Parental Diploid | 4.97±0.25$^{b}$ |
| Tetraploid : | |
| B60.3H8 | 6.28±0.24$^{a}$ |
| P1T22 | 6.03±0.14$^{a}$ |
| P3T5 | 5.73±0.17$^{a}$ |

The numbers within the same columns followed by the same uppercase letter(s) are not significantly different at P≤0.05, as determined by Duncan's Multiple Range Test

Table 3. Length and diameter of roots of *S. rebaudiana*

| Clone | Root size | |
|---|---|---|
| | Length (cm) | Diameter of tap root (mm) |
| Parental Diploid | 17.26±0.54$^{b}$ | 1.55±0.03$^{c}$ |
| Tetraploid : | | |
| B60.3H8 | 18.70±0.65$^{a}$ | 1.80±0.05$^{b}$ |
| P1T22 | 11.78±0.22$^{c}$ | 1.97±0.03$^{a}$ |
| P3T5 | 12.24±0.39$^{c}$ | 1.76±0.03$^{b}$ |

The numbers within the same columns followed by the same uppercase letter(s) are not significantly different at P≤0.05, as determined by Duncan's Multiple Range Test

Table 1. Length, width, and area of *S. rebaudiana* leaf

| Clone | Leaf length (cm) | Leaf width (cm) | Leaf area (cm$^2$) | Leaf color |
|---|---|---|---|---|
| Parental diploid | 6.48±0.10$^{b}$ | 2.29±0.05$^{c}$ | 8.12±0.29$^{c}$ | 5 GY 3/2 (dark olive green) |
| Tetraploid: | | | | |
| B60.3H8 | 7.06±0.11$^{a}$ | 2.94±0.09$^{ab}$ | 11.22±0.46$^{b}$ | 7.5 GY 3/4 (moderate olive green) |
| P1T22 | 7.12±0.13$^{a}$ | 2.88±0.08$^{b}$ | 11.80±0.53$^{ab}$ | 7.5 GY 3/4 (moderate olive green) |
| P3T5 | 7.39±0.14$^{a}$ | 3.15±0.08$^{a}$ | 12.83±0.52$^{a}$ | 7.5 GY 3/4 (moderate olive green) |

The numbers within the same columns followed by the same uppercase letter(s) are not significantly different at P≤0.05, as determined by Duncan's Multiple Range Test

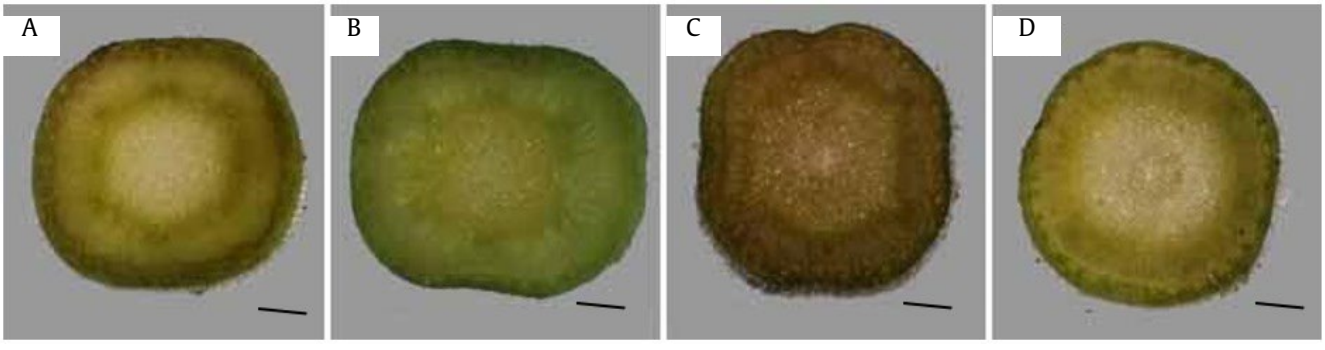



Figure 3. Cross section of *S. rebaudiana* stem. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 1 mm

### 3.1.5. Flower Morphology

Stevia flower structure was classified as a paniculate compound flower. The inflorescence was arranged and composed of 5 single flowers, wrapped by 5 circled protective leaves (*involucrum*), encasing the base of the flower compound to form a saucer (*corymb*) called capitulum. All clones, both diploid and tetraploid, had the same shape and arrangement of the flower head (Figures 5A-D).

The five protective leaves (*involucrum*) intertwined at the base and were arranged to resemble a star from the top sight. It was green, with a coarse texture with fine hairs. All clones, both diploid and tetraploid, had the same shape and color of flower involucrum (Figure 6).

The single flower, both in the parental diploid and tetraploid clones, had the same components, i.e., the base of the flower (*capitulum*), the floral tube, the ovary, the stamen, and the pistil. The floral tube is directly joined to the carpel. The floral tube and the ovary were yellowish-green (Figures 7A-D).

The crown (corolla) of an individual flower, in both diploid and tetraploid clones, had the same structure and purplish-white color. The shape of the crown in individual flowers resembled a star, with 5 strands of a crown attached directly from the base of the floral tube (Figures 8A-D).


Figure 4. Fibrous roots of *S. rebaudiana*. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5

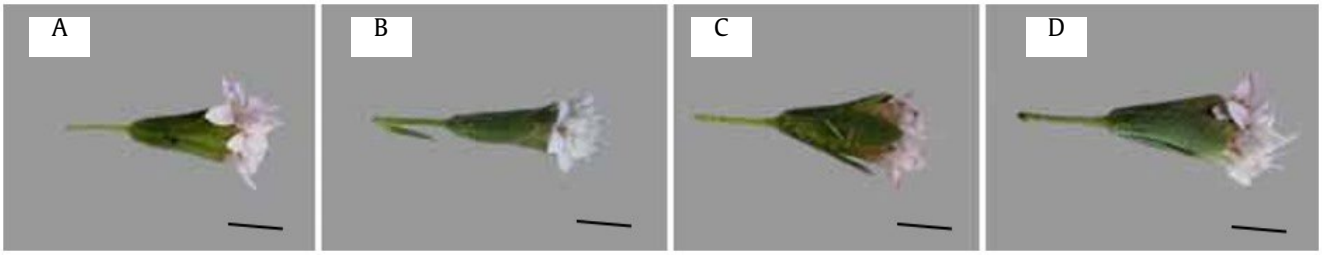



Figure 5. Inflorescence capitulum of *S. rebaudiana*. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 2.5 mm

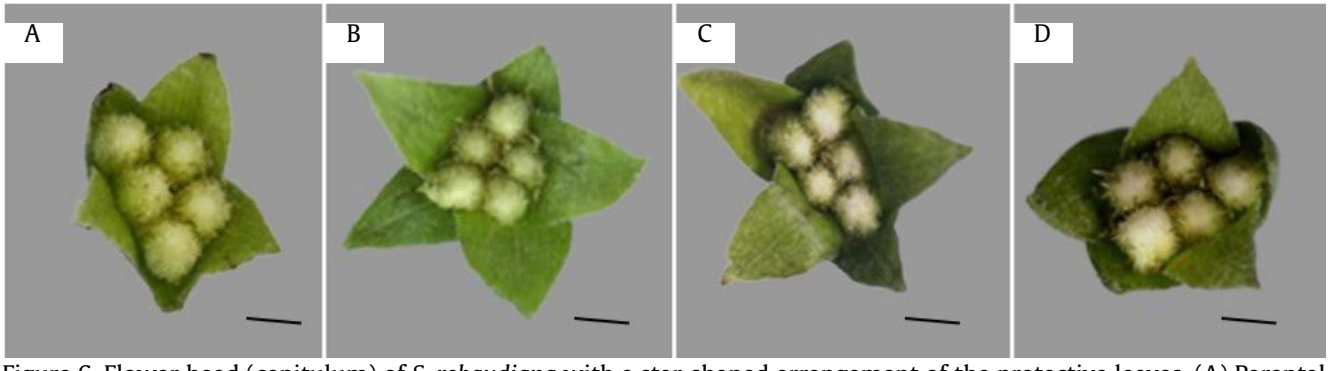



Figure 6. Flower head (capitulum) of *S. rebaudiana* with a star-shaped arrangement of the protective leaves. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 1 mm

The pistil of the diploid clone (Figure 9A) and the tetraploid clones (Figures 9B-D) had the same shape, thin in size, with a stigma having two hooks (*bilobus*). The stylus was divided in the middle into two-three branches, surrounded by the stamens. The pistil was green at the bottom and white at the top (stigma).

The stamen of the diploid (Figure 10A) and the tetraploid clones (Figures 10B-D) were thin in shape like spears, at the center of which there were sections like interlocking wings (connate in the middle) forming tubes. The stamens were separated from each other, sticking from the bottom of the flower and surrounding the pistil.

The diploid clone had different flowering times from the tetraploid clones (Table 4). The diploids were earlier to flower than the tetraploid clones. The diploid produced the highest number of flowers compared to tetraploids but the smallest in size of

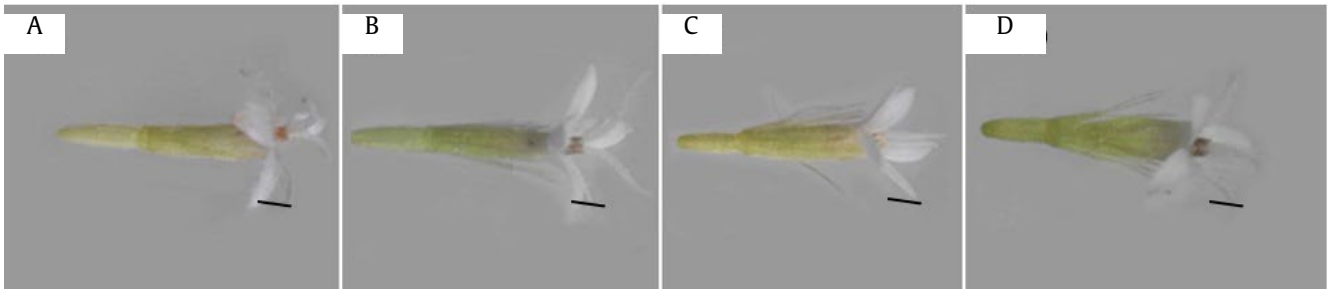



Figure 7. Single flower of *S. rebaudiana*. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 1 mm


Figure 8. The flower crown (corolla) of *S. rebaudiana*. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 1mm

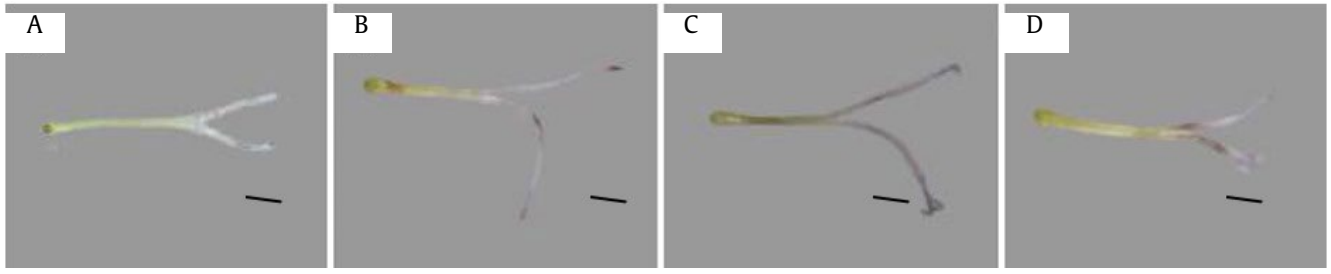



Figure 9. *S. rebaudiana* pistil. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 1mm

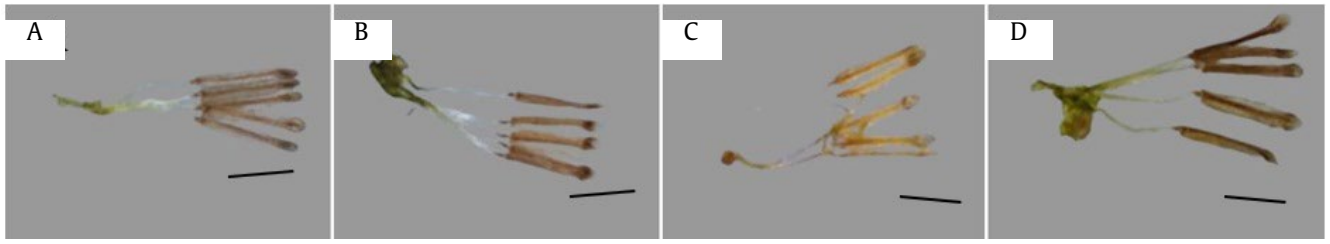



Figure 10. *S. rebaudiana* stamen. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 1mm.

single flowers. Tetraploid P3T5 clone had the smallest number of flowers but the biggest size of a single flower and stalk. Tetraploid B60.3H8 clone started to flower in the latest time range, the smallest size of a single flower and stalk but had the most abundant flowers among the tetraploids. Tetraploid P1T22 clone had a medium size of single flowers and in the number of flowers.

### 3.1.6. Fruit and Seed Morphology

Diploid and all the tetraploid clones had the same fruit type, i.e., achene fruit with fur-like fur horns called *papus*. The polyploidization did not affect the fruit morphology (Figures 11A-D). Stevia had both fruits with seeds or seedless. Fruit with seeds was dark-colored, while the seedless fruits were either bright-colored or transparent. At 12 WAP, the diploid clone produced the most abundant fruits compared to the tetraploid clones. The smallest number of fruits was found in the P3T5 clone. The percentage of fruits containing seeds was much higher in the diploid clone than in the tetraploids. Almost all tetraploids did not produce seeds (Table 5).

## 3.2. Plant Anatomical Characteristics

### 3.2.1. Leaf Anatomy

The leaf vein in all clones was *trinerved*. The repatriation of the leaf has three-leaf bones (*costa*) arising from the base, with the secondary leaf bone finely forming like nets (*reticulate venation*) (Figures 12A-D).

All clones, both control (parental clone) and the three tetraploid clones, had the same anatomical arrangement as shown in the leaf blade anatomy. The leaf consisted of upper and lower epidermis cells, palisade, and spongy tissue. Vascular bundles consisted of xylem and phloem vessels. (Figures 13A-D).

Table 4. Flowering time, number of flowers per plant, length of a single flower, and length of the flower stalk of *S. rebaudiana*

| Clone | Time of flowering (WAP) | Number of flowers per plant | Length of a single flower (mm) | Length of flower stalk (mm) |
|---|---|---|---|---|
| Parental diploid | 6–9 | 3 545.60±277.31$^{a}$ | 14.38±0.015$^{d}$ | 5.27±0.014$^{c}$ |
| Tetraploid: | | | | |
| B60.3H8 | 8–10 | 1 108.60±162.37$^{b}$ | 15.03±0.015$^{c}$ | 5.70±0.010$^{b}$ |
| P1T22 | 7–8 | 975.80±99.04$^{c}$ | 16.53±0.019$^{b}$ | 6.87±0.020$^{a}$ |
| P3T5 | 7–8 | 494.80±64.48$^{d}$ | 17.25±0.022$^{a}$ | 6.60±0.019$^{a}$ |

The numbers within the same columns followed by the same letter are not significantly different at P≤0.05, as determined by Duncan's Multiple Range Test

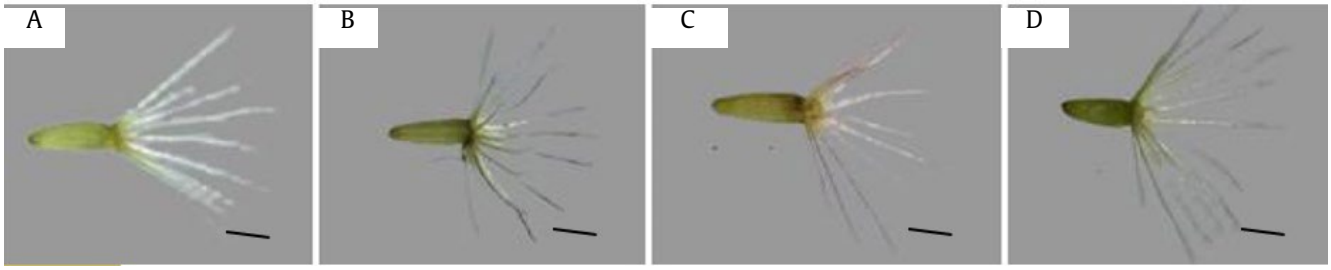



Figure 11. *S. rebaudiana* fruits. (A) parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 1mm

Table 5. The length, quantity, and percentage of fruits with seeds and without seeds of *S. rebaudiana*

| Clone | Fruit length (mm) | Number of fruits per plant | Percentage (%) | |
|---|---|---|---|---|
| | | | Fruits containing seeds | Fruits without seeds |
| Parental diploid | 2.53±0.11$^{a}$ | 17728±1386.53$^{a}$ | 4.49 | 95.51 |
| Tetraploid: | | | | |
| B60.3H8 | 2.77±0.22$^{a}$ | 5543±811.87$^{b}$ | 0.03 | 99.97 |
| P1T22 | 2.74±0.15$^{a}$ | 4879±495.19$^{bc}$ | 0.03 | 99.97 |
| P3T5 | 2.59±0.08$^{a}$ | 2474±322.42$^{c}$ | 0.02 | 99.98 |

The numbers within the same columns followed by the same letters are not significantly different at P≤0.05, as determined by Duncan's Multiple Range Test

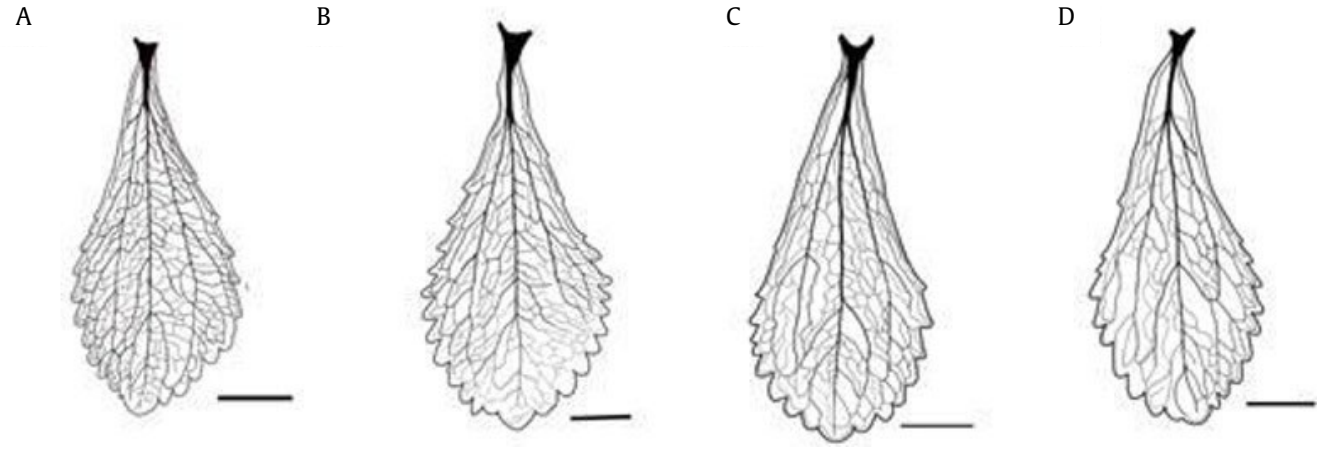



Figure 12. Leaf venation of *S. rebaudiana.* (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Scale = 1 cm

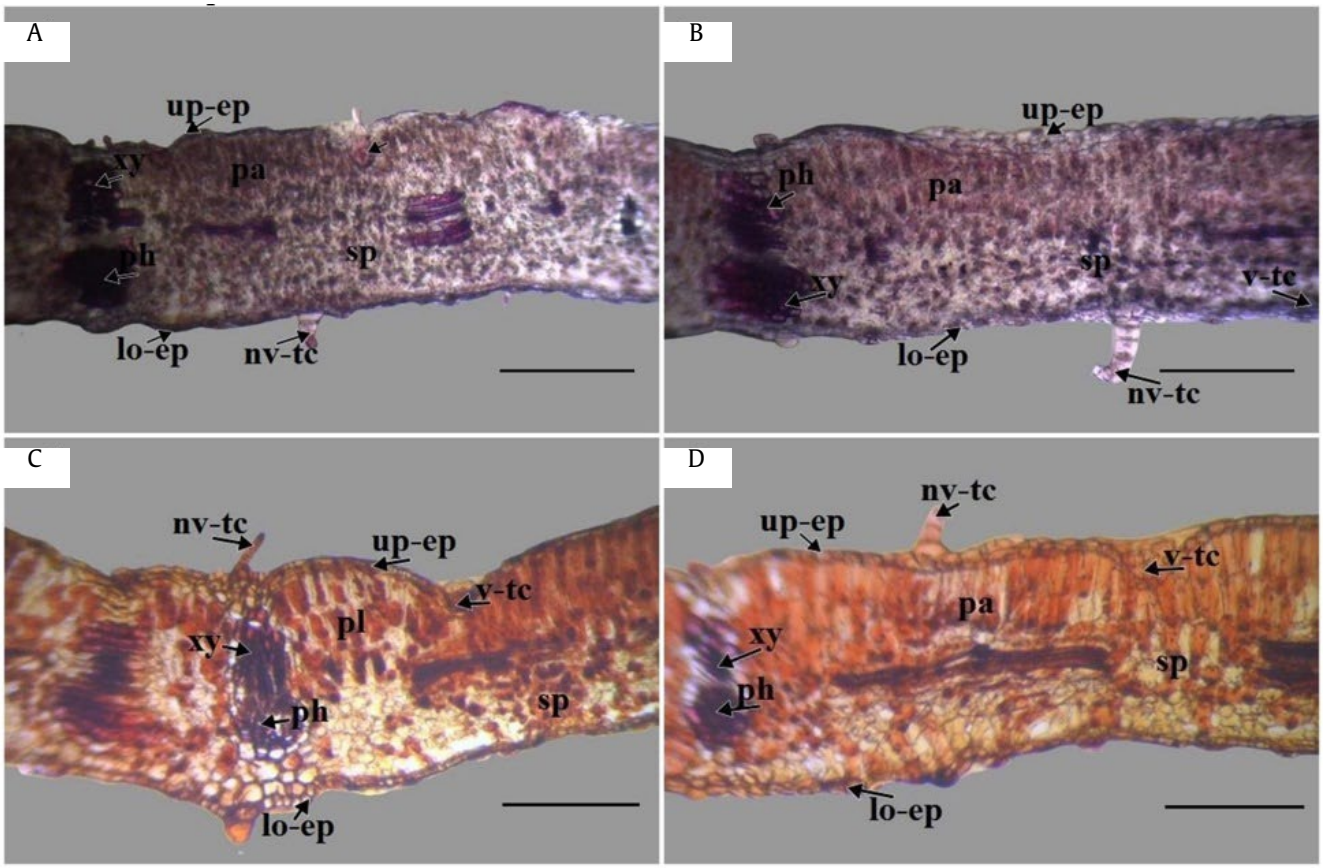



Figure 13. Anatomy of *S. rebaudiana* leaf lamina. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. tc: trichome, nv-tc: non vascular trichome, v-tc: vascular trichome, ep: epidermis, up-ep: upper epidermis, lo-ep: lower epidermis, ph: phloem, xy: xylem, sp: spongy mesophyll, pa: palisade mesophyll. cc: collenchyma, pc: parenchyma. 1: leaf costa, 2: leaf lamina. mag 40x. Scale: 0.5 mm

Our finding indicates that three tetraploid Stevia clones had thicker leaves, statistically different from the parental diploid clone. Tetraploid clone P3T5 was thicker than B60.3H8, P1T22, and diploid clones. The thicker was P3T5, and the thinnest was the diploid parental clone (Table 6). The thickest upper and lower epidermal tissues were found in the tetraploid clone P1T22. However, it was not significantly different from B60.3H8 and P3T5 clones. All tetraploid clones had significantly thicker epidermal cells than the diploid plant. The thickest palisade and spongy mesophyll were found in clone P1T22, and the thinnest was in the parental diploid clone (Table 6).

Table 6. Average of leaf thickness and leaf tissue arrangement of *S. rebaudiana* leaf

| Clone | Leaf thickness (mm) | The thickness of leaf tissue arrangement (µm) | | | |
|---|---|---|---|---|---|
| | | Upper epidermis | Lower epidermis | Palisade mesophyll | Spongy mesophyll |
| Parental diploid | 0.40±0.007$^{c}$ | 34.12±2.68$^{b}$ | 33.11±3.07$^{b}$ | 269.12±7.34$^{c}$ | 241.13±9.15$^{b}$ |
| Tetraploid: | | | | | |
| B60.3H8 | 0.46±0.008$^{b}$ | 47.17±2.03$^{a}$ | 47.17±2.61$^{a}$ | 309.83±11.55$^{ab}$ | 267.99±10.58$^{ab}$ |
| P1T22 | 0.44±0.011$^{b}$ | 48.27±1.87$^{a}$ | 48.46±1.69$^{a}$ | 344.99±12.02$^{a}$ | 313.96±17.22$^{a}$ |
| P3T5 | 0.52±0.013$^{a}$ | 47.38±1.90$^{a}$ | 45.99±2.16$^{a}$ | 305.33±16.61$^{bc}$ | 276.01±19.82$^{ab}$ |

The numbers within the same columns followed by the same uppercase letter(s) are not significantly different at P≤0.05, as determined by Duncan's Multiple Range Test.

### 3.2.2. Stem Anatomy

The transverse section of the stevia stem was round. All clones, both diploid and the three tetraploid clones, had the exact arrangement of the stem tissue. The anatomical structure of the stem consisted of epidermal tissue, cortical tissue, phloem vascular bundles, xylem vascular bundles, cambium, and pith (Figures 14A-D).

The thickness of the stem tissue in the three tetraploid clones was greater than that in the parental clone (Table 7). The thickness of the epidermis and cortex in all three tetraploid clones was higher and significantly different from that of the diploid clone. The thickest xylem was found in tetraploid clone B60.3H8, significantly different from that of the P1T22, P3T5, and diploid clones. The thickest phloem was found in P1T22 and P3T5 clones, significantly different from B60.3H8 and control clones. The thinnest epidermis, cortex, xylem, and phloem tissues were found in the diploid clone (Table 7).

### 3.2.3. Root Anatomy

The cross-section of the roots shows that the diploid Stevia clone (Figure 15A) and the three tetraploid clones (Figures 15B-D) had the same shape and arrangement of root tissue, namely irregularly rounded. The anatomical structure of the root consisted of the epidermis, sclerenchyma, cortex, endodermis, pericycle, vascular bundles (phloem and xylem), and pith. The thickness of the root tissue showed that the epidermis and sclerenchyma tissue of the tetraploid clone P3T5 was not significantly different from B60.3H8 but significantly different from P1T22 and diploid clones. The thickest cortex, endodermis, and vascular bundles were found in tetraploid P3T5, significantly different from B60.3H8, P1T22, and the diploid clones. The thinnest tissue size was found in the diploid clone (Table 8). The pith diameter of B60.3H8 was not significantly different from that of clone P3T5 but significantly different from P1T22 and diploid clones. The minor pith diameter was found in the diploid clone (Table 8).

## 3.3. Principal Component Analysis (PCA) of Morphological and Anatomical characteristics

The PCA analysis of quantitative data related to the morphology of the leaf, stem, roots, flower, and fruit is presented in Figure 16. The results showed that the diploid plant differed from 3 tetraploid clones in the length of roots, number of fruits, number of flowers per plant, and fruits containing seeds. Other characteristics were more dominant to tetraploids. In contrast, the PCA of the anatomical characteristics showed that all tetraploid clones differed from their parental diploid plant (Figure 17). They were distinct in the thickness of the leaf, leaf epidermis, leaf mesophyll, stem epidermis, cortex, xylem and phloem of the stem, root epidermis, root sclerenchyma, cortex and endodermis of the root, vascular bundle, and diameter of the pith.

## 3.4. Ploidy Level Confirmation

Confirmation of the ploidy level of Stevia diploid and tetraploid clones determined by flow cytometry is presented in Figure 18 and Table 9. The histogram showed that the spectral peaks of the diploid clones were around channel 100. Meanwhile, the tetraploid clones B60.3H8, P1T22 and P3T5 were around channel 200 (Figure 18). The mean X-peak of diploid and three tetraploid clones was presented in Table 9.

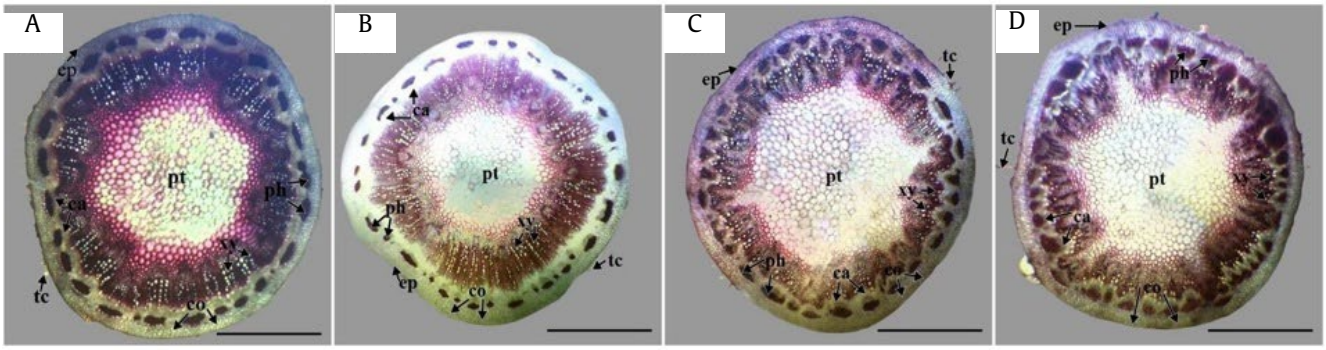



Figure 14. Anatomy of *S. rebaudiana* stem. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. tc: trichoma, ep: epidermis, co:cortex, ph: phloem, xy: xylem, pt: pith. mag 40x. Scale: 1 mm

Table 7. Stem thickness of *S. rebaudiana*

| Clone | Thickness (µm) | | | |
|---|---|---|---|---|
| | Epidermis | Cortex | Xylem | Phloem |
| Parental diploid | 22.30±2.02[b] | 153.30±5.74[b] | 573.07±31.18[c] | 141.93±4.57[c] |
| Tetraploid: | | | | |
| B60.3H8 | 47.45±4.16[a] | 353.94±27.94[a] | 1187.06±45.00[a] | 171.87±5.82[b] |
| P1T22 | 59.84±5.89[a] | 360.86±18.49[a] | 753.61±19.20[b] | 209.15±9.01[a] |
| P3T5 | 48.70±5.71[a] | 305.41±14.98[a] | 633.25±31.07[c] | 194.11±5.80[a] |

The numbers within the same columns followed by the same uppercase letter(s) are not significantly different at P≤0.05, as determined by Duncan's Multiple Range Test.

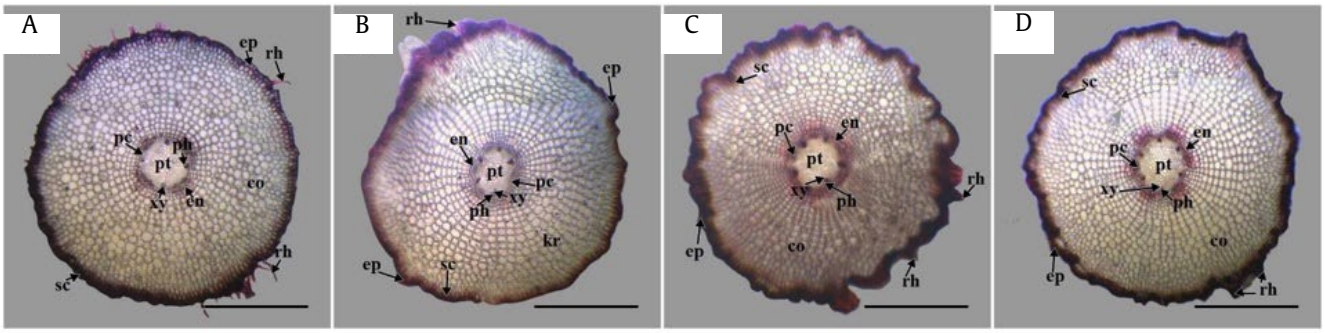



Figure 15. Anatomy of *S. rebaudiana* root. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5. Rh: root hairs, ep: epidermis, en: endodermis, ph: phloem, xy: xylem, co: cortex, sc: sclerenchyma, pc: parenchyma, pt: pith. mag 40x. Scale: 0.5 mm

Table 8. Length and diameter of roots tissue of *S. rebaudiana*

| Clone | Thickness (µm) | | | | | Diameter of pith |
|---|---|---|---|---|---|---|
| | Epidermis | Sclerenchyma | Cortex | Endodermis | Vascular bundle | |
| Parental diploid | 35.40±2.05[b] | 38.62±2.14[b] | 691.59±46.48[b] | 30.47±1.79[b] | 42.06±3.13[b] | 465.10±6.82[b] |
| Tetraploid: | | | | | | |
| B60.3H8 | 53.35±2.26[a] | 58.47±4.98[a] | 810.00±43.46[a] | 33.61±2.66[ab] | 47.33±4.20[b] | 686.42±29.87[a] |
| P1T22 | 37.97±2.42[b] | 38.78±4.25[b] | 746.72±21.67[b] | 30.68±2.09[b] | 42.79±4.83[b] | 485.38±11.73[b] |
| P3T5 | 58.35±1.86[a] | 58.36±0.66[a] | 1016.17±48.33[a] | 41.43±1.96[a] | 67.89±4.03[a] | 658.00±8.17[a] |

The numbers within the same columns followed by the same uppercase letter are not significantly different at P≤0.05, as determined by Duncan's Multiple Range Test

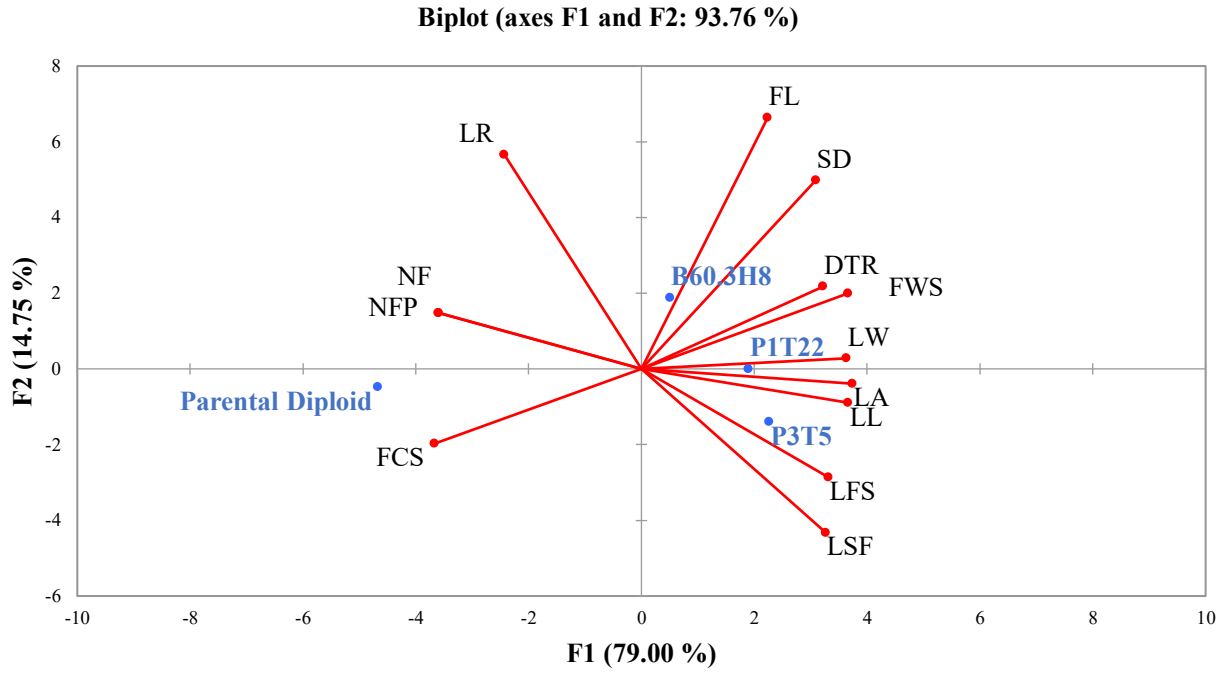



Figure 16. Biplot of Principal Component Analysis (PCA) of morphological characteristics between three tetraploid *Stevia rebaudiana* compared with their parental diploid plant. (LL: leaf length (cm), LW: leaf width (cm), LA: leaf area ($cm^2$), SD: stem diameter, LR: length of root (cm), DTR: diameter of tap root (mm), NFP: number of flowers per plant, LSF: length of single flower (mm), LFS: length of flower stalk (mm), FL: fruit length (mm), NF: number of fruits, FCS: fruits containing seeds, FWS: fruits without seeds)

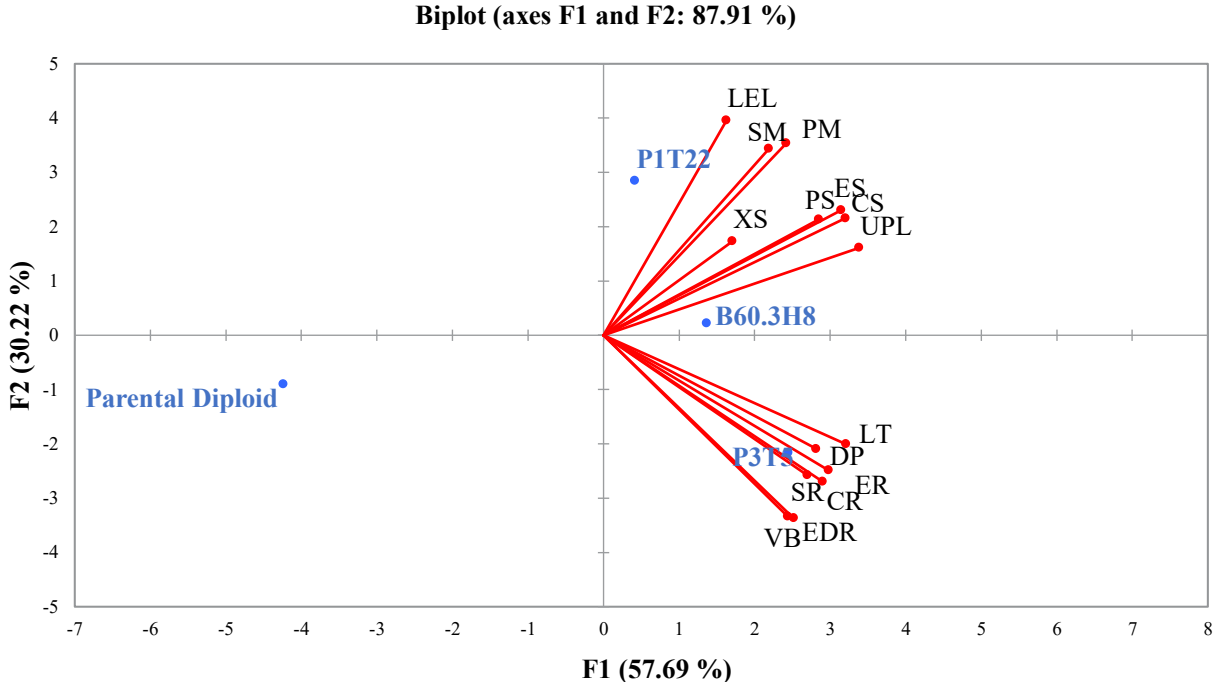
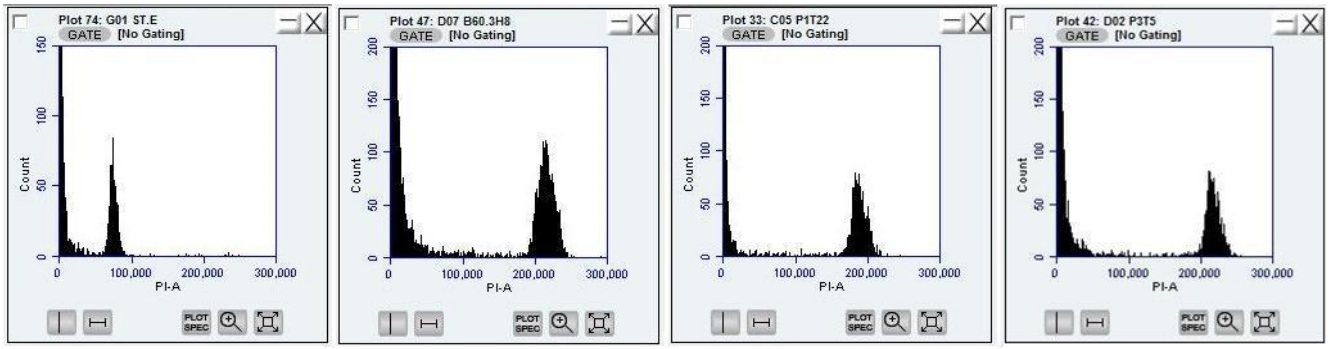



Figure 17. Biplot of Principal Component Analysis (PCA) of anatomical characteristics between three tetraploid *Stevia rebaudiana* compared with their parental diploid plant. (LT: leaf thickness (mm), UPL: upper epidermis of leaf, LEL: lower epidermis of leaf, PM: palisade mesophyll, SM: spongy mesophyll, ES: epidermis of stem, CS: cortex of stem, XS: xylem of stem, PS: phloem of stem, ER: epidermis of root, SR: schlerenchyma of root, CR: cortex of root, EDR: endodermis of root, VB: vascular bundle, DP: diameter of pith)




A B C D

Figure 18. Histogram of *S. rebaudiana* shoot analyzed by flow cytometry. (A) Parental diploid, (B) tetraploid B60.3H8, (C) tetraploid P1T22, (D) tetraploid P3T5

Table 9. Ploidy level of *S. rebaudiana* clones by flow cytometry analysis

| Clone | Mean-X peak | CV (%) | Ploidy level |
|---|---|---|---|
| Parental Diploid | 95.84 | 5.62 | Diploid (2n = 2x) |
| Tetraploid : | | | |
| B60.3H8 | 169.38 | 6.46 | Tetraploid (2n = 4x) |
| P1T22 | 189.62 | 5.35 | Tetraploid (2n = 4x) |
| P3T5 | 209.27 | 4.62 | Tetraploid (2n = 4x) |

## 4. Discussion

Morphological and anatomical characteristics of tetraploid *Stevia rebaudiana* were compared to its parental diploid plant. Our previous finding showed that Stevia habitus, both diploid and tetraploids, were not different (Adabiyah *et al.* 2019). They were categorized as perennial herbaceous, meaning that polyploidization does not permanently modify the plant habitus but with some variation in the size of the leaf, roots, and flowers. Pijnacker *et al.* (1990) reported some alterations of plant phenotypes in potato plants (*Solanum phureja* and *Solanum brevidens*) caused by somaclonal mutation. However, some did not change the plant's stature (*habitus*).

Our results showed that tetraploid Stevia had a bigger leaf size, root, and flower than diploid parental clones (Tables 1–6 and Figure 17). Similarly, in *Arabidopsis thaliana*, the ploidy level altered phenotypic and plant agronomic characteristics (Corneillie *et al.* 2019). The induction of polyploidization on Stevia using colchicine caused an increase in the DNA content (Mahdi *et al.* 2018; Rameshing *et al.* 2015; Yadav *et al.* 2013). Increased DNA content in mesophyll cells can increase cell volume (Warner and Edwards 1993). The indirect effects of higher ploidy are numerous and include changes in nuclear size, cell size, and the size of plant organs, leading to an increased photosynthetic rate. The size increase gave greater net photosynthetic $CO_2$ uptake (Dudits *et al.* 2016).

The leaves of Stevia on the upper surface (*adaxial*) were darker in color compared to the lower surface (*abaxial*) and downy (*pilosus*) on both surfaces. The tetraploid clones had an even darker leaf color than the diploid ones (Table 1). At the same age, the tetraploid plants of *Echinacea purpurea* had a darker leaf color than the diploids, presumably due to increased chloroplasts and chlorophyll content (unpublished data). The increased chloroplasts and chlorophyll content were in line with the higher plant ploidy (Abdoli *et al.* 2013).

Histological analysis of Stevia plants was observed based on the shape and anatomical structure of the leaf, stem, and roots. The results showed that all the control diploid and the three tetraploid clones had the same anatomical type and arrangement but differed in the thickness of the constituent tissues. Dunn and Lindstrom (2007) stated that leaf, stem, and root thickness could be the best phenotype indicator to distinguish various characteristics in polyploid Buddleja plant species.

Our findings showed that three tetraploid clones had leaf thickness and leaf tissue thickness more remarkable than the control clones, similar to *Buddleja* polyploids (Dunn and Lindstrom 2007). Polyploidization affects the size of cells and tissues that make up Stevia leaves, resulting in thicker and broader leaf sizes (Table 1). Wardana *et al.* (2018) reported that tetraploid lilies have thicker leaves than their diploid leaves. The size of the upper and lower epidermis, spongy tissue, and vascular bundles on tetraploid lilies had a greater thickness.

The increased rate of photosynthesis could happen if there were an increase in the volume of leaf mesophyll cells in plants with a high ploidy level (Tomas *et al.* 2013). Increased DNA content in mesophyll cells could affect the cell volume (Warner

and Edwards 1993; Wang *et al.* 2017). In *Robinia pseudoacacia* plants, the size of the mesophyll tissue was larger due to the enlarged size of the vacuoles and chloroplasts. Therefore, the presence of large cell space in the chloroplast, a larger thylakoid structure, causes the rate of photosynthesis to increase (Liu *et al.* 2012).

Our study revealed tetraploid Stevia showed enlarged stem diameter, darker stem color, and denser smooth hairs on the stem surface (Figure 3 and Table 2). A similar result was reported by Zhang *et al.* (2018), that tetraploid Stevia had shorter and straighter stems with larger and thicker leaves, which supported the plants in increasing an efficient photosynthetic capacity. Besides, large and sturdy stems can strongly support the plant so that the plant is not easily damaged by wind and rain (Singh and Jauhar 2019). However, polyploidy does not permanently enlarge plant organs. Yadav *et al.* (2013) reported that polyploid Stevia had a stem diameter smaller than the diploid plants. In addition, the length and diameter of the stem were greater in *Erianthus arundinaceus* tetraploid plants (Yan *et al.* 2016); also, the root diameter was thicker, but the root length was shorter and darker in color, in *Salvia miltiorrhiza*, compared to the diploid plants (Chen *et al.* 2018).

The size of the stem and root tissues of the three tetraploid Stevia clones was also more significant than that of the control clones (Tables 2 and 3). In the stems of tetraploid plants, there was a significant increase in secondary growth (cambium growth), increasing stem diameter. Dudits *et al.* (2016) proved that the cross-section of the tetraploid *Salix viminalis* stem in the older section had wood formation between the primary and secondary xylem rings, so this plant had thicker stems significantly compared to the diploid control plant.

The root of Stevia was fibrous with a long and thin root shape like yarns (*filiform*). Polyploidization caused changes in the diameter of the roots but not the root length. Our observations showed that the root diameter of tetraploid Stevia was larger and that it can be assumed that the tetraploid clones had a more vigorous rooting so that the plant was more upright than the diploid clones.

In roots, the increase in genome size in *S. viminalis* caused significant differences in root anatomy between diploid and tetraploid plants (Dudits *et al.* 2016). Cortical cells in tetraploid *S. viminalis* had a larger size. In our study, the thickness of the root tissues of tetraploid Stevia plants, namely the epidermis, sclerenchyma, cortex, endodermis, vascular bundle, and stele, was larger than that of diploid plants, so that the diameter of the tetraploid roots became more extensive. Polyploid plants with larger stems and root systems could absorb nutrients and water from the soil more efficiently, even in a dry environment (Corneillie *et al.* 2019).

Tetraploidization in Stevia plants did not affect the flower's shape, type, and color but resulted in a long time to initiate flowers, fewer flowers, and longer single flower and stalk compared to the diploid plants (Table 4). Hegde *et al.* (2015) also proved that diploid Stevia had an early flowering time on day 39 compared to day 73 for tetraploid clones; the tetraploids had larger flower sizes and fewer flowers than the parental diploid plants. The delay of flowering in Stevia becomes an expected growth parameter as it extends the vegetative growth period and is expected to have a denser canopy (Hegde *et al.* 2015). The plants generated from polyploidization showed increased gene activity and enzyme diversity, lower transpiration and photosynthetic rate, and late flowering than diploid plants (Levin 1983).

Tetraploid clones had fewer and more seedless fruits than the parental diploid clone (Table 5). Furthermore, polyploidization could cause higher abnormalities (Oliveira *et al.* 2004). Yadav *et al.* (2013) reported that tetraploid Stevia produced fewer flowers than the diploids, and some of its flowers had stamens with a smaller amount of pollen. It may explain why tetraploid plants have many seedless fruits. The tetraploid plant of *Echinacea purpurea* produced a larger diameter of pollen and flower, longer flower, and greater seed weight than the diploid plant (Abdoli *et al.* 2013). Lawrence (1980) added that polyploidization caused an imbalanced formation of gametes during the process of meiosis to form pollen, resulting in a high degree of pollen infertility. That case may cause fertilization not to occur, leading to the absence of seeds in tetraploid Stevia.

Polyploidization occurs randomly and spontaneously; it causes a massive effect on the overall genome, such as a change in the number of chromosomes, heterosis, meiosis recombination, and epigenetic and phenotypic modifications. It can modify the diversity of biochemical compounds in the plant (Finigan *et al.* 2012; Paule *et al.* 2017; Ramsey and Schemske 1998). Adabiyah *et al.* (2019) stated that the content of stevioside and rebaudioside-A of tetraploid clones was higher than the parental diploid clone. In several other cases, polyploidization produced plants with higher vigor and resistance against extreme climatic conditions, such as in *Ranunculus kuepferi* (Schinkel *et al.* 2016),

*Andrographis paniculata* (Surochita and Debasree 2018) and in *Medicago* spp. (Innes *et al.* 2020).

Our findings showed that the diploid and all three tetraploid Stevia clones still had stable ploidy levels when grown in the field (Figure 18 and Table 9) compared to their *in vitro* parent stock plant (Adabiyah 2019). The previous study indicated that the growth of tetraploid B60.3H8 was superior to the diploid and two other tetraploid clones (Adabiyah 2019; Adabiyah *et al.* 2019). Chemicals mutagen could inhibit the *in vitro* growth of tetraploid taro buds, resulting in fewer buds than diploid plants, but all genotypes kept stable ploidy levels at that stage (Ermayanti *et al.* 2018; Wulansari *et al.* 2016). The induction of polyploidy using colchicine in various concentrations also decreased plant survival and the growth of axillary buds and shoots of *in vitro Trachyspermum ammi* (Noori *et al.* 2017). Colchicine also inhibited the growth of Arabidopsis roots (Kim *et al.* 2018).

In conclusion, our finding gives more information that the tetraploid Stevia differed in morphology and anatomy characteristics compared to diploid parental plants. Phenotypically, the size of tetraploids is bigger than diploid plants.

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