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In vitro Micropropagation of *Stevia rebaudiana* Bertoni in Malaysia

Ummi Nur Ain Abdul Razak, Chong Boon Ong[*], Tiew Sing Yu and Li Kiaw Lau
Faculty of Biotechnology; Twintech International University College of Technology; Sri Damansara Business Park; Kuala Lumpur – Malaysia

ABSTRACT

Stevia rebaudiana *Bertoni is a medicinal plants and commercially use as non-caloric sweetener for diabetic patient. In the present study, a protocol was developed for* in vitro *micropropagation using 6-benzylamino purine (BAP) and Kinetin (Kn) for the formation of multiple shoot proliferation and Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA) for the induction of roots. Maximum shoot formation (7.82 ± 0.7 shoots per explants) was observed on a Murashige and Skoog (MS) medium supplemented with 0.5 mg L^{-1} BAP and 0.25 mg L^{-1} Kn. The maximum number of roots (30.12 ± 2.1 roots per explants) was obtained on a MS medium containing 1.0 mg L^{-1} IBA. The well rooted plantlets were successfully weaned and acclimatized in plant soil with survival rate of 83.3 %.*

Key words: *MS medium, non-caloric sweetener, proliferation*

INTRODUCTION

Stevia rebaudiana (Bert.) Bertoni is a perennial herb, belonging to family Asteraceae. The leaves of Stevia are source of diterpene glycosides, such as stevioside and rebaudiosides, which are estimated to be 100–300 times sweeter than sucrose (Tanaka 1982). The plant originated from the northern regions of South America and grows wild in the highlands of Amambay, located at Iguac district, a border between Brazil and Paraguay. It is being commercially cultivated in China, Taiwan, Thailand, Korea, Japan, India and Malaysia (Jain et al. 2009). Stevia are known as natural non-caloric sweetener because of the presence of stevioside in its leaves. It has been used in a wide range of processed foods as a substitute for conventional sugars, or artificial dietetics especially in Japan (Handro and Ferreira 1989).

Stevia was recommended for diabetic patients because it is non-calorie sweetener and is approved by the Food and Drug Administration (FDA) as a dietary supplement (Bespalhok-Filho and Hattori 1997). According to Summon et al. (2008), the powdered form of stevia leaves possessed both hypoglycemic and body weight reducing effect without any adverse effects for diabetic patients. Stevia leaf extract has the ability to reduce the blood sugar level up to 35.2 % within 6 to 8 h of ingestion (Oviedo 1971). Stevia also can act as an antimicrobial in pharmacological studies and according to Debnath (2008), the crude leaf chloroform extract of *S. rebaudiana* leaves can inhibit *Staphylococcus aureus*, *Streptococcus mutans*, *Bacillus subtilis* and *Escherichia coli*. The large-scale cultivation of stevia plants is important for the human health because of the many benefits it gives.

[*]Author for correspondence: ongchongboon@yahoo.com

Stevia can be propagated using the seed, or stem cutting. However, propagation by the seed is not efficient due to low fertility (Tadhani et al. 2006). Propagation using the seed also causes great variability on stevioside level and composition (Nakamura and Tamura 1985). Seed germination is often poor and rates below 10% are common (Miyazaki and Wantabe 1974). The stem cutting method has limitations such as low number of new plants and destruction of the donor plant. Micropropagation, or *in vitro* culture appears to be the best method to overcome those problems and has the potential to produce large quantity of stevia plantlets in short time. Uddin et al. (2006) established *in vitro* propagation from the leaf, nodal and inter-nodal segments of *S. rebaudiana* Bertoni by using certain plant growth regulators in the medium. Stevia can form multiple shoots from the nodal explants and appears to be suitable for large- scale production. However, there are many varieties and clones of stevia. In Malaysia, the leaves of stevia are lanceolate to oblanceolate in shape and have tiny white florets (Raji and Osman 2012) compared to other countries such as Canada, where the leaves shape range from oblanceolate to ovate to spatulate (Tan et al. 2008). In spite of there being several protocols of micropropagation of *S. rebaudiana*, different clones or varieties of a same species, demonstrate different behaviours in *in vitro* culture. Therefore, it is necessary to adapt this protocol to material genetic specifics (Carvalho et al. 2001). The tissue culture is also a powerful tool that can accelerate the genetic breeding (Alves et al. 2011). This study was undertaken to develop a rapid *in vitro* propagation protocol of *S. rebaudiana* for local environment of Malaysia.

MATERIAL AND METHODS

Plant Material and Surface Sterilization

The plant samples were collected from three months old plants from a nursery at Sungai Buloh, Selangor, Malaysia. The nodal segments comprised the explants. After excision, the explants were placed under running tap water for 15 min. Then, the nodal segments were brushed and soaked in a detergent for 5 min, rinsed with distilled water and surface sterilized with 70% (v/v) ethanol for a few seconds and moved to the laminar flow cabinet. Under laminar flow, the nodal segments were further surface sterilized by immersion in 5% (v/v) sodium hypochlorite for 5 min, rinsed with sterile distilled water three times, followed by immersion in 0.1 % (w/v) mercury (II) chloride, ($HgCl_2$) for 5 min and rinsed with sterile distilled water a further three times.

Culture condition

MS (Murashige and Skoog 1962) basal medium with 30 g L^{-1} of sucrose was used and solidified using 7.5 g L^{-1} of agar. MS medium was supplemented with various concentrations of plant regulators such as 6-benzylamino purine (BAP), Kinetin (Kn), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA). The pH of the media were adjusted to 5.75 – 5.80 before adding the agar and then autoclaved at 120°C for 20 min. All the cultures were incubated in an air-conditioned growth room at 25 ± 2°C with a photoperiod 16 h light and 8 h dark with 25 µMol m^{-2} s^{-1} of light intensity.

Shoot and root induction

For shoot induction and multiplication, the nodal segments were inoculated on the MS medium, supplemented with various combinations of BAP, Kn and a combination of both. The regenerated isolated shoots were used as explants for root induction. Microshoots of about 2-3 cm in length were excised and inoculated on MS medium with IAA, IBA and NAA. The MS medium without any hormones (MS0) was used as a control for both the test.

Acclimatization

Rooted plantlets grown on the MS medium supplemented with 1.0 mg L^{-1} of IBA were taken out from the culture vessels and their roots were carefully washed with distilled water. Then, the rooted plantlets were transferred into black poly planter bags filled with one of three types of soils, which were Soil A, Soil B and a combination of both (1:1). Soil A contained red mineral soil, black soil, cocopeat and vermicompost while Soil B contained top soil, cocopeat, black ash and chicken manure compost. The plantlets were covered with transparent polythene plastics and placed in the growth room at 28 ± 2°C with 70-90% humidity under low light intensity for three weeks. The transparent plastic was opened and the plantlets were placed in the ambient room temperature for two weeks. After that, the plantlets were transferred to field and placed under shade cover for four weeks to avoid direct sunlight.

Data Analysis

Various growth data were recorded such as the number of multiple shoot formation, shoot length, number of roots, root length, and survival of plants during acclimatization. All the studies were made with ten replicates and the mean and standard errors were calculating by using IBM SPSS Statistics software (IBM 2011).

RESULTS AND DISCUSSION

Shoot induction

The highest shoots production was found in the MS medium supplemented with 0.5 mg L^{-1} BAP and 0.25 mg L^{-1} Kn (7.82 ± 0.7) after four weeks of cultivation (Table 1). The combination of low concentration of BAP and low concentration of Kn induced high number of multiple shoots. Higher concentration of BAP resulted decreasing multiple shoots formation of stevia (Sivaram and Mukudan 2003). According to Tadhani et al. (2006), the maximum number of shoots were achieved on MS medium supplemented with 0.6 mg L^{-1} of BAP. However, the nodal segments inoculated in the MS medium supplemented with 1.0 mg L^{-1} Kn produced the longest shoots lengths (5.05 ± 1.0 cm). Das et al. (2011) also reported the longest shoots lengths of stevia when Kn was present in the medium.

Table 1- Effect of various concentrations of PGRs on multiple shoots induction from nod segment of *Stevia rebaudiana* after 4 weeks of cultivation.

Concentration of PGRs (mg L^{-1})		Length of shoots (cm)	Number of shoots
BAP	**Kn**	**Mean ± SE**	**Mean ± SE**
0	0.2	3.50 ± 0.2	3.52 ± 0.5
0	0.5	4.67 ± 0.3	2.10 ± 0.3
0	1.0	5.05 ± 0.2	2.21 ± 0.3
0	1.5	2.90 ± 0.4	2.71 ± 0.7
0.2	0	1.28 ± 0.1	6.12 ± 0.3
0.5	0	1.31 ± 0.1	6.60 ± 0.3
1.0	0	1.41 ± 0.1	6.31 ± 0.5
1.5	0	1.40 ± 0.1	6.10 ± 0.6
0.5	**0.25**	**1.92 ± 0.1**	**7.82 ± 0.7**
1.0	0.25	1.41 ± 0.1	6.90 ± 0.3
1.5	0.25	0.92 ± 0.1	3.60 ± 0.5
2.0	0.25	0.61 ± 0.1	2.30 ± 0.2
0	0	1.20 ± 0.2	2.21 ± 0.2

SE standard errors.

The various concentrations of the hormones used in this study induced the proliferation of multiple shoots derived from the nodal segments of *S. rebaudiana*. Kn was less effective at inducing multiple shoots compared to BAP. According to Rafiq et al. (2007), no specific increase in multiple shoot formation occurred when the explants were cultured on Kn based media. Similar results were found in *Vriesea scalaris*, where Kn alone had no effect in *in vitro* multiplication (Silva et al. 2009). Nevertheless, Kn can be more effective to induce multiple shoot when combined with BAP (Sharuti et al. 2011). According to Anbaznagan et al. (2010), the combination of BAP and Kn work well for booth shoot proliferation and their elongation from the nodal explants.

Root induction

The maximum number of roots (30.12 ± 2.1) induced was observed in the MS medium supplemented with 1.0 mg L^{-1} of IBA. However, the longest root (5.40 ± 0.3 cm) was observed in the MS medium supplemented with 0.5 mg L^{-1} of IAA. The roots in the IBA-containing medium were thick and strong but short. The roots in IAA-containing medium were thin but long. The presence of NAA in the medium induced high number of roots but was inefficient in inducing long roots (Table 2).

Table 2 - Effect of various concentrations of PGRs on root induction from microshoots of *Stevia rebaudiana* after 4 weeks of cultivation.

Concentration of PGRs (mg L^{-1})			Length of roots (cm)	Number of roots
IAA	**NAA**	**IBA**	**Mean ±SE**	**Mean ± SE**
0.2	0	0	5.03 ± 0.2	10.30 ± 1.2
0.5	0	0	5.40 ± 0.3	8.60 ± 1.1
1.0	0	0	4.40 ± 0.1	14.70 ± 1.4
1.5	0	0	5.01 ± 0.1	13.01 ± 1.1
0	0.2	0	3.30 ± 0.4	14.40 ± 0.8
0	0.5	0	2.69 ± 0.3	16.10 ± 1.0
0	1.0	0	2.06 ± 0.1	14.90 ± 1.4
0	1.5	0	1.30 ± 0.1	14.40 ± 0.7
0	0	0.2	2.94 ± 0.3	21.03 ± 0.8
0	0	0.5	2.70 ± 0.2	25.30 ± 0.6
0	**0**	**1.0**	**2.39 ± 0.1**	**30.12 ± 0.6**
0	0	1.5	1.86 ± 0.2	28.30 ± 0.4
0	0	0	2.98 ± 0.4	4.40 ± 1.4

SE standard errors

Hwang (2006) reported the maximum numbers of roots using a treatment of 1.0 mg L^{-1} IBA in the MS medium with up to 100 % rooting. Tadhani et al. (2006) also obtained the highest rate of root induction in 1.0 mg/L IBA medium. Root length was decreased in IBA containing medium and this was accentuated as the IBA concentration increased (Trauttman and Visser 1990). The IBA

seems to be the best auxin in *S. rebaudiana* to the initiation of the root induction. The potential of IBA in root induction has also been reported in many species (Epstein et al. 1993).

Acclimatization

The well-rooted stevia plantlets were shifted into Soil A, which were established in natural condition with a survival rate of 83.3% (Table 3).

Table 3 - Acclimatization of well-developed rooted *in vitro Stevia rebaudiana* in different types of soil after 4 weeks of cultivation.

Soil	Rate of plant survival (%)
Soil A	83.3
Soil B	0
Soil A : Soil B (1:1)	33.3

However, no plants survived when they were planted into Soil B, which was too sandy and failed to maintain the moisture of the soil. Soil A contained the mixture of red mineral soil and showed greater water retention ability and good aeration than the other soil used. The use of a sufficiently porous substratum that allows adequate drainage and aeration has been recommended for fast acclimatization of *in vitro* regenerated plants (Dunstan and Turner 1984).

The use of transparent polythene during acclimatization can improve the survival plants by controlling the humidity and can help in adaptation of the plants into new environment. According to Kramarenko (1999), *in vitro* hardening of explants can be an alternative that could bridge the transplanting shock and speed up the whole propagation procedure. In the current investigation, the plastic covering was essential for the successful weaning. A good example of this *in vitro* hardening was observed in *Eucalyptus saligna*, where the culture flasks were opened for three days (with the addition of distilled water to avoid the dehydration of culture medium and explants). This increased 50% survival rate compared to the control (flasks closed) during acclimatization (Silva et al. 2011).

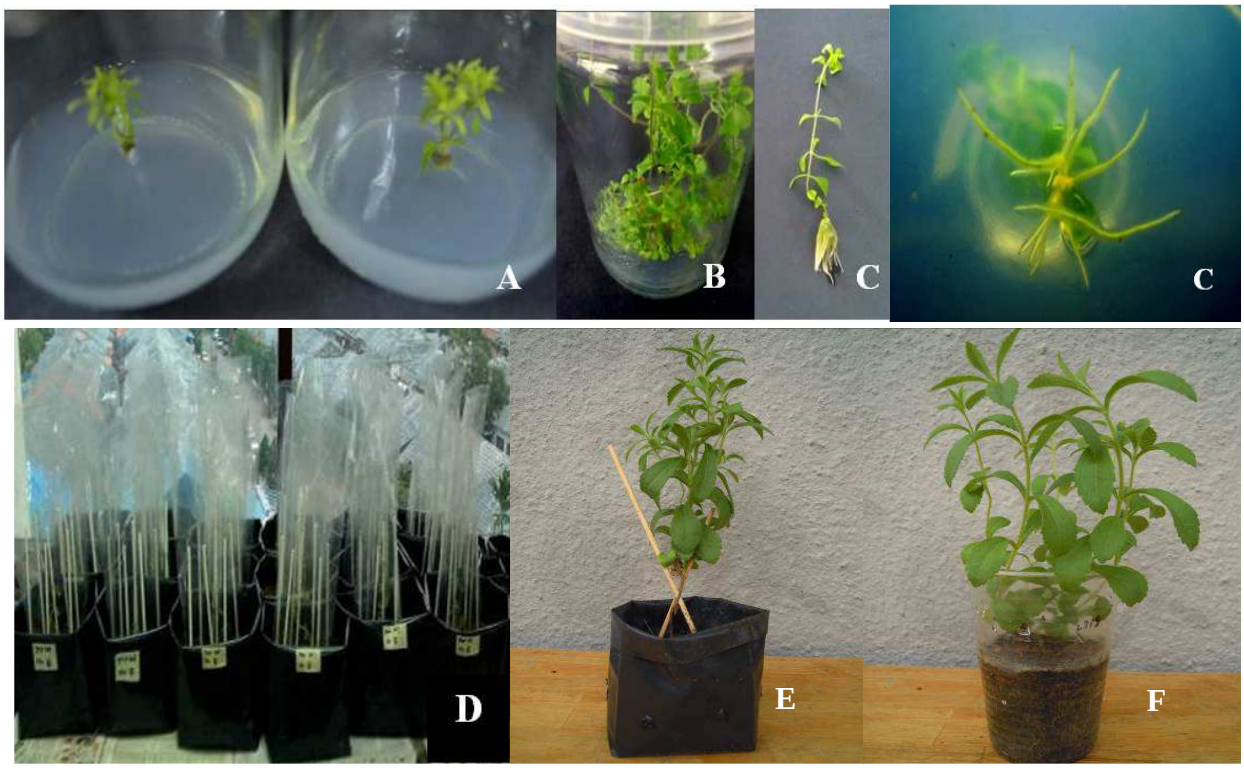

Figure 1 - (A) Initiation of multiple shoot formation of *Stevia rebaudiana* (B) Development of more number of multiple shoot after 4 weeks (C) Root formation from regenerated shoot (D) Transplantation of regenerated plantlets in black poly planter bag (E) Establishment of regenerated plants (F) Well-grown regenerated *S. rebaudiana* Bertoni plant.

CONCLUSIONS

A suitable protocol and composition was developed for rapid *in vitro* micropopagation of *S. rebaudiana* Bertoni clone from Malaysia. The results proved the possibility of *in vitro* propagation of *S. rebaudiana* clone cultivation in Malaysia, demonstrating to be an alternative to increase the production at commercial scale compared to the conventional propagation.

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