Fundamental and Applied Agriculture
Vol. 5(2), pp. 243–247: 2020
doi: 10.5455/faa.103572

Plant Biotechnology
SHORT COMMUNICATION

In vitro responses of *Stevia rebaudiana* (Bert) to MS basal medium supplemented with 6-benzylaminopurine and indole-3-butyric acid

Nor Asikin Asmuni[1], **Mansor Hakiman** [1,2*]

[1]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, Selangor, Malaysia
[2]Institute of Tropical Forestry and Forest Products, Universiti Putra Malaysia, Selangor, Malaysia

ARTICLE INFORMATION

Article History
Submitted: 01 May 2020
Accepted: 29 May 2020
First online: 31 May 2020

Academic Editor
Totan Kumar Ghosh
tkbsmrau@yahoo.com

*Corresponding Author
Mansor Hakiman
mhakiman@upm.edu.my

ABSTRACT

Propagation of *S. rebaudiana* Bertoni can be done by seed germination and stem cutting. Seed germination of this plant is not efficient due to low fertility and having heterogeneous population, thus producing unstable sweetening level. Therefore, micropropagation can overcome this problem by producing large number of plantlets within a short time. In this effort, the effect of different concentrations of plant growth regulators; auxin and cytokinin were observed to find out the multiplication responses of *S. rebaudiana in vitro*. The nodal segments were used as explant and inoculated onto full strength of Murashige and Skoog (MS) media. The medium was supplemented with different concentrations of 6-benzylaminopurine (BAP); 0, 1, 2, 3 and 4 mg L^{-1} and indole-3-butyric acid (IBA); 0, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}. After six weeks of culture, the data regarding number and length of shoots, number and length of roots and callus percentage were recorded. The results showed that among the treatment combinations, 3.0 mg L^{-1} BAP produced the highest number of shoots per explant and application of 1.5 mg L^{-1} IBA showed the best performance for rooting.

Keywords: 6-benzylaminopurine, indole-3-butyric acid, *in vitro* responses, *Stevia rebaudiana*

Cite this article: Asmuni NA, Hakiman M. 2020. *In vitro* responses of *Stevia rebaudiana* (Bert) to MS basal medium supplemented with 6-benzylaminopurine and indole-3-butyric acid. Fundamental and Applied Agriculture 5(2): 243–247. doi: 10.5455/faa.103572

1 Introduction

Stevia rebaudiana Bertoni is a medicinal perennial plant under Asteraceae family that is native to Paraguay and Brazil. Japan was the first Asian country that commercialized stevioside as natural sweetener in food and drug industry (Puri et al., 2011). Since then, the cultivation of *S. rebaudiana* expanding to other Asian countries including China, Thailand and Malaysia. *S. rebaudiana* commonly known as sweet weed, sweet leaf, sweet herbs and honey leaf because the plant contains 'sweet' compounds which is estimated to be 300 times sweeter than sucrose (Jitendra et al., 2012, Fig. 1a,b). Stevioside and rebaudioside A are the most abundant compounds that are responsible for the sweetness of the plants (Taleie et al., 2012). The natives of Paraguay used the leaves to sweeten their traditional drinks (Goyal et al., 2009). Nowadays, *S. rebaudiana* extracts have been widely used as sugar alternative and natural sweetener in food and beverages, medicine and also dietetic supplements.

Recently, there are many ongoing medicinal studies on *S. rebaudiana* that proved the therapeutic values of this plant such as antihyperglycemic, antihypertensive, anti-inflammatory, antioxidant, and antimicrobial activities (Gupta et al., 2014). In Malaysia, the continuous and increasing demands for *S. rebaudiana* products has been observed as people are going to shift their routine dietary into a healthier lifestyle. *S. rebaudiana* extracts and its purified compounds have been commercialized into various products. The local companies that produced stevia products had to import stevia extract or powder from China because

the production of *S. rebaudiana* in this county is still limited to meet the consumer demand. So it is very important to cultivate stevia plants by local growers in order to achieve sufficient supply of stevia.

Conventional propagation of *S. rebaudiana* can be done by two ways; seed or stem cutting. Seed propagation of *S. rebaudiana* is not efficient as the seeds have low viability and producing heterogeneous population resulting variability in important characteristics such as the sweetening level (Taleie et al., 2012). Another propagation technique by using stem cutting seems more efficient than seed propagation as the cuttings respond positively after 3-4 weeks after planting in favorable condition. However, stem cutting has limitations such as lower number of plant can be obtained from a single donor plant, limited stock of stevia stem, high labor and time consuming (Sivaram and Mukundan, 2003). Micropropagation can overcome these problems because it can produce large number of stevia plant within a short period of time. In Malaysia, there are several varieties and clones of *S. rebaudiana* which respond differently in *in vitro* propagation (Razak et al., 2014). So it is very important to determine the best protocol that can be used by majority of the varieties or clones to maximize the production of plantlets. To aid the growth of explants, plant growth regulator can be supplemented to the medium. Cytokinin and auxin have been widely used in *in vitro* propagation to induce shoot and root of explants. Auxin helps in promoting cell division, cell expansion, organization of meristem to unorganized tissue (callus) or defined organ (usually roots) and vascular differentiation (Gaspar et al., 1996). Meanwhile cytokinin aid in release apical dominance, cell division and induce adventitious bud formation (Gaspar et al., 1996). Auxin and cytokinin interaction is really important in plant tissue culture. Higher auxin to cytokinin ratio induce roots while lower auxin to cytokinin ratio increase shoot proliferation (Bernula et al., 2019).

Thus the objectives of this study were to determine the effect of different concentration levels of cytokinin, 6-benzylaminopurine (BAP) and auxin; indole-3-butyric acid (IBA) on *in vitro* responses of *Stevia rebaudiana*. Here we claim a suitable protocol for micropropagation of *S. rebaudiana* that fits the local environment and helps rapid multiplication of this potential medicinal herb which should be necessary to meet the demands of country people and aiding further researches regarding *S. rebaudiana*.

2 Materials and Methods

2.1 Plant materials and location of study

The explants used in this experiment were obtained from Ladang 10, Taman Pertanian Universiti, Universiti Putra Malaysia (UPM). The internodes were used as explants taken from 6-8 nodes from the shoot tip. This study was conducted at Plant Tissue Culture Laboratory, Department of Crop Science, Faculty of Agriculture, UPM.

2.2 Preparation of treatment media

Full strength of MS medium was prepared according to Murashige and Skoog (1962). Each conical flasks containing MS medium were supplemented with different concentration levels of IBA; 0, 0.5, 1.0, 1.5, 2.0 mg L^{-1} and BAP; 0, 1, 2, 3, 4 mg L^{-1}. After that, the pH was adjusted to 5.7 - 5.8 with sodium hydroxide (NaOH) and hydrochloric acid (HCl) followed by addition of 5.5 g L^{-1} Gelrite. All flasks were autoclaved at 121 °C at 0.103 MPa for 20 min. After autoclaving, the medium was allowed to solidify at room temperature prior to inoculation.

2.3 Surface sterilization and inoculation

Explants used in this experiment were the internodes of stevia plants. The internodes were washed under running tap water for about 10-15 min with the addition of a few drops of Tween 20 to remove all the debris and eliminate bacteria and fungus spores. Next, the internodes were surface sterilized with 30% of Clorox® and then rinsed with sterile distilled water. Then the nodes were further surface sterilized once again with 20% of Clorox® and rinsed again for the second time with sterile distilled water. The tip of the single internodes was excised (1 cm) by using scalpel and then transferred onto respective treatment medium. All the culture were kept in culture room at 24 $\pm$ 2 °C, 16:8 photoperiod (days to night ratio) and light intensity, 25 µMol $m^{-1}s^{-1}$. After 6 weeks of culture, number of shoots and roots, length of shoots and roots and callus percentage were recorded.

2.4 Statistical analysis

This study was conducted by using Completely Randomized Design (CRD) in 25 treatments with 5 replications each. The data obtained were subjected to analysis of variance (ANOVA) and means were separated using Tukey's Studentized Range (HSD) Test at $P \leq 0.05$ for comparison between treatment combination means. The analysis was run using Statistical Analysis System (SAS 9.4) computer program.

3 Results and Discussion

The results of treatment combination of IBA and BAP at different concentration levels on average mean number of shoots explant^{-1} was significantly difference. The nodal explants inoculated on MS medium fortified with 3.0 mg L^{-1} BAP in combination with

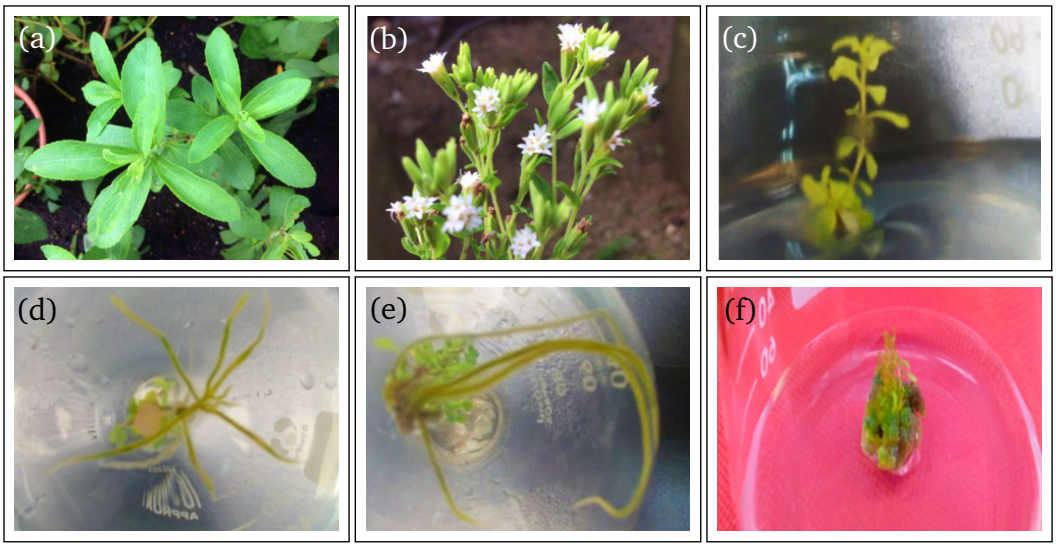

Figure 1. (a) Example of *Stevia rebaudiana* plant, (b) white flowers of *S. rebaudiana*, (c) low number of shoots and short length of shoots of *S. rebaudiana* cultured on MS without IBA and BAP (control, T1), (d) high number of root of *S. rebaudiana* cultured in MS supplemented with 1.5 mg L^{-1} IBA without any BAP (T16), (e) longest length of root of *S. rebaudiana* cultured in MS supplemented with 1.0 mg L^{-1} IBA without any BAP (T11), and (f) growth of callus of *S. rebaudiana* in MS supplemented with 1.0 mg L^{-1} BAP + 1.5 mg L^{-1} IBA (T17).

Table 1. Effect of different concentrations of IBA and BAP on growth parameters of *Stevia rebaudiana* after 6 weeks of culture

Treatment	PGR conc. (mg L^{-1})		No. of shoots	Length of	No. of roots	Length of	Callus
	BAP	IBA	explant^{-1}	shoots (cm)	explant^{-1}	roots (cm)	percentage
T1	0.0	0.0	1.4 e	1.46 cdef	nd	nd	nd
T2	1.0	0.0	1.6 e	0.82 ghij	nd	nd	nd
T3	2.0	0.0	3.4 abcd	1.74 bcd	nd	nd	nd
T4	3.0	0.0	4.4 a	1.28 defg	nd	nd	nd
T5	4.0	0.0	3.4 abcd	0.80 ghij	nd	nd	nd
T6	0.0	0.5	3.0 abcde	2.12 b	nd	nd	nd
T7	1.0	0.5	1.8 de	0.86 ghij	nd	nd	80 b
T8	2.0	0.5	4.2 ab	0.96 fghij	nd	nd	100 a
T9	3.0	0.5	4.4 a	0.74 ghij	nd	nd	nd
T10	4.0	0.5	3.8 abc	1.04 fghij	nd	nd	nd
T11	0.0	1.0	1.4 e	3.50 a	4.0b	2.28 a	nd
T12	1.0	1.0	1.4 e	1.18 defgh	nd	nd	60 c
T13	2.0	1.0	2.4 cde	1.96 bc	nd	nd	100 a
T14	3.0	1.0	2.6 bcde	1.64 bcde	nd	nd	nd
T15	4.0	1.0	2.4 cde	1.70 bcd	nd	nd	nd
T16	0.0	1.5	1.6 e	2.96 a	5.6a	1.5b	nd
T17	1.0	1.5	1.8d e	1.12 efghi	nd	nd	100 a
T18	2.0	1.5	2.4 cde	0.78 ghij	nd	nd	100 a
T19	3.0	1.5	3.6 abc	1.12 efghi	nd	nd	100 a
T20	4.0	1.5	2.4 cde	0.78 ghij	nd	nd	100 a
T21	0.0	2.0	1.4 e	1.08 efghij	nd	nd	100 a
T22	1.0	2.0	1.4 e	0.58 ij	nd	nd	100 a
T23	2.0	2.0	1.8 de	0.66 hij	nd	nd	100 a
T24	3.0	2.0	1.4 e	0.52 j	nd	nd	100 a
T25	4.0	2.0	1.4 e	0.70 hij	nd	nd	100 a

Means with the same letter column are not significantly different by Tukey's Studentized Range (HSD) Test at $P \leq 0.05$; nd = not developed.

0 and 0.5 mg L^{-1} IBA (T4 and T9, respectively) produced the highest number of shoots explant^{-1} with similar average mean of 4.4 number of shoots explant^{-1} (Table 1). But these two treatments combination is not significantly difference with T3, T5, T6, T8, T10 and T19. From 0 - 3.0 mg L^{-1} BAP without any IBA (T1 - T4), the number of shoots increased but when the medium supplied with 4 mg L^{-1} BAP, the number of shoots per explant was declined and appeared smaller. This result is in agreement with study reported by Jitendra et al. (2012) who found out that 3.0 mg L^{-1} BAP evoked the best response compared to other treatments.

There was a significant different between treatments combination of IBA and BAP at different concentrations on the average mean length of the shoots of *S. rebaudiana*. From Table 1, the maximum length of shoot observed at 1.0 mg L^{-1} IBA with average mean 3.5 cm (T11). Razak et al. (2014) demonstrated 1.0 mg L^{-1} kinetin produced longest shoot length with average mean (5.05 cm $\pm$ 1.0 cm). From this data, it proved that 1.0 mg L^{-1} of auxin (IBA and kinetin) induced shoot elongation. But T11 was not significantly different with T16. T24 (3.0 mg L^{-1} BAP + 2.0 mg L^{-1} IBA) showed the shortest shoots with average mean 0.52 cm. These might be due to the presence of high concentration of auxin in the medium that suppressed the induction of shoots.

In the case of root formation, no significant effects of the treatment combinations of BAP and IBA was found rather sole application of IBA at 1.0 mg L^{-1} or 1.5 mg L^{-1} that contributed to root formation (Table 1). Positive results for *in vitro* root formation can be observed in MS medium supplemented with 1.0 mg L^{-1} or 1.5 mg L^{-1} IBA only without the presence of BAP (T11 and T16). The highest number of roots explant^{-1} produced in 1.5 mg L^{-1} IBA with average mean of 5.6 number of roots explant^{-1}. Treatments with 1.0 mg L^{-1} IBA showed lower number of roots explant^{-1} with mean of 4.0 number of roots explant^{-1}. According to Ahmadiyan et al. (2014), 2.0 mg L^{-1} IBA can produce roots but low number of roots explant^{-1} produced. However, in this present study, medium supplemented with 2.0 mg L^{-1} IBA did not produced any root formation. Ahmadiyan et al. (2014) further explained that lower concentration of auxin can induce growth and cell division but higher concentration of the same auxin might cause death to the plant. However, low concentration of IBA at 0.5 mg L^{-1} did not produced any root formation. This is related to the formation of callus which inhibits the root production. Due to genetic variation in plants, supplementation of auxin to the basal medium might induce root and/or callus formation.

There was significant difference between treatment combinations of IBA and BAP at different concentrations on the mean length of roots. The MS medium supplemented with 1.0 mg L^{-1} IBA showed longest root formation with mean 2.28 cm meanwhile 1.5 mg L^{-1} IBA showed shorter mean length of roots; 1.5 cm. Hence, it can be concluded that MS supplemented with 1.5 mg L^{-1} IBA can produce more number of roots explant^{-1} (Fig. 1d) but shorter in the length of the root while MS supplemented with 1.0 mg L^{-1} IBA produce less number of roots explant^{-1} but longer in length (Fig. 1e).

There was significant difference between treatments combination of IBA and BAP on the callus percentage of *S. rebaudiana*. From Table 1, data showed that not all the treatments gave positive result to the development of callus. Treatments with 0.5 and 1.0 mg L^{-1} IBA only showed development of callus with the presence of 1.0 and 2.0 mg L^{-1} BAP. When concentration of BAP is too low; 0 mg L^{-1} BAP or too high; 3.0 and 4.0 mg L^{-1} BAP combined with 0.5 mg L^{-1} and 1.0 mg L^{-1} IBA, no callus was developed. Meanwhile, all treatments combination of 1.5 and 2.0 mg L^{-1} IBA except T16 gave positive results to the development of callus with combination of all concentrations of BAP.

4 Conclusion

High concentration of cytokinin (BAP) induces more shoot formation while auxin (IBA) induces the initiation of rooting. For the indirect regeneration, combination of auxin and cytokinin are mandatory for the initiation of callus. However further investigations involving other phytohormones and plant parts are needed to fine tune the protocol of micropropagation of *S. rebaudiana*.

Acknowledgments

The authors wish to thank the Ministry of Education Malaysia and Universiti Putra Malaysia for financing the present study through Translational Research Program, 5526700 and Putra Grant – Putra Young Initiative, 9629000.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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The Official Journal of the **Farm to Fork Foundation**
ISSN: 2518–2021 (print)
ISSN: 2415–4474 (electronic)
http://www.f2ffoundation.org/faa