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CALLOGENESIS AND PLANT REGENERATION VIA *IN VITRO* CULTURE OF *STEVIA REBAUDIANA* EXPLANTS

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ABSTRACT

Tissue culture technique became one of the most important tools in plant breeding. Callus is important for rapid mass multiplication, generation of variability, cell suspension culture, preservation of cell line culture and production of secondary metabolites. Therefore, this study was carried out on stevia plant for establishing efficient methods of formation and regeneration of callus. Three different explants (shoot tip, leaves and nodal segments) and Murashige and Skoog medium (MS) with different concentrations and combinations of growth regulators (PGRs) were used to determine which explant is the most suitable for callus induction and regeneration in the presence of different PGRs. All studied media induced callus for all explants, but MSc_4 (MS+1.0 mgl^{-1} 2,4-D +0.75 mgl^{-1} NAA) gave the highest values of callus fresh weight. Only, calli obtained from Msc_4 callus induction medium gave the best response to regenerate a sufficient number of shoots. Half strength MS medium with 1 mgl^{-1}IBA was found to be the optimum medium for root formation. It gave a good root formation (88.67%), highest roots number/shoot (6.24), and highest root length (2.90cm).

Key words: ***Stevia rebaudiana, Explants, Plant growth regulators, Callus formation, plant regeneration.***

INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a perennial herbal plant belongs to *Asteraceae* family. It is native to tropical and subtropical regions of North and South Americas. In natural conditions stevia grows in a form of a shrub and reaches even 1 m of height (Sivaram and Mukundan 2003). Stevia is a short-day plant and its flowering is induced when days become shorter. However, a long photoperiod stimulates leaf growth and steviol glycoside production. A crucial characteristic of stevia plant is its ability to synthesize a group of chemical compounds with low-caloric and sweet taste, i.e. diterpenoid steviol glycoside (SGs) which is highly sweet compound, being 300 times sweeter than sucrose, non-toxic and non-mutagenic in nature (Geuns 2003).The distribution of SGs in plant organs differs and leaves have the most abundant concentrations of Stevioside and other SGs. The major SGs of *S. rebaudiana* are Stevioside (4–13% w/w), Rebaudioside A (2–4% w/w), Rebaudioside C (1–2% w/w) and Dulcoside A (0.4–0.7% w/w) (Melis *et al* 2009). Some of the SGs have also therapeutic properties (Megeji *et al* 2005 and Thiyagarajan and Venkatachalam 2012). In stevia, seed germination is poor commonly due to infertile seed (Kumar 2013) and small endosperm (Yadav *et al* 2011). In addition, propagation by seeds does not allow the production of homogeneous populations, which

lead to great variability in sweetening composition and its levels (Tamura *et al* 1984 and Nakamura and Tamura 1985).Vegetative propagation through the direct planting of stem cuttings in the field has limited success due to poor rooting (Pande and Gupta 2013 and Khalil *et al* 2014). Hence, Plant tissue culture technique is one such biotechnological approach, which will improve both qualitatively and quantitatively the productivity of stevia plant as well as free disease plants. It has contributed many biotechnologies, mostly for the propagation of problematic species (species having problems in their natural propagation), rapid propagation of selected and superior genotype and also genetic mutants through somaclonal variation as well as synthesis of secondary metabolites, which is an important part of food fragrance and pharmaceutical industries. On the other hand, callus masses can sometimes yield the highest amount of secondary metabolites (Das *et al* 2010). Parts like leaves, nodes and shoot tips from *Stevia* can be used to raise plants *in vitro* (Naz and Hashmi 2008). *In vitro* callus formation and regeneration of *Stevia rebaudiana* has received little attention, but improvement in the yield of stevioside needs further attention. A Few studies on callus formation and plant regeneration of stevia have been reported by Naz and Hashmi (2008), Patel and Shah (2009), Preethi *et al* (2011), Singh *et al* (2011), Singh *et al* (2012). Therefore, the objective of the present study was to establish an efficient method of callus induction and plant regeneration of a new sweetening crop plant (*Stevia*) using different explants with different growth media.

MATERIALS AND METHODS

Plant Materials

The present study was conducted at the Plant Tissue Culture Laboratory, Sugar Crops Research Institute, Agricultural Research Center (ARC), Giza, Egypt during the period from 2014 to 2016. Seedlings of *Stevia rebaudiana* var. Spanti were kindly obtained from Sugar Crops Research Institute (SCRI), ARC, Ministry of Agriculture, Giza, Egypt, which were grown under greenhouse conditions.

Equipment sterilization and preparation of stock solution

The glass wares were soaked in 5% chromic acid overnight and then washed in running tap water followed by rinsing with double distilled water. The cleaned glass wares were then dried in forced draft hot air oven at 100°C temperature and were stored in dust free cupboards before use. Clean and dry glass wares were used for all kinds of tissue culture studies throughout the present investigation. The stock solution of auxins; Naphthalene acetic acid (NAA), Indole-3-butyric acid (IBA) and 2,4-Dichlorophenoxy acetic acid (2,4-D) were dissolved separately in minimal

quantity of redistilled ethyl alcohol and the volume was made up with double distilled water. The stock solution of cytokinins: Benzyl adenine or 6-Benzyl amino purine (BAP) was dissolved by adding few drops of 0.1 N HCl and volume was made up with double distilled water. All the above stock solutions were stored in refrigerator at 5-6°C then kept back to the ambient temperature before using them for the media preparation.

Explant sterilization and medium preparation

Shoot tips, axillary nodes and leaves were excised and washed under running tap water and then submerged in tap water with a few drops of Tween-20 in a flask with shacking by hand for 5 min followed by rinsing in tap water to remove the soap. Explants were surface sterilized by immersing in an aseptic solution of 20% Clorox (5 % Sodium hypochlorite) concentration for 5 minutes, Under aseptic condition inside the culture cabinet (Laminar Air Flow Hood) by using sterilized instruments; explants were surface sterilized with 70 % (v/v) ethanol for 30 seconds and subsequently with 0.15% mercuric chloride ($HgCl_2$) solution for 1 min followed by repeated washing (3-4 times) with sterile distilled water to remove all traces of $HgCl_2$. The sterilized explants (shoot tips , axillary nodes and leaves) were trimmed (0.5-1.0 cm) at the base and cultured with the cut surface in contact with contained 4.43 gl^{-1} MS basal salt mixtures and vitamins (Murashige and Skoog 1962) supplemented with 0.1 gl^{-1}myo-inositol and 30.0 gl^{-1} sucrose. Plant growth regulators (PGRs) from stock solutions were added to the media for all cultures. The pH of the medium was adjusted to 5.6 - 5.8 with 0.1 N KOH and 0.1 N HCl before gelling with agar, then the medium was heated on the hot plate magnetic stirrer for 20 min followed by adding Agar 7 gl^{-1}. Then, the medium was distributed into the culture jars, where each jar volume 500 ml contained (30-40 ml medium for each jar) and closed with polypropylene scrow cap then sterilized. The medium was autoclaved at 121°C and 1.2–1.3 kg/cm^2 pressure for 20 min.

Culture conditions

The cultures were maintained in an air conditioned culture room at 25±2°C under 16 h per day photoperiod and 8 hr dark period which provided by cool white fluorescent lamps (light intensity 2000 Lux) measured by (Traceable Dual- Range light meter). All media were solidified with7 gl^{-1}Agar. The media were made up in distilled water; the pH was adjusted to 5.8 and sterilized by autoclaving for 20 minutes at 121 °C and 15 psi.

Callus induction

For callus induction; shoot tips, axillary nodes and leaves were used as explants. These explants were cultured on MS (Murashige and Skoog 1962) medium with five different PGR's combinations (Table1).

Table1. Different combinations of medium and explants type used for callus induction.

Medium code	Media	References
MSc_1	MS + 3mgl^{-1} 2,4-D	SalimUddin *et al* (2006)
MSc_2	MS + 2.0 mgl^{-1} BAP+ 2.0 mgl^{-1} NAA	Patel and Shah (2009)
MSc_3	MS + 1.0 mgl^{-1} BA + 1.5 mgl^{-1} NAA	Moktaduzzaman and Rahman (2009)
MSc_4	MS + 1.0 mgl^{-1} 2,4-D + 0.75 mgl^{-1} NAA	Gupta *et al* (2010)
MSc_5	MS + 2mgl^{-1} 2,4-D + 2mgl^{-1} BA	Ahmad *et al* (2011), Khalil *et al* (2014)

Plant regeneration

To regenerate plants from callus, four types of MS medium different in their growth regulators' combinations were used (Table 2). Callus was transferred as a cluster on the surface of regeneration medium containing MS medium with 0.1gl^{-1}myo-inositol, 1 ml MS Mix vitamin, 3% sucrose. The clusters from callus were incubated at 25- $^{+}$ 1°C under a photoperiod of 16 hr/day and 3000 Lux for 60 days. The regenerated shoots which reach 2-4 cm in length (after 42 days from culture) in each callus tissue were calculated.

Table 2. Different hormones combination used for callus regeneration.

Code No.	Media	References
1	MS + 2mgl^{-1} BA	Ahmad *et al* (2011), Preethi *et al* (2011)
2	MS + 2mgl^{-1} BAP + 0.2mgl^{-1} NAA	Patel and Shah (2009)
3	MS + 1.8 mg l^{-1} BA + 0.12 mg l^{-1} NAA	Moktaduzzaman and Rahman (2009)
4	MS + 0.5mgl^{-1} BAP + 0.1mgl^{-1} NAA	Singh *et al* (2011)

Shoot proliferation and multiplication

Shoots were transferred to medium consisted of MS basal salts supplemented with 3% sucrose, 0.1gl^{-1}myo-inositol, 1 mgl^{-1}GA_3 , 1 ml MS Mix vitamin and 7 gl^{-1} Agar and the pH was adjusted to 5.6-5.7 for shoot proliferation and multiplication.

Rooting stage

Shoots with 2 to 4 cm in length that regenerated from in vitro cultured explants were rooted. The medium was supplemented with 30 gl-1sucrose and 7 gl-1agar in the presence of different concentrations of auxins like Indole Butyric acid (IBA) to monitor the initiation and quality of adventitious roots on the regenerated shoots (Table 3).

Table 3. Rooting medium strength (Full and Half) with different IBA concentrations.

MS strength		References
Half MS	Full MS	
Control		Singh *et al* (2017)
IBA		
0.5 mgl^{-1}		Hassanen and Khalil (2013), El-Motaleb *et al* (2013)
1.0 mgl^{-1}		Mirniam *et al* (2010), Mehta *et al* (2012)
1.5 mgl^{-1}		Mubarak *et al* (2009), Moktaduzzaman and Rahman (2009)

Statistical analysis

Data were analysed using SPSS computer program V.10 (1999) based on Completely Randomized Design (CRD) with four replicates. Means were compared using Duncan's multiple range test (DMRT) (Duncan 1955) at 5% level of probability.

RESULTS AND DISCUSSION

Callus induction

Data in Table (4) showed that the three tested media (MSc_1, MSc_4 and MSc_5) produced high callus induction percent (100%) for the three explants (leaf, shoot tip and node), while the other two media (MSc_2 and MSc_3) gave the lowest induction percent that ranged between 70 and 95%. For callus fresh weight , MSc_4 gave the highest values with leaf (0.633g) , shoot tip (0.549g) and node (0.494g), followed by MSc_1 (0.610 , 0.446 and 0.412g, respectively), while MSc_2 gave the lowest values with shoot tip (0.344g) , leaf (0.326g) and node (0.308g). Regarding the quantity indicator of callus and its color, MSc_4 and MSc_1 were the best media for both traits and leaf explant was the best type compared to other two explants (Table 5). It could be noticed that MSc_4 ($1mgl^{-1}$ 2,4 D + $0.75mgl^{-1}$ NAA) was the best medium for callus induction (100%) and fresh weight, while leaf explant was the best type for this trait. Similar results were obtained by Gupta *et el* (2010) who found that combination of 1mg/l 2,4-D and 0.75mg/l NAA was the best for callus induction (100%) observed from leaf explant which also produced the highest fresh weight.

Table 4. Effect of explant type and growth medium on callus induction and its growth parameters of *Stevia rebaudiana.*

Explant type	Media	Callusing			
		Callus induction %	Callus fresh weight (g)	Indicator to callus quantity	Callus color
Node	MSc_1	100[a]	0.412[d]	+++	Yellow tends to green
	MSc_2	85[d]	0.346[e]	++	Yellow tends to green
	MSc_3	70[f]	0.308[e]	+	Mainly brownish
	MSc_4	100[a]	0.494[c]	+++	Green
	MSc_5	100[a]	0.351e	++	Yellow
Shoot tip	MSc_1	100[a]	0.446[c]	+++	Yellow tends to green
	MSc_2	95[b]	0.395[d]	++	Yellow tends to green
	MSc_3	90[c]	0.344[e]	++	Yellow
	MSc_4	100[a]	0.549[b]	+++	Green
	MSc_5	100[a]	0.374[d]	++	Yellow
Leaf	MSc_1	100[a]	0.610[a]	++++	Green
	MSc_2	90[c]	0.370[de]	++	Yellow tends to green
	MSc_3	80[e]	0.326[e]	+	Mainly brownish
	MSc_4	100[a]	0.633[a]	++++	Green
	MSc_5	100[a]	0.386[d]	++	Yellow

+, ++, +++, ++++ **denotes low, moderate, elevated and excellent callus, respectively.**

Regeneration of callus

Data presented in Table (5) showed that MS medium with four different combination of growth regulators were used for shoot formation. All calli which produced from callus induction medium were transplanted in different combinations of BA and NAA to evaluate the number of shoots per callus, the average length of shoots and regeneration response%. The obtained data revealed that there were no response to regenerate any shoots from callus source (callus induction media) which produced from MSc_1, MSc_2, MSc_3 and MSc_5 medium (Table 4).

Table 6. Effect of different hormones' combinations on regeneration response%, shoots number and shoot length of *Stevia rebaudiana*.

Callus induct-ion media	Regeneration media (BA+NAA)mgl^{-1}	No. of total shoots/culture	Average length of shoots/culture (cm)	Regener-ation response%
MSc_1	MS+2mgl^{-1}BA	0.00	0.00	0.00
	MS+2mgl^{-1}BAP+0.2mgl^{-1}NAA	0.00	0.00	0.00
	MS+1.8mgl^{-1}BA+0.12mg l^{-1} NAA	0.00	0.00	0.00
	MS+0.5mgl^{-1}BAP+0.1mgl^{-1} NAA	0.00	0.00	0.00
MSc_2	MS+2mgl^{-1}BA	0.00	0.00	0.00
	MS+2mgl^{-1}BAP+0.2mgl^{-1}NAA	0.00	0.00	0.00
	MS+1.8mgl^{-1}BA+0.12mg l^{-1} NAA	0.00	0.00	0.00
	MS+0.5mgl^{-1}BAP+0.1mgl^{-1} NAA	0.00	0.00	0.00
MSc_3	MS+2mgl^{-1}BA	0.00	0.00	0.00
	MS+2mgl^{-1}BAP+0.2mgl^{-1}NAA	0.00	0.00	0.00
	MS+1.8mgl^{-1}BA+0.12mg l^{-1} NAA	0.00	0.00	0.00
	MS+0.5mgl^{-1}BAP+0.1mgl^{-1} NAA	0.00	0.00	0.00
MSc_4	MS+2mgl^{-1}BA	5.27^{b}	3.68^{b}	82^{b}
	MS+2mgl^{-1}BAP+0.2mgl^{-1}NAA	8.24^{a}	4.82^{a}	100^{a}
	MS+1.8mgl^{-1}BA+0.12mg l^{-1} NAA	3.67^{c}	2.93^{c}	74^{c}
	MS+0.5mgl^{-1}BAP+0.1mgl^{-1} NAA	2.94^{d}	2.09^{d}	60^{d}
MSc_5	MS+2mgl^{-1}BA	0.00	0.00	0.00
	MS+2mgl^{-1}BAP+0.2mgl^{-1}NAA	0.00	0.00	0.00
	MS+1.8mgl^{-1}BA+0.12mgl^{-1} NAA	0.00	0.00	0.00
	MS+0.5mgl^{-1}BAP+0.1mgl^{-1} NAA	0.00	0.00	0.00

On the other hand, calli obtained from Msc_4 callus induction medium gave the best response to regenerate a sufficient number of shoots with all studied regeneration medium. The highest response to regeneration was 100% with 2mgl^{-1} BAP + 0.2mgl^{-1} NAA as a high values of number of shoots per callus and the average length of shoots (8.24 shoots, 4.82cm) and the lowest response to regenerate was 60% with 0.5mgl^{-1} BAP + 0.1mgl^{-1} NAA as a low values of number of shoots per callus and the average length of shoots (2.94 shoots, 2.09cm). Therefore, MSc_4 callus induction medium was recommended to be used as a good callus induction source for plantlet production on MS supplemented with 2mgl^{-1} BA + 0.2mgl^{-1} NAA as regeneration medium with three types of explants (node , shoot tip and leaf). Similar results were obtained by Patel and Shah (2009) who noticed higher regeneration frequency with MS medium supplemented with 2mgl^{-1} BAP + 0.2 mgl^{-1} NAA.

Root formation

Adventitious rooting is a critical factor for successful production of elite clones and an important step in the plant species vegetative propagation, according to Davis and Haissig (1994). Interaction effects of MS strengths, concentrations of IBA on root formation, number of roots per plantlet and length of roots are shown in (Table 6). In present study, regenerated shoots (1- 1.5 cm long) were cultured on two MS basal strength media containing different concentrations of IBA (0.5, 1.0 and 1.5mgl^{-1}). Application of IBA significantly increased roots formation percent, roots number/shoot and root length (Table 7). Root formation and number of roots per plantlet in Half MS medium (77.12%, 4.65, respectively) were significantly higher than in Full MS medium (72.27%, 3.86 respectively). Half strength MS medium with 1mgl^{-1}IBA was found to be the optimum medium for root formation. It gave a good root formation (88.67%), highest roots number/shoot (6.24), and highest root length (2.90 cm). Similar results were obtained by Mehta *et al* (2012), who reported the positive role of IBA during *in vitro* rooting of *Stevia rebaudiana.* Also, Hwang (2006) and Tadhani *et al* (2006) stated that the maximum number of roots was obtained with 1.0 mgl^{-1} IBA in the MS medium.

Table 6. Effect of medium strength and different IBA concentrations on percentage of root formation, No of roots and roots length of *Stevia rebaudiana*.

Traits	Root formation%		Mean	No. of roots		Mean	Root length (cm)		Mean
MS strength	Half MS	Full MS		Half MS	Full MS		Half MS	Full MS	
Control	66.54^{A}	63.33^{A}	64.94^{c}	2.64^{A}	2.25^{A}	2.45^{d}	2.27^{A}	2.50^{A}	2.39^{c}
IBA mgl^{-1}									
0.5	75.33^{A}	69.74^{B}	72.54^{b}	3.98^{A}	3.21^{B}	3.60^{c}	2.05^{A}	2.29^{A}	2.17^{c}
1.0	88.67^{A}	83.17^{B}	85.92^{a}	6.24^{A}	5.45^{B}	5.85^{a}	2.90^{A}	2.81^{A}	2.86^{a}
1.5	77.95^{A}	72.85^{B}	75.4^{b}	5.73^{A}	4.54^{B}	5.14^{a}	2.58^{A}	2.45^{A}	2.52^{ab}
Mean	77.12^{A}	72.27^{B}	74.70^{b}	4.65^{A}	3.86^{B}	4.26^{b}	2.45^{A}	2.51^{A}	2.48^{bc}

Different capital letters denote significant difference between the two media, while lower case letters denote differences among hormone concentration across both media.

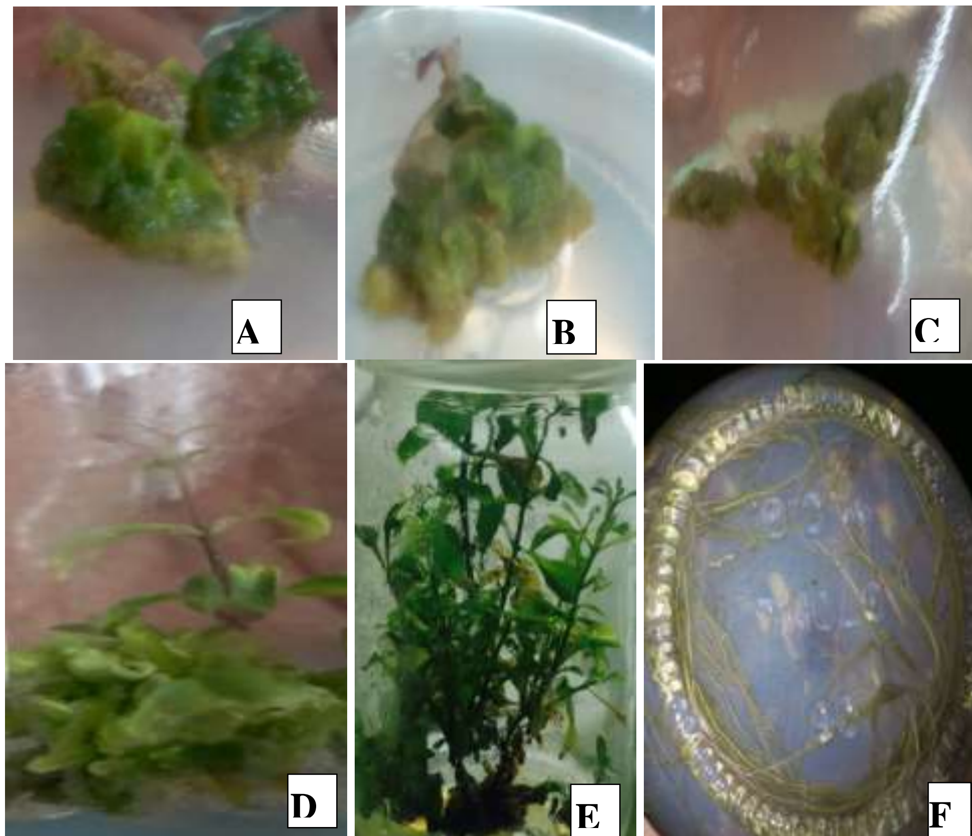

Fig. 1. A, B, C) callus induction (A-leaf explant; B-shoot tip explant; C-node explant); D)regeneration callus; E)proliferation; F)root formation.

CONCLUSION

On the basis of findings achieved in this research study, it is concluded that leaf explant give maximum callus induction response at 1 mgl^{-1} 2, 4-D+ 0.75 mgl^{-1} NAA in MS medium. Results also revealed that maximum shoots induction and shoots elongation was observed when 2mgl^{-1} BAP + 0.2mgl^{-1} NAA added to MS medium. IBA concentration 1 mgl^{-1} in half MS medium resulted in maximum roots per in vitro grown shoot.

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تكوين الكالس واستيلاد النباتات فى مزارع الأنسجة من عزلات نباتية لنبات الاستيفيا

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أصبحت تقنية زراعة الأنسجة واحدة من أهم الأدوات فى تربية النباتات. يعتبر الكالس مهم فى الإكثار الخضرى المتعدد السريع، توليد التباين، المزارع الخلوية، الحفاظ على زراعة السلالات وإنتاج المركبات الثانوية. لذلك تم إجراء هذه الدراسة على نبات الإستيفيا لتحديد الطرق الفعالة لتكوين الكالس و أستيلاد النباتات منه. تم استخدام ثلاثة أنواع من العزلات النباتية (القمة النامية، الأوراق و البراعم العقدية الجانبية) و بيئات مختلفة من موراشيج و سكووج (MS) مع تركيزات مختلفة من منظمات النمو (PGRs) لتحديد ما هى العزلة النباتية الأكثر ملاءمة لتكوين الكالس و أستيلاد النباتات منه فى وجود الهرمونات النباتية المختلفة. جميع البيئات المدروسة أنتجت الكالس لجميع العزلات النباتية. و لكن بيئة MSc_4 (MS + ١ملجم/لتر ٢،٤ داي كلوروفينوكسي أسيتك أسيد + ٠,٧٥ ملجم/لتر نفثالين أسيتك أسيد) أعطت أعلى قيم للوزن الطازج للكالس. الكالس الوحيد الناتج من بيئة (MSc_4) لتحفيز تكوين الكالس أعطى أفضل أستجابة لاستيلاد عدد كاف من السيقان الخضرية. و قد وجد أن نصف تركيز بيئة موراشيج و سكووج مع ١ملجم/لتر أندول بيوتريك أسيد هى أفضل بيئة لتكوين الجذور. و قد أعطت أفضل نسبة تكوين للجذور بنسبة (٨٨,٦٧%)، أعلى عدد جذور على الساق (٦,٢٤) و أعلى طول للجذر (٢,٩٠ سم).

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