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AN EFFICIENT PROTOCOAL FOR CLONAL PROPAGATION OF THE RECENTLY INTRODUCED SWEETENER PLANT (*STEVIA REBAUDIANA* BERTONI) IN EGYPT

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ABSTRCT

Stevia rebaudiana Bertoni, is a perennial herb belongs to the Asteraceae family. The plant has strong sweet taste and very few calories. It is estimated to be 300 times sweeter than sugar cane. Seed germination is notably very poor due to infertile small size seeds and their self incompatibility and lower number of vegetative cutting. It is a newly introduced plant in Egypt, So, the present investigation was carried out to standardize a protocol for *in vitro* mass propagation and acclimatization of *Stevia rebaudiana* plants. In establishment stage MS medium supplemented with 0.25mg/l BAP or 0.25mg/l Kn in combination with PP_{333} enhanced shoot number significantly (10.00 and 5.50 shoots/explant, respectively). In multiplication stage, adding growth retardant (PP_{333}, CCC and PEG) as well as AC positively affected growth parameters. In rooting and acclimatization stages auxins especially IAA and NAA played an important role in combination with PP_{333} and AC.

Keywards: *Stevia rebaudiana*, tissue culture, BAP, Kn, IAA, NAA, 2, 4-D, PP_{333}, CCC, PEG, AC, shoot tip necrosis and acclimatization

INTRODUCTION

Stevia rebaudiana Bertoni, is a perennial herb belongs to the Asteraceae family. It is a tall herb native of Eastern Paraguay widely used in Latin America (Liu and Li, 1995, Chalapathi and Thimmegowda, 1997 and Andolfi *et a*l., 2006). It is being cultivated in Japan, Taiwan, Philippines, Hawaii, Malaysia and overall South America for food and pharmaceutical products. It is a natural sweetener plant known as “Sweet Weed”, “Sweet Leaf”, “Sweet Herbs” and “Honey Leaf”, which is estimated to be 300 times sweeter than sugar cane. The plant contains *Stevioside, Rebaudiside , Rebaudioside C, Dulcoside A*, with strong sweet taste but with very few calories. Consequently Stevia is potentially extremely useful for food industry and dietary treatment (Montoro *et al*., 2009). It can be added to tea and coffee, cooked or baked goods, processed foods and beverages, fruit juices, tobacco products, pastries, chewing gum and sherbets (Brandle and Rosa, 1992). The leaf of *Stevia rebaudiana* has antimicrobial and antitumor activities which may have a role in pharmaceutical and preservatives industries**.** Stevia is helpful for hypoglycemia and diabetes because it nourishes pancreas and thereby helps to restore its normal function **(**Soejarto *et al*., 1983, Konoshima and Takasaki, 2002**;** Jayaraman *et al*., 2008 and Subudhi and Ghosh, 2008).

Seed germination is notably very poor in stevia due to infertile seed, small size seed and their self incompatibility and vegetative propagation is limited by lower number of individuals and instability of plantlets produced from stem cutting,

so, Tissue culture is the most reliable method of Stevia micropropagation through shoot tip or axillary bud culture allow recovery of genetically stable and true to type progeny within short time duration (Sakaguchi and Kan, 1982, Miyagawa and Fujioka, 1986, Midmore and Rank, 2002, Darekar, 2004 and Ahmed *et al*.,2007).

Optimal shoot initiation was achieved on medium containing 0.3mg/l kn. While in the case of *Stevia rebaudiana* multiplication, BAP was superior to all other hormonal treatments for shoot proliferation. Shoots number were maximized with increasing of BAP concentrations through subcultures, while, shoot length and growth vigor were negatively affected by both increasing BAP concentration and subculture number (Hossain *et al*., 2008, Ibrahim *et. al.,* 2008, Ibrahim *et al*., 2010 and Taware *et al*., 2010).

MS (Murashige and Skoog,1962) medium supplemented with 4.0 mg/l kinetin showed maximum shoot formation response (Tadhani *et al*., 2006). The nodal explants revealed good results with BAP and NAA 2mg/l for establishment. For multiplication MS basal medium containing BAP 2mg/l showed the formation of multiple shoots up to 10. When these shootlets were subcultured in the same medium good number of multiple shoots was observed (Sri Murali *et al*., 2011). No specific increase in multiple shoot formation was observed on different concentrations of BAP and Kn in combination with 0.2 and 0.5 mg/l IAA. In all treatments, maximum shoot formation was observed by supplementing 2.0 mg/1 BAP. The highest shoot length 3.73±0.14cm/microshoot was observed on MS medium containing 2.0mg/l Kn and 0.25mg/l IAA after 15 days of inoculation plant growth regulators promoted shoot formation, In contrast 0.5 mg/l NAA caused the maximum root formation in nodular stem sections of *S. rebaudiana*. Furthermore, survival rate of regenerated plants were 92 and 83% during hardening and shifting to green house (Rafiq *et al*., 2007).

Shootlets were regenerated from nodal explants of *Stevia rebaudiana* Bertoni through axillary shoot proliferation. The induction of multiple shoots from nodal segments was the highest in MS medium supplemented with 1.5 mg/l BAP+0.5 mg/l Kn (Ahmed *et al*., 2007).

Maximum shoots/explant was observed at the BAP concentration of 3.0 mg/l with the further increase in concentration of BAP the number of shoot/explant was decreased which may be due to the toxicity of BAP at higher concentration. But the shoot length was maximized under similar treatment. Root length and root number/explant were maximized on medium with 2.5 mg/l IBA (Jena *et al*., 2009).

The percentage of shoots that formed roots and number of roots/shoot significantly varied at different concentrations of IBA, IAA and NAA. However, auxins concentrations had a positive effect on the number of roots/shoot and it was higher in the case of IAA than IBA and NAA (Ahmed *et al*., 2007, Rafiq *et al*., 2007 and Ashish *et al*., 2010**).** Full MS was superior to half MS. Full MS supplemented with NAA (1.5 mg/l) was the best medium for rooting of microcuttings (Hossain *et al*., 2008). Root induction was observed in hormone free MS basal medium, but best rooting response (96% with 7 roots/plant) was obtained on full strength MS medium supplemented with 1.0 mg/l NAA (AAMIR *et al*., 2010). On other study, root induction was observed on MS medium supplemented with 2.0 mg/l IBA (Taware *et al*., 2010). Carbon was initially

added to tissue culture media in an attempt to simulate soil conditions; today it is routinely included in many tissue culture media formulations. The benefits of using activated carbon often include greater plant survival, greater plant growth, and improved plant quality and vigor (Van Winkle and Pullman, 1995).

The protocol for *in vitro* propagation of *Stevia rebaudiana* is scanty (Sivaram and Mukundan, 2003; Uddin *et al*., 2006; Ibrahim *et al*., 2008), and it is a newly introduced plant in Egypt, So, the present investigation was carried out to standardize a protocol for *in vitro* mass propagation and establishment of vigor acclitamized *Stevia rebaudiana***.**

MATERIALS AND METHODS

Stevia rebaudiana shoots (its height about 30cm) were collected from the nursery of Genetic Engineering and Biotechnology Research Institute (GEBRI), Menofiya University, Sadat City, Egypt. In vitro investigation were initiated by divided shoots into small explants containing two nodes (about 3cm) which were washed by rinsing in running tap water and detergent for one minute. The explants were sterilized in laminar air flow chamber according to Ibrahim *et al.,* (2010). After sterilization, explants were carefully washed with sterilized water three times, then, two explants were cultivated in 250ml jars contained MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of 6benzylaminopurine (BAP) or kinetin (Kn) (0.0, 0.25, 0.50, 0.75 and 1.00mg/l) individual or in combination with either 0.2mg/l puclobutrazol (PP_{333}) or 2g/l activated charcoal (AC). Shoots number, shoot length, nodes number, growth vigor and shoot tip necrosis were recorded at the end of establishment stage (about 30 days of cultivation) according to the methods described by Pottino (1981). Stevia shoots; which resulted from the previous stage, were divided and subcultured on MS medium supplemented with 0.5mg/l BAP individual as a control and different concentrations of growth retardant (low, mediate and high concentration) ie., puclobutrazol (PP_{333})(0.2, 0.4 and 0.8mg/l), chlorocolinchlorid (CCC)(0.36, 0.72 and 1.18mg/l), polyethylene glycol (PEG) (0.2, 0.4 and 0.8mg/l) and activated charcoal (AC) as adsorbance agent (1, 2 and 3g/l). After two subcultures in the same media with interval periods 21 days for subculture, shoots number/initial expant, shoot length, nodes number/shoot and leaves number/shoot were recorded. Then, *Stevia rebaudiana* shoots were divided in to explants containing two nodes and cultivated on rooting media which consisted of MS medium supplemented with different auxin types (IAA, 2,4-D or NAA) and concentrations (0.0, 0.2, 0.4 and 0.6mg/l) individual or in combination with 0.4mg/l PP_{333} or 1g/l AC. After about 30 days, shoots number, shoot length, nodes number, roots number and root length were recorded. The cultures were incubated at a temperature of 25±2°C and 16h photoperiod and light intensity 2000 lux during establishment stage and multiplication stage, light intensity increased to 3000 lux during rooting stage. Plantlets were acclimatized according to Ibrahim *et al*., (2010) with some modification, plantlets were washed with

warm water carefully, then dipped in antifungal solution (1g/l Rhizolex for five minutes) for protection of Soil–borne diseases i.e., *Rhizoctonia solni* and *fuxarium oxysprioum* and were cultured in the recommended cultured medium. Number of successful acclimatized pantalets was recorded after 30 days.

Statistical analysis: Data were statically analyzed by one or two factorial complete randomized design using SAS (1988) package. Each treatment was represented as six replicate jars with two explants per jar. Differences among various treatments were compared using the least significant differences (LSD) test at 5% level according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

Stevia rebaudiana clonal propagation stages are affected by many factors namely, genotype of mother plant, nutrients, sugar types and concentrations, plant growth regulators and physical growth factors (light, temperature, pH and aeration). Growth regulators are the maestro of micropropagation process. Some effects of growth regulators and growth retardant will be discussed (BAP, Kn, IAA, NAA, 2, 4-D, PP_{333}, AC, PEG and CCC) during initiation, multiplication and rooting stages.

Results in Table (1) and Fig. (1) revealed that both BAP concentrations individual or in combination with PP_{333} or AC significantly affected shoots number. The highest shoots number was resulted from 0.25mg/l BAP (5.71 shoots/explant). There were no significant difference between shoots number of control and PP_{333} (5.00 and 4.68 shoots/explant, respectively), while, adding AC to the media resulted in significant minimizing shoots number (3.90 shoots/explant). Regarding the interaction between BAP concentrations and PP_{333} or AC, MS medium supplemented with 0.25mg/l BAP in combination with PP_{333} enhanced shoot number significantly (10.00 shoots/explant). Shoot length of *Stevia rebaudiana* was significantly affected by BAP concentrations. MS medium supplemented with 0.25mg/l BAP gave the highest shoot length. Regarding the effect of PP_{333} and AC, AC significantly maximized *Stevia rebaudiana* shoot length followed by PP_{333} and control (8.18, 6.37 and 5.15cm, respectively). Concerning the interaction between BAP concentrations and PP_{333} or AC, the highest shoots of *Stevia rebaudiana* were obtained from MS media supplemented with 0.00mg/l BAP in combination with PP_{333}, 0.50 and 1.00mg/l BAP in combination with AC (9.28, 9.25 and 8.88cm, respectively). Also, nodes number was significantly affected by BAP concentrations. While, there were no significant differences of nodes number in presence of PP_{333} or AC compared with control. Interaction revealed that MS supplemented with 0.50mg/l BAP in combination with AC significantly maximized nodes number (4.00nodes/shoot), the same result was observed on basal MS medium supplemented with PP_{333} (3.88 nodes/shoot). Growth vigor gave the same trend of nodes number. Shoot tip necrosis was observed after about 23 days, the differences between BAP concentrations individual or in combination with PP_{333} or AC were not significant; this phenomenon appear due to ethylene accumulation in culture containers

which may resulted from plant metabolism and lead to unavailable of calcium in culture medium.

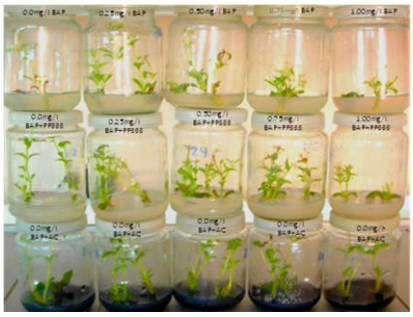

Fig. (1): Effect of balance among BAP concentrations, growth retardant (PP_{333}) or activated charcoal on *Stevia rebaudiana* Bertoni during establishment stage

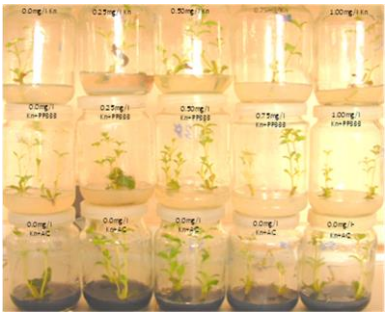

Fig.(2): Effect of balance among Kn concentrations, anti gebrillen (PP333) or activated charcoal on Stevia rebaudiana Bertoni during establishment stage.

Data in Table (2) and Fig.(2) showed that Kn concentrations affect shoots number of *Stevia rebaudiana* Kn at concentrations 0.25 and 0.50mg/l maximized shoots number (4.50 and 4.17shoots/explant, respectively). Adding PP_{333} or Ac to MS media did not significantly affected shoot number. Concerning the interaction between Kn concentrations and PP_{333} or AC, MS media supplemented with 0.25mg/l Kn in combination with PP_{333} gave the highest shoots number (5.50shoots/explant). Shoot length was affected significantly with Kn concentrations individual or in combination with PP_{333} or AC. The highest shoot length obtained from Ms media supplemented with 1.00mg/l Kn individual or in combination with AC (10.00 and 9.25cm, respectively). Nodes number and growth vigor significantly affected by Kn concentrations individual or in combination with PP_{333} or AC. Shoot tip necrosis did not affected by Kn concentrations. Adding PP_{333} or AC to MS media significantly decreased shoot tip necrosis compared with control, adding Ac to MS media minimized shoot tip necrosis (1.35). Interaction between Kn concentrations and PP_{333} or AC revealed that 0.25, 0.50mg/l Kn combined with AC minimized shoot tip necrosis (1.00, 1.25 and 1.25, respectively). These results may be due to the adsorption activity of AC which may results in adsorption of ethylene which affected calcium absorption as well as shoot tip necrosis phenomena which results from decreasing in calcium available for plant; which play essential role in cell wall structure. The unavailability may be resulted from the effect of accumulation of ethylene in the culture container and join with calcium leading to turn it into unavailable form. Results agree with those reported by Van Winkle and Pullman, (1995).

Shoots of *Stevia rebaudiana* were divided into micro-cuttings consisted of two nodes each micro-cutting replanted on MS medium supplemented with different growth retardant; Paclobutrazol (PP_{333}), Chlorocholinechloride (CCC) and Polyethylene glycol (PEG) as well as activated charcoal (AC) as adsorbent substance at various concentrations (control, low, mediate and high concentration).

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Two micro-cuttings were planted in each jar. Data in Table (3) and Fig. (3) revealed that PEG possessed the highest shoots number followed by PP_{333} (7.44 and 7.39shoots/explant, respectively) with no significant differences between them. Regarding concentrations effect, there were no significant differences between control, growth retardant and AC. Concerning interaction, PP_{333} at high concentration possessed the highest shoot number (10.67shoots/explant), while the lowest one was obtained on MS medium supplemented with high concentration of AC (2.83shoots/explant).

Concerning data discussed shoot length; data revealed that the highest shoot length was obtained on AC followed by PP_{333}, CCC and PEG (8.72, 6.14, 5.35 and 4.83cm, respectively). Regarding concentrations of growth retardant and AC, data cleared that no significant differences was observed between high and mediate concentrations of growth retardant and control, while low concentration possessed significant decreasing of shoot length (5.83cm). Inter action data cleared that AC at high concentration, possessed the highest shoot length (10.66cm) while the lowest shoot length was obtained on MS medium supplemented with PEG at mediate concentration (3.66cm).

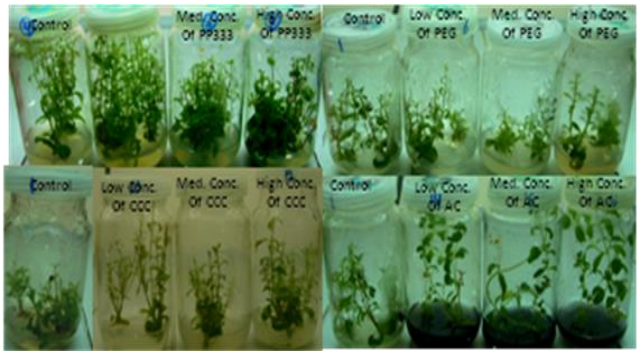

Fig. (3): Effect of various concentrations of growth retardant (PP_{333}, CCC or PEG) or AC on multiplication parameters of *Stevia rebaudiana* Bertoni.

Also, growth retardant and AC affected nodes and leaves number significantly; PP_{333} resulted in the highest nodes and leaves number followed by PEG, CCC and activated charcoal (14.61, 12.94, 11.49 and 9.35nodes/shoot, respectively). Regarding concentrations of growth retardant, no significant differences were observed among all of them. Inter action data cleared that PP_{333} at high concentration possessed the highest nodes and leaves number (21.44nodes/shoot and 43.33leaves/shoot, respectively). While the lowest nodes number was obtained on MS medium supplemented with AC at high concentration (7.33nodes/shoot and 14.67leaves/shoot, respectively).

Table (3): Effect of various concentrations of growth retardant (PP_{333}, CCC or PEG) or AC on multiplication parameters of *Stevia rebaudiana* Bertoni.

Concentrations	Control	Low concentration	Mediate concentration	High concentration	Mean
Growth retardant	**Shoot number/initial expant**				
PP_{333}	7.17	5.58	6.17	10.67	7.39
CCC	7.17	7.33	8.17	4.67	6.83
PEG	7.17	8.75	6.92	6.92	7.44
AC	7.17	3.50	3.67	2.83	4.29
Mean	7.17	7.60	7.04	7.29	
LSD:	A	B	A*B		
at 0.05	0.70	NS	0.86		
	Shoot length (cm)				
PP_{333}	6.41	5.08	6.25	6.83	6.14
CCC	6.41	4.83	5.75	4.41	5.35
PEG	6.41	5.41	3.66	3.83	4.83
AC	6.41	8.00	9.83	10.66	8.72
Mean	6.41	5.83	6.37	6.43	
LSD:	A	B	A*B		
at 0.05	0.40	0.40	0.81		
	Nodes number/shoot				
PP_{333}	12.28	10.00	14.72	21.44	14.61
CCC	12.28	12.72	13.33	7.61	11.49
PEG	12.28	17.44	11.83	10.22	12.94
AC	12.28	8.67	9.11	7.33	9.35
Mean	12.28	12.21	12.25	11.65	
LSD:	A	B	A*B		
at 0.05	2.00	NS	3.00		
	Leaves number/shoot				
PP_{333}	24.22	20.00	29.44	43.33	29.25
CCC	24.22	25.22	26.67	15.22	22.83
PEG	24.22	34.89	23.67	20.44	25.80
AC	24.22	17.33	18.22	14.67	18.61
Mean	24.22	24.36	24.50	23.42	
LSD:	A	B	A*B		
at 0.05	3.76	NS	7.51		
	Growth vigor				
PP_{333}	3.67	3.17	3.33	4.50	3.67
CCC	3.67	2.67	3.33	2.67	3.08
PEG	3.67	3.17	2.00	2.33	2.80
AC	3.67	4.50	5.00	5.00	4.54
Mean	3.67	3.38	3.41	3.62	
LSD:	A	B	A*B		
at 0.05	0.27	0.27	0.55		

***Growth vigor was determined according to method described by Pottino (1981)**
Where:
PP_{333}=puclobutrazol at low (0.2mg/l), mediate (0.4mg/l) and high (0.8mg/l) concentrations.
CCC=chlorocolinchlorid at low (0.36mg/l), mediate (0.72mg/l) and high (1.18mg/l) concentrations. PEG= polyethylene glycol at low (0.2mg/l), mediate (0.4/l) and high (0.8mg/l) concentrations.
AC=activated charcoal at low (1g/l), mediate (2g/l) and high (3g/l) concentrations.

Growth vigor of shoots under growth retardant and AC treatments showed that the highest growth vigor was obtained on activated charcoal (4.54) followed by PP_{333}, CCC and PEG (4.17, 3.34 and 2.79, respectively).

Regarding concentrations, there were no significant differences among shoot lengths of mediate and high s as well as control (3.41, 3.62 and 3.67, respectively). Inter action data cleared that AC at both mediate and high concentrations possessed the highest growth vigor (5.00and 5.00, respectively), while the lowest growth vigor was obtained on MS medium supplemented with PEG at mediate concentration (2.00).

Results in Table (4) and Fig.(4) discuss effect of auxin types concentrations individual or in combination with growth retard agent (Paclobutrazol, PP_{333}) or activated charcoal (AC) on rooting and acclimatization of *Stevia rebaudiana*. Results showed that plantlet length of *Stevia. rebaudiana* possessed its highest values on M.S. media supplemented with no auxin, 0.2mg/l IAA and 0.2mg/l NAA (8.79, 8.46 and8.50cm, respectively).

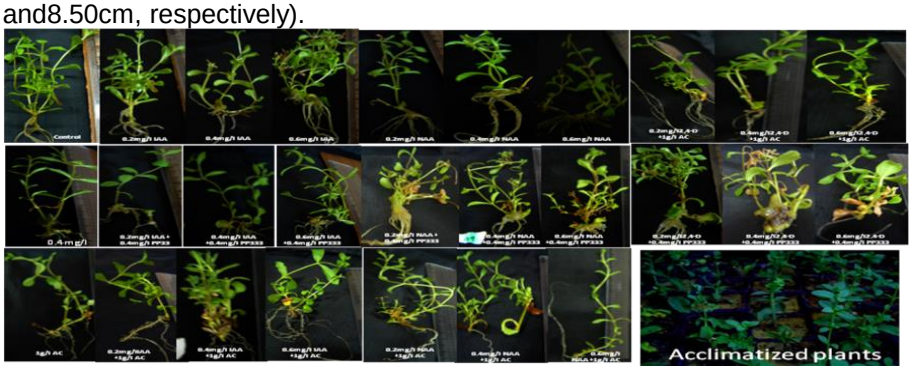

Fig. (4): Effect of balance among concentrations of auxin types and growth retardant (Paclobutrazol PP_{333}) and activated charcoal (AC) on root formation and acclimatization of *Stevia rebaudiana* Bertoni.

Where: BAP=6benzylaminopurine, Kn=Kinetin,IAA= Indole-3-acetic acid, 2,4-D= Dichlorophenoxyacetic acid and NAA= α-Naphthaleneacetic acid PP333= Paclobutrazol and AC =activated charcoal CCC=chlorocolinchlor and PEG= polyethylene glycol

Adding PP_{333} and AC to MS media resulted in significant plantlet length enhancement compared with control. These results may be due to their effect in reducing multiplication which may resulted in save nutrient medium as well as growth regulators which play role in cell division and plantlet elongation. Interaction between concentrations of auxin types and PP_{333} or AC revealed that basal MS medium supplemented with PP_{333} possessed the highest plantlet length followed by MS medium supplemented with 0.4mg/l 2, 4-D in combination with AC, MS media supplemented with 0.2mg/l IAA and 0.2 or 0.6mg/l NAA in combination with PP_{333} (11.75 and 11.50cm, 10.75, 10.25 and 10.25cm respectively). Concerning nodes number, auxin types affected nodes number, NAA possessed the highest values followed by IAA and 2, 4-D, respectively. Nodes number was negatively affected by auxins concentrations.

The highest nodes number resulted from Ms medium supplemented with 0.2 and 0.6mg/l IAA individual and 0.2mg/l NAA in combination with AC or PP_{333} (12.7, 14.0, 13.3 and 12.4nodes/plantlet, respectively). Roots number were enhancement by MS medium supplemented with 0.2mg/l NAA (15.00roots/plantlet) followed by control (13.00roots/plantlet). MS additives reduced roots number compared with control. The inter action between concentrations of auxin types and MS additives cleared that the highest roots number were possessed when MS medium was supplemented with 0.6 and 0.2mg/l NAA individual, 0.6mg/l IAA individual, 0.2mg/l NAA in combination with AC and basal MS medium, with no significant differences between them (21.50, 19.00, 19.00, 16.00 and 17.00roots/plantlets, respectively). Root length was affected by concentrations of auxin types, 0.2mg/l NAA possessed the tallest roots followed by control and 0.2mg/l IAA (8.67, 6.75 and 5.83cm, respectively). Regarding the effect of PP_{333} and AC, root length positively affected by adding AC and PP33 compared with control (5.75, 3.75 and 3.58cm, respectively). Inter action data cleared that Ms medium supplemented with 0.4mg/l 2,4-D in combination with AC resulted in the tallest root length followed by 0.2mg/l NAA in combination with both Ac and PP_{333} (12.00, 11.50 and 9.00cm, respectively). Regarding the number of successful acclimatized plantlets, auxin types showed clear difference: IAA and NAA positively affected successful of acclimatized plantlets while 2, 4-D negatively affected, this result may be due to the formation of calli which were enhanced in presence of 2, 4-D. Calli lead to bad contact between shoots and formed roots on it. Also, it seems that the vascular system between shoot and the roots formed from callus is absent, so the plantlet failed in acclimatization. Concentrations of auxin types significantly affected successful of acclimatized plantlets, 02mg/l NAA possessed the highest successful of acclimatized plantlets. Regarding MS additives effects, PP_{333} was dominance. Inter action between auxin types concentrations and MS additives, MS media supplemented with 0.2mg/ NAA individual and in combination with AC or PP_{333} gave the highest successful of acclimatized plantlets (28.00, 26.00 and 25.00). Results came in agreement with Hossain *et al*, 2008, Ibrahim *et al*., 2010, Taware *et al*., 2010, Sri Murali *et al*., 2011 and Preethi *et al*., 2011).

Conclusion

Stevia rebaudiana Bertoni clonal propagation strongly affected by growth regulators (cytokinins; BAP and Kn, and auxins; IAA, NAA and 2,4-D) as well as growth retardant (PP_{333}, PEG and CCC) and activated charcoal through the various stages of tissue culture. Adding BAP to establishment and multiplication media and IAA or NAA to rooting medium plus PP_{333} or AC enhanced Stevia growth and the ability of acclimatization.

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بروتوكول فعال للاكثار الدقيق للنبات التحلية المدخل مؤخرا (ستيفيا ريبديانا بيرتونى) فى مصر

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يعد نبات الستيفيا من الاعشاب الحولية والتى تنتمى للعائلة المركبة. يتميز النبات بطعمه الشديد الحلاوة مع قلة السعرات الحرارية الناتجة منه. وللنبات قوة تحلية تصل الى ٣٠٠ مثل قوة التحلية التى نحصل عليها من قصب السكر. يلاحظ ان انبات بذور الستيفيا نادرا نتيجة عقم البذور وحجمها الصغير ووجود ظاهرة عدم التوافق الذاتى وكذلك يعد عدد النباتات التى يمكن الحصول عليها عن طريق الاكثار الخضرى بالعقل قليل. أدخل نبات الستيفيا حديثا لمصر، لذلك أجريت هذه الدراسة بهدف الحصول على بروتوكول لاكثار الستيفيا ريبديانا بأعداد كبيرة معمليا و اقلمتها. ادى استخدام بيئة موراشيجى وسكوج بالاضافة الى ٠.٢٥ملجم/ل بنزيل امينو بيورين او ٠.٢٥ ملجم/ل كينتين بالاضافة الى البكلوبترازول الى زيادة عدد الافرع معنويا فى مرحلة التأسيس (١٠,٠و ٥٠,٠فرع/منفصل نباتى، على الترتيب).اضافة مثبطات النمو (PP333-CCC-PEG) وكذلك الفحم النشط اثناء مرحلة التضاعف كان لها تأثير ايجابى على القياسات المختلفة للنمو. للأكسينات دور مهم فى مرحلة التجذير والاقلمة بالضافة لدور البكلوبترازول والفحم النشط

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Table (1): Effect of balance among BAP concentrations, growth retard agent (PP_{333}) or activated charcoal on *Stevia rebaudiana* Bertoni during establishment stage.

IS additive	Cont.	PP_{333}	AC	Mean	Cont.	PP_{333}	AC	Mean	Cont.	PP_{333}	AC	Mean	Con.	PP_{333}	AC	Mean	cont.	PP_{333}	AC	Mean
BAP(mg/l	Shoot number/explant				Shoot length (cm)				Nodes number/shoot				Growth vigor				Shoot tip necrosis			
0.0	2.25	4.75	3.75	3.58	5.00	9.28	6.78	7.02	3.25	3.88	3.13	3.42	2.50	4.00	2.75	2.08	1.25	1.00	1.50	1.25
0.25	2.87	10.00	4.25	5.71	7.88	6.93	8.25	7.69	3.75	3.75	3.38	3.63	2.75	3.00	3.63	3.13	1.75	1.25	1.50	1.50
0.50	2.00	7.50	3.50	4.33	5.00	5.50	9.25	6.58	2.75	3.00	4.00	3.25	1.75	3.00	4.25	3.00	1.75	1.50	1.00	1.42
0.75	5.75	4.75	4.50	5.00	3.88	5.13	7.75	5.59	3.25	3.00	3.75	3.37	2.88	3.25	3.50	3.21	1.25	1.50	1.50	1.42
1.00	4.75	3.50	3.75	4.00	4.00	5.00	8.88	5,96	2.50	3.00	3.50	3.00	2.25	2.50	3.38	2.71	1.00	2.00	1.25	1.42
Mean	5.00	4.68	3.90		5.15	6.37	8.18		3.10	3.33	3.55		2.43	3.15	3.50		1.40	1.45	1.35	
LSD:	A	B	AxB		A	B	AxB		A	B	AxB		A	B	AxB		A	B	AxB	
At 0.05	1.03	0.80	1.78		0.80	0.62	1.38		0.49	NS	0.85		0.39	0.30	0.68		0.39	NS	0.67	

***Growth vigor: and shoot tip necrosis were determined according to method described by Pottino (1981)**
Where: BAP=6benzylaminopurine PP_{333}=Paclubutrazol and AC= Activated charcoal.

Table (2): Effect of balance among Kn concentrations, anti gebrillen (PP_{333}) or activated charcoal on *Stevia rebaudiana* Bertoni during establishment stage.

MS additives	Cont.	PP_{333}	AC	Mean	Cont.	PP_{333}	AC	Mean	Cont.	PP_{333}	AC	Mean	Con.	PP_{333}	AC	Mean	cont.	PP_{333}	AC	Mean
Kn (mg/l)	Shoot number/explant				Shoot length (cm)				Nodes number/shoot				Growth vigor				Shoot tip necrosis			
0.0	2.25	4.00	2.75	3.00	5.00	8.50	8.00	7.17	3.25	3.75	4.00	3.67	2.50	3.50	4.25	3.42	1.25	1.75	1.75	1.58
0.25	4.50	5.50	3.50	4.50	8.50	6.00	9.13	7.88	3.75	4.00	4.25	4.00	3.63	3.00	4.00	3.54	1.75	2.00	1.00	1.58
0.50	4.25	4.25	4.00	4.17	7.23	7.63	7.38	7.41	4.00	4.25	5.00	4.42	3.75	3.75	3.88	3.79	1.00	1.75	1.25	1.33
0.75	4.58	2.75	3.75	3.69	6.88	7.03	9.00	7.64	3.50	3.25	4.00	3.58	3.25	3.13	4.50	3.63	1.25	1.50	1.50	1.42

1.00	4.00	3.50	4.25	3.92	10.00	7.75	9.25	9.00	5.00	3.00	3.25	3.75	5.00	3.00	3.25	3.75	1.00	2.00	1.25	1.42
Mean	4.15	3.85	4.30		7.52	7.38	8.55		3.90	3.65	4.10		3.63	3.28	3.98		1.25	1.80	1.35	
LSD:	A	B	AxB		A	B	AxB		A	B	AxB		A	B	AxB		A	B	AxB	
At 0.05	0.79	NS	1.37		1.02	0.79	1.77		0.57	0.44	0.97		0.37	0.29	0.64		NS	0.28	0.62	

***Growth vigor: and shoot tip necrosis were determined according to method described by Pottino (1981)**
Where: Kn=Kinetin, PP_{333}=Paclubutrazol and AC= Activated charcoal.

Table (4): Effect of balance among concentrations of auxin types and growth retar dant (Paclobutrazol PP_{333}) and activated charcoal (AC) on root formation and acclimatization of *Stevia rebaudiana* Bertoni.

Auxin conc. (mg/l) (A)	MS additives (B)	Plantlet length (cm)				Nodes number/plantlet				Roots number/plantlet				Root length (cm)				No. of successful acclimatized plantlets			
		Control	PP_{333}	AC	Mean	Control	PP_{333}	AC	Mean	Control	PP_{333}	AC	Mean	Control	PP_{333}	AC	Mean	Control	PP_{333}	AC	Mean
Control		7.38	11.75	7.25	8.79	9.9	7.0	5.9	7.6	17.50	7.50	14.00	13.00	6.25	7.00	7.00	6.75	20.00	22.00	23.00	21.67
IAA	0.2	8.13	10.75	6.50	8.46	12.7	7.0	6.6	8.8	6.30	9.30	6.50	7.37	7.00	6.25	4.25	5.83	19.00	24.00	20.00	21.00
	0.4	5.88	9.75	3.25	6.29	6.8	11.5	6.3	8.2	3.00	8.80	2.50	4.77	1.75	3.50	1.50	2.25	10.00	24.00	11.00	15.00
	0.6	5.75	6.50	7.00	6.42	14.0	5.8	8.0	9.3	19.00	5.30	3.80	9.37	4.00	1.75	3.00	2.92	26.00	20.00	14.00	20.00
2,4-D	0.2	4.75	2.50	7.75	5.00	5.1	4.7	5.4	5.1	1.00	1.00	1.00	1.00	2.25	1.00	3.75	2.33	1.00	1.00	4.00	2.00
	0.4	3.75	1.88	11.50	5.71	3.3	2.3	9.4	5.0	1.00	1.00	12.00	4.67	1.00	1.00	12.00	4.67	1.00	1.00	1.00	1.00
	0.6	6.75	2.50	9.50	6.25	7.0	3.3	6.4	5.6	1.00	1.00	2.00	1.33	1.00	1.00	2.25	1.42	1.00	1.00	1.00	1.00
NAA	0.2	6.50	10.25	8.75	8.50	8.6	12.4	13.3	11.4	19.00	10.00	16.00	15.00	5.50	9.00	11.50	8.67	26.00	25.00	28.00	26.33
	0.4	4.25	9.50	7.25	7.00	3.5	9.9	11.3	8.3	1.00	10.50	8.50	6.67	1.00	5.50	8.50	5.00	15.00	16.00	19.00	16.67
	0.6	6.25	10.25	7.25	7.92	7.3	9.6	7.3	8.1	21.50	3.80	2.50	9.27	6.00	1.50	3.75	3.75	25.00	20.00	17.00	20.67
Mean		5.94	7.56	7.60		7.8	7.4	8.0		9.03	5.82	6.88		3.58	3.75	5.75		14.40	15.40	13.80	
LSD:		**A**	**B**	**AxB**		**A**	**B**	**AxB**		**A**	**B**	**AxB**		**A**	**B**	**AxB**		A	B	AxB	
at 0.05		**2.24**	**1.23**	**3.88**		**2.44**	**NS**	**4.23**		**2.40**	**1.50**	**4.20**		1.72	NS	2.97		2.80	1.05	3.88	

Where: IAA= Indole-3-acetic acid, 2,4-D= Dichlorophenoxyacetic acid and NAA= α-Naphthaleneacetic acid
PP333= Paclobutrazol and AC =activated charcoal

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