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Steviol Glycosides Induced in Vitro from Three Stevia Genotypes Successfully Planted in Egypt

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Abstract

Stevia is considered a promising plant in Egypt according to the suitable soil and climate. Vegetative propagation of stevia is needed to encourage spreading distinguished genotypes in Egypt (Morita, Eirete and Brazilian genotype).For this target, micropropagation could be a beneficial new technique. Our study aimed to establish a tissue culture protocol for three genotypes of stevia plant in Egypt. Moreover, salinity tolerance assessment had been done for the three genotypes. In this investigation, Benzylaminopurine (BAP) with various concentrations (0.00, 0.25, 0.50, 1.0 mg/l) was studied for multiplication as well as various medium strengths. In addition, Indole butyric acid (IBA) with concentrations of 0.0, 0.25, 0.5, 1.0 and 2.0 mg/l were studied for rooting stage. Various concentrations of NaCl were used to study the tolerance ability of the three genotypes used in this research; 0.0, 25, 50and 100 mM. Our results assured that enhancing multiplication rate had been taken place with all used concentrations of BAP comparing with the control (free of BAP). Meanwhile, 1.5 of Murashige and Skoog medium (MS) strength enhanced all growth parameters. Rooting percentage as well as root number highly rose at 1.0 mg/l IBA. Morita genotype showed superior response in multiplication and rooting stage followed by other genotypes. According to salinity tolerance, Eirete was the most tolerated genotype. Nutrient, carbohydrates and proline contents of the three stevia genotypes were determined according to salinity stress. In addition, HPLC analysis showed steviol glycosides in vitro production and their raising production according to salinity stress.

Keywords: High-performance liquid chromatography (HPLC), Micropropagation, Proline, Salinity, Stevia, Stevioside.

Abbreviations

BAP Benzylaminopurine
IBA Indole butyric acid
HPLC High-performance liquid chromatography

Introduction

Stevia rebaudianais a South American plant native to Paraguay. It is aperennial plant belongs to the family Asteraceae. It is commonly known as stevia, sweetleaf, candyleaf or sugarleaf due to its sweet taste. It is one of the most important medicinal plant and widely used as an alternative for traditional sugar. Its commercial cultivation has spread to Japan, Southeast Asia, USA and Middle East, additional to mildly tropical or moderate climates as it prefers warm, moist and sunny conditions [1].

Stevia has been already consumed as a sweetener for centuries in some countries like Japan, China, Brazil, Indonesia, Tanzania and Korea [1].The powder of the stevia leaf sweetness is as higher as20 to 25 times than sugar. Moreover, the pure extract of stevia (stevioside) is 300 times sweeter than cane sugar [2]. Due to the sweetening properties of its leaf with non-caloric in human consuming, stevia has gained attention for food industries [3] and nutrition processes [4]. The World Health Organization expects stevia to represent 20% of the amount of sweeteners used globally.

In fact, its leaf includes diterpene glycosides, such as steviolbioside, rubsoside, rebaudioside A, B, C, D, E and F, dulcoside and stevioside which give its sweetening flavor [5]. Additionally, it has a strong antimicrobial properties [6] and therapeutic agents such as anti-hyperglycemic, anti-hypersensitive [7, 8] and prevention of dental decay that are important for pharmacological industries. Fortunately, the soil and climate of Egypt is suitable for the cultivation of stevia, as the climate is as close as possible to the country of origin; Paraguay. It grows at a temperature range between 20 to 40 degrees Celsius and does not need much water to irrigate the plant, opposed to sugar cane and beet. At present, there are at least 5,000 acres planted with stevia in Egypt. To provide large numbers of stevia plants for expanding, tissue culture technique proved to be a suitable tool for it. Thus, plant tissue culture is not only an important tool for fundamental research such as plant physiology and gene discovery, but it is also of direct commercial interest for mass micropropagation of plants, plant conservation and breeding, genetic transformation, genome editing, production of secondary metabolites, etc. [9, 10, 11].

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The aim of this investigation is conducting a protocol for stevia micropropagation, assessing the suitability of three genotypes for tissue culture processing as well as salinity tolerance and its efficiency on stevioides production for the three cultivars under investigation.

Materials and Methods

The present study was conducted in 2020 to 2021 at the Tissue Culture Technique Lab, Central Laboratories Network, National Research Centre, Dokki, Giza, Egypt. Preparing samples was conducted at Sugar Crops Research Institute (SCRI), Agricultural Research Center (ARC), Egypt. Extraction was applied at Medicinal and Aromatic Plants Research Dep., NRC, Egypt.

1. Samples collection

Vegetative shoots of three genotypes of stevia (*Stevia rebaudiana* Bertoni): two new genotypes "Morita" (C1) and "Eirete"(C2) in addition to the Brazilian genotype (C3; locally cultivated in Egypt from 15 years) were collected and brought to the lab.

2. Micropropagation

2.1. Establishment stage

Stevia shoots from the three genotypes under investigation were leaves detached. Shoots were divided into one-node-explants. These explants took 20 min washing with tap water then sterilized under aseptic conditions. Quick immersing (one second) in mercuric chloride at 0.1 mg/l then soaking in 20% Clorox (sodium hypochlorite 5%) were used for sterilization [12].Explants were cultured individually on MS medium [13] as a basal medium. Cytokinin (6- Benzylaminopurine at 0.5 mg /l; BAP), carbon source (sucrose at 30 g/l) and solidifying agent (agar at 6.0 g/l) were added to the medium. The medium pH was adjusted to 5.7 then autoclaved at 121°C and 15 Ib/in2 for 20 minutes. The cultured explants were incubated at average temperature of 23± 2°C and under 16 hours of light; at 1000 lux by cool fluorescent lamps and 8 hours of darkness per day [14].

2.2. Multiplication stage

After establishing stage, responded explants were recultured on MS medium with some modifications. Subculturing was done regularly at four weeks intervals whereas; the following experiments were implemented:

2.2.1. Effect of 6- benzylaminopurine (BAP) concentrations on *in vitro* culture of three stevia genotypes

BAP at different concentrations (0.00, 0.25, 0.50 and 1.0 mg/l) were studied to estimate its effect on multiplication of the three genotype. Average shoots number, shoots length, leaf number were determined.

2.2.2. Effect of medium strength on *in vitro* culture of three stevia genotypes

Multiplied shoots were cultured into full or one and half strength of MS medium to detect the highly inducing medium strength for growth vigor and differentiation.BAP was added at 1.0 mg/l. Average differentiated shoots number, shoots length, leaf number were determined.

2.3. Rooting stage: Effect of indole butyric acid (IBA) concentrations on *in vitro* rooting of three stevia genotypes

The proliferated shoots (3-4 cm, in length) were cultured on rooting medium consisted of MS at 3/4 strength and indole butyric acid (IBA) with different concentrations (0.0, 0.25, 0.5, 1.0 and 2.0 mg/l).Average shoots length, root number, root length and rooting percentage were determined.

3. Tolerance to NaCl treatments

Various concentrations of NaCl were used to study the tolerance ability of the stevia three genotypes used in this research; 0.0, 25, 50, 100 and 200 mM. After four subcultures in the same concentrations; it was found that the fifth concentration was lethal for the three genotypes so it was neglected (Table 1).

Table 1: The studied combinations of salinity with the three genotypes

Symbol	Genotype	NaCl (mM)
C1T1	Morita	00.0
C1T2	Morita	25.0
C1T3	Morita	50.0
C1T4	Morita	100.0
C2T1	Eirete	00.0
C2T2	Eirete	25.0
C2T3	Eirete	50.0
C2T4	Eirete	100.0

C3T1	Brazilian genotype	00.0
C3T2	Brazilian genotype	25.0
C3T3	Brazilian genotype	50.0
C3T4	Brazilian genotype	100.0

3.1. Effect of NaCl concentrations on in vitro growth of three stevia genotypes

Three clusters from multiplication stage were cultured in each jar. All replicates were recultured for four subcultures. Average shoots number, shoots length (cm), leaf area (score), fresh weight (g), dry weight (g) and growth vigor (score) were determined.

3.2.Chemical and biochemical analysis

3.2.1. Effect of NaCl concentrations on shoots total carbohydrates and proline

The total carbohydrates contents were determined in stevia shoots of each treatment with the method described by Dubois et al. [15]. Proline content was measured in fresh shoots using the method of Bates et al. [16].

3.2.2. Effect of NaCl concentrations on NPK shoots content

Total content of nitrogen (N) and phosphorus (P) in dried shoots of each treatment were determined using the methods described by the Anonymous [17]. Potassium (K)was extracted by acid digestion according to Cottenie et al. [18] and determined by UV-VIS spectrophotometer (T60 UV-Visible Spectrophotometer PG INSTUMENTS) using a Perkin–Elmeras mentioned by Gonzalez et al. [19].

3.2.3. Effect of NaCl concentrations on glycosides contents

a. Extraction and purification of the steviol glycosides.

Five hundred milligrams of dried shoots of the three genotypes under investigation were extracted three times with 5 mL of water in a boiling water bath (100˚C) for 30 min each time. Extracts were leaved to reach the room temperature then centrifuged at 2500 × g, 10˚C for 10 minutes. The aqueous phases were transferred to a 25 mL volumetric flask and filled to capacity. The solution was filtered through a 0.45 lm membrane filter before HPLC analysis [20].

b. High-performance liquid chromatography (HPLC) analysis

Extraction of stevia sweeteners were carried out by 0.5 g of dried stevia in vitro shoots from the three genotypes in each treatment. Samples were ground and dissolved in 0.5 ml methanol (95%) and put in shaking then heated for 30 minutes at 70°C thereafter kept in room temperature for cooling. After that, filtration was done using a filter paper (RC membrane filters from IVA, Meerbusch, Germany that were 0.45 lm by 25 mm.). Activated charcoal was used for filtration. Stevia extract has been described for their quantification by high performance liquid chromatography (HPLC Agilent 1200 infinity series).

3.3. Principal Component Analysis (PCA)

PCA method described by Harman analysis [21]. was followed in the extraction of the components. PCA was performed the values of the first five components were selected the related clusters were plotted based on the main components**.**

4. Statistical analysis

Two-ways completely randomized design was used in this investigation. Each treatment was replicated three times and each replicate contained 5 jars. Means were compared at a 5 % level of significance, according to the method described by Snedecor and Cochran [22].

Results

1. Micropropagation:

1.1. Multiplication stage

Data in Table 2 revealed that C1surpassed other studied genotypes in multiplication rate as it showed the highest number of shoots, leaves and shoot length, in the presence of multiplication medium. C2 followed C1 in all parameters then C3. BAP showed a significant result giving the highest number of shoots at 1.0 mg/l, regardless genotype factor. The highest shoot number was observed in C1 with 1.0 mg/l BAP. Meanwhile, the highest shoot length and number of leaves were occurred with the control treatment.

Table 2: Effect of BAP concentrations on in vitro multiplication of three stevia genotypes

BAP	Shoot number				Shoot length				Leaf number			
(mg/l)	C1	C2	C3	M	C1	C2	C3	M	C1	C2	C3	M
0.0	31.33^{i}	21.44^{k}	19.11^{l}	23.96D	4.11^{a}	3.5^{b}	3.11^{d}	3.57A	2.50^{ef}	2.02^{h}	2.50^{ef}	3.07A
0.25	43.21^{g}	35.51^{h}	30.22^{j}	36.31C	3.33^{c}	3.12^{cd}	2.85^{e}	3.1B	2.63^{de}	2.12^{gh}	2.80^{cd}	2.86B
0.50	68.50^{b}	56.67^{e}	50.24^{f}	58.47B	3.00^{cd}	2.86^{e}	2.55^{g}	2.8C	3.26^{ab}	2.33^{fg}	3.00^{bc}	2.52C
1.00	73.33^{a}	65.33^{c}	61.34^{d}	66.67A	2.84^{ef}	2.63^{fg}	2.33^{h}	2.6D	3.37^{a}	2.45^{ef}	3.40^{a}	2.34D
M	54.09A	44.74B	40.23C		3.32A	3.03B	2.71C		2.94A	2.93B	2.23C	

Means with different letters within each parameter were significantly different at 5% level.

Data in Table 3 shows that the higher strength of Murashige and Skoog medium (1.5 MS) was better than the full strength as it resulted more number of shoots (over the double) compared with the full strength. Similarly, 1.5 MS gave the highest shoot length and leaf number. Moreover, C1 surpassed other studied genotypes in shoot number while, C3 showed more improvement in growth; as shoot length and leaf number, compared with others in response to the higher strength of MS.

Table 3: Effect of Murashige and Skoog medium (MS) strength on in vitro growth of three stevia genotypes

MS strength	Shoot number				Shoot length				Leaf number			
	C1	C2	C3	M	C1	C2	C3	M	C1	C2	C3	M
Full MS	73.34^{d}	69.67^{e}	61.21^{f}	68.07B	2.86^{d}	2.65^{e}	2.35^{f}	2.62B	3.07^{d}	2.45^{e}	3.14^{c}	2.89B
1.5 MS	169.0^{a}	140.0^{b}	133.23^{c}	147.41A	2.94^{c}	3.94^{b}	4.77^{a}	3.88A	3.28^{b}	2.46^{e}	3.50^{a}	3.08A
M	121.17A	104.84B	97.22C		2.9C	3.3B	3.56A		3.18B	2.46C	3.32A	

Means with different letters within each parameter were significantly different at 5% level.

1.2. Rooting stage

Data in Table 4 shows that C1 surpassed other genotypes in rooting percentage and root length. Meanwhile, C3 was the highest genotype in achieving roots per plant. Data also shows that1.0 mg/l IBA gave the highest rooting percentage followed by the concentration of 0.5 mg/l. The highest number of roots was achieved with IBA at 1.0 and 0.5 mg/l. Regarding root length, IBA at 0.5 mg/l surpassed other treatments giving the highest root length followed by IBA at 1.0 mg/l.

Table 4: Effect of IBA concentrations on in vitro rooting of three stevia genotypes

IBA (mg/l)	Rooting %				Root number				Root length			
	C1	C2	C3	M	C1	C2	C3	M	C1	C2	C3	M
0.00	100.0	33.33	86.67	73.33	4.60^{h}	2.40^{i}	4.57^{h}	3.86D	4.61^{e}	0.63^{k}	1.18^{j}	2.14E
0.25	100.0	46.67	93.33	80.00	5.80^{g}	4.56^{h}	7.00^{ef}	5.79C	5.24^{d}	1.92^{h}	1.76^{i}	2.97C
0.5	100.0	60.0	100.0	86.67	6.47^{f}	7.00^{ef}	10.13^{a}	7.87A	7.21^{a}	2.45^{g}	3.90^{f}	4.52A
1.0	100.0	73.33	100.0	91.11	7.27^{de}	7.00^{ef}	9.27^{b}	7.85A	6.84^{b}	3.80^{f}	2.25^{g}	4.30B
2.0	100.0	53.33	100.0	84.44	7.67^{d}	4.38^{h}	8.67^{c}	6.91B	5.75^{c}	1.06^{j}	1.77^{hi}	2.86D
M	100.0	53.33	96.00		6.36B	5.07C	7.93A		5.93A	1.97C	2.17B	

Means with different letters within each parameter were significantly different at 5% level.

2. Salinity tolerance:

2.1. Effect of salinity on stevia genotypes in vitro growth

Data in Table 5 revealed that NaCl treatments affected stevia genotypes in vitro growth. C2 was more tolerant to sodium chloride than other genotypes giving the highest number of shoots, shoot length, growth vigor and fresh weight. Meanwhile, C3 gave the highest leaf area and dry weight under sodium chloride stress. It obvious that number of shoots, shoot length, growth vigor and fresh weight were inhibited due to NaCl treatments while; the trend was inverted with leaf area and dry weight.

Table 5: Effect of NaCl on in vitro growth of three stevia genotypes

NaCl	Shoot number				Shoot length				Leaf area				Growth vigor				F.W. (g)				D.W./100 g F.W.			
(mM)	C1	C2	C3	M	C1	C2	C3	M	C1	C2	C3	M	C1	C2	C3	M	C1	C2	C3	M	C1	C2	C3	M
0.0	200.0^{a}	274.67^{a}	210.0^{d}	254.89A	11.0^{ab}	12.0^{a}	9.0^{cde}	10.67A	3.73^{g}	4.0^{e}	4.51^{b}	4.08C	5.0^{a}	5.0^{a}	4.5^{b}	4.83A	52.0^{b}	60.0^{a}	52.67^{b}	54.89A	7.82^{b}	7.68^{b}	8.36^{f}	7.95D
25	190.0^{c}	240.0^{b}	213.33^{c}	214.44B	10.0^{bc}	9.5^{bcd}	8.5^{de}	9.47B	4.50^{b}	4.52^{b}	5.0^{a}	4.67B	5.0^{a}	4.9^{ab}	4.4^{b}	4.77A	33.63^{e}	59.9^{a}	52.5^{b}	48.68B	8.68^{a}	7.77^{b}	9.4^{d}	8.62C
50	140.0^{f}	160.0^{e}	105.0^{f}	135.00C	9.5^{cd}	8.0^{de}	5.5^{fg}	7.67C	4.53^{b}	5.0^{a}	5.0^{a}	4.85A	4.0^{c}	4.5^{b}	4.3^{b}	4.67B	31.23^{f}	50.17^{c}	43.77^{d}	41.72C	9.29^{d}	8.63^{c}	10.14^{c}	9.35B
100	40.0^{g}	65.0^{h}	53.67^{i}	52.89D	8.0	7.0^{e}	5.5^{fg}	6.83D	4.92^{b}	5.0^{a}	5.0^{a}	4.97A	2.0^{e}	3.0^{d}	4.0^{c}	3.01C	20.02^{g}	31.17^{f}	28.43^{g}	26.54D	9.32^{d}	13.62^{a}	16.8^{a}	13.25A
M	162.50B	184.92A	145.5C		9.63A	9.23A	7.13B		4.43C	4.63B	4.88A		4.0B	4.35A	4.3A		34.22C	50.31A	44.34B		8.78C	9.43B	11.18A	

Means with different letters within each parameter were significantly different at 5% level.

2.2. Effect of salinity on NPK, carbohydrates and proline contents

NaCl treatments affected stevia shoot contents. Data in Table 6 indicated that C1 surpassed other genotypes in N, K and carbohydrates percentages while, C3 showed higher P and proline and equality with C1 in N percentages. Treatment with NaCl at 50 mM led to increase in N and P% in stevia shoots. Increasing the concentration of NaCl decreased K and increased proline percentages, gradually. On the other hand, carbohydrates % increased with the lower concentration of NaCl and then decreased with increasing its concentration.

Table 6: Effect of NaCl on neutral, carbohydrates and proline contents of three stevia genotypes

NaCl(mM)	N (%)				P(%)				K(%)				Carbohydrates(%)				Proline	
	C1	C2	C3	M	C1	C2	C3	M	C1	C2	C3	M	C1	C2	C3	M	C1	C2
0.0	4.00^{F}	3.73^{F}	4.07^{F}	3.93C	0.21^{I}	0.21^{I}	0.25^{E}	0.22C	8.0^{A}	7.3^{E}	11.3^{A}	8.87A	24.95^{C}	22.81^{E}	26.13^{E}	24.63B	0.97^{D}	0.08^{F}
25	3.93^{F}	3.56^{F}	3.90^{F}	3.80D	0.27^{H}	0.21^{I}	0.23^{F}	0.24B	7.3^{E}	6.6^{G}	6.3^{H}	6.73C	23.47^{G}	25.65^{B}	27.01^{C}	25.38A	0.12^{E}	0.15^{E}
50	4.54^{E}	3.76^{F}	4.74^{E}	4.35A	0.26^{I}	0.28^{H}	0.32^{C}	0.29A	9.8^{A}	6.3^{H}	7.6^{C}	7.90B	22.51^{H}	26.88^{B}	19.97^{K}	23.12D	0.21^{C}	0.31^{C}
100	4.64^{E}	4.00^{F}	4.33^{F}	4.32B	0.29^{G}	0.27^{H}	0.30^{D}	0.29A	5.^{D}	9.0^{B}	5.4^{I}	6.77C	26.79^{D}	21.46^{J}	24.47^{F}	24.24C	0.32^{B}	0.90^{A}
M	4.28A	3.76B	4.26A		0.26B	0.24C	0.28A		7.75A	7.30C	7.65B		24.43A	24.20B	24.40A		0.19C	0.38B

Means with different letters within each parameter were significantly different at 5% level.

2.3. Effect of NaCl on steviol glycosides components

Data in **Figure (1)** show that the tested stevia genotypes varied in stevioside% content with a superiority of the genotype Eirete (C2) recording 40% which is 16.38 % and 23.99% higher than that given by the two others genotypes; Morita and Brazilian, respectively. On other hand, the Brazilian genotype (C3) recorded the highest percentage of steviol and Rep A as compared to other genotypes, recording 6.06 % and 9.67 % higher than that given by Morita and Eiretein steviol% and 13.34% and 8.92% with Rep A, respectively (Fig. 1).

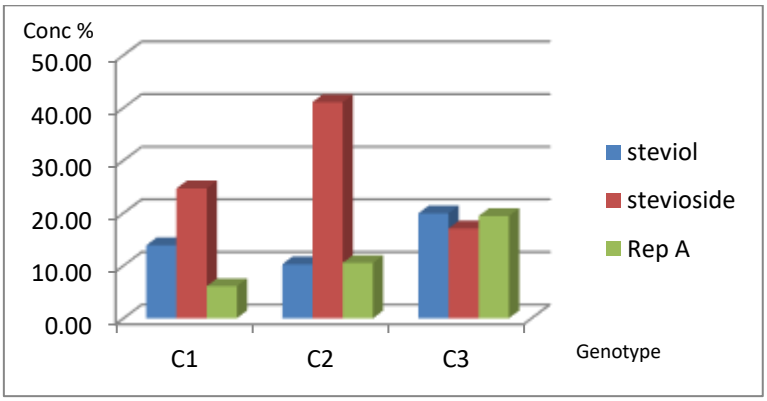


Fig. 1. Stevia genotypes and their variation in stevioside, rebaudioside A and steviol%

The results indicated that NaCl had a marked influence on stevioside% in stevia. It was found that applying NaCl at 25 mM resulted in the highest stevioside%, which led to a noteworthy increase of 9.22%, 6.85% and 2.10% higher than that given by 0, 50 and 100mM (Fig.2).

By contrast, stevioside% was affected by the interaction between stevia genotypes and NaCl levels. There was a variance among the three genotypes, Eirete (C2) surpassed Morita (C1) and Brazilian genotype (C3) significantly, when they were supplied with T4 (Fig 3). The results cleared that applying T4 resulted in a major increase in steviol% with Brazilian genotype (C3) compared with others genotypes and treatments (Fig. 3).

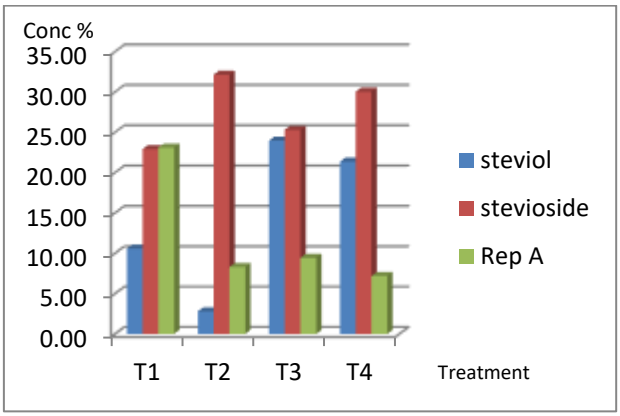


Fig. 2. Different concentrations of NaCl and their impact on stevioside, rebaudioside A and steviol %

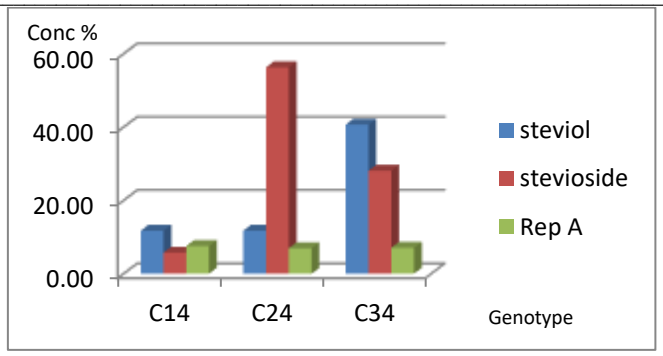


Fig. 3. The effect of applying NaCl at 100 Mm on genotypes content of stevioside, rebaudioside A and steviol %

The Steviol percentage increased by NaCl concentrations up to 50 mM in two genotypes; Eirete (C2) and Brazilian genotype (C3) and after this level displayed a decrease in Eirete. By contrast, it was found that Morita genotype (C1) applying non NaCl (0 mM) resulted in the highest steviol% (Fig.4).

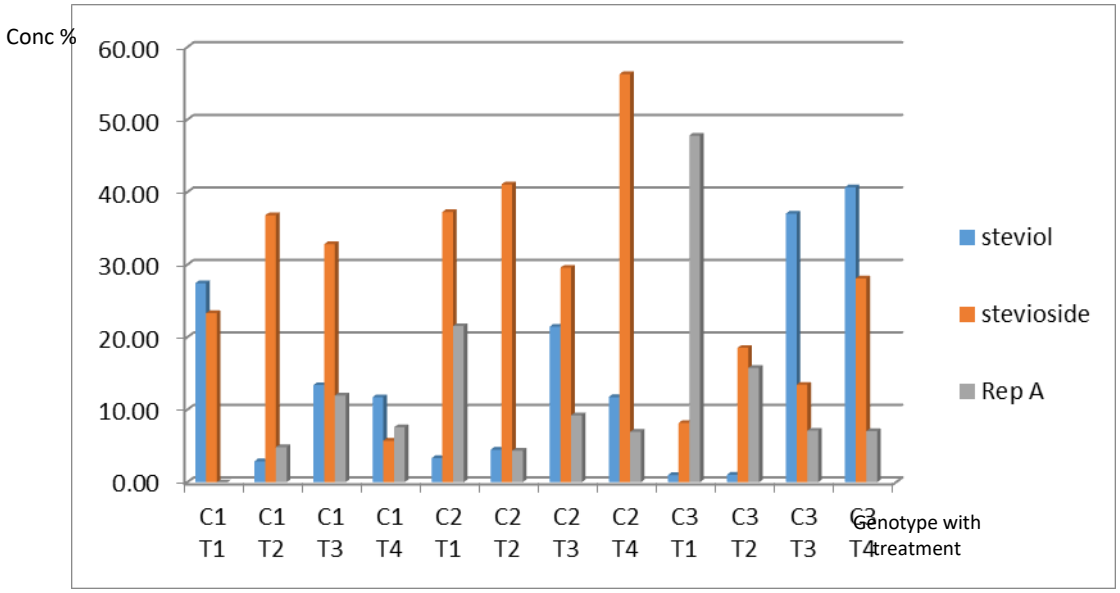
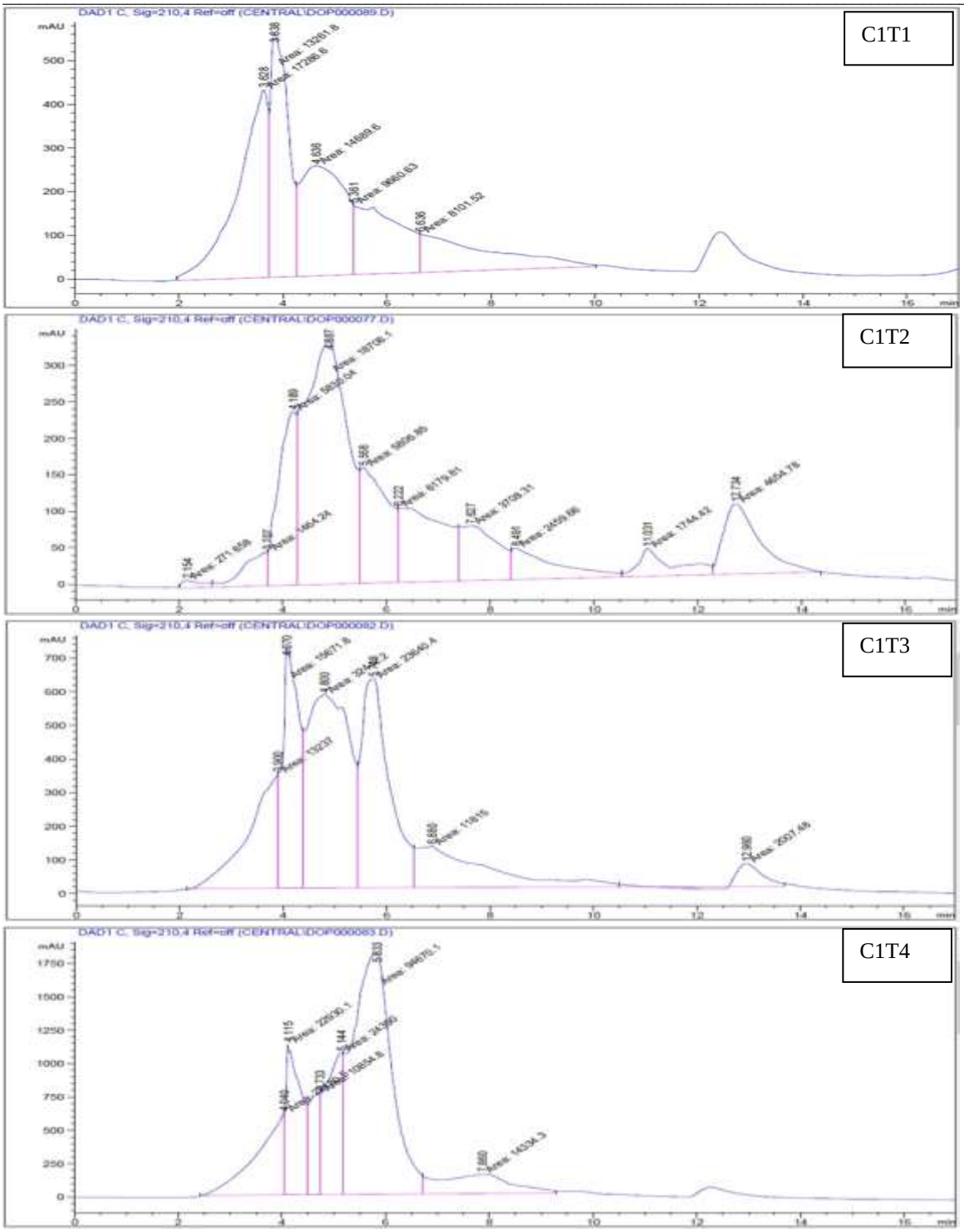


Fig. 4. The effect of the interaction between different concentrations of NaCl on the three genotypes

In general, the stevioside concentrations were observed permanently higher than the rebaudioside A content in the all samples except Morita genotype at 100 mM of NaCl (CIT4) and Brazilian genotype at 0.0 mM of NaCl (C3T1)(Fig. 5).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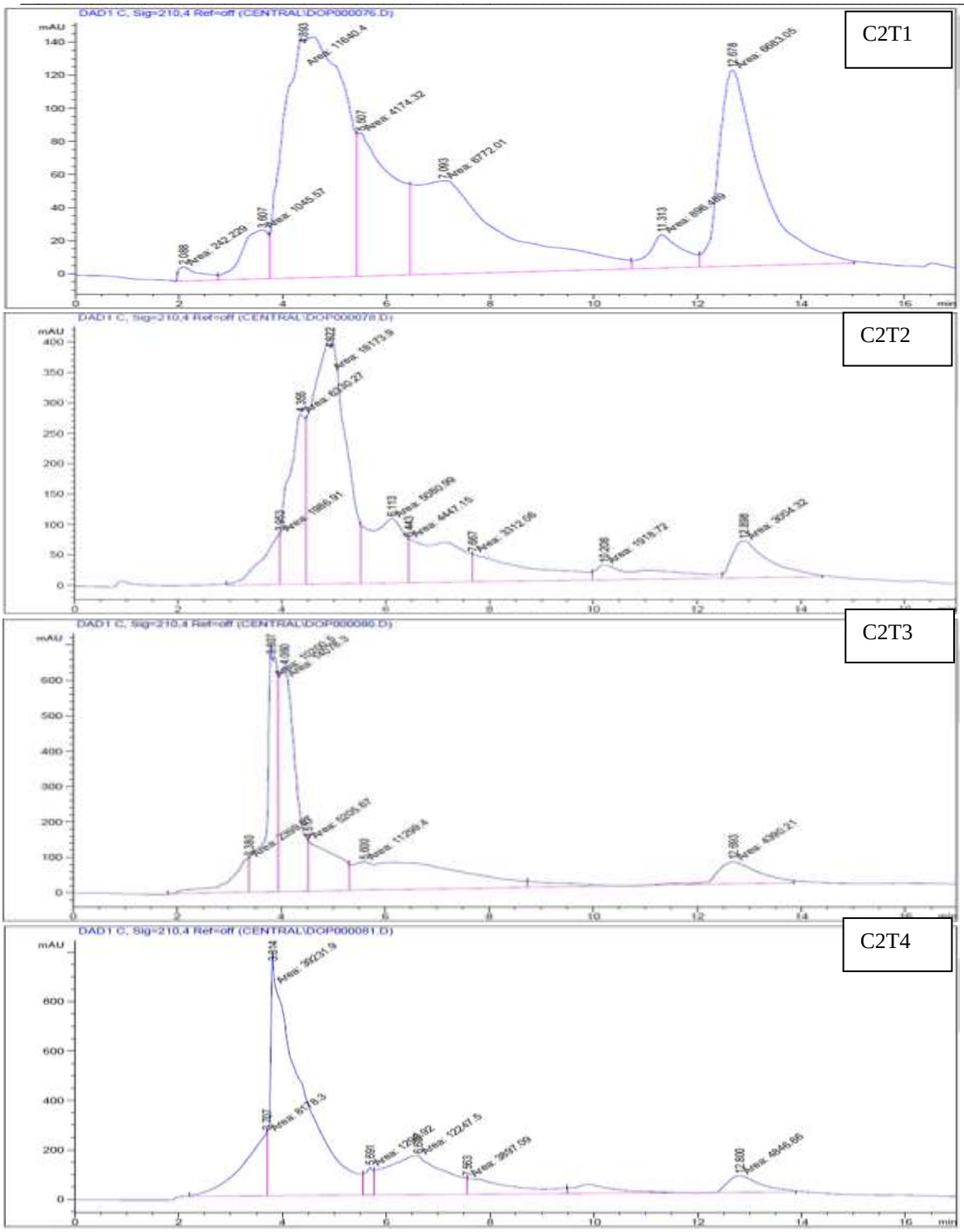
C1T2
C1T3
C1T4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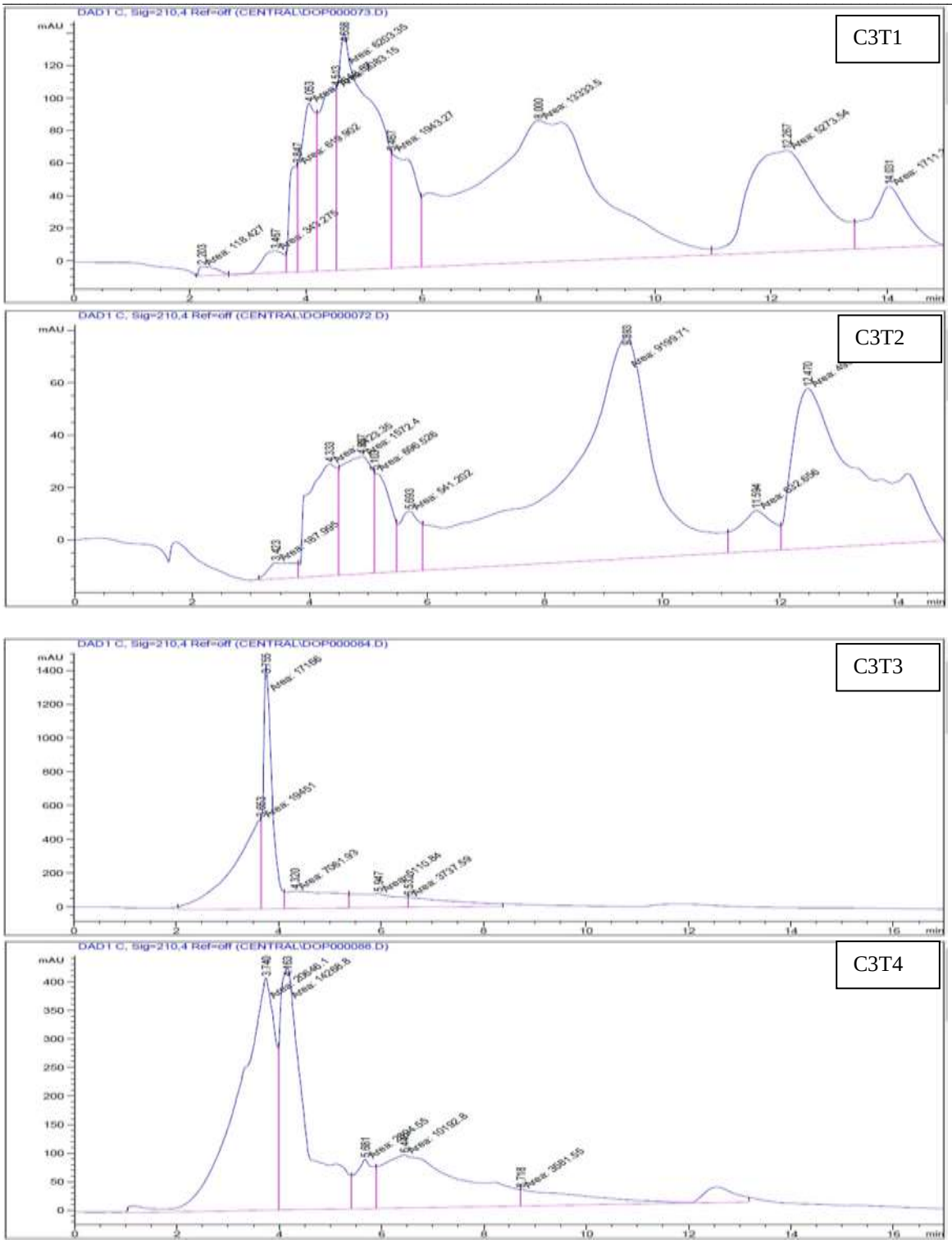

Fig.5. Stevioside concentrations due to various genotype and NaCl concentrations

2.4.The Principal component analysis (PCA)

The PCA aggregated the treatments into groups and remained scattered in all four quadrants based on the phenotypic characters. The treatments Morita + 0.0mMNaCl, Eirete + 25 mM, Morita + 25 mM and Eirete+0.0 mM were placed at superior positions from the origin in the PCA biplot. PCA results were demonstrated in groups of treatments to estimate relationships among treatments (Figure 6). Table 7 present PCA that out of the eight components, the first four components explained the majority of the total variation and contributed88.9% of the total variation among the varieties.

Table 7: Principal component analysis of measured characters in stevia in vitro shoots

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Stevioside	0.006	0.564	-0.675	0.016	0.173	-0.19	0.146	0.325
Rep A	0.147	-0.628	-0.13	0.327	0.55	-0.162	0.241	0.212
Steviol	-0.236	0.346	0.662	0.277	0.083	-0.288	0.152	0.307
Shoot number	0.46	0.086	-0.008	0.103	-0.067	-0.031	-0.117	-0.504
Shoot length	0.387	0.059	0.203	-0.411	0.303	0.386	-0.373	0.489
Leaf area	-0.378	-0.201	-0.192	0.342	-0.385	0.495	-0.176	0.297
Growth vigor	0.365	0.274	0.094	0.441	0.051	0.561	0.442	-0.053
F.W. (g)	0.383	0.021	-0.03	0.507	-0.238	-0.331	-0.541	0.213
D.W./100 g F.W.	-0.372	0.203	-0.025	0.261	0.597	0.186	-0.476	-0.355
Eigenvalue	4.5167	1.51	641.058	00.91	380.56	460.21	460.13	890.0733
Proportion	50.2	16.8	11.8	10.2	6.3	2.4	1.5	0.8
Cumulative	50.2	67	78.8	88.9	95.2	97.6	99.1	100

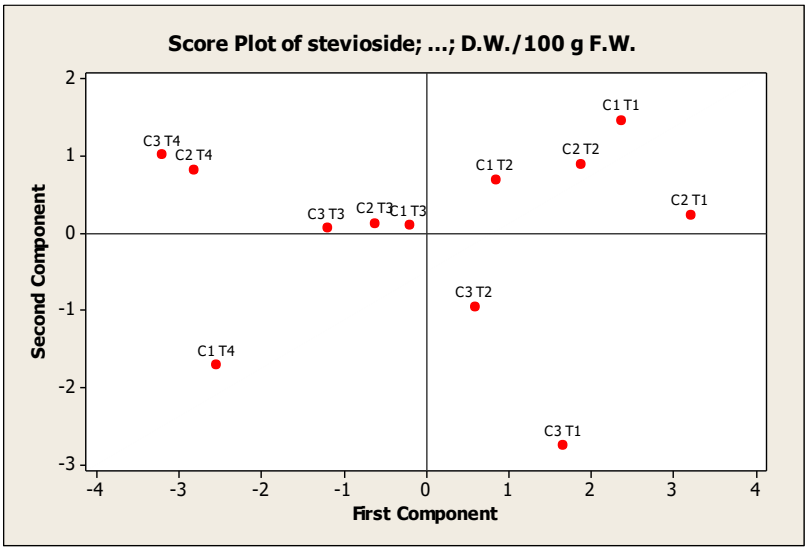

Fig.6. Principal component analysis of measured traits in the three stevia genotypes

Discussion

Data in our investigation showed that all concentrations of BAP induced stevia multiplication rate compared with the control. The highest average shoots and leaves number was occurred with 1.0 mg/l BAP. Similarly, Jadid et al [23] assured that BAP at 1.0 induced the highest shoot multiplication in stevia culture. Meanwhile, AbdAlhady [24] claimed that the highest number of stevia shoots was proliferated with2.0 mg/l BAP and0.5 mg/l kinetin supplemented medium. In addition, Amen et al [25] found that banana in vitro multiplication was enhanced with BAP supplementation at 3.0mg/l.

Results also indicated that, one and half MS strength gave the highest average number of shoots, length of shoots and number of leaves of in vitro stevia culture. Similarly, Hassan et al [26] indicated that one and half MS strength was superior for multiplication and growth vigor of plum cv. “Santa Rosa”. Meanwhile, Taha [11] assured that full MS strength was superior for goji shoot multiplication.

Our results showed that IBA at 1.0 mg/l highly rose the rooting percentage and root number per stevia rooted shoots. Similarly, AbdAlhady [24] assured that 1.0 or 2.0 mg/l IBA induced 100% rooting for stevia shoots. Meanwhile, Javed et al [27] claimed that the best root induction for stevia was occurred by 0.5 mg/l of indole acetic acid treatment. Also Jadid et al [23] observed roots at medium free of plant growth regulators.

Plant genotypes usually differ from each other in response to in vitro medium and plant growth regulators. Morita genotype showed superior response in multiplication and rooting stage followed by Eirete then Brazilian genotype. Similarly, Rodriguéz-Páez et al [28] found that L020, L102, and Morita II stevia genotypes differed with each other in due to growth hormones needed for shoot multiplication. They found that 1 µM 6-benzylaminopurine (BAP), 1 µM BAP and 0.5 µM naphthalene acetic acid

(NAA) or 2 μM BAP and 0.5 μM NAA were superior for L020, L102 or Morita II, respectively. Otherwise, according to salinity tolerance, Eirete was the most tolerated genotype in our research.

In our study, nutrients, carbohydrates and proline contents of three stevia genotypes were determined according to salinity treatments. Nitrogen and phosphorus were increased while potassium decreased with NaCl treatments. NaCl stress reduced plant height, leaf fresh and dry weight, chlorophyll a, b, and total chlorophyll as well as K+ content in stevia root and shoot organs under greenhouse conditions [29].

In our study, carbohydrates accumulated in stevia shoots due to salinity stress at 25 mM then reduced gradually with increasing NaCl concentration. Similarly, many studies assured these changes due to NaCl and its concentration in stevia [30, 29].Increasing carbohydrates due to salinity stress supports that sugars have a role as osmoprotectant that stabilizes cellular membranes and maintains turgor pressure as amino acids [31].

Many authors have reported the increase in proline accumulation, as our study did, under salt stress in different plants such as jojoba in vitro plants [32, 33], date palm in vitro plants [34, 35] and stevia greenhouse plants [27].

Recently, up to 60 steviol glycosides (SGs) have been identified in stevia [36]. Stevioside (Stev) and rebaudioside A (Reb A) are the most profuse and representative compounds of Steviol glycosides presenting in stevia [37]. According to Abou-Arab et al [38]; Lemus-Mondaca et al (2012) and Ribeiro et al [39], the stevioside content observed higher than the rebaudioside A concentrations. Generally, Steviol percentage increased by NaCl concentrations up to 50 mM in two genotypes; Eirete (C2) and Brazilian genotype (C3) and after this level it displayed a decrease in Eirete genotype. According to Cantabella et al [40] and Shahverdi et al [27], low concentration of NaCl stress caused increase in Steviol glycosides content (Stev, Reb-A, and Stev+Reb-A). The same results were obtained from Ceunen and Geuns [41]. They claimed that these metabolites play a role as osmoprotectant molecules towards stress conditions in stevia plant and there is a great contribution of these metabolites to osmotic adjustment.

PCA objects to resolve the total variation of a set of characters into linear, independent composite characters, which successively maximize variability in the data [42]. Considering a minimum threshold eigenvalue of one, the four principal components (PCs) accounted for a cumulative of about 88.9% of the whole phenotypic diversity observed among the varieties. These results are in harmony with Massaoudou et al [43]; Abo Elenen et al [44] and Mehareb et al [45] who stated four principal components with eigenvalues more than one, which explained > 75% of the total variation for the traits.

Conclusion

In this study, a successful completed in vitro protocol was established for stevia plant. One and half MS strength with BAP at 1.0 mg/l gave the best shoot number for the three genotypes. Stevia proved to be moderate tolerant to NaCl. Eirete (C2) proved to be the best salinity tolerated genotype and the highest producer for stevioside. Low salinity level enhanced steviol glycosides components production.

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