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Induction and morpho-chemical characterization of *Stevia rebaudiana* colchiploids

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ABSTRACT

A study was conducted to produce higher biomass-yielding stevia *Stevia rebaudiana* (Bertoni) with larger leaf size and high glycoside content. To improve leaf yield and sweet glycoside content found in the leaves of the plant, polyploidy was induced by treating the seeds with colchicine. Treatment of seeds with 0.2% colchicine and above (up to 0.6%) reduced the survival rate of stevia young seedlings to below 50%. Maximum number of polyploids (tetraploids) were obtained with 0.6% colchicine treatment for 24 hr; mixoploidy was observed at 0.2% colchicine treatment for 6 hr. It however needs further experimentation for more precise information. The nuclear DNA content (2C-value of diploid *Stevia rebaudiana* variety Madhuguna) was estimated by flow-cytometry (FCM) to be 2.72pg. Polyploids C7-3-4, C7-3-5, C9-1-1 and C9-4-3 could be distinguished from diploid control plants as they had double the DNA content, suggesting autotetraploidy. Chromosome count in root-tip cells of polyploids (2n=44) confirmed their tetraploid status as compared with the control (2n=22). The autotetraploids in stevia had significantly increased leaf size, thickness and chlorophyll content and reduced internode length. Tetraploid stevia genotypes are important germplasm for further improvement of stevia biomass and glycoside content.

Key words: Autotetraploid, Colchiploid, Flow-cytometry, Mixoploid, Scanning Electron Microscopy, *Stevia rebaudiana*

Stevia [*Stevia rebaudiana* (Bertoni)], a perennial herb of the Asteraceae family, has been recognized worldwide for its excellent sweetening property. Leaves of stevia produce diterpene glycosides (stevioside and rebaudiosides), which are high-potency sweeteners (300 times sweeter than sucrose) and substitutes for sugar (Megeji *et al.* 2005, Rajasekaran *et al.* 2007). Stevia is a natural source of non-calorie sweetener and an alternative to the synthetic sweetening agents that are presently available in the market for diabetic patients and body weight conscious consumers. Stevia is important especially in the context of the proclivity of the society towards more natural foods (Brandle and Rosa 1992, Kamalakannan *et al.* 2007).

Manipulation of ploidy is a valuable tool and has long been used in plant breeding programmes to improve agronomic yield, particularly biomass production. Colchicine has been widely used for polyploidization in plants ever since it was first reported to have this property (Blakeslee and Avery 1937). Colchicine disrupts polymerization of microtubules, which, in turn, disrupts spindle-fiber development and mitosis (Bartels and Hilton 1973). Cells arrested at metaphase may recover and enter the next mitotic cycle with twice as many chromosomes. Polyploids have successfully been induced earlier in stevia via colchicine (Valois 1992) and studied for different morphological characters (Oliveira *et al.* 2004). Polyploids often generate variants that may contain useful characteristics. Improved adaptability of individuals and increased organ and cell sizes are usually associated with polyploidy (Guerra 1988). 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 diploid stevia genotypes have low biomass productivity and steviol glycoside content. Polyploids often generate variants that may contain useful characteristics. Induction of polyploidy has been observed to improve desirable traits (Gandhi and Patil 1997, Romero- Aranda *et al.* 1997, Pinheiro *et al.* 2000, Aleza *et al.* 2009, Kanchanapoom and Koarapatchaikul 2012). Therefore, in an attempt to produce high biomass-yielding stevia with high glycoside content, experiments were undertaken to induce

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polyploidy in stevia using colchicine treatment. Here, we report the induction and morphological characterization of autotetraploid *Stevia rebaudiana* and discuss its potential benefits for breeding programmes.

MATERIALS AND METHODS

Seeds of open-pollinated stevia breeding line Madhuguna, being maintained at Stevia Breeding Field at Chandpur Research Farm, Institute of Himalayan Bioresource Technology (CSIR), Palampur (Himachal Pradesh), were used for induction of polyploidy. Pappus was removed from dark stevia seeds and the seeds were soaked in distilled water for 24 hr. The imbibed seeds were treated with different concentrations of colchicine aqueous solution 0.01, 0.05, 0.1, 0.2, 0.4 and 0.6% (Table 1); 1% dimethyl sulfoxide (DMSO) was added to each solution. Two-hundred seeds per Petri dish were subjected to treatment durations of 6, 18 and 24 hr. Petri dishes were sealed with parafilm and incubated at 22° C. After the treatment, seeds were first washed under running tap water and then three times with distilled water and grown on the seed germinating paper at 22°C in a BOD incubator (model-Caltan, Narang Scientific Works Pvt Ltd, India). Then Petri dishes were transferred to a polyhouse under natural lighting and partial shade for 2 days. The treated seedlings were transferred to 10 cm-diameter pots containing plant growth mixture comprising soil, sand and farmyard manure (FYM) in the ratio of 1:1:1 (N 216.4 kg/ha, P_2O_5 16.8 kg/ha and K_2O 154.8 kg/ha) and the surviving seedlings were transplanted in the field after 30 days and a recommended dose of fertilizer ($N:P_2O_5:K_2O$=110:45:45 kg/ha) was given. Seedling mortality was determined. After three months of transfer of plants to soil, young leaves were collected for ploidy estimation through flow cytometry and root tips of treated plants were used to confirm ploidy via cytology.

Because the number of treated plants for screening for polyploidy was large, flow cytometry was used to estimate the ploidy level. Flow cytometry is used only if the number of plants to be screened is large. Dolezel *et al.* (2007) described a method for ploidy analysis and estimation of nuclear DNA content in plants through flow cytometry. Young leaves of all the colchicine-treated plants, along with control plants, of stevia were used for flow cytometric measurements. Nuclear suspensions were prepared according to Dolezel *et al.* (1994) with certain modifications (sample size was increased to 100 mg of young leaf tissue). Approximately 100 mg of young leaf tissue was chopped up, in a plastic Petri dish, with a sharp razor blade for approximately 60s in 1 ml of woody plant (WP) nuclei-isolation buffer (Loureiro *et al.* 2007) containing 200 mM TrisHCl, 4 mM $MgCl_2.6H_2O$, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10mM sodium metabisulfite, 1% PVP-10, and 0.5% (v/v) TritonX-100; pH was 7.2. Some modifications to Loureiro *et al.* (2007) method were made. Plants of tomato (*Lycopersicon esculentum*) variety Stupicke with nuclear DNA content 1.96 pg/2C, maize (*Zea mays*) variety CE 777 (5.43pg/2C) and pea (*Pisum sativum*) variety Ctirad (9.09 pg/2C) grown from seeds provided by the Institute of Experimental Botany, Olomouc, Czech Republic, were used as internal reference standards to estimate the nuclear DNA content of stevia (Dolezel *et al.* 1992). Internal reference standards are the specific crop varieties with known nuclear DNA content for estimating nuclear DNA content of an unknown sample.

In comparison to other buffer solutions, viz. Galbraith's buffer (Galbraith *et al.* 1983), Tris $MgCl_2$ buffer (Pfosser *et al.* 1995) and Otto's buffer (Otto 1990), the WP nuclei-isolation buffer (Loureiro *et al.* 2007) with modifications performed best with regard to nuclear isolation in stevia, tomato, maize and pea. Therefore, it was used in this study for estimation of DNA content and ploidy in stevia. The leaf tissues of stevia and internal reference standards were chopped simultaneously in the buffer and the resulting homogenate was filtered through a 41 mm nylon filter to remove the debris. Nuclei were stained with 60 µl of 1 mg/ml propidium iodide (PI) and 4 µl of 50 µg/ml RNase-A was added to nuclear suspension to prevent staining of double-stranded RNA. Samples were incubated in a water-bath at 37°C for 30 min. After incubation, samples were analyzed in a flow cytometer for ploidy determination.

Relative fluorescence intensity of stained nuclei was determined using fluorescence-activated cell sorting (FACS) station (BD FACS Calibur System of Becton and Dickinson Company, New Jersey, USA). In each sample, 10 000 nuclei were analyzed at a rate of about 50–60 nuclei/second by measuring PI fluorescence area signals (FL2-A) using Becton and Dickinson Cell Quest Pro software. Forward light scatter channel (FSC) parameters were adjusted to generate a histogram of FL2-A mean peak position of G_0/G_1 (nuclei). To screen for ploidy variations, peaks of sample and internal standard were determined by analyzing the data on Becton and Dickinson Cell Quest Pro software. Prior to analysis, the instrument was checked for linearity with fluorescent beads and the amplification settings were kept constant throughout the experiment. The nuclear DNA content (pg) of the sample

Table 1 Effect of colchicine on the survival of seedlings in stevia

Colchicine conc. (%)	Seedling survival (%)		
	Duration of the colchicine treatment		
	6 hr	18 hr	24 hr
Control	78.0	78.5	72.5
0.01	75.5	75.0	76.0
0.05	74.5	66.0	60.0
0.1	66.5	60.5	56.5
0.2	39.0	37.0	38.0
0.4	10.0	11.0	7.0
0.6	6.0	3.0	2.5

was calculated from the fluorescence value according to Loureiro *et al.* (2007):

$$\text{Sample DNA content (pg)} = \frac{\text{Sample 2C peak mean}}{\text{Reference 2C peak mean}} \times \text{Reference DNA content (pg)}$$

Chromosome counts were made on root tips of untreated (control) plants and polyploids induced by colchicine treatment. Actively growing root tips, about 5mm in length, were excised and pre-treated with 0.1% colchicine at 20°C for three hours. The root tips were washed and transferred to fixative solution [ethanol:acetic acid (3:1, v/v)] for at least 24 hr at 4°C and then transferred to absolute alcohol till further analysis. The fixed root tips were hydrolyzed in 1N HCl for 10 min at 60°C and transferred to 2% pectinase solution for 45 min at room temperature. After hydrolysis, root tips were stained with 0.5% aceto-carmine staining solution in dark at room temperature for about 3 hr. Over-stained cytoplasm was de-stained by applying a drop of a mixture of 1 part 1% aceto-carmine and 1 part 1N HC1. The stained tips of roots were cut, placed on glass slides and squashed beneath cover slips in one or two drops of 45% acetic acid and glycerin (10:1). The slides were placed between two pieces of folded filter paper and a moderate thumb pressure was applied to flatten the cells for observation under the microscope. Chromosomes were counted at the metaphase stage from at least five root tips of diploid (control) and putative tetraploid plants. Pollen viability was examined under a microscope after staining the pollen with aceto-carmine stain.

Abaxial leaf portion of diploid control and tetraploid stevia plants was characterized, using a scanning electron microscope (S-3400N, Hitachi, Japan), for stoma size and count and glandular and non-glandular trichomes. The leaves were thoroughly washed (five times) with distilled water to remove dust particles. Small pieces (2 mm^2) of leaves were mounted on an aluminum stub using a double-sided carbon tape and partially dried under vacuum to remove the surface-adsorbed water. The pieces were coated with gold by sputter coating unit at 10 Pascal vacuum for 10s (E1010 ion sputter Hitachi, Japan). Images were captured on VP-SEM mode at desired magnification using 20.0 kV acceleration voltage. The number and size of the stomata were determined using third leaf of the primary shoot from the apex of field-grown plants. The stomata, glandular and non-glandular trichome densities were calculated by taking an average of five different microscopic fields in scanning electron microscope images.

Observations were made in colchicine treatments to determine seedling mortality. Data of different treatment durations were pooled and LD_{50} value was determined by regression analysis, considering x (colchicine treatment) as an independent variable and y (mortality) as a dependent variable. Morphological variations were recorded in tetraploids and mixoploids with respect to leaf (length, width, thickness and chlorophyll content), stomata (length, width and density), trichome (density of glandular and non-glandular trichomes) and agronomic (plant height, stem thickness, internode length and primary shoots) characteristics. Except for plant height, stem thickness and number of primary shoots in colchiploids, all the recorded data were based on five observations, whereas data with respect to these traits in control were based on observations of 10 plants, for which flow cytometry was used. The glycoside profile (stevioside and rebaudioside-A) of plants was estimated through HPLC analysis from dried leaf samples. Stevioside and rebaudioside-A contents in the leaves of the plants were estimated on a dry weight basis (Table 3). Morphological and chemical data of polyploids were analyzed in comparison with control plants of diploid origin using Student's t-test.

RESULTS AND DISCUSSION

Based on regression analysis, LD_{50} value of colchicine treatment in stevia was 0.23%, beyond which there was a drastic reduction in seedling survival (Table 1, Fig 1). Significant differences were observed among different colchicine treatments on the basis of the t-test (Table 1). In flow cytometric studies, the peak of tomato variety Stupicke (1.96 pg/2C) appeared closest to 2C peak of stevia (diploid) control as compared with maize variety CE 777 (5.43 pg/2C) and pea variety Ctirad (9.09 pg/2C). Using tomato as the internal reference standard and based on average of 10 observations, the relative fluorescence intensity (RFI) of nuclei of diploid stevia was estimated to be 2.72 pg/2C. Using histogram peaks of tomato (M1) as the internal reference standard, the peaks (M2) of putative stevia tetraploids (C7-3-4, C7-3-5, C9-1-1 and C9-4-3) had approximately twice the fluorescence value of stevia control plants. In the case of mixoploid plants, one 2C peak of tomato (M1), along with two peaks of the stevia sample (2C at M2 and 4C at M3), appeared in C8-3-4, C10-1-3 and C10-1-4.

Induction and selection of polyploid stevia

Polyploidy is often induced in plants of economic interest to produce variability that can improve the yields. Variations in chromosome number of the genus *Stevia* have been reported; e g n=11 (Guerra 1988), whereas Galiano (1987)

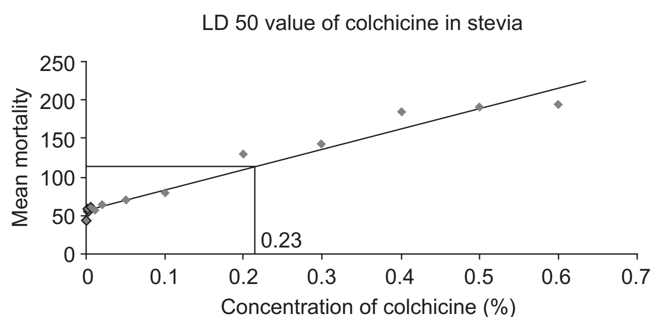

Fig 1 Effect of colchicine on seedling mortality in stevia

considered *Stevia* as a multibasic genus with x=11, 12 and 17. Frederico *et al.* (1996) considered its basic chromosome number to be x=11, with variations in the form of numerical (aneuploidy and polyploidy) and structural changes, mainly pericentric inversions. The chromosome number of 2n=22 (n=11) in *Stevia rebaudiana*, as previously reported (Guerra 1988, Frederico *et al.* 1996, Oliveira *et al.* 2004), was confirmed here through cytological observations for diploid control plants (Table 2 and Fig 2a). However, chromosome number in root tips of colchiploid cytotypes (C7-3-4, C7-3-5, C9-1-1 and C9-4-3) was observed to be 2n=44 (Table 2 and Fig 2b) and represented tetraploid *S. rebaudiana*, whereas in root tips of mixoploid plants (C8-3-4, C10-1-3 and C10-1-4), chromosome counts of 2n=22 and 2n=44 were observed. Mixoploid plants showed both peaks (diploid and tetraploid) along with internal reference standard.

Four tetraploids and three mixoploids were generated by colchicine treatments, whereas control plants maintained the diploid status. Colchicine treatment of 0.2% for 6 hr had a desirable effect relative to polyploid induction, but the number of mixoploids produced was high as compared with the number of tetraploids (Table 2). However, colchicine treatment of 0.6% for 24 hr produced two tetraploids but no mixoploid from a total of only five surviving plants. The present study suggested that identification of polyploids at lower doses of colchicine would be difficult because a large number of treated plants would need to be screened. Polyploidization was obtained at higher doses of colchicine treatment, which corresponded with 0.2-0.6% concentration of colchicine for 6 to 24 hr, unlike earlier reports (Valois 1992, Oliveira *et al.* 2004), where polyploidization corresponded to the lowest colchicine concentrations. The variability of the ploidy level within the same treatment may be because of the different cell division stages of the cells at which colchicine treatment took effect and subsequent initiation of new shoots from those cells. Although a number of cells in meristematic tissue may become polyploidized, many others may be unaffected and remain diploid. The plants with both normal and polyploidized cells intermixed are regarded as mixoploids or chimeras (Wan *et al.* 1989).

Table 2 Plants identified for changed ploidy level

Plant No.	Colchicine(%) /Duration (hr)	Chromosome count (2n)	FCM estimation of ploidy level
Control	-	22	Diploid
C-7-3-4	0.6/24 hr	44	Tetraploid
C-7-3-5	0.6/24 hr	44	Tetraploid
C-9-4-3	0.4/24 hr	44	Tetraploid
C-9-1-1	0.2/6 hr	44	Tetraploid
C-8-3-4	0.2/6 hr	22, 44	Mixoploid
C-10-1-3	0.2/6 hr	22, 44	Mixoploid
C-10-1-4	0.2/6 hr	22, 44	Mixoploid

The leaves of chimeras/mixoploids were deformed and asymmetric in shape. This may be because in chimeric plants some portion of the leaf or plant may have tetraploid origin, whereas other portions may have diploid origin. The mixoploids/chimeric plants have poor ploidy stability, as leaves induced from the partially tetraploid shoots may later be replaced by vegetative growth from diploid sectors (Vanstechelman *et al.* 2010). Clonal division of mixoploids may allow for isolation of stable polyploid sectors, which may subsequently be maintained vegetatively.

Because 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. The leaves of the diploid control were small, lanceolate to oblanceolate, oblong, serrate above the middle and slightly folded upwards, whereas those of tetraploids were round, large and thick (Fig 2c). Polyploidy in stevia was observed to positively affect leaf shape, size, thickness and ultimately biomass yield of the plants. Compared with diploids, the tetraploid plants had larger leaves. Also, lower internode length, as observed in stevia polyploids, may result in corresponding increase in number

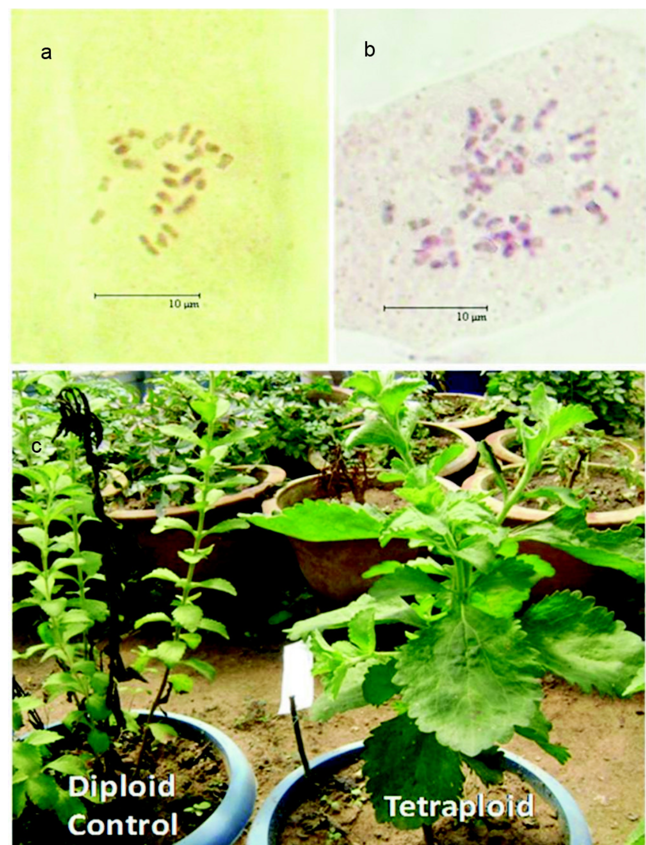

Fig 2 Chromosome counts from root tips of stevia a) diploid (2n=22) and b) tetraploid (2n=44) plant and c) morphological variation among diploid control and tetraploid plant (C7-3-4) developed through colchicine treatment

of leaves per shoot, thereby improving the yielding ability of the plant. However, plant height and number of primary shoots are directly correlated with an increase in biomass; therefore, a decrease in these characters of stevia polyploids may lead to reduction in yielding ability of the plants. Highly significant differences were earlier reported among diploid and polyploid strains with a positive correlation between the level of ploidy and the morphological features examined (Oliveira *et al.* 2004).

Characterization of polyploids

Based on Student's t-test, significant differences were observed for the morphological traits of polyploids analyzed in comparison with diploid control plants under the same growing conditions (Table 3). The glandular trichome was peltate or deeply sunken in the leaf epidermis. The average glandular and non-glandular trichome density was higher in diploid plants (29.3 and 35.0, respectively) compared with tetraploids and mixoploids obtained (16.51 and 19.84, respectively) (Fig 3). The average stomata size of diploid plants was 21.16×15.98 µm (length×width) and that of tetraploids ranged from 32.5×23.2 to 36.31×26.5 µm. The average density of stomata per unit area on the diploid stevia

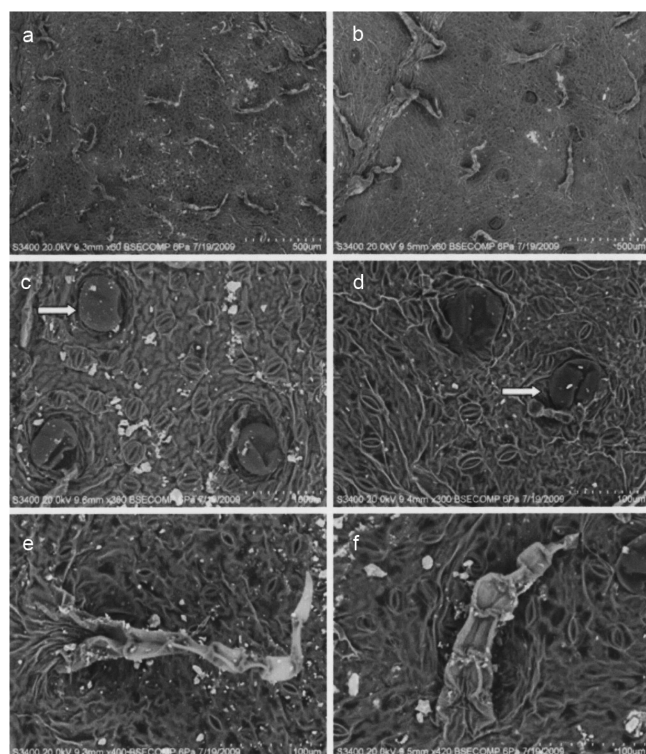

Fig 3 SEM micrographs of *Stevia rebaudiana* showing glandular and non-glandular trichome density at X60 a) in diploid, b) C7-3-4 tetraploid plant, c) size and density of glandular trichome at X300 in diploid, d) C7-3-4 tetraploid plant, e) on-glandular trichome shape and size at X400 in diploid, f) C7-3-4 tetraploid plant

Table 3 Variability for different agronomic characteristics and glycoside profile among diploid (control) and tetraploid plants

Plant No.	Leaf length (cm)	Leaf width (cm)	Leaf thickness (mm)	Chloro-phyll content	Stomata length (µm)	Stomata width (µm)	Stomata no./unit area	Glandular trichome no./unit area	Non-glandular trichome/unit area	Plant height (cm)	Stem thickness (mm)	Inter-node length (cm)	Primary shoots	Stev. (%)	Reb- A (%)
C-7-3-4	10.2	7.4	0.71	33.5	34.6	24.5	21.3	14.0	13.0	28.3	5.1	3.5	10	6.30	2.55
C-7-3-5	8.2	5.0	0.75	29.8	32.5	23.2	28.0	17.0	18.3	30.5	4.38	3.2	13	8.85	0.13
C-9-1-1	7.9	5.2	0.65	28.3	36.3	26.5	22.3	13.0	15.3	36.4	6.25	2.5	11	2.10	1.07
C-9-4-3	8.5	6.2	0.69	24.8	33.5	24.1	24.0	14.3	17.0	41.6	5.35	2.4	17	7.45	2.20
C-8-3-4	9.8	7.6	0.73	30.5	34.6	24.3	26.0	15.0	19.3	33.4	4.8	3.4	7	2.81	1.91
C-10-1-3	6.4	3.1	0.58	22.9	28.8	20.6	35.3	19.0	25.0	38.5	5.48	2.4	17	5.30	2.40
C-10-1-4	7.2	4.2	0.62	20.9	30.1	21.4	33.0	23.3	31.0	40.2	6.48	4.7	9	5.60	2.60
Mean	8.3	5.5	0.67	27.2	32.9	23.5	27.1	16.5	19.8	35.6	5.4	3.2	12	5.5	1.84
Control Mean	4.4	2.1	0.32	21.5	21.1	15.9	37.0	29.3	35.0	58.8	3.5	4.1	21	5.36	2.27
Difference	3.9	3.4	0.35	5.7	11.8	7.6	9.9	12.8	15.2	23.2	1.9	0.9	9.0	0.13	0.43
SE	0.19	0.24	0.01	0.65	0.38	0.29	0.76	0.52	0.88	0.72	0.11	0.12	0.55	0.34	0.13
LSD	0.47	0.57	0.02	1.58	0.93	0.69	1.86	1.26	2.16	1.75	0.26	0.29	1.35	0.83	0.32

Difference (±) = Treatment mean – control mean

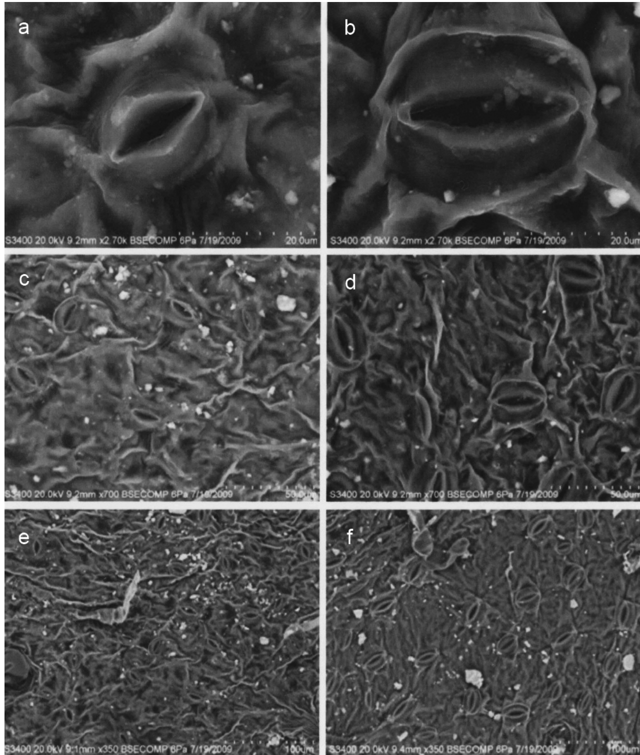

Fig 4 SEM micrographs of *Stevia rebaudiana* abaxial leaves surfaces a) stomata of diploid control at X270k, b) C7-3-4 tetraploid plant, c) stomata of diploid control at X700, d) tetraploid, e) showing stomata count at lower magnification X350 of diploid plant and f) stomata of tetraploid C7-3-4 plant

plants was 37.0, whereas that in the tetraploids was 24.3 (Fig 4). The mean values of stomatal size and density, glandular and non-glandular trichome density were intermediate in C8-3-4, C10-1-3 and C10-1-4, as the mixoploid plants contained both diploid and tetraploid tissues in their leaves (Table 3).

There was significant increase in leaf length and width in polyploids C7-3-4 and C8-3-4, leaf thickness in C7-3-5 and C8-3-4 and chlorophyll content in C7-3-4 and C9-1-1. Among polyploids, there was a decrease in stomata number and frequency of glandular and non-glandular trichomes per unit area but an increase in size of stomata as compared with the control. Maximum stomatal length and width were observed in C9-1-1, whereas highest density of stomata per unit area was observed in C10-1-3, which was at par with the control. The frequency of glandular and non-glandular trichomes per unit area was highest in C10-1-4 but lower than that in the control. Also, there was a significant decrease in plant height, internode length and number of primary shoots but an increase in stem thickness (Table 3). Low internode lengths were recorded in C9-1-1, C9-4-3 and C10-1-3. The highest stevioside content was obtained in C7-3-5, but it was poor in rebaudioside-A content, whereas C7-3-4, C9-4-3, C10-1-3 and C10-1-4 had more rebaudioside-A content compared with the control. The results obtained here open up new possibilities for breeding programs of *Stevia rebaudiana* because polyploid individuals have shown a higher content of stevioside than diploid plants and the selection of such plants for commercial production could possibly increase the level of these compounds.

There was restricted flowering in polyploid plants and fertility of the flowers was also affected. Compared with control plants, flowering in tetraploids and mixoploids was adversely affected. A few inflorescences were formed and the anthers lack pollen formation in tetraploids, whereas viable pollen was observed in the diploids by use of aceto-carmine stain and therefore, normal meiosis can be inferred in the control plants. Sterile seeds were formed in the tetraploid plants in comparison with normal seed formation in control plants. Sterility in stevia polyploids has earlier been reported (Oliveira *et al.* 2004), where lower tetrad normality rate was observed in tetraploid plants than in diploids. Lawrence (1980) postulated that because of irregularities that produced unbalanced gametes during meiosis, polyploids with an odd number of chromosome sets had a high level of sterility. A few viable seeds were obtained in the mixoploid plants C8-3-4 and C10-1-3, which germinated. There is a possibility that the diploid sector of the chimeric plants C8-3-4 and C10-1-3 formed seeds, whereas the polyploid sector produced sterility. However, the chromosome count of the germinated seedlings needs to be ascertained to determine their ploidy level because stevia is a perennial plant, some degree of viable pollen formation may take place in the polyploids during subsequent season of growth the following year. Diploid and polyploid plants lost their vegetative cover with the onset of winters and showed re-sprouting during spring season.

CONCLUSION

The induction of tetraploids in stevia confirmed the effectiveness of colchicine as a polyploidizing agent. The tetraploids obtained in this experiment generated immense variability for morphological characteristics. The polyploids need to be multiplied and evaluated further for glycoside profile as well as agronomic traits and the promising genotypes have potential use as parents in breeding programmes. Breeding of stevia at the polyploid level will generate new variations with respect to biomass and steviol-glycoside content, which may greatly benefit stevia improvement. Triploids of *Stevia rebaudiana* can be produced by mating tetraploid as female and diploid as male parent. Based on regression analysis, LD_{50} value of colchicine treatment in stevia was 0.23%, beyond which there was a drastic reduction in seedling survival. Treatment with 0.6% colchicine for 24 hr was most effective for inducing polyploidy; but at 0.2% colchicine treatment for 6 hr more mixoploids were observed. The nuclear DNA content (2C-value of diploid *Stevia rebaudiana* variety

Madhuguna) was estimated by flow-cytometry to be 2.72pg. The autotetraploids in stevia had significantly increased leaf size, thickness and chlorophyll content and reduced internode length.

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