Physico-Chemical Properties of Chromatin, Proline Content; and Induction of Polyploidy in *Stevia rebaudiana* (Bertoni)

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ABSTRACT

This work aims at investigating Physico-chemical properties of purified chromatin isolated from *Stevia rebaudiana*(Bertoni) and estimation of proline content before and after induction of polyploidy, to achieve such purpose, isolation of chromatin was carried out and purified, temperature of melting, chemical composition of chromatin,proline content were investigated, polyploidy was proven to be successful. Analysis of data obtained from the physico-chemical properties showed that differential gene expression was detected, given an evidence that the tested treatments were genetically different. However, tow polyploidy plants were observed and it is possible to propagate this martial through tissue culture. This materials provides a good and fretful approach for plant breeder.

INTRODUCTION

Stevia (*Steviarebaudiana*)is an herbaceous perennial used normally as a natural herbal sweetener (Shibata *et al*., 1991). Stevia leaves contain several chemicals called glycosides. These glycosides collectively give 100 to 300 times the sweetness compared with sucrose and are extracted and refined from plants without chemical or enzymatic modifications. The two main glycosides are Stevioside (traditionally 5-10% of the dry weight of leaves) and Rebaudioside A (2-4%)(Dzyuba1998).These glycosides are the sweetest compounds. There are also other related compounds including RebaudiosideC (1-2%),Duclosid A and C as well as minor glycosides including flavonoid glycosides; coumarins; cinnamic acids; phenylpropanoids and some essential oils (Kinghorn1987 andKohda*et al*., 1976).

Stevia products have some advantages from a food technology point of view. It has no calories; it is suitable for diabetics; does not cause tooth decay; it is heat stable for cooking and baking; easily soluble in water; stable in food stuffs and natural sweetener (Ikan*et al.,* 1993).In Egypt. The gap between sugar production and consumption is widening. In November 2013 Egypt had produced 2 million tons of sugar and had been imported 1.2 million tons. (USAD2013). At the same time, five grams of stevioside can substitute one Kg of table sugar. Thus, the cultivation of Stevia in Egypt as a main natural sweetener may reduce the gap between sugar production and consumption. Moreover, water requirements for the growth of sugarcane plants are relatively quite high, Sugarcane plant needs three times the water amount compared with Stevia, which requires approximately 300 m^3 water / feddan. Also, Stevia can grow under different climatic conditions and in different type of soils (Jia1984). So, there is a great interest on the strategical agriculture plan for Stevia cultivation in the new land reclamation projects to meet the demands of the Egyptian markets and generate income for the growers.The genus stevia shows great variations in chromosomenumber. The chromosome number of *Stevia rebaudiana*(2n=22) (Frederico*et al.,* 1996). The induction of polyploidy to improve agronomic yields is a process commonly used in plants of economic interest (Allard 1960) and has been applied to other species, such as coffee (Cruz *et al.,*1993) and orange (Romero-Aranda*et al*., 1997).

MATERIALS AND METHODS

The plant material used in the present study consisted of one variety, named *Stevia rebaudiana* (Bertoni) c.v. Sponti (2n = 22).This variety was used as a negative control obtained from Sugar Crops Research Institute (SCRI); Agricultural Research Center (ARC); Ministry of Agriculture, Egypt.

Seeds and buds treatment

Colchicine treatments were divided into two groups:

a- Seeds treatment:

Seeds were treated with different concentrations aqueous solution of colchicine (0.01(B_1), 0.05(B_2), 0.1(B_3), 0.25(B_4)) and 0.5%(B_5), seven-hundred seeds per Petri dish were subjected to treatment duration 18 hours in addition to the control treatment(received water only)Bc seeds were firstly washed under running tap water and then three times with distilled water (Figure. 1)(Valois 1992)

b- buds treatment:

Buds at one month age was sprayed with five concentrations of colchicine, (0.01(S_1), 0.05(S_2), 0.1(S_3), 0.25(S_4)) and 0.5%(S_5), for 18 hours and the control treatment. (Sc)

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The treated seeds of Stevia plants were cultivated in December (2013), in planting trays (petmose: sand :clay)(1:1:1) at the green house of Sabahia Agricultural Research Station (Figure. 2). All trays were kept under temperature ranging from 16 to 36°C and photoperiod for13-16 hours with 6000 lux fluorescent day lights. After one month of cultivation plants were transferred to pots (Figure 3. (a,b)), after three months of cultivation leaves of some plants were cut and subjected to the various analyses:

1. Analysis of Stevia sweeteners by HPLC

Stevia sweeteners were extracted with Methanol (MeOH 95%) method as that described by (Kolb *et al.,* 2001) then estimated at the Central Laboratory, Faculty of Science, Alexandria University. Stevia leaves extracts were separated and identified on HPLC.

Figure 1. Seeds treatment with colchicine

Figure 2. Seeds cultivation in planting trays

Figure 3 a,b. Transfer of plants

2. Cytological technique

The flower buds were used to study the chromosome behavior at meiosis. The Feulgen technique was used in this study. (Feulgen and Rossenbeck 1924).

The microsporocytes of the treatments were examined at late diakinesis and first metaphase stage of meiosis soon after preparation to determine the chromosome number and the frequencies of different types of chromosomal associations.

To estimate the pollen viability of the studied treatments, the technique was described by Moreira and Gurgel (1941) were used.

3. Physico-chemical properties of chromatin:

The chromatin was isolated by the method described by Fellenberg and Schomer (1975).

3.1. Estimation of melting temperature (Tm value)

Heating was carried out in diluted saline solution (0.0014 M Nacl + 0.00025 M Tris-Hcl, pH 7.2). Melting temperature of the chromatin was recorded at 260 nm as well as 280nm by Ultraviolet Spectrophotometer Schimadsu uv-160, while heating was done by temperature program controller, Schimadsu TB-85, giving a temperature rate of 1°C / min. In order to estimate Tm-value, the procedure was the same as that described by Bonner *et al*., (1968) and El-Sharnoby,(2009). From each extraction two estimations were calculated. The absorbance of all investigated samples was recorded every minute at 260 nm as well as at 280 nm. The relative absorbances were calculated as described by Spang& Platt (1972) and Seehy*et al*., (1990). Relative Absorbance = AG/A 50°C, where AG is the absorbance at a given temperature and A 50°C is the absorbance at 50 °C.

RESULTS AND DISCUSSION

1. Treatment and cultivation:

Table (1) shows the percentage of survived plants after treatment with Colchicinefor each of seeds and buds.

Table (1) illustratedthat; the Colchicine treatments were lethal at concentrations 0.1, 0.25 and 0.5%in both seeds and buds treatments.

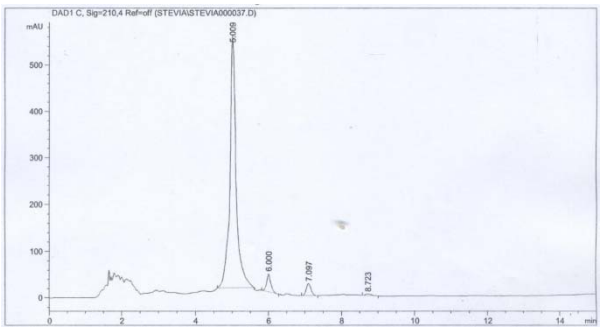
2. Chemical analysis for Stevia sweeteners:

Chang and Hung (1981) reported that a simple method for purifying sweeteners extract of dry Stevia leaves was developed in which active carbon was used. The adsorbed sweeteners on active carbon were then desorbed from the active carbon with aqueous ethanol. The elute was colorless and pure sweetener was obtained. This method facilitated the transfer of the sweetener from the aqueous solution, which is easier to concentrate and to have the sweeteners crystallize out. Finally the sweeteners were analyzed by HPLC to give about 90 -92 % of the initial concentration. Therefore, it can be concluded from an economical view point that the Stevia sweeteners concentration on active carbon should be used, in order to obtain an elute of higher Stevia sweeteners concentrations.

Stevia sweeteners were analyzed and determined employing the High Performance Liquid Chromatography HPLC (Figure4). The data obtained are given in Table (2). From this Table there were differences between the different treatments. The Concentration of total Stevia sweeteners (mg. sweeteners/g. dried leaves) were increased to be (100.31) in treatment (S.2), and decreased to be (49.73) in treatment (B.1) and (76.36) in treatment (B.2). These Results gave an evidence that these treatments are different and differential gene function was observed.

Table 1. Percentages of survived plants after Seeds (S) and buds (B)treatment

Treatment	Survived Plants (%) after one month	Survived Plants (%) after two months	Treatment	Survived Plants (%) after one month	Survived Plants (%) after two months
S.C	5.2	4.1	B.C	55.8	48.5
S.1	6.6	5.2	B.1	41.6	37.2
S.2	6.1	3	B.2	43.1	36.3
S.3	6.5	-	B.3	19	-
S.4	3.4	-	B.4	6.8	-
S.5	0.6	-	B.5	7.6	-



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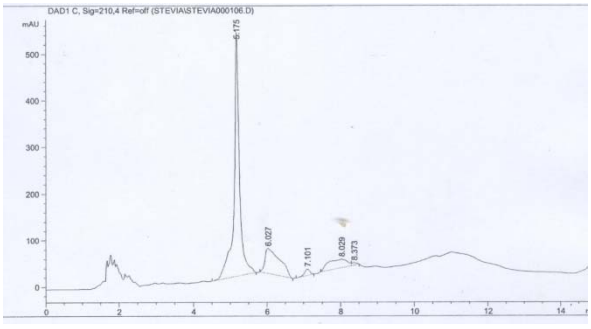
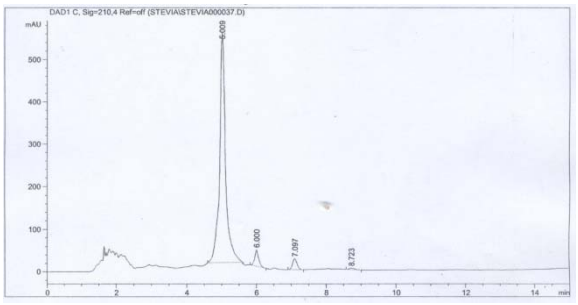
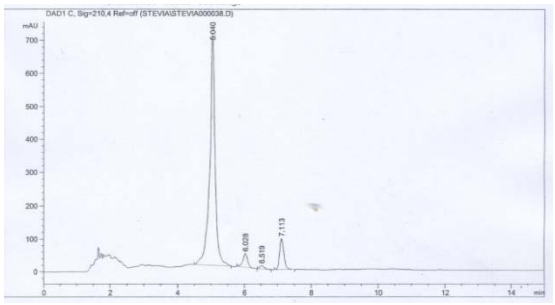


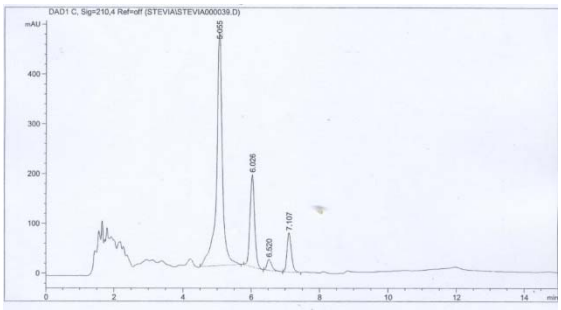
S.C



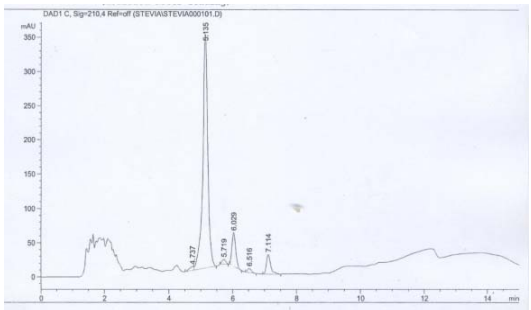
S.1



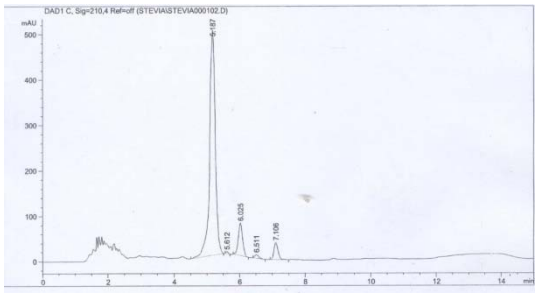
S.2



B.C



B.1



B.2

Figure 4. HBLC of six stevia treatments of both seeds and buds

Table 2. Concentration of Stevia sweeteners

Sweeteners	S.C	S.1	S.2	B.C	B.1	B.2
**Steviolbioside	58.29	75.76	85.77	62.05	40.8	64.61
**Stevioside	16.78	3.26	4.44	18.63	4.33	7.10
**Rebaudioside C	-	-	0.99	2.253	0.54	0.72
**Rebaudioside A	1.47	2.51	9.10	7.55	2.87	3.70
**Unknown component	7.95	-	-	-	1.19	0.23
**Total	84.49	81.54	100.31	98.04	49.73	76.36

**mg. sweeteners/g. dried leaves.

3. Cytological analysis

Meiotic and mitotic studies:

Table 3. Chromosome number in PMCs (pollen mother cells)of the studied treatments

Treatments	Chromosome Number
S.C	22
S.1	22
S.2	22
B.C	22
B.1	22
B.2	44

Regarding chromosome numbers cytological examination showed that induction of polyploidy was observed in treatment (B.2) (Table3), since 44chromosomes were obtained and giant pollen grain observed and photographed as shown in figure (5).

Wang and Wang (1998) used deferent methods for ploidy determination in sugar beet (*Beta vulgaris* L.). These methods included measurements of leaf stomatal size (longer and diameter), stomatal density, chloroplast number in stomatal guard cells, pollen size and somatic chromosome counting. All these methods were conducted among a diploid population, and its equivalent triploid and tetraploid population.

The number of chromosomes also is a controversial subject in this species. For example, Monteiro (1980, 1982), Frederico*et al.* (1996), Oliveira *et al.* (2004) and Raina*et al.,*(2013) have reported 2n=22 chromosomes for this species.

Several workers studied different plants pollen grain (Smith-Huerta &Vasek, 1984, Vitagliano&Viti, 1989, Shivanna*et al.,* 1991, Oddone, 1997, Acar*et al.*, 2010, Abdullateef1 *et al.* 2012 and Raina*et al.,* 2013).

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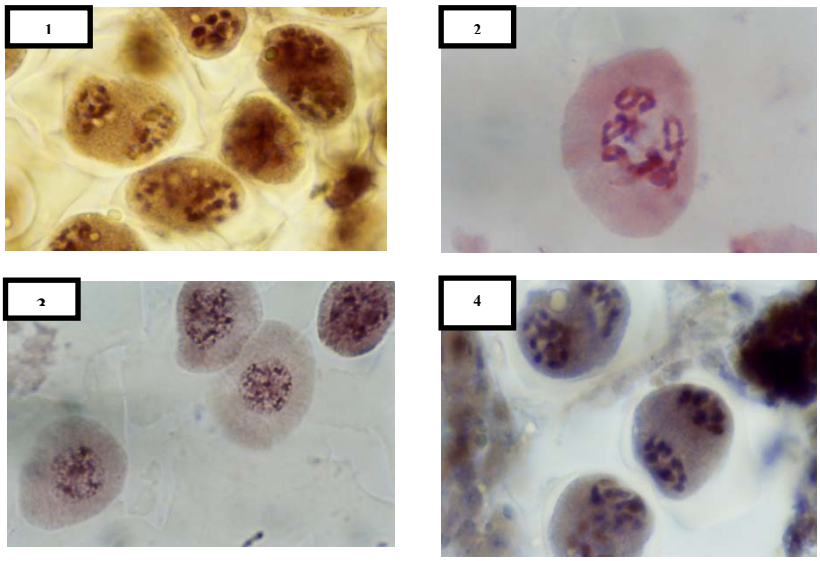

Figure 5. Photomicrographs showing (1)secondary meiotic division polar viewin treatment S.C. (2) secondary association in treatment B.1. (3) abnormal quartetin treatmentB.2.(4) secondary meiotic division in treatment B.1.

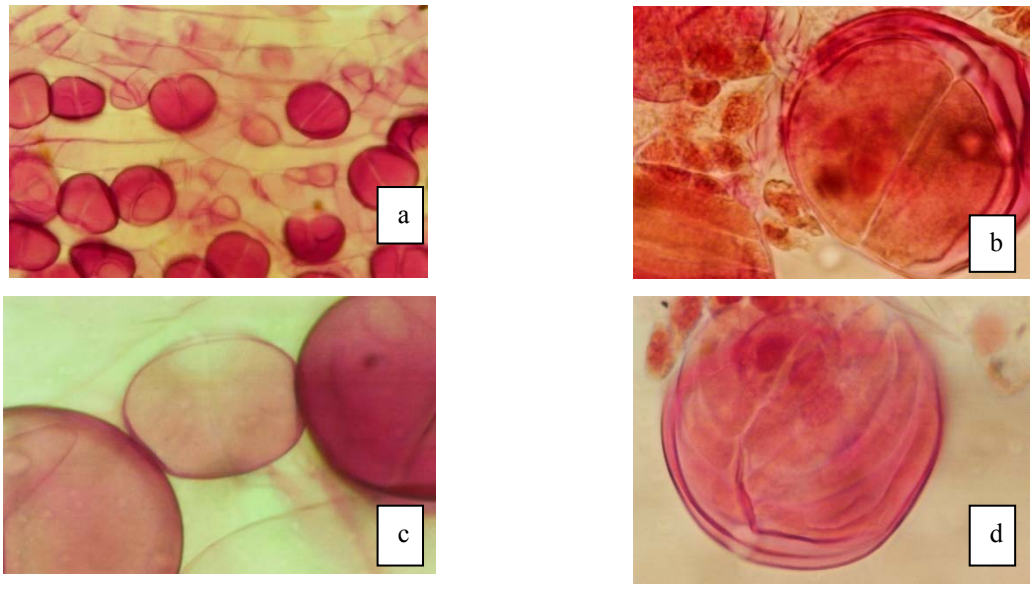

Figure 6. a-Photomicrograph showing pollen grain in Control (S.C).b- Photomicrograph showing giant pollen grain in treatment (B.1).c- Photomicrograph showing aborted pollen grain (in the middle) in treatment (S.1).d-Photomicrograph showing giant pollen grain in treatment (B.2)

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4. Physico-chemical properties of chromatin

The present investigation revealed that stevia leaves are a good source for chromatin. In addition, the method used was found to be successful for chromatin isolation.The chromatins of these six treatments were proven to be pure enough to subject for testing the melting temperature. Table (4) illustrates the ultraviolet absorption spectrum of purified chromatin isolated from the treatments S.C, S.1, S.2, B.C, B.1 and B.2.

Table (5) represent the Tm values for each chromatin of the six treatments of *Stevia rebaudiana* understudy.

Ultraviolet absorption spectra of all used samples of chromatin were shown to be acceptable. Melting of chromatin was carried out at 260 nm as well as 280 nm and showed differences in melting temperature of chromatin isolated from different treatments understudy, giving an evidence that transcriptional activity of chromatin was different, Tm values at 260nm ranged from 72.5ºC for treatment B.1 to 77 ºC for treatment B.C; At 280nm, the Tm values ranged from 77.5 ºC for treatments S.2 and B.1 to 80 ºC for treatment B.C.

Table 4. Ultraviolet absorption spectra of purified chromatin isolated from the six *Stevia rebaudiana* treatments

Treatments	Wave length (nm)					
	240	**250**	**260**	**280**	**300**	**320**
S.C	120	200	280	190	98	28
S.1	100	120	180	150	92	16
S.2	110	130	190	140	62	18
B.C	140	180	220	200	110	25
B.1	122	190	270	200	92	18
B.2	200	300	320	280	110	22

Table 5. Tm and Δ T m values at 260 nm and280 nm f or the purified chromatin of six treatments of *Stevia rebaudiana*

Treatments	Tm : 260nm	Tm : 280nm	Δ T m [280-260]
S.C	74.5	79.5	5
S.1	76	78.5	2.5
S.2	75.5	77.5	2
B.C	77	80	3
B.1	72.5	77.5	5
B.2	75	78	3

Also, Δ Tm values were differed from one treatment to another and ranged from 2 ºC for treatment S.2 to 5 ºC for treatments S.C and B.1 (Table, 5). Sehyet al.(1990) reported that an alteration of 0.5 ± ºC in Tm-value is considered to be a significant difference. A comparison of Tm-values at 260nm and 280nm makes it possible to differentiate the cohesion of hydrogen bonds in deoxyribonucleic acid regions rich in adenine-thymine and guanine-cytosine, since the former more strongly absorb the wave length 260nm, while the latter absorb the wave length 280nm more strongly, (Fellenberg, 1974 and Seehy, *et al*. 1990).

The amounts of total proteins; histone and non-histone proteins relative to DNA were estimated in the purified chromatin of the six treatments understudy (Table, 6).

Data obtained from the chemical composition of purified chromatin relative to DNA at seeds treatment showed that the treatment S.2 displayed histones higher than that of other treatments while the control S.C represents the lowest value of histones, on the other hand the control B.C displayed histones higher than the other treatments while B.1 treatment represents the lowest value of histones at buds treatment (Table, 6).

Repressed and Active fraction of genome (RFG) & (AFG) illustrated in Table (7)

The repressed fraction of genome ranged from 66.70 to 83.00% for seeds treatment S.C and S.2; respectively, while the active fraction of genome ranged from 17.00 to 33.30% for seeds treatment S.2 and S.C; respectively. On the other hand repressed fraction of genome at buds treatments ranged from 63.70 to 79.30% for B.1 and B.C; respectively, while the active fraction of genome ranged from 20.70 to 36.30% for B.C and B.1; respectively (Table, 7).

The tested samples could be arranged, according to the transcriptional activity based upon non-histone proteins (relative to DNA) would be arranged as follows: S.C > S.1 > S.2 & B.2 > B.1 > B.C of seeds and buds treatments; respectively. Furthermore, they, according to their histones (as a function for transcriptional activity) might be ranked as follows: S.C < S.1 < S.2 & B.1 < B.2 < B.C of seeds and buds treatments; respectively. These results, however, gave a strong evidence that the purified chromatin of the seeds treatment S.C displayed:

1- low Tm-value at 260nm;
2- low histones, relative to DNA (1), and;
3- higher non-histones (percentage of total proteins) and;
4- high active fraction of genome for transcription.

While at buds treatment B.1 displayed:

1- low Tm-value at 260nm as well as at 280nm;
2- low histones, relative to DNA (1), and;
3- high active fraction of genome for transcription

The results obtained from this part of the present investigation revealed, from a molecular genetics point of view, that these seeds and buds treatment are different.These results is in agreement with that reported by several workers(Bonner, 1976,Seehy*et al*, 1990 and Ghonema,2010).

Table 6. Chemical compositions of the purified chromatin for the six treatments of *Stevia rebaudiana*(relative to DNA).

Treatments	DNA	Total proteins (µg/ml)	Histones (µg/ml)	Non-histones (µg/ml)
S.C	1	2.22	.90	1.31
S.1	1	1.42	1.05	0.36
S.2	1	1.37	1.12	0.25
B.C	1	1.24	1.07	0.16
B.1	1	1.77	0.86	0.91
B.2	1	2.28	0.95	1.33

Table 7. Repressed and Active fraction of genome (RFG) & (AFG),

Treatmnts	RFG	AFG
S.C	66.70	33.30
S.1	77.80	22.20
S.2	83.00	17.00
B.C	79.30	20.70
B.1	63.70	36.30
B.2	70.40	29.60

5. Proline:

Regarding the determination of Proline content, the data obtained are shown in Table (8). Proline was found to range from 17.5µg / g in treatment S.2 to 45.95µg / g in treatment B.2. Such a result gave an evidence that these Stevia treatments are genetically different or at least gene expression of the amino acid proline displayed differential expression.

Table 8. Concentrations of the amino acid proline in the different treatments of Stevia

*Concentration µg/g	Treatments
21.7	S.C
20.2	S.1
17.5	S.2
20.6	B.C
18.11	B.1
45.95	B.2

* µg / g dry weight

It was suggested that proline is acting as a compatible cytoplasmic solute, balancing an accumulation of salts outside of the cytoplasm. (Vetberge& Stewart, 2001). The results obtained from this bioassay is in agreement with that reported by several workers (e.g. Chu et al 1973, Aspinall&Pa1eg, 1976; Buhl & Stewart, 1983; Stewart et al; 1986; Ueda et al. (2001) and Voteberg& Stewart (2001).

Proline plays an important role in plants for stress tolerance, (Stewart 1978; Voetberg and Stewart 2001). Proline was increased to a steady state concentration in response to salt treatments (Voetberg and Stewart, 2001 and El-Sharnoby, 2009). These authors, stated that proline levels in salt shocked leaves, decreased to near control levels within 24 hours of relief of stress.

The data previously obtained indicated differential gene expression-with respect to Proline, at the same environmental conditions. In conclusion, the present study revealed, at the different levels of study, that the tested treatments are different. This conclusion, however, might be usefully used in selection and evaluative purposes in breeding program of Stevia.

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الملخص العربي

الخواص الفزيوكيماويه للكروماتين ومحتوي الحامض الاميني البرولين واستحداث التضاعف في نبات الاستيفيا

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هدفت هذه الدراسه الي دراسة الخواص الفزيوكيماويه للكروماتين المنقي والمستخاص من نبات الاستيفيا وكذلك محتوي الاوراق من الحامض الاميني برولينوارتباط ذلك بتضاعف الكروموسومات المستحدث بواسطه الكولشسين، ولتحقيق هذا الهدف تم اختيار الصنف المسمي sponti وزراعتة والمعامله بتركيزات مختلفه من الكولشسين للبذور وكذلك للبراعم. ولدراسه الخواص الفزيوكيماويه تم جمع الاوراق واجراء التكنيك اللازم لذلك لتقدير درجه حراره انصهار الكروماتين وكذلك المكونات الكيماويه للادنا وبروتينات كروموسوميه كليه وبروتينات هستونيه وبروتنيات لا هستونيه علي طول موجه قدرها ٢٦٠ و٢٨٠nm كما تم تقدير الحامض الاميني برولين من اوراق نباتات عاديه ومقارنتها باوراق نباتات تم استحداث التضاعف بها. وكذلك تحليل تركيز مكونات التحليه في الاوراق وكذلك فحص الكروموسومات وحبوب اللقاح في النباتات المعامله والغير معامله بالكولشسين. هذا وقد اظهرت النتائج وجود تضاعف عند تركيز ٠,٠٥%. وقد انعكس ذلك علي تضاعف كميه البرولين وكذلك عدد الكروموسومات والخلايا وحجم حبوب اللقاح. وقد اوضحت النتائج المتحصل عليها انه يمكن استخدام النباتات الناتجه عن التضاعف بعد اكثارها بمزارع الانسجه في برامج التربيه والانتخاب، وهو يقدم ميزه هائله للمربي.