Abstract
Mutation and manipulation of ploidy were tried on stevia (2n=22) to develop higher content of steviol glycosides. Different stevia mutants were developed using Colchicine using 0.25%, 0.50%, 0.75%, 1.0%, 1.50%, and 2.5% and they were tested for their DNA content to ascertain the change in ploidy. Ploidy level was identified by flow cytometry analysis and steviol glycoside content in the leaves was determined by HPLC. Some polyploids showed two times increase in the percentage of stevioside as well rebaudioside-A, compared to control. Thus, induction of polyploidy in stevia confirmed the effectiveness of colchicines as a polyploidizing agent creating new variants with higher steviol glycosides (stevioside and rebaudioside-A) content contributing to crop improvement in stevia.

Key words : Stevioside, mixaploid, colchicine.

Introduction
The sugars along with sweetening qualities also have been found to contribute calories, which can lead to obesity, a risk factor for some chronic diseases such as diabetes mellitus, hypertension, cardiovascular diseases, etc. Hence, the craving for sweetness led man to discover several forms of alternative intense sweeteners which have made possible to offer consumers the sweet taste without the calories (1). The worldwide demand for high-potency sweeteners is expected to increase, especially with the new practice of blending different sweeteners.

Stevia rebaudiana Bertoni (2n=22) stands out among more than 150 species because of its intense sweetness. It is a perennial, endemic shrub belonging to the Asteraceae family (2, 3). Leaves of stevia contain around 10 sweetening glycosides, of which stevioside (5–10%), rebaudioside-A (2-4%), and rebaudioside- B, C, D and dulcoside are important. This sweetener does not metabolize in the human body, but pass through the digestive process without chemically breaking down. This property makes stevia safe for those who need to control their blood sugar level (4). Steviosides \((\text{C}_{38}\text{H}_{60}\text{O}_{18})\) are the major compounds (60-70% of the total glycosides content) and are assessed as being 110-270 times sweeter than sugar, responsible for the bitter aftertaste. The other compound rebaudioside-A (\((\text{C}_{44}\text{H}_{70}\text{O}_{23})\)), is usually present as 30-40% of total sweeteners and has the sweetest taste, assessed as 180-400 times sweeter than sugar with no bitter aftertaste (5).

Development of new varieties of stevia with a higher content of Steviol Glycosides is the primary aim in this crop. Polyploids have successfully been induced earlier in stevia via colchicine and induction of polyploidy has been observed to improve desirable traits (6). Polyploid individuals have a higher content of stevioside than diploid plants and the selection of plants for commercial production could possibly increase the level of these compounds (7).
The main objective of this study was to identify the mutants with high steviol glycosides content through induction of polyploids in stevia using different colchicine treatment. Polyploids of *Stevia rebaudiana* were developed in 2012 using colchicine as the mutagen. The ploidy level was confirmed through flowcytometry at the University of Agricultural Sciences, Bangalore in 2012 and analysis of the leaf samples recorded higher Stevioside and Rebaudioside-A content through High Performance Liquid Chromatography (HPLC) (8).

**Materials and Methods**

**Plant material:** The *S. rebaudiana* plants were treated with colchicine (0.25%, 0.50%, 0.75%, 1.0%, 1.50%, and 2.5 %) at the Dept. of Plant Biotechnology, University of Agriculture Sciences, Bangalore in the year 2012. These polyploidy induced each plants were separately propagated by means of cuttings and planted in separate plots, the experiment was conducted during 2013-2014 at Sugandhavana, Department of Horticulture, University of Agricultural Sciences, Bangalore.

**Preparation of sample:** Leaves were harvested after 120 days of planting and separated from stevia stem. Leaves were dried at room temperature for 4 days and then made into fine powder in blender. The powder was sieved using 100 micron mesh. Then one gram of leaf powder was taken for extraction from each replication. 20 ml of HPLC grade methanol was added and kept on stirring in water bath at 60° for two minutes. Later filtered with whatman filter paper and repeated the process by washing it for 4 times in methanol. The filtrates were taken in a measuring flask and the volume of remaining extract was made to 100ml by adding HPLC grade methanol. The filtrate was again filtered using nylon membrane filter paper and the solution obtained was used for HPLC analysis.

**Separation was performed on a column: NH₃C18 column (250 mm × 4.6 mm). The mobile phase consisted of sonicated HPLC grade acetonitrile: water (75:25). The analysis was performed at 25° C with 1ml/min flow rate at detection wavelength of 210nm and Injection volume is set for 20µl (9).**

**Standard preparation:** The samples of standard stevioside of pure form (95%) obtained from Natural Remedies India, and Rebaudioside-A (96%) obtained from Sigma Aldrich Pvt Ltd, USA. The reference stevioside compound of 2 mg quantity was dissolved in 1 ml of HPLC grade methanol to make stock solution of 2000 ppm (concentration of 2 mg ml⁻¹). From this stock solution, serial dilution of 1mg ml⁻¹, 0.5mg ml⁻¹ and 0.25 mg ml⁻¹ was made. Similarly reference rebaudioside-A stock solution and serial dilutions were made to obtain the standard calibration curve.

**Analysis of samples:** Extracted leaves samples were injected (20 µl) in port with the help of 25 µl syringe. Before injection the syringe was thoroughly rinsed with HPLC grade methanol and then with the sample. The run time was adjusted for 20 minutes throughout the experiment. Stevioside and rebaudiosides were identified in chromatogram with a peak by means of retention time.

**Calculation**

Stevioside and Rebaudioside content (100%) was calculated by using the formula (10):

\[
\text{Area of sample} \times \frac{\text{standard wt. (in mg)}}{\text{x sample dilution} \times \text{Purity of standards}} \times 100
\]

\[
\text{Area of the standard} \times \frac{\text{standard dilution}}{\text{x sample weight (in mg)}} \times 100
\]

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Results and Discussion
Calibration Curve
Fig. 1A. The calibration curve of stevioside standard:- linear regression

A particular peak with retention time of 8.2 minute for stevioside and 12.8 minute for rebaudioside-A was identified as the standard concentration peak of stevioside and rebaudioside-A. Calibration curve was prepared on graph by putting area on x-axis and concentration on y-axis and presented in figure-1A and 1B.

Quantitative analysis of diploids and polyploids of stevia for steviol glycosides
The stevioside and rebaudioside-A was estimated by High Performance Liquid Chromatography (HPLC), from untreated and different colchicine treated stevia plants. Highest stevioside content of 13.50% was observed in mixaploid- T7, followed by tetraploid- T9 (11.77%). Highest rebaudioside-A content of 6.21% was recorded in tetraploid- T9, followed by mixaploid- T7 (5.94%), triploids and treated diploids, while the least was recorded in untreated diploid (Table-1, figure- 2A, 2B, 2C, 2D and 2E). Treated diploids showed higher stevioside content compared to that of untreated control diploid. Colchicine concentration may also influence the glycoside content because the variation was observed in plants of similar ploidy.

In the present study, ploidy level positively influenced the stevioside and rebaudioside content. Manipulation of ploidy is a valuable tool and has long been used in plant breeding programmes to improve agronomic yield, particularly biomass production. In addition, with the doubling of the gene products and increased dosage effect, polyploids provide a wider germplasm base than diploids for breeding purposes.

The colchicine treated plants showed higher stevioside content than the control, stevioside content in the leaves of different polyploids of stevia plants ranged from 5.57% to 14.98% (8). Leaves are the economic part where steviol glycoside synthesis takes place, improvement in leaf characters will have a direct influence on yield as well as glycoside content of the plant (11). Glycoside synthesis is reduced at

Steviol Glycosides in Stevia
### Table 1. Stevioside and Rebaudioside-A contents of *Stevia rebaudiana*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Colchicine (%) used</th>
<th>Polyploidy level</th>
<th>Stevioside Content (%)</th>
<th>Rebaudioside-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>0.00</td>
<td>Untreated diploid</td>
<td>06.80</td>
<td>3.32</td>
</tr>
<tr>
<td>T₁</td>
<td>0.25</td>
<td>Treated diploid</td>
<td>08.50</td>
<td>4.83</td>
</tr>
<tr>
<td>T₂</td>
<td>1.00</td>
<td>Treated diploid</td>
<td>07.85</td>
<td>4.65</td>
</tr>
<tr>
<td>T₃</td>
<td>2.50</td>
<td>Treated diploid</td>
<td>07.98</td>
<td>4.25</td>
</tr>
<tr>
<td>T₄</td>
<td>1.50</td>
<td>Triploid</td>
<td>10.79</td>
<td>5.65</td>
</tr>
<tr>
<td>T₅</td>
<td>2.00</td>
<td>Triploid</td>
<td>11.01</td>
<td>4.94</td>
</tr>
<tr>
<td>T₆</td>
<td>2.50</td>
<td>Triploid</td>
<td>11.76</td>
<td>5.89</td>
</tr>
<tr>
<td>T₇</td>
<td>0.50</td>
<td>Mixaploid</td>
<td>13.50</td>
<td>5.94</td>
</tr>
<tr>
<td>T₈</td>
<td>1.00</td>
<td>Tetraploid</td>
<td>10.48</td>
<td>5.26</td>
</tr>
<tr>
<td>T₉</td>
<td>1.50</td>
<td>Tetraploid</td>
<td>11.77</td>
<td>6.21</td>
</tr>
</tbody>
</table>

**Fig. 2A.** HPLC profile of Stevioside and Rebaudioside-A of tetraploid plants (T₉)

**Fig. 2B.** HPLC profile of Stevioside and Rebaudioside-A of mixaploid plants (T₇)
or just before flowering, delayed flowering in mixaploid allows more time for steviol glycoside accumulation. The results are in conformity with findings (12) in stevia.

According to a study conducted by different researchers that the stevioside and rebaudioside content varied from 2-10 % and 1-7.12% respectively (13). It varied from location to location, stage of harvest, method of extraction of steviosides, etc. Under Indian conditions stevioside concentration was about 9.08% of the dry weight of leaves (14). The glycoside quality of stevia is improved by changing the ploidy level. Stevioside content is influenced by both leaf surface and number of roots; however, the leaf surface has more influence on stevioside content than the number of roots (15).
Breeding of stevia at the ploidy level and induction of polyploidy will generate new variants and can be used in further crop improvement program in stevia with improved glycoside profile particularly rebaudioside-A which is most preferred glycoside has no bitter after taste.

References