

CYTOGENETICAL AND BIOCHEMICAL EVALUATION OF DIFFERENT GENOTYPES OF STEVIA PLANTS USED AS A NATURAL SWEETENER

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ABSTRACT

Seven *Stevia rebaudiana* Bertoni genotypes were investigated in this work. In order to achieve such a purpose, chromosome behavior at mitosis as well as at meiosis, pollen fertility, their content of the amino acid proline, and some agronomic traits were precisely studied. The obtained results showed that the chromosome number is $2n=22$ as mitotic examination revealed and 11 bivalents as indicated by meiosis were observed at diakinesis. Number of branch ranged from 4 to 12, plant height from 8.3 to 28.6 cm and number of leaves ranged from 9.3 to 17.2, Number of heads ranged from 1.1 to 4.2. The amino acid proline ranged from 1.18 to 8.2 mg/g dry leaves. Stickiness of chromosomes and presence of 2-3 nucleoli / cell might be indicated that this genotypes are a result of polyploidy.

Keywords. *Stevia rebaudiana*, morphological, chromosome stickiness and tappetum.

INTRODUCTION

Stevia rebaudiana Bertoni, is one of the new world genera of the family compositae and is related to Sunflower and Chicory. The number of species within this genus is ranged from 150 to 300 (King & Robinson, 1972). *Stevia* combines with other herbs to enhance the nutritive values (Soejarto *et al.*, 1982 ; David 1996 and El Sharnoby, 2009).

It is a perennial herb belonging to the Asteraceae family. The leaves of *Stevia* are source of diterpene glycosides, such as stevioside and rebaudioside A and C which are estimated to be 110–270 times sweeter than sucrose (Uddin *et al.* 2006; Megeji *et al.* 2003 and El Sharnoby, 2009). *Stevia* products have some advantages from a food technology stand point, it has no calories, and so it is suitable for diabetics, don't cause tooth decay, is a heat stable for cooking and baking, easily soluble in water, stable in foodstuffs and natural sweetener. These glycosides are extracted and refined from plants without chemical or enzymatic modifications. On the other hand, *Stevia* has several properties over other sweeteners, it combines with other herbs to enhance the nutritive values (Seehy, 2003 and El- Sharnoby, 2009). Originally, the natural of *Stevia* is Paraguay.. The number of species within this genus ranges from 150 to 300 (King and Robinson, 1972 and El Sharnoby, 2009).

Today the use of products and extracts of *Stevia* is widespread throughout the world. The extracts of *Stevia* are used in China, Taiwan, Thailand, South Korea, Brazil, Malaysia, Indonesia, Argentina, Paraguay, Philippines, and Vietnam (Soejarto *et al* 1982, and David 1996). The extracts also are used in some arabic countries (eg. Egypt and Saudia Arabian)

Egypt, in 1994 has registered Stevia as natural sweetener for use in food and pharmaceutical products. The main advantage of Stevia use as natural sweeteners is for diabetics.

This study, however, was planned to determine the chromosome number and behaviour, pollen fertility, proline content and some agronomic traits. Proline content in leaves was estimated as an indicator for salinity and drought tolerance.

MATERIALS AND METHODS

Materials used in this work were kindly obtained from Biotechnology Laboratory, Department of Genetics, Fac. Agric. Alexandria University and Sabbhia Experimental Station, Institute of Sugar Crops, Agricultural Research Center (ARC), Egypt.

Agronomic traits

Agronomic traits were recorded and analyzed using a Randomized Complete Block Design (RCBD) with 10 replicates for each genotype and Least Significant Difference Test (LSD) was used for comparison between means.

Determination of Proline content

Proline content was estimated in the Central Laboratory, Faculty of Agriculture; Alexandria University. According to the procedure described by Hamilton (1962), 4 ml of 3.5% sulfa salicylic acid were added, to one gram leaves, homogenized and centrifuged at 3000 rpm for 5 min. The supernatant was saved and used for proline analysis using Beckman CL Amino Acid Analyzer 119cl.

Cytological studies

Floral buds, and root tips were collected. For cytological examination the microsporocytes (immature heads) from each genotype were collected and fixed in glacial acetic acid for one hr. and then are transferred in fresh Carnoy's for killing and fixation. The fixed materials were, then transferred to a solution of 70% ethanol and stored in a refrigerator at 4 C^o until usage. The chromosomes stainability was achieved according to (Eid 1958, 1963) and Fachinnetto *et al.*, (2008). The cytological preparations were made following the well-known aceto-carmine smear technique. Staining of pollen mother cells (PMC) was done according to the procedure described by McClintock (1929) and Darlington and La-Cour (1962). Diakinesis stages were examined to determine the total number of chromosomes and meiotic as well as mitotic stages were photographed when needed.

RESULTS

Regarding the tested agronomic traits (1) illustrated the observed results.

No. of branches: this character ranged from 4 (Genotyp-6) to 12 (genotype-1). This values means that a difference between genotypes (up to 3 fold increase was achieved.

Plant height: ranged between 8.3 cm to 28.6 cm giving avidness that more than 3.5 fold increases was detected.

No. of Leaves: it was found to be ranged from 8.2 (Genotype -3) to 17.2 (Genotype-1). However, comparing this result, one can conclude that genotype-1 was proven to be higher in number of branch, plant height, and number of leaves. This result, however, might be taken in consideration in breeding program and in general. The genotypes 1, 4 and 5 were found to be the best in this evaluation

Table (1): Description of six characters of the seven *Stevia* genotypes which used in this study.

Genotype	No. of branch	Plant height Cm X±S.D%	No. of leaves on branch X±S.D%	Leaf length Cm X±S.D%	Leaf width Cm X±S.D%	No. of heads X±S.D%
Genot-1	12	14.2±11.23	17.2±12.98	4.9±1.74	2.0±0.48	3.4±4.44
Genot-2	6	13.3±10.97	12.3±8.5	4.4±1.07	1.4±0.37	2.1±2.67
Genot-3	9	8.30±4.36	8.2±4.18	3.5±0.67	1.5±0.44	2.2±1.32
Genot-4	11	28.6±11.91	14.9±3.9	4.2±0.70	2.0±0.25	4.2±2.10
Genot-5	10	16.60±8.34	13.8±2.39	4.5±0.51	2.0±0.33	1.7±2.10
Genot-6	4	10.8±4.44	10.5±1.91	4.4±0.50	2.0±0.22	1.7±1.14
Genot-7	8	12.0±7.74	9.3±3.01	4.4±0.67	1.6±0.32	1.1±1.23

Means (X) and standard deviation (S.D%) for number of branches, plant height, number of leaves on branch, leaf length, leaf width, and number of heads in studied genotypes of *Stevia rebaudiana* Bertoni.

Pollen fertility: Estimation of pollen fertility for the seven genotypes in shown in (table, 2). Pollen fertility ranged from 89.01 to 98.1 for the genotypes 5 & 4, respectively. However, this pollen fertility was not affected by Stickiness noted from the cytological examination.

Table (2): Genotypes of *Stevia* and pollen fertility

Genotype	No. pollen grains examined	No.viable pollen grains	% fertility
1	2340	2111	90.21
2	3210	3117	97.10
3	1998	1882	94.19
4	2010	1972	98.10
5	3002	2672	89.01
6	2450	2310	94.28
7	2320	2070	89.22

Figures (1, 2, and 3) show different stages of meiosis and mitosis and multinucleolei. Table (3) shows the result obtained from the estimation of proline content in *Stevia* leaves of the different genotypes. Proline was found to be ranged from 1.18 (genotype, 6) to 8.2 (genotype, 1). This result means that more than 7 – fold increases in proline content had achieved.

Table (3): Genotypes of *Stevia* and amino* acid proline

Genotype	Proline	Genotype	Proline
1	8.2	5	2.2
2	4.1	6	1.18
3	2.8	7	4.06
4	4.12		

*mg/ g dry weight leaves

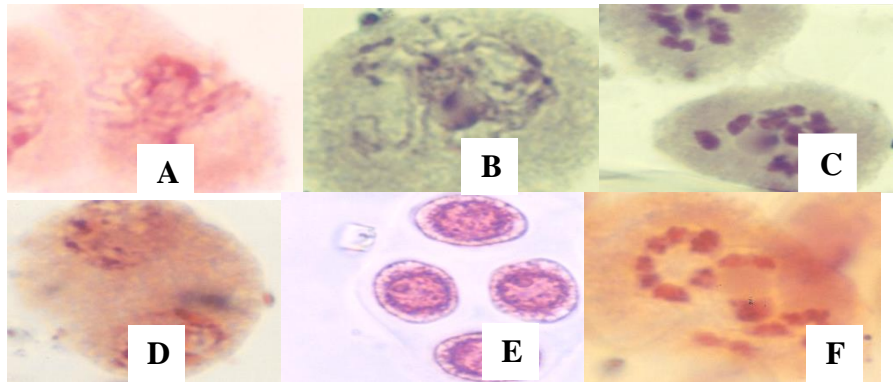


Figure (1): A and B photomicrographs of Pachytene stage of meiosis with heteropycnosis of *Stevia rebaudiana*, C photograph of diakinesis stage with 11 bivalents, D photograph of meiotic Telophase stage, E photomicrograph of quarted stage and in F photomicrograph showing stickiness aberration to bivalents in diakinesis stage.

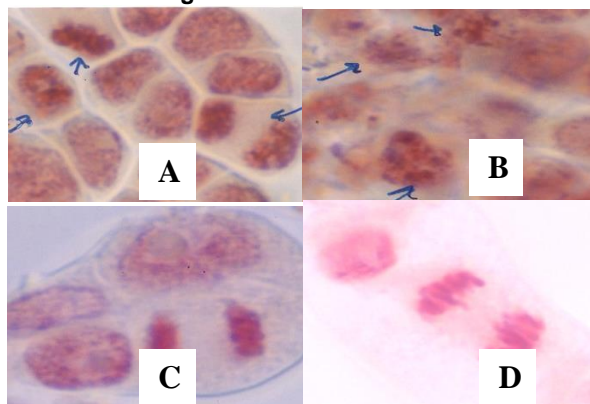


Figure (2): Photomicrographs of mitotic division stages; in cells of Tappetum layer with high mitotic activity in photomicrographs A and B, while in C showing mitotic Telophase stage in Tappetum layer, and in D showing the Telophase stage in cells of root tip.

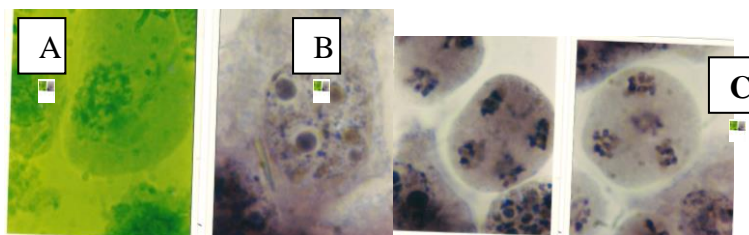


Figure (3): Photomicrographs showing (A) multinucleolei, (B) heterochromatin and (C) second telophase.

DISCUSSION

In conclusion, the present investigation revealed that the chromosome number of *Stevia* is $2n=22$ and 11 bivalents in meiosis were observed. Normal chromosome behavior with high degree of stickiness was observed with 2-3 nucleoli/ nucleolus giving an evidence that the tested genotypes are genetically different and this genotype came from polyploidy. Investigation of some agronomic traits proved that the tested genotypes are genetically different. Examination of pollen fertility revealed that this phenomenon was not affected by stickiness. It was suggested that proline is acting as a compatible cytoplasmic solute, balancing an accumulation of salts outside of the cytoplasm (Vetberg and Stewart, 2001). The results obtained from this bioassay is in agreement with that reported by several workers (e.g. Chu *et al.*, 1973, Aspinall and Paleg, 1976; Buhl and Stewart, 1983; Stewart *et al.*; 1986; Ueda *et al.*; 2001, and Votberg & Stewart, 2001). Wilting caused an increased conversion of glutamate to other products. In nonstarved leaves, conversion to organic acids as well as to proline was increased. In starved leaves, wilting caused an increase in the conversion of glutamate to glutamine, aspartate, asparagines, and organic acids.

Estimation of the amino acid proline as an indicator for salinity and/ or drought tolerance presented an evidence that the tested genotypes displayed a wide range for this character, and accordingly it might be employed and used in evaluative purposes and breeding program. Assessment of genotoxicity of *Stevia* extract has been precisely carried out (Seehy, 2003), who employed a variety of short-term genotoxic bioassays using yeast, mice, rat and human lymphocytes. It was found that this extract is negative clastogen, negative mutagen on yeast, mice, rat and human genome.

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التقييم الوراثي الخلوي والكيموحيوي لطرز وراثية مختلفة من نبات الإستيفيا والمستخدمة كمحلى طبيعي

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اجري هذا البحث بغرض دراسة تقييم تراكيب وراثية مختلفة والمستخدمة من الإستيفيا والمستخدمة كمحلى طبيعي.

نظرا للفجوة الكبيرة بين انتاج السكر والاستهلاك فقد تقدم العلماء وادخلوا مستخلص اوراق نبات الإستيفيا كمحلى طبيعي حيث يتميز بصفر كالورى وحلاوة تصل لاكثر من 250 مرة بالمقارنة بالسكروز. وقد دلت الابحاث على ان هذا المستخلص له القدرة على خفض الجليسيريدات الثلاثية وضغط الدم وتم ادخال هذا النبات فى جمهورية مصر العربية منذ فترة قصيرة.

يهدف البحث الحالى الى تقييم سبع تراكيب من هذا النبات لمعرفة العدد الكروموسومى وسلوك الكروموسومات والقاء نظرة على النشأة ودراسة محتوى هذه الاوراق من الحمض الامينالبرولين الذى يمكن الاستدلال به على المقاومة للملوحة والجفاف ولتحقيق هذا الهدف تم قياس بعض الصفات المحصولية كارتفاع النبات وعدد الافرع وعدد الاوراق ودراسة السلوك الكروموسومى وكذلك العدد فى الانقسامين الميوزونوالمبيوزى. وقياس حيوية حبوب اللقاح وكذلك قياس مستوى الحمض الامينالبرولين. وقد اظهرت النتائج ان هناك اختلافا جوهريا فى الصفات المحصولية المدروسة وتميز الانقسامين الميوزونوالمبيوزى بسلوك طبيعى رغم وجود لزوجة كروموسومية وظهور عدد من 2-3 نوية /نواة دالا ذلك على ان منشأ هذا النبات من التضاعف. وتراوحت حيوية حبوب اللقاح من 89.1% الى 98.1% أظهر تحليل الحمض الامينى برولين تفاوتاً كبيراً (1.18 - 8.2 مجم/جمورقة جافة) مما يسهل الامر فى الاغراض التقييمية وبرامج التربية.

تستخلص الدراسة الحالية ان التراكيب قيد الدراسة الحالية أظهرت انها مختلفة وراثيا وانها نشأت من التضاعف (الخطى) وان بعضها محتوى عالى من الحمض الامينى برولين مما يسهل توظيفها فى برامج التربية لتحمل الملوحة والجفاف.

Keywords: *Stevia rebaudiana*, morphological, chromosome stickiness and tappetum.

قام بتحكيم البحث

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