KKU Res. J. 2014; 19(Supplement Issue): 172-180
http : //resjournal.kku.ac.th

Investigation of Effective *In Vitro* Propagation Media for *Stevia rebaudiana* Bertoni

Nguyen Thanh Huy and Tran Thi Xuan Mai*

Plant Genetic Engineering Laboratory, Biotechnology Research and Development Institute, Can Tho University
** Correspondent author: nolan.huy.nguyen@gmail.com*

Abstract

Stevia rebaudiana Bertoni, a natural non-caloric sweetener, can be used as a sugar substitute for diabetic patients. Conventionally, it is propagated by seeds or stem cuttings which has a limitation of quality and quantity of the seed material. In this study a protocol for shoot regeneration of Stevia from nodal segments, callus formation from leaf explants and plant regeneration from callus were investigated. The results showed that Murashige and Skoog (MS) medium containing vitamins supplemented with 0.2 mg/l NAA and 0.15 mg/l BAP was found to be an efficient medium for not only shooting with 100% of multiple shoot proliferation from nodal segments but also callusing with 92.6% of nodular callus induction from leaf explants; however, the callus failed to differentiate into shoots. The highest rooting was obtained (83.33%) in MS medium (basal salt mixtures) supplemented with 0.5 mg/l IAA. The rooting plantlets were successfully acclimatized in the mixture of 1 soil: 1 sand: 1 decomposed rice straw (v/v) with survival rate of 100% after 3 weeks and no observable morphological abnormalities. This developed protocol could be used efficiently for large scale production of *Stevia rebaudiana* Bertoni through *in vitro* propagation.

Keywords: BAP, IAA, MS medium, NAA, *Stevia rebaudiana* Bertoni.

1. Introduction

Stevia (*Stevia rebaudiana* Bertoni), also known as sugar leaf, honey leaf, or sweet weed, is perennial shrub belonging to genus Stevia, family Asteraceae. Stevia is said to be a natural sweetener due to stevioside compounds found mostly in leaf content. Steviosides can be used as a sugar replacement for patients suffering from diabetes, obesity, hypertension, or on-diet people since they are 300 times sweeter than sugarcane, delicious, and non-caloric.

The demand on Stevia is increasing in recent years accompanying with the significant rising rate of the mentioned diseases. Consequently, large-scale production of this valuable herb is expanded in many provinces of Vietnam requiring much investment for not only growing techniques but also a large number of homogeneous and disease-free plantlets. Propagation by seeds, however, is very poor and usually results in great variability in features like sweetening levels and composition. Moreover, vegetative propagation can be done from stem nodes but limitation in number is a facing problem (Guruchandran and Sasikumar, 2013). Plant tissue culture is thus an alternative way for rapid and mass production of Stevia.

2. Materials and Methods

Leaves and nodal segments were collected from *in vitro* Stevia sample provided by Biotechnology Institute of Ha Noi, Vietnam.

Half- or full-strength Murashige and Skoog (MS) medium with or without vitamins (Duchefa Biochemie) was similarly added with 20 g/l sucrose and solidified with 8 g/l agar. Different concentrations of cytokinin (N6-benzylaminopurine (BAP), 2,4-Dichlorophenoxyacetic acid (2,4-D), Kinetin) and auxin (Indol-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA)) singly or in combination were used for callus induction and shoot proliferation. To induce root formation, IAA or activated charcoal was supplemented to the media. The pH was then adjusted to 5.8 before autoclaving at 121^{o}C and 1 atm pressure for 20 minutes. All the cultures were placed under stable conditions at 27±1^{o}C for a photoperiod of 16 hours per day with 1000 lux light intensity.

2.1 The effects of different media on callus induction of Stevia leaves

These following media were used for callus induction: C1: MS basal salt mixtures+1 mg/l Kinetin+ 2 mg/l 2,4-D; C2: MS basal salt mixtures+0.75 mg/l NAA+ 1 mg/l 2,4-D; C3: MS basal salt mixtures+3 mg/l 2,4-D; C4: MS basal salt mixtures+0.5 mg/l BAP; C5: MS including vitamins+0.15 mg/l BAP+0.2 mg/l NAA; C6: MS including vitamins+0.2 mg/l BAP+0.5 mg/l IAA.

Leaf explants were cut into small pieces (approx. 0.3 - 0.5 cm^2) and placed on different media (C1, C2, C3, C4, C5 and C6) with the dorsal side being in contact with the medium surface.

There were totally 3 replicates for each treatment, 3 Petri dishes for a replicate and 9 leaf explants cultured on each Erlenmeyer flask.

For each treatment, the percentage of explants forming callus was recorded.

2.2 The effects of different media on shoot proliferation from nodal segments.

Nodal segments with the length being 1 cm were excised from in vitro saplings and then cultured on different media (S1: MS basal salt mixtures+0.2 mg/l NAA+0.15 mg/l BAP, S2: MS including vitamins+0.2 mg/l NAA+0.15 mg/l BAP, S3: MS basal salt mixtures+3.5 mg/l BAP) to determine the most appropriate for shoot multiplication.

There were 3 replicates for each treatment, 3 Erlenmeyer flasks for a replicate and 4 explants cultured on each bottle.

The percentage of shooting explants, the number of shoots per explant, and average shoot length were recorded.

2.3 The effects of different media on root formation.

Produced shoots from nodal explants with the length being at least 4 cm were transferred to 3 different root-induced media: R1: MS basal salt mixtures+ 0.5 mg/l IAA; R2: 1/2MS basal salt mixtures+100 mg/l activated charcoal; R3: MS including vitamins+0.5 mg/l IAA. The experiment was set up with 3 replicates for each treatment, 3 Erlenmeyer flasks for a replicate and 4 explants cultured on each Erlenmeyer flask. In each treatment, the percentage of plantlets emerging roots, the number of roots per plantlet, and average root length were recorded.

2.4 Acclimatization

After 50 days cultured on the rooting medium, rooted plants were taken out of the Erlenmeyer flasks and removed agar under tap water. The plantlets were then transplanted to plastic glasses containing mixture of soil, sand and decomposed rice straw in the ratio of 1:1:1 (v/v/v). During the first week, plantlets were covered with plastic bags and kept under well-managed conditions (temperature 27±1^{o}C, light intensity 1000 lux, 16-hour illumination per day). In the second week, plastic bags were bored to allow air flow passing inside. From the third week onward,

non-covered plantlets were transferred to the greenhouse. The survival rate was recorded after 3 weeks.

2.5 Data analysis

All data collected were assessed by analysis of variance for factorial complete randomized design (CRD) using computer software Statgraphics Centurion XV. Duncan's Multiple Range Test (DMRT) was applied for means separation.

3. Results and Discussion

3.1 Callus induction

Calli were initiated from Stevia leaves in C1, C4 and C5 after 3 weeks cultured but morphology varied greatly between treatments (Table 1). It can be clearly seen that MS medium (including vitamins) in combination with BA and NAA at concentration 0.15 mg/l and 0.2 mg/l, respectively (C5) was the most effective formula with 92.6% of explants developing profuse yellow calli, statistically different from the others at 95% confidence level. Also, under stereoscopic magnifier (10x1.6x1), well-going process of differentiation could be witnessed in this medium, with globular structures appearing at some points of the callus (Figure1). Regardless which kinds or concentrations of phytohormones supplemented, callus, however, was failed to form in MS medium (basal salt mixtures) (C1, C2, C3, C4) since explants cultured in these media responded at low rate or even showed no response. C1 resulted in poor amount of calli which appeared in only 1/3 of explants while observation under the stereoscope showed no sign of differentiation. Following C1, C4 got only 3.7% of explants forming callus. Although the colour of calli in this treatment was nearly the same as that of C5, yellow, the amount was far less than in C5 and only the leaf edges formed callus.

The combination of 2 mg/l 2,4-D and 1 mg/l Kinetin used to be applied by Sairkar *et al.* (2009) in order to produce calli from Stevia leaves. The results, nevertheless, were much different from the present study, 88% compared to 33.33%. As taking a reference to the study of Gupta *et al.* (2010), NAA and 2,4-D at concentration 0.75 mg/l and 1 mg/l, respectively, resulted in maximum rate of callusing when using MS medium with vitamins, totally in contrary to the result of C2 (0.00%). In addition, BAP and

Table 1. The effects of different media on callusing of Stevia leaves after 21 days.

Treatment	The percentage of explants forming callus (%)	Morphology of callus
C1	33.33[b]	Poor white callus
C2	0.00[d]	Unswollen leaves
C3	0.00[d]	Unswollen leaves
C4	3.70[c]	Poor yellow callus
C5	92.60[a]	Profuse yellow callus
C6	0.00[d]	Swollen leaves

a, b, c, d: means followed by the same letters in the same column were not significantly different ($p<0.05$).

C1: MS basal salt mixtures+1 mg/l Kinetin+2 mg/l 2,4-D

C2: MS basal salt mixtures+0.75 mg/l NAA+1 mg/l 2,4-D

C3: MS basal salt mixtures+3 mg/l 2,4-D

C4: MS basal salt mixtures+0.5 mg/l BAP

C5: MS including vitamins+0.15 mg/l BAP+0.2 mg/l NAA

C6: MS including vitamins+0.2 mg/l BAP+0.5 mg/l IAA

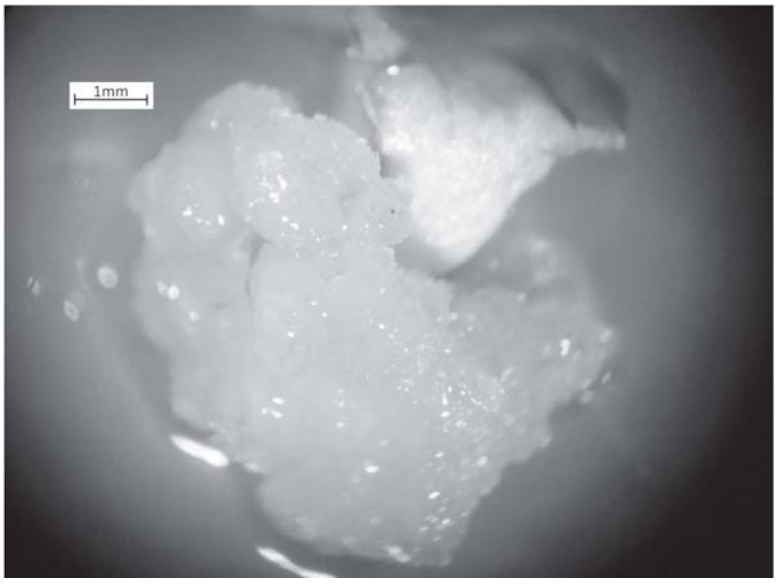

Figure 1. Callus formed in treatment C5 after 21 days under stereoscope at magnification 10x1.6x1.
C5: MS including vitamins+0.15 mg/l BAP+0.2 mg/l NAA

IAA were supplemented to MS (including vitamins) by Danh (2010) to obtain callus from *in-vitro*-grown tomato leaves; 85.53% was recorded but no explants responded as carrying on Stevia. However, instead of IAA, NAA (0.2 mg/l) mixed to MS (including vitamins) with BAP (0.15 mg/l) in C5 created more suitable environment than the others for Stevia leaves to form callus. Such great variations in the results may be due to the endogenous phytohormone contents in plants, their uptake, types of auxin and cytokinin used and their mode of action (Gupta *et al*., 2010). Also, a conclusion can be drawn that vitamins are in demand when Stevia leaves dedifferentiates to callus.

A concerned problem was the browning of callus. In treatments such as C2 or C3, browning occurred at earlier time, the end of the second week, while it could be delayed to fourth week in C5. According to study of Gupta *et al.* (2010), MS medium with 2 mg/l NAA was used for callus multiplication. However, all subcultured calli rapidly darkened and died after 1 week. Furthermore, addition of activated charcoal was applied as many studies done. Activated charcoal plays role in adsorption of inhibitory compounds from the medium, adsorption of growth regulators from the culture medium or darkening of the medium (Saad and Elshahed, 2012). In this research, activated charcoal was added to the medium at concentration 3 g/l and all the other components unchanged. The results showed that activated could not maintain the callus growth since all explants continued to turn brown and died out. The maintenance of callus plays an important role in *in vitro* propagation through callusing. Good maintenance makes sure that globular structures have enough time to develop totally, creates a background for better shoot induction in the next stage. In the research of Das *et al*. (2006), NAA and BAP added to ½ MS medium resulted in good callus nourishment but the concentrations were used at 1 mg/l and 1-2 mg/l, respectively, much higher than 0.2 mg/l and 0.15 mg/l in this experiment.

Table 2. The results of shoot multiplication from Stevia nodal segments after 21 days.

Treatments	Number of shoots per explant	Shoot length (cm)
S1	2.14[b]	0.36[c]
S2	2.24[b]	1.15[a]
S3	3.24[a]	0.80[b]

a, b, c: means followed by the same letters in the same column were not significantly different ($p<0.05$).

S1: MS basal salt mixtures+0.2 mg/l NAA+0.15 mg/l BAP

S2: MS including vitamins+0.2 mg/l NAA+0.15 mg/l BAP

S3: MS basal salt mixtures+3.5 mg/l BAP

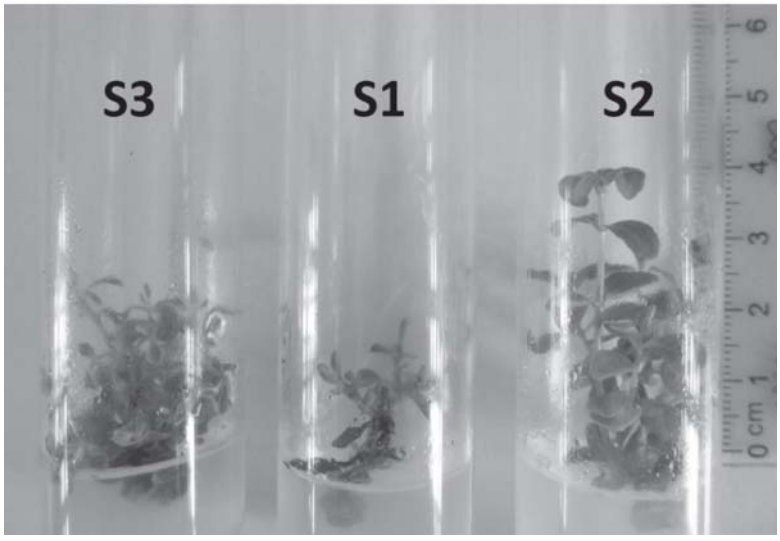

Figure 2. Shoots multiplication from Stevia nodal segments cultured in different media.

S1: MS basal salt mixtures+0.2 mg/l NAA+0.15 mg/l BAP.

S2: MS including vitamins+0.2 mg/l NAA+0.15 mg/l BAP.

S3: MS basal salt mixtures+3.5 mg/l BAP.

Figure 3. The morphology of Stevia shoots grown in different media under stereoscope at magnification 1.6x10x0.65.

S1: MS basal salt mixtures+0.2 mg/l NAA+0.15 mg/l BAP.

S2: MS including vitamins+0.2 mg/l NAA+0.15 mg/l BAP.

S3: MS basal salt mixtures+3.5 mg/l BAP.

3.2 Shoot proliferation

After 3 days of culture, 100% of nodal explants containing lateral buds in all 3 media produced multiple shoots. Nevertheless, great differences in both number of shoot and length were obvious as presented in Table 2 and Figure 2. BAP singly used at concentration of 3.5 mg/l with MS medium (basal salt mixtures) (S3) resulted in the largest number of shoots per explant (3.24 shoots/explant), significantly different from S1 and S2 (2.24 and 2.14, respectively) at 95% confidence level. Regarding the shoot length, however, nodal segments inoculated on MS medium (including vitamins) and the combination of NAA (0.2 mg/l) and BAP (0.15 mg/l) in S2 developed the longest shoot, being 1.15 cm, much higher than that of S3 (0.8 cm) and without-vitamin S1 (0.36 cm) (Table 2). At a closer look, observation under the stereoscope clearly showed the morphological differences between 3 treatments. S2 possessed the most natural-like appearance (hairy green obovate leaves) while S3 developed unhairy bright green oblanceolate leaves. Although S1 also had obovate leaves, the leaf size was smaller than S2's with yellowish green colour (Figure 3).

Hossain *et al.* (2008) reported that MS medium added with 1 mg/l BAP resulted in 1.8 shoots per explant and 7.25-cm average length. At the concentration 3 mg/l of BAP, the number of shoot increased to 3.4±0.58 while the length dropped to 6.51±0.76 (Jitendra *et al*, 2012). The type of cytokinin was the most important factor affecting shoot multiplication. The highest shoot multiplication rate was obtained from single stem node segment cultured on medium supplemented with BAP. Increasing BA concentration promoted shoot multiplication (Abd Alhady, 2011). However, as comparing to the results of S3 in experiment 2, it is clear that an increase in BAP concentration could promote the rate of shoot proliferation but there was a certain limitation. Obviously, the number of shoots slightly decreased to 3.24 shoots per explant cultured at concentration 3.5 mg/l BAP. Moreover, the rising of shoot number might affect the length since the nutrients taken up were distributed to more shoots.

In all 3 treatments, from a single nodal segment, there were only 2 shoots developing at the node after 1 week. The third and other shoots would appear later, at the end of the second week. It was seen that shoots nearly stopped growing after 5 weeks cultured, they remained stunted regardless of subculturing or not. Due to such problem, 4-cm-and-above shoots were transferred to rooting media in order to help the plants take up nutrients more efficiently. When those plantlets got stronger, they would be ready for a new round of shoot multiplication.

3.3 Root formation

By comparison of different media for root formation, the results showed that shoots from the previous experiment emerged root in all 3 treatments at different frequency. As shown in Table 3, root grew the most rapidly in half-strength MS basal salt mixtures containing 100 mg/l activated charcoal (R2). After 25 days, the longest roots (7.02 cm in average), significantly different from R1 and R3 (2.2 cm and 1.39 cm, respectively), were found in R2. Regarding the number of roots per plant and the percentage of saplings emerging roots, however, R1, which was MS (Basal salts mixtures) added with IAA at the concentration of 0.5 mg/l, resulted in 9.57 roots and 83.33%, much larger than recorded figures of R2 and R3 (MS medium including vitamins). Under the same stereoscopic magnification being at 1.6x10x1, there were noticeable differences in the morphology of roots from 3 treatments. R1 and R3 tended to develop thick and hairy fibrous roots while R2 gave rise to far less hairy and thin taproots (Figure 4 and Figure 5). Lastly, in all 3 treatments, especially R1, most of the plants grew faster in height and leaf size, many auxiliary shoots were elongated.

MS medium supplemented with 0.5 mg/l IAA was used by Ojha *et al.* (2010) for rooting but the obtained results were quite higher than R1 (98.1% compared to 83.33%). Such difference can be explained by the

difference in length of the shoots cultured. The results of this study were also in agreement with Hossain *et al.*' (2008), MS medium showed better capability to promote root growth in both number and length compared to ½ MS. Besides, the experiments of Hossain *et al.* recorded that the majority of rooting plants rapidly died after 30-day culturing in rooting media which were MS or ½ MS media supplemented with NAA or BAP. In this research, such limitation was successfully solved as the type of auxin used was changed to IAA and vitamins was removed. After 50 days, plantlets still grew well, height and leaf size continued to increase.

3.4 Acclimatization

At the end of the third week, after being moved to the greenhouse and totally removed plastic bags, maximum survival rate (100%) of plantlets was obtained. New shoots formed while a significant increase in the height was observed.

After attaining the rapid in vitro multiplication rate, successful acclimatization or establishment of tissue culture-raised propagules in the soil, was the key parameter of a micropropagation protocol (Ahmed *et al.*, 2007). By applying this method, the survival rate of plantlets reached 100%, higher than the results of Hossain *et al.* (2008) (69% survivals in mixture including soil and sand in a ratio of 1:1 (v/v), respectively, under stable temperature 30°C) and Das *et al.* (2011) (93.09% survivals in aseptic sand and decreased to 82.14% as being transplanted to mixture including soil, sand and farm yard manure in a ratio of 1:1:1 (v/v/v), respectively).

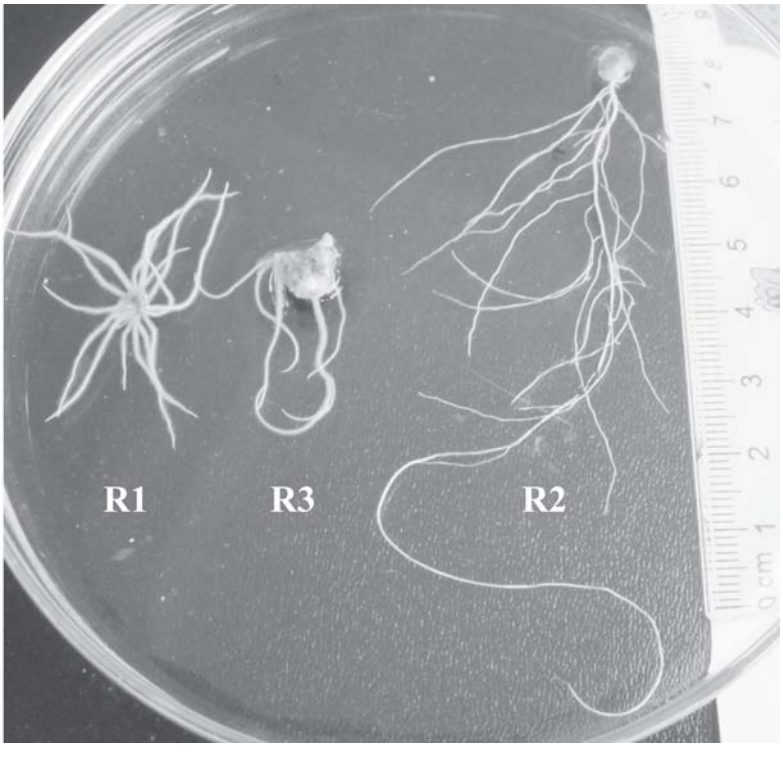

Figure 4. Formation of Stevia roots in different media after 25 days

R1: MS basal salt mixtures+ 0.5 mg/l IAA

R2: ½ MS basal salt mixtures+100 mg/l activated charcoal

R3: MS including vitamins+0.5 mg/l IAA.

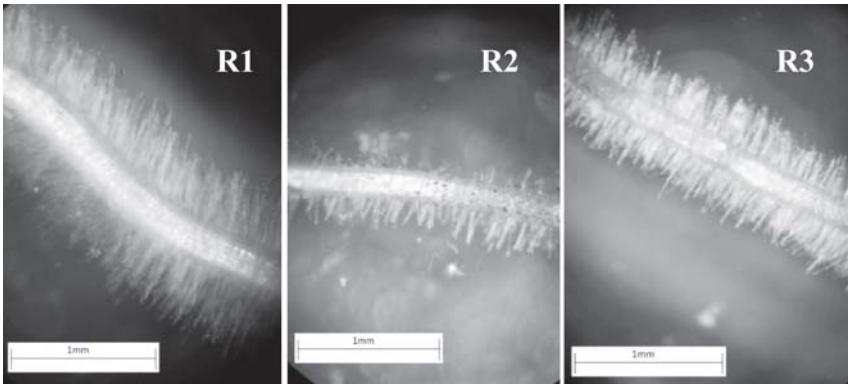

Figure 5. Morphology of Stevia roots grown in different media under stereoscope at magnification 1.6x10x4.

R1: MS basal salt mixtures+ 0.5 mg/l IAA

R2: MS basal salt mixtures+100 mg/l activated charcoal

R3: MS including vitamins+0.5 mg/l IAA.

Table 3. Root induction of Stevia shoots grown in different media after 25 days.

Treatments	The percentage of explants developing roots (%)	Number of roots per plant	Root length (cm)	The morphology of roots
R1	83.33[a]	9.57[a]	2.20[b]	Hairy, thick fibrous roots
R2	58.33[b]	1.67[c]	7.02[a]	Less hairy, thin taproots
R3	33.33[c]	3.50[b]	1.39[c]	Hairy, thick fibrous roots

a, b, c: means followed by the same letters in the same column were not significantly different ($p<0.05$).

R1: MS basal salt mixtures+ 0.5 mg/l IAA

R2: MS basal salt mixtures+100 mg/l activated charcoal

R3: MS including vitamins+0.5 mg/l IAA

4. Conclusion

The research "Investigation of effective media for *in vitro* propagation of *Stevia rebaudiana* Bertoni" was carried out and successfully obtained the complete process for plant tissue culture of Stevia. MS medium (including vitamins) supplemented with 0.2 mg/l NAA and 0.15 mg/l BAP was the best for the formation of leaf-derived callus and shoot multiplication from nodal segments. Rooting was done the most effectively in MS medium (basal salt mixtures) including 0.5 mg/l IAA and finally, 100% of plantlets survived in the mixture of soil, sand and decomposed rice straw in the ratio of 1:1:1 (v/v/v), respectively after 3 weeks transferred to the greenhouse. Suggestions

1) The problem of browning will need to be controlled and the medium composition for the multiplication of callus should be studied

2) Shoots can be induced from leaf-derived callus in further research.

5. Acknowledgement

We are grateful to the Plant Genetics Engineering Laboratory, Biotechnology Research and Development Institute, Can Tho University for both financial support and providing necessary facilities for our research.

6. References

(1) Abd Alhady MRA. Micropropagation of *Stevia rebaudiana* Bertoni. A new Sweetening Crop in Egypt. Global Journal of Biotechnology & Biochemistry. 2011; 6(4): 178-182.

(2) Ahmed MB, Salahin M, Karim R, Razvy MA, Hannan MM, Sultana R, Hossain M, Islam R. An Efficient Method for *in vitro* Clonal Propagation of a Newly Introduced Sweetener Plant (*Stevia rebaudiana* Bertoni.) in Bangladesh. American-Eurasian Journal of Scientific Research. 2007; 2(2): 121-125.

(3) Danh X. *In vitro* propagation of tomato (*Lycopersicum esculentum* Miller) [BSc thesis]. Can Tho: Can Tho University; 2010. Vietnamese.

(4) Das A, Gantait S, Mandal N. Micropropation of an Elite Medicinal Plant: *Stevia rebaudiana* Bert. International Journal of Agricultural Research. 2011; 6(1): 40-48.

(5) Das K, Dang R, Rajasekharan PE. Establishment and maintenance of callus of *Stevia rebaudiana* Bertoni under aseptic environment. Natural Product Radiance. 2006 Oct; 5(5): 373-376.

(6) Gupta P, Sharma S, Saxena S. Callusing in *Stevia rebaudiana* (Natural Sweetener) for Steviol Glycoside Production. International Journal of Agricultural and Biological Science. 2010; 1(1): 30-34.

(7) Guruchandran V, Sasikumar C. Organogenic plant regeneration via callus induction in *Stevia rebaudiana* Bert. International Journal of Current Microbiology and Applied Sciences. 2013; 2(2): 56-61.

(8) Hossain MA, Shamim Kabir AHM, Jahan TA, Hasan MN. Micropropagation of Stevia. Int. J. Sustain. Crop Prod. 2008 Jun; 3(4): 1-9.

(9) Jitendra M, Monika S, Ratan SD, Priyanka G, Priyanka S, Kiran DJ. Micropropagation of an Antidiabetic Plant- *Stevia rebaudiana* Bertoni, (Natural Sweetener) in Hadoti Region of South-East Rajasthan, India. Journal of Biological Sciences. 2012 Jul; 1(3): 37-42.

(10) Ojha A, Sharma VN, Sharma V. An efficient protocol for *in vitro* clonal propagation of natural sweetener plant (*Stevia rebaudiana* Bertoni). African Journal of Plant Science. 2010 Aug; 4(8): 319-321.

(11) Sairkar P, Chandravanshi MK, Shukla NP, Mehrotra NN. Mass production of an economically important medicinal plant *Stevia rebaudiana* using *in vitro* propagation techniques. Journal of Medicinal Plants Research. 2009 Apr; 3(4): 266-270.

(12) Saad AIM, Elshahed AM. Recent Advances in Plant *in vitro* Culture. InTech publisher; 2012.