ISSN 0258-7122 (Print), 2408-8293 (Online)
Bangladesh J. Agril. Res. 50(1): 13-22, March 2025

DEVELOPMENT OF IN VITRO PROPAGATION PROTOCOL FOR Stevia (*Stevia rebaudiana* Bertoni)

T. NUSRAT[1], Z. TASNIM[2], T. K. GHOSH[3], S. M. N. ISLAM[4*] AND M. A. HAQUE[5*]

Abstract

Stevia rebaudiana Bertoni an anti-diabetic medicinal herb is cultivated throughout the world for economic and medicinal values. However, conventional propagation methods are inefficient due to seed dormancy and slow growth rates. In this study, *in vitro* propagation protocol has been developed through indirect organogenesis for the production of healthy plantlets. Nodal and leaf explants were sterilized and cultured on MS medium containing various concentration and combination of Plant Growth Hormone (PGRs). Shoot induction was highest (100%) with Kinetin (0.5 mg L^{-1}) while shoot proliferation (14.57 shoots/explant) was improved with Kinetin (1.0 mg L^{-1}) + 6-Benzylaminopurine (BAP) (0.2 mg L^{-1}). The highest rooting (100%) was observed in the MS medium supplemented with Indole-3-acetic acid (IAA) at 0.2 mg L^{-1} and Naphthalene Acetic Acid (NAA) at 0.5 mg L^{-1}. Hardened plantlets were successfully acclimatized to the sand and showed the maximum survival rate (87.5%) with similar morphology to the mother plants. Therefore, this study offers a valuable alternative to overcome the limitations of conventional propagation methods contributing to the commercial production of *Stevia rebaudiana* in Bangladesh and similar agro-climatic regions.

Key words: Nodal explant, Indirect organogenesis, *In vitro* propagation, *Stevia rebaudiana*

Introduction

Stevia is a non-caloric natural sweet herb originated in Paraguay. Stevia leaves accumulate bioactive compounds, Steviol glycosides (SGs) (less than 1% of each leaf), which is 250-300 times sweeter than commercial sucrose (Ashwell, 2015). In 2008, the Food and Drug Administration approved SGs as a safer and healthier sugar substitute for diabetes and obesity because they stimulate insulin secretion and reduce blood glucose levels (Marcinek and Krejpcio, 2015). The increasing trend of global diabetes and obesity also increases the global importance of natural sweeteners. For this reason, stevia has become more prevalent as a significant crop in several developed and developing countries worldwide for food and

[1,4&5]Institute of Biotechnology and Genetic Engineering (IBGE), Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur-1706, [2]Department of Agroforestry and Environment, BSMRAU, Gazipur-1706, and [3]Department of Crop Botany, BSMRAU, Gazipur-1706, Bangladesh.

pharmaceutical use (Hossain *et al*. 2017). Clonal propagation was widely used commercial propagation method. Nevertheless, stevia cultivation is not profitable due to high labor costs, large input stocks, and obtaining quality planting materials are the main limitations. The micropropagation of plants by using shoot tips or axillary buds can provide genetically uniform plants. Various reports have been published on successful multiplication and *in vitro* rhizogenesis (Singh *et al*., 2017; Rasouli *et al*., 2021). Subsequently, the maximum survivability of *in vitro* regenerated stevia plantlets has been reported by many researchers (Soliman *et al*., 2014) but their success in the hardening process is low. Therefore, this study aimed to optimize the *in vitro* propagation protocol of *S. rebaudiana* to achieve the higher survival rate of acclimatized plants for large-scale propagation.

Materials and Methods

Explant preparation and culture conditions

Healthy shoots with nodal buds (5-6 cm) were collected from field-grown Stevia genotype at Bangladesh Sugarcrop Research Institute (BSRI), Ishwardi, Pabna, Bangladesh. After washing under running tap water for 15-30 minutes, explants were resized to 1.5 -2.5 cm. Then the explants were surface sterilized using 0.1% $HgCl_2$ and 1% Sodium hypochlorite and ethanol for different time duration. The explants were rinsed with sterile distilled water (SDW) and cultured on MS (Murashige and Skoog, 1962) medium supplemented with 3 % sucrose and 0.7 % agar and BAP (0.2 mg L^{-1}) and 2,4 D (1 mg L^{-1}). The medium was adjusted at 5.7 and autoclaved at 121°C for 20 min. Cultures were incubated at 25±2ºC, 55-60% humidity under florescent light (33.73 µmol $m^{-2}s^{-1}$) intensity for 16/8 hours (light/dark cycle).

Shoot induction and shoot proliferation

Surface sterilized nodal explants (0.5-1.5 cm) were cultured on MS medium containing different concentrations of BAP or Kin (0.5, 1.0, 1.5, 2, 2.5 mg L^{-1}) for shoot induction, as both BAP and Kin are crucial for cell division, shoot initiation. Multiple shoots were induced on MS medium with BAP and Kin at different concentrations (0.5, 1.0, and 1.5 mg L^{-1}).

In vitro root formation

In this stage, elongated shoots (2-5 cm in length and about 8–12 leaves) were transferred onto MS medium supplemented with different concentrations of auxin (0.5, 1.0, 1.5, 2.0, and 2.5 mg L^{-1} of IAA, Indole-3-butyric acid (IBA) and NAA each.

Acclimatization

Healthy-rooted plantlets (3 to 4 cm) with expanded leaves were transferred in the controlled environment for approximately 2-3 days for initial adaptation (Figure 3B). Afterward, the plantlets were gently removed from the culture tubes so that the roots were not damaged. Then, the plantlets were washed and dipped in 0.1% Bavistin, transplanted into plastic pots containing one of the following substrates: Garden soil, Sand, Cocopeat, Sand + Garden soil (1:1), Sand + Cocopeat (1:1), Garden soil + Cocopeat (1:1). After two weeks, polythene bag were gradually removed to acclimatize the plantlets under natural conditions.

Statistical analysis

All statistical analyses were performed with Statistix-10 and the Microsoft Excel 2010. All the experiments were set up in a completely randomized design (CRD) with four replicates with 12 explants per replication. Data represent the mean± SE (n = 4) and were analyzed with one-way ANOVA, mean values were separated by the Tukey's HSD. All statistical analyses were carried out with Statistix-10 (Trial version) and the Microsoft Office Excel 2010 program package.

Results and Discussion

Effect of cytokinins on direct organogenesis

Cytokinins are the most influential group of endogenous hormones for all plant species. In this study, the induction of healthy green shoots was observed from nodal explant after four weeks of culture. The MS medium supplemented with Kinetin at 0.5 mg L^{-1} induces the highest percent of shoots (100%) compared to the hormone free medium. (Fig. 1, Table 1). Similarly, Razak *et al*. (2014) found lower concentration of kinetin for highest number of shoot production. Shoot number is also affected by Cytokinin concentration. Kin at 1.0 mg L^{-1} produced a maximum of seven shoots from nodal explant within one week of culture. Increasing the concentration of Kin from 1.0 to 2.5 mg L^{-1} decreased the establishment percentage at a 5 % level. Among the different BAP, concentrations applied with MS medium, only BAP at 0.5 mg L^{-1} induced maximum percentages (91.6%) of shoot bud with 4.42 shoots/explant. As shown in Table 1, increasing the concentration of BAP from 0.5 to 2.5 mg L^{-1} significantly decreased the establishment rate. However, the higher concentrations of BAP caused the earlier blooming (5th day) with hyper hydrous buds. The shoot induction study showed that both cytokinin performed well and were almost equally effective. Considering the importance of shoot multiplication, it is beneficial and economical to use Kin (as lower concentration is very effective in induction of shoots and no. of shoots) in MS media for micropropagation instead of other cytokinin (Abu-Romman *et al*., 2015).

Table 1. Effects of MS medium with cytokinins at various concentrations on cultures establishment from nodal explant of *Stevia rebaudiana*

Plant growth regulators (mg L^{-1})	Days to shoot bud initiation	Shoot induction (%)	Shoot number $explant^{-1}$
Control	15.25±0.27^{a}	31.24±2.08^{e}	1.05±0.17^{h}
BAP (0.2)	11.18±0.65^{b}	64.57±2.08^{c}	3.5±0.18d^{e}
BAP (0.5)	9.43±0.25^{c}	91.66±0^{a}	4.42±0.12^{c}
BAP (1.0)	8.0±0.54^{cd}	81.24±2.08^{b}	5.42±0.12^{b}
BAP (1.5)	5.42±0.14^{f}	72.91±2.08^{bc}	3.67±0.28^{d}
BAP (2.0)	5.25±0.21^{f}	64.57±2.08^{c}	2.62±0.18^{f}
BAP(2.5)	6.06±0.12^{ef}	47.91±2.08^{d}	1.67±0.18^{g}
Kin (0.2)	6.18±0.27^{ef}	97.91±2.08^{a}	3.17±0.09^{e}
Kin (0.5)	7.37±0.21^{de}	100±0^{a}	5.32±0.17^{b}
Kin (1.0)	6±0.27^{ef}	95.83±2.4^{a}	7.22±0.15^{a}
Kin (1.5)	5.43±0.21^{f}	77.08±2.08^{b}	5.55±0.12^{b}
Kin (2.0)	5.18±0.18^{f}	66.66±0^{c}	4.2±0.14^{c}
Kin (2.5)	7.18±0.12^{de}	45.83±2.4^{d}	5.2±0.14^{b}

Data represent the mean± SE (n = 12, replication = 4) after 4 weeks of culture. Mean data in each column followed by the same letter in superscript are not significantly different at $P \leq 0.05$ as determined by Tukey's HSD test.

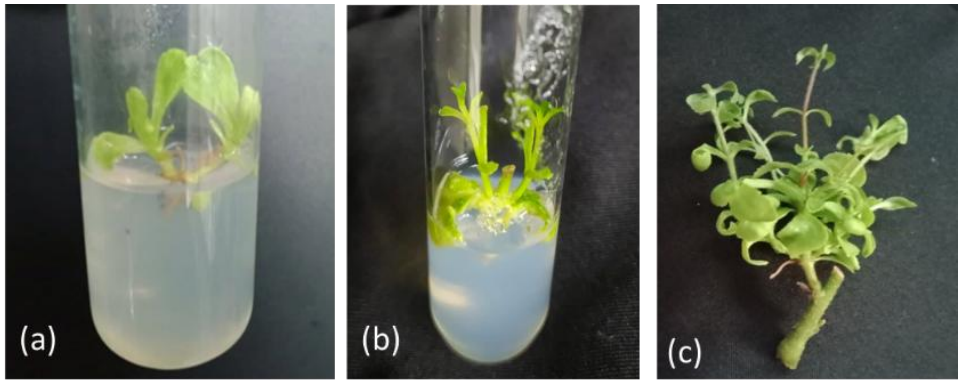

Fig. 1. Shoot induction of *S. rebaudiana* (a) BAP (2.0 mg L^{-1}); (b) Kin (0.2 mg L^{-1}); (c) Kin (0.5 mg L^{-1}).

Effects of PGRs on shoot proliferation

Following the shoot induction, 2 cm long shoots were transferred on the shoot proliferation medium. MS medium with Kin 1.0 (mg L^{-1}) and BAP (0.2 mg L^{-1}) was found to be the best for shoot proliferation (95%) with 14.57 shoots/culture. MS medium supplemented with 0.5 mg L^{-1} BAP was recorded maximum number of shoots (7.12 shoots/explant) compared to all the BAP treatments tested (Fig. 2, Table 2). The shoot proliferation frequency, number of shoot buds, and length of shoots were negatively affected by increasing the Kin and BAP concentration to 1.5 mg L^{-1}. Similar effects also observed with on MS medium supplemented with higher concentration of Kin (1.5 mg L^{-1}) compared to the other Kin treatments with maximum number of shoots (12.52 shoots/explant). However, increasing concentrations of Kin from 1.0 to 1.5 mg L^{-1} significantly increased the shoot number (Figure 2). Das *et al*. (2011) reported Kin (2.0 mg L^{-1}) resulted most effective in multiple shoot proliferation in stevia. Moreover, Razak *et al*. (2014) observed that BAP at 1.5 mg L^{-1} with Kinetin showed the maximum response for shoot proliferation. Considering the importance of shoot proliferation, it is beneficial and economical to use a combination of Kin (1.0 mg L^{-1}) and BAP (0.5 mg L^{-1}) micro propagation.

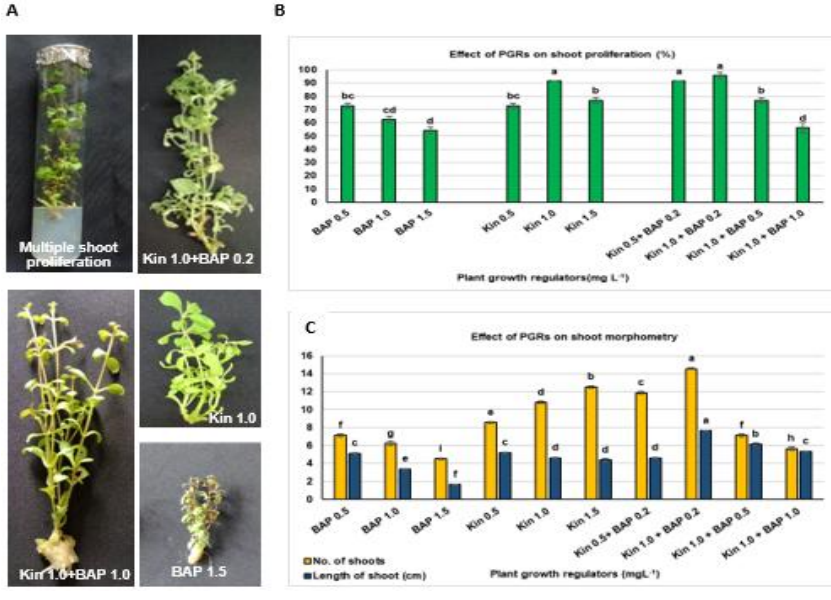

Fig. 2. Effects of growth regulators combination on shoot proliferation from micro shoots of *S. rebaudiana* (A) Morphology (B) percentage of shoot proliferation and (C) shoot morphometry from micro shoots. The photos in (A) were taken after 4 weeks of culture.

Data represent the mean± SE (n = 12, replication = 4) and mean data followed by the same letter in superscript are not significantly different at $P < 0.05$ as determined by Tukey's HSD test.

In vitro rooting

Root induction is one of the important limiting factors in plant propagation. In this study, MS-medium supplemented with 0.2 mgL^{-1} of IAA induces significantly highest percent (100%) root with maximum number of roots and root length obtained was 10.13 and 5.35cm roots/shoot, respectively compared to control (27%) (Figure 3a, Table 2). This result is in agrees with Soliman *et al*. (2014), and Yucesan *et al*. (2016), who found the highest rooting on MS medium augmented with low concentration of IAA. However, Yesmin (2019) found MS medium containing lower concentration of IBA is best for rooting. MS media fortified with intermediate concentration of IAA and IBA were found to produce more thick and long roots (IBA 1.0 mg L-1) than lower and higher concentrations (IBA 1.5 mg L^{-1}).

Table 2. Effects of auxins (IAA, IBA, NAA) on the rooting of *S. rebaudiana*

Plant growth regulators (mg L^{-1})	Days to rooting	Rooting (%)	Root number explant $^{-1}$	Root length (cm)
Control	25.12±.31^{a}	27.08±2.08^{f}	2.23±0.01^{f}	3.01±0.20^{c}
IAA (0.2)	15.25±.27^{b}	100±0.00^{a}	10.13±0.06^{a}	5.35±0.23^{a}
IAA (0.5)	11.18±.65^{cd}	81.24±2.08^{b}	8.37±0.12^{b}	3.14±0.12^{c}
IAA (1.0)	10.18±.27^{cd}	66.66±0.00^{c}	6.56±0.01^{c}	1.97±0.17^{f}
IAA (1.5)	11.56±.21^{c}	47.91±2.08^{e}	3.46±0.10^{e}	1.32±0.09^{g}
IAA (2.0)	15.18±.29^{b}	12.49±2.40^{h}	1.53±0.01^{g}	0.7±0.08^{h}
IBA (0.2)	10.18±.27^{cd}	77.08±2.08^{b}	3.66±0.07^{d}	1.87±0.09^{f}
IBA (0.5)	9.18±.12de	64.57±2.08^{c}	6.57±0.06^{c}	2.65±0.12^{d}
IBA (1.0)	8±.54^{e}	54.16±2.4d^{e}	3.66±0.07^{de}	2.37±0.18^{de}
IBA (1.5)	12.25±.25^{c}	47.91±2.08^{e}	2.24±0.01^{f}	1.15±0.25^{g}
IBA (2.0)	10.93±.59^{cd}	22.91±2.08f^{g}	0.98±0.07^{h}	0.79±0.07^{h}
NAA (0.2)	11.81±.10^{c}	81.24±2.08^{b}	3.71±0.08^{d}	2.47±0.12^{de}
NAA (0.5)	9.31±.23de	64.57±2.08^{c}	8.34±0.02^{b}	4.17±0.09^{b}
NAA (1.0)	10.93±.59^{cd}	58.33±0.00^{cd}	3.67±0.09^{d}	2.21±0.04^{ef}
NAA (1.5)	11.12±.63^{cd}	27.08±2.08^{f}	1.53±0.02^{g}	1.44±0.02^{g}
NAA (2.0)	11±.60^{cd}	14.57±2.08^{gh}	0.86±0.02^{h}	0.48±0.02^{h}

Data represent the mean± SE (n = 12, replication = 4) after 4 weeks of culture. Mean data in each column followed by the same letter in superscript are not significantly different at $P < 0.05$ as determined by Tukey's HSD test.

Although, IBA at a lower concentration (0.5 mg L^{-1}) shows an average number of shoots per explant. NAA alone (0.2 mg L^{-1}) also showed better rooting (82.3%), a maximum of 8.34 roots per shoot after 12 days of cultivation as compared to IBA augmented media. The rooting percentage decreased with increasing the concentration of all auxins. However, with the higher concentration of IBA, IAA at 2.0 mg L^{-1} and NAA (1.5 and 2.0 mg L^{-1}) were found less effective in percent root induction. But maximum shoot length with the least number of roots per plantlet was observed when the MS medium was augmented with 2.0 mg L^{-1} of IBA. From root induction study, it is observed that MS media supplemented with all three auxins at lower concentrations (individually) performed well and were almost equally effective. Therefore, considering the importance of plant survival, it is beneficial and economical to use IAA in MS media for micro propagation instead of other auxins.

Acclimatization

The acclimatization of *in vitro* regenerated plants was the most difficult and labor consuming step because the newly transplanted plantlets were highly susceptible to fungal contamination. Garden soil alone has no beneficial effects on in vitro-raised stevia plantlets due to necrosis and contamination. Higher survival values (87.5%) were recorded on the sand with the average root number (17.5), most extended root length (8.41cm), and leaves (105) per plant (Table 3, Fig. 3C). However, 43% of plantlets survived with lower root numbers on garden soil. These results agree with Sreedhar *et al*. (2008), who successfully established (more than 90%) *in vitro* stevia plantlets on the sand. The different mixtures of sand: coco peat (1:1), sand: garden soil (2:1), and garden soil: coco peat (1:1) also influenced plant height and the number of leaves. It was found that after transplanting on sand: garden soil and sand: coco peat, 75% and 65% of adapted plants survived respectively, with an average number of leaves of 80 and 52 per plant. The Rest of the substrates had inhibiting effects on growth and development, resulting in low acclimatization rates.

Fig. 3. Effect of different hardening media on plantlets survival during acclimatization of *S. rebaudiana* (A) Morphology of in vitro rooted plantlets (B) Acclimatization of plantlet in culture room; (C) Ex vitro plant on sand with greater number of leaves after successful establishment under natural field conditions.

Coco peat alone (33.3%) or mixing with the garden soil (35.5%) proved to be the least beneficial growth medium compared to sand (87%). Because, with frequent misting, water logging occurred around the plantlets, and they started to rot, leading to reduced survival. However, plant heights are significantly taller than ther plant heights. There was no detectable variation among the potted regenerants of *S. rebaudiana* plants and mother plants concerning morphological and growth characteristics. The in vitro- derived plantlets have a characteristic feature of a poorly developed epicuticular waxy layer. This leads to uncontrolled foliar water loss when the plants are taken out from the culture vessels. However, when the plants are kept at high humidity conditions, they synthesize more epicuticular wax, which enhances survival success during acclimatization. Besides, the in-vitro raised plants have poorly developed epicuticular waxes, more stomata per unit area, and raised guard cells with wide openings, which result in more transpiration losses and less survival of plantlets.

Table 3. Effects of different hardening media on the acclimatization of *S. rebaudiana*

Hardening media	Survival rate (%)	Plantlets height (cm)	Root number explant $^{-1}$	Root length (cm)	Leaf numbers
Garden soil	45.83±2.4^{c}	17.27±0.11^{c}	7.57±0.11^{c}	3.47±0.02^{f}	43.27±0.87^{d}
Sand	87.50±4.17^{a}	20.6±0.03^{b}	17.47±0.08^{a}	8.41±0.04^{a}	105.09±1.25^{a}
Cocopeat	33.33±3.40^{c}	25.5±0.06^{a}	6.37±0.18^{d}	5.63±0.06^{d}	24.58±0.87^{e}
Sand + Garden soil (1:1)	75.00±00^{ab}	14.33±0.03^{f}	13.85±0.06^{b}	6.56±0.34^{b}	80.42±5.16^{b}
Sand + Cocopeat (1:1)	64.57±2.08^{b}	16.43±0.03^{d}	6.82±0.37c^{d}	6.35±0.23^{c}	51.9±0.89^{c}
Garden soil + Cocopeat (1:1)	35.41±3.99^{c}	15.54±0.21^{e}	5.37±0.16e	3.92±0.01^{e}	47.25±1.3^{cd}

Data represent the mean± SE (n = 12, replication = 4) after six weeks of culture. Bold characters denote significant responses and values. Mean data in each column followed by the same letter in superscript are not significantly different at $P < 0.05$ as determined by Tukey's HSD test.

Conclusion

This study developed an efficient micro-propagation protocol for *Stevia rebaudiana* using tissue culture techniques. During the proliferation stage, optimal shoot growth was achieved using low concentrations of BAP (0.2 mg L^{-1}) and Kinetin (1.0 mg L^{-1}), while rooting was induced with low concentrations of IAA

(0.2 mg L^{-1}) and NAA (0.5 mg L^{-1}). Then, the regenerated plantlets were acclimatized on various hardening media and evaluated under field conditions. Quantitative evaluations confirmed that plants grown in sand showed morphological characteristics identical to the mother plant. These findings suggest that this protocol is highly suitable for the large-scale commercial propagation of stevia and efficient alternative to conventional cultivation propagation techniques.

Acknowledgement

This study was funded by the National Science and Technology Fellowship, Bangladesh. Authors are thankful to Kuasha Mahmud, Bangladesh Sugarcrop Research Institute, Sheikh Mansoor, Sher-e Kashmir University of Agricultural Sciences and Technology, India.

References

Abu-Romman, S. M., Al-Hadid, K. A. & Arabiyyat, A. R. 2015. Kinetin is the most effective cytokinin on shoot multiplication from cucumber. *Journal of Agricultural Science* **7(10),** 159.

Ashwell, M. 2015. Stevia, nature's zero-calorie sustainable sweetener: A new player in the fight against obesity. Nutrition today **50(3),** 129-134.

Das, A., S. Gantait and N. Mandal. 2011. Micro propagation of an elite medicinal plant: *Stevia rebaudiana Bertoni*. *International Journal of Agricultural Research* 6: 40-48.

Hossain, M. F., M.T. Islam, M.A. Islam and S. Akhtar. 2017. Cultivation and uses of stevia (*Stevia rebaudiana Bertoni*): A review. *African Journal of Food, Agriculture, Nutrition and Development* **17:** 12745-12757.

Marcinek, K. and Z. Krejpcio. 2015. *Stevia rebaudiana* Bertoni-chemical composition and functional properties. *Acta Scientiarum Polonorum Technologia Alimentaria* **14:** 145-152.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15:** 473-497.

Rasouli, D., S. Werbrouck, B. Maleki, H. Jafary and V. Schurdi-Levraud. 2021. Elicitor-induced *in vitro* shoot multiplication and steviol glycosides production in *Stevia rebaudiana*. *South African Journal of Botany* **137:** 265-271.

Razak, U. N. A. A., C. B. Ong, T. S. Yu and L. K. Lau. 2014. *In vitro* micropropagation of *Stevia rebaudiana* Bertoni in Malaysia. *Brazilian Archives of Biology and Technology* **57:** 23-28.

Singh, M., V. Saharan, D. Rajpurohit, Y. Sen, A. Joshi and A. Sharma. 2017. Thidiazuron induced direct shoot organogenesis in *Stevia rebaudiana* and assessment of clonal fidelity of regenerated plants by RAPD and ISSR. *International Journal of Current Microbial Applied Science* **6:** 1690-1702.

Soliman, H. I. A., E. M. R. Metwali and O. A. H. Almaghrabi. 2014. Micropropagation of *Stevia rebaudiana* Betroni and assessment of genetic stability of *in vitro* regenerated

plants using inter simple sequence repeat (ISSR) marker. *Plant biotechnology* **31:** 249-256.

Sreedhar, R. V., L. Venkatachalam, R. Thimmaraju, N. Bhagyalakshmi, M.S. Narayan and G. A. Ravishankar. 2008. Direct organogenesis from leaf explants of *Stevia rebaudiana* and cultivation in bioreactor. *Biologia Plantarum* **52:** 355-360.

Yesmin, S. 2019. *In vitro* micropropagation of *Stevia rebaudiana* Bertoni. *Plant Tissue Culture and Biotechnology* **29(2):** 277-284.

Yücesan, B., A. Mohammed, R. Büyükgöçmen, C. Altuğ, O. Kavas, S. Gürel and E. Gürel. 2016. *In vitro* and *ex vitro* propagation of *Stevia rebaudiana* Bertoni with high Rebaudioside-A content—A commercial scale application. *Scientia Horticulturae* **203:** 20-28.