Development of a protocol for *in vitro* regeneration of *Stevia rebaudiana* Bertoni

ABSTRACT

The experiment was conducted to find out an effective combinations and concentrations of plant growth regulators for *in vitro* regeneration of *Stevia rebaudiana* Bertoni, a medicinally important, zero-calorie value, sweet tasted and an antidiabetic herb. Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of BAP and NAA were used for shoot regeneration using shoot tip as explant. The highest shoot regeneration percentage (83.33%) was obtained from the MS medium supplemented with 1.0 mg/l BAP + 2.0 mg/l NAA followed by 72.92% of shoot regeneration from MS medium supplemented with 3.0 mg/l BAP + 3.0 mg/l NAA. The lowest percentage (29.17%) of shoot regeneration was observed in MS medium with 0 mg/l BAP + 1.0 mg/l NAA. Significant numbers of healthy and vigorous shootlets were proliferated showing normal phenotypes. The *in vitro* proliferated shoots were inoculated on MS medium supplemented individually with 0, 1.0, 6.0 mg/l IBA for root initiation. Among the treatments used for root initiation, the highest percentage (75 %) of root formation was observed in MS medium supplemented with 6 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l NAA followed by 71.43 % in MS medium with 6 mg/l IBA + 3.0 mg/l BAP + 3.0 mg/l NAA. In contrast, the lowest percentage (14.28 %) of root formation was obtained in MS medium supplemented with 6 mg/l IBA + 0 mg/l BAP + 1.0 mg/l NAA. For mass production of healthy Stevia plantlets, MS media supplemented with 1.0 mg/l BAP + 2.0 mg/l NAA, in combination with MS media supplemented with 6 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l NAA could be used for further proliferation of the plantlets for commercial purpose.

Key words: *Stevia rebaudiana*, *in vitro* regeneration, plant growth regulator, MS media.

INTRODUCTION

*"Stevia rebaudiana*Bertoni belongs to the family Asteraceae. It is one of 154 members of the genus Stevia and one of only two species that produce sweet steviol glycosides. Dry leaves of *Stevia rebaudiana* are 300 to 450 times more sweeter than sucrose" (Chang *et al.*, 2007; Yu and Shi, 2009; Silvia and Luciana, 2013) and "also contain flavonoids, vitamins, tannins, phytosterols, minerals, alkaloids, hydroxycynnamic acid, essential oils and other miscellaneous compounds with antimicrobial as well as antioxidant properties" (Tadhani and Subhash, 2006; Darabpour*et al.*, 2010; Muanda*et al.*, 2011; Mondaca*et al.*, 2012; Rieck, 2012; Galbis*et al.*, 2014). "Consumption of dry leaves of *Stevia rebaudiana*Bertoni positively influences the human health by regulating hypotension, enhancing antiglycaemic activity, preventing dental cavity, decreasing heart rate or improving digestion and gastrointestinal functioning" (Lee *et al.*, 2001; Midmore and Rank, 2002; Jeppesen*et al.*, 2003; Gupta *et al.*, 2013). "With the current demand for food supplements having low carbohydrate, minimum calorie and low sugar content large scale production of *Stevia rebaudiana*Bertoni is needed. But seeds of *Stevia rebaudiana*Bertoni show a very low germination percentage" (Felippe and Lucas, 1971; Monteiro, 1980; Toffler and Orio, 1981), and "vegetative propagation through cuttings is limited by the small number of individuals" (Sakaguchi and Kan, 1982)."The conventional approaches to stevia mass propagation or multiplication are less effective, impulsive, time-consuming, and unreliable.Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition" (Nakamura and Tamura, 1985). "Commercial cultivation requires a homogeneous range of improved plants; however, plants germinated from seeds exhibit variability. Field conditions exhibit significant variation due to external environmental factors, including plant pathogens, temperature, drought, and waterlogging, resulting in differences in composition and sweetening levels" (Nakamura and Tamura, 1985).

Considering the prospects of commercial propagation of Stevia, the present investigation was commenced to develop a simple, rapid, and economical, reproducible and high frequency regeneration protocol from shoot tips of *Stevia rebaudiana*Bertonito be used in large scale propagation. This experiment also focused on optimizing suitable concentrations and combinations of commonly available plant growth regulators for effective proliferation of shoots and roots.

MATERIALS AND METHODS

The experiment was conducted during the period from January 2023 to November 2023in the Plant Tissue Culture laboratory, Department of Biotechnology, Bangladesh Agricultural University (BAU), Mymensingh.Shoot tips of healthy, vigorous *Stevia rebaudiana*Bertonifield grown plants were used as explant. Explants were washed in running tap water and then treated with 2% savlonwith constant shaking for 5-6 minutes and washed thoroughly with distilled water. Then the explants were taken under laminar air flow cabinet. The surface sterilization of the explants were done with 0.1% mercuric chloride solution and 1-2 drops of Tween-20 to remove the superficial dust particles as well as fungal and bacterial spores for 5 minutes under aseptic condition followed by washing 5-6 times with sterilized distilled water and finally shoot tips were inoculated aseptically for *in vitro* propagation on culture medium. MS (Murashige and Skoog, 1962) medium was used as culture medium and sixteen different combinations of NAA (1-Naphthaleneacetic acid) and BAP (6-Benzylaminopurine) were used (Table 1) for shoot induction(Table 1).

Table 1: Treatments with different concentrations of BAP and NAA for shoot initiation

Treatment	Concentration of growth regulator (BAP+NAA)mg/l	Treatment	Concentration of growth regulator (BAP+NAA)mg/l
T1	0+0	T9	2+0
T2	0+1	T10	2+1
T3	0+2	T11	2+2
T4	0+3	T12	2+3
T5	1+0	T13	3+0
T6	1+1	T14	3+1
T7	1+2	T15	3+2
T8	1+3	T16	3+3

The culture vessels with inoculated explants were incubated both in dark and light (the photoperiod was maintained as 16 hours light and 8 hours dark) in a temperature controlled growth room (25 ± 1°C). Subculturewas performed within same concentration at regular intervaluntil explants showed sufficient shootlet formation. When the plantletsshowed proper growth they were rescued aseptically from the culture vessels and were separated from each other and again subcultured on culture vessels with freshly prepared root induction medium containingIndole-3-butyric acid (IBA) (0, 1, 6 mg/l) along with sixteen shoot proliferation treatments for consistent growth both shoots and roots.To investigate the effect of different treatments on *in vitro* regeneration of *Stevia rebaudiana*, data were collected on number of shoots per culture, shoot length, percentage of shoot regeneration, days to shoot initiation, number of roots per plantlet, percentage of root induction and days to root initiation. Then percentage of shoot regeneration and root induction was calculated on the basis of following formula:

$$\text{Percentage of shoot regeneration} = \frac{\text{Number of explants showing shoot}}{\text{Number of inoculated explants}} \times 100$$

$$\text{Percentage of root induction} = \frac{\text{Number of shoots with root}}{\text{Number of inoculated shoots}} \times 100$$

The recorded data were statistically analyzed using Microsoft Statistical (MSTAT) programme and Microsoft Excel wherever applicable. The experiment followed Completely Randomized Design (CRD) design and data for the characters under the present study were statistically analyzed following analysis of variance (ANOVA) technique. The analysis of variance was performed and means were compared by Least Significant Difference (LSD) test at 5% level of probability for results (Gomez and Gomez, 1984) and the mean differences were adjusted by DMRT (Ducan's Multiple Range Test) and the ranking was indicated by letters.

RESULTS AND DISCUSSION

Effect of BAP and NAA on shoot regeneration in *Stevia rebaudiana*Bertoni

Shoot initiation of*Stevia rebaudiana*was significantly influenced by different concentrations of BAP and NAA. After inoculation of explant in the culture medium, shootlets were proliferated. The highest number of shootlets per culture (4.170) was observed in 1.0 mg/l BAP + 2.0 mg/l NAA supplemented MS medium whereas explants inoculated in MS medium without growth regulator did not proliferate any shootlets until 30 days after inoculation (Table 2).

Laribi*et al.* (2012) found in their experiment that BAP (1 mg l^{-1}) and IAA (0.25 mg l^{-1}) combination was superior for multiple shoot bud induction (4.25 shoots).BAP was identified as the most effective cytokinin for promoting multiple shoot regeneration in the experiment of Thiyagarajan and Venkatachalam (2012). Our experiment uses combination of NAA and BAP, that’s why the shoot regeneration is satisfactory. Shulgina*et al.* (2021) experimented on Stevia considering impacts of both PGPRs and light effect on regeneration *in vitro*, they found that *S. rebaudiana* microshoots, cultured on MS media containing 1.0 mg L^{-1} 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} IAA (Indole-3-acetic acid)with red monochrome light treatments, increased the multiplication coefficient by 30% compared with controls (white light, media without PGRs).

Rodriguéz-Páez et al. (2024) studied with 3 elite genotypes of Stevia to generate more efficient regeneration protocol. Their method employed various combinations of cytokinins and auxins following organogenesis protocols like ours. The findings demonstrated that when cultures were cultivated on a basal medium MS supplemented with 1 μM 6-benzylaminopurine (BAP), the best shoot multiplication (17.3 shoots per explant) for L020 was obtained. The best treatment for Morita II was an MS treated with 2 μM BAP and 0.5 μM NAA, which produced 16.4 shoots per explant, whereas the best therapy for L102 was an MS supplemented with 1 μM BAP and 0.5 μM naphthalene acetic acid (NAA), which produced excellent shoot multiplication (18.5 shoots per explant). This work demonstrated the important influence of genotype on tissue culture, especially in shoot multiplication, by successfully achieving micropropagation for promising *S. rebaudiana* genotypes.

Rezvankhah et al. (2022) also searched for better protocol of Stevia economic micropropagation and understood that combination of 0.02 mg/l NAA and 0.05 mg/l BAP in a bioreactor resulted best for shoot generation along with stevioside, a metabolite.

Alani et al. (2023) stated that in their experiment, best combination for number of shoots explant^{-1} was found to be (0.4 BAP + 0.2 Kinetin mgl^{-1}) for shoot tips explants.

Uddin *et al.* (2006) observed multiple shoots from modified MS medium with 1.0 mg/l BAP. Sivaram and Mukundan (2003) used BA (2 mg/l) and IAA (1 mg/l) to regenerate shoots from shoot tips, nodal and leaf segments of *Stevia rebaudiana*Bertoni. Sritonkum (1995) found that MS medium supplemented with 12.0 mg/l Kinetin was good for shoot multiplication from shoot tips.

The highest shoot regeneration percentage (83.33%) was obtained when the MS medium was supplemented with 1.0 mg/l BAP + 2.0 mg/l NAA followed by 72.92% of shoot regeneration from 3.0 mg/l BAP and 3.0 mg/l NAA. The lowest percentage (29.17 %) of shoot regeneration was found in 0 mg/l BAP + 1.0 mg/l NAA (treatment 2) and MS medium without growth regulator did not show any response. The data revealed that percentage of shoot regeneration gradually increased with increasing concentrations of NAA (Fig.1).

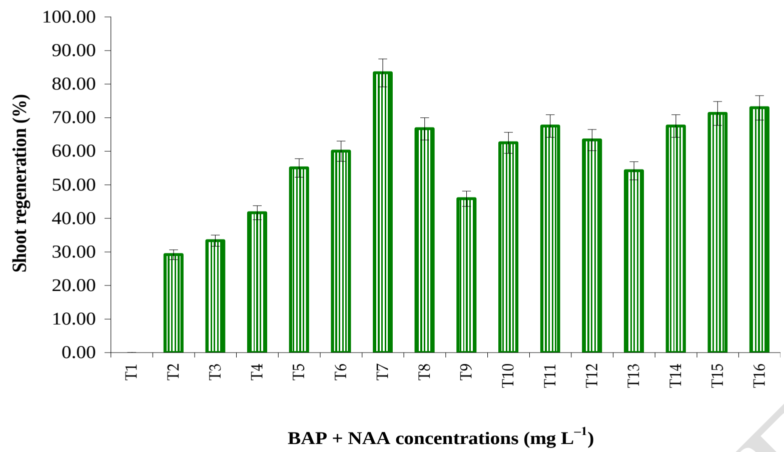

Figure 1 : Effect of different concentrations and combinations of BAP and NAA onpercentage of shoot regeneration in *Stevia rebaudiana* Bertoni

Ali *et al.* (2010) observed the maximum shoot formation when BAP was used in combination with kinetin. Sridhar and Aswath (2014) also observed the best result with the combination of BAP and Kinetin for shoot regeneration. The longest shoot (6.53 cm) was produced while MS medium was supplemented with 1.0 mg/l BAP + 2.0 mg/l NAA and the shortest shoot (1.28 cm) was produced in MS medium with 0 mg/l BAP + 1.0 mg/l NAA (Figure 2). The shortest time (10 days) required for shoot initiation when the explants were cultured in MS medium with 1.0 mg/l BAP + 2.0 mg/l NAA whereas the longest time (32 days) was observed when the culture medium was supplemented with 0.0 mg/l BAP + 1.0 mg/l NAA (Table 2). It was found that small amount of BAP in combination with increasing amount of NAA (0, 1.0, 2.0 and 3.0 mg/l respectively) increased percentage of shoot regeneration and shoot lengthin *Stevia rebaudiana*Bertoni at a certain limit and then decreased the percentage of shoot regeneration and shoot lengthgradually(Figure 1 and 3). This may happen as most of the plant hormones are functional at very low concentrations and a balance of BAP and NAA promotes the highest shoot regeneration.

Table 2: Effect of different treatments on various shoot characteristics of *Stevia rebaudiana*

Treatments	**No. of shootlets culture^{-1}**	**Shoot regeneration (%)**	**Shoot length (cm)**	**Days required for shoot initiation**
T1	**0.000 l**	**0.000 l**	**0.000 l**	-
T2	1.430 k	29.17 k	1.280 k	**32.00 a**
T3	1.670 j	33.33 j	2.410 j	28.00 b
T4	2.000 i	41.67 i	3.120 i	26.30 c
T5	2.780 g	55.00 g	4.180 g	25.00 d
T6	3.000 f	60.00 f	4.680 f	19.20 gh
T7	**4.170 a**	**83.33 a**	**6.530 a**	**10.00 j**
T8	3.330 d	66.66 d	4.790 f	21.50 f
T9	2.140 h	45.83 h	3.960 h	25.10 d
T10	3.130 e	62.50 e	4.970 e	18.50 hi
T11	3.330 d	67.50 d	5.030 de	22.00 f
T12	3.180 e	63.33 e	5.110 d	**17.50 i**
T13	2.860 g	54.17 g	4.210 g	24.20 de
T14	3.330 d	67.50 d	4.790 f	23.50 e
T15	3.460 c	71.25 c	5.510 c	19.70 g
T16	**3.640 b**	**72.92 b**	**6.180 b**	18.40 hi
LSD (0.05)	**0.1179**	**1.374**	**0.1179**	**1.01**
CV (%)	**2.57**	**1.51**	**1.67**	**2.93**

Level of significance	**	**	**	**

****=** Significant at 1**%** level of probability, ($p \leq 0.01$)

Figure 2: Plate 1-2: Shoot proliferation in Stevia rebaudiana in MS medium supplemented with of 1.0 mg/l BAP + 1.0 mg/l NAA

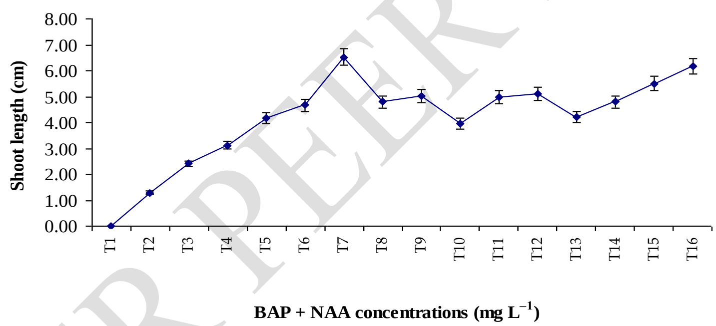

Figure 3 : Effect of different concentrations of BAP and NAA on shoot length of *Stevia rebaudiana*

Root formation in regenerated shoots

Each Single shootlet of *in vitro* proliferated shoots were carefully isolated and inoculated on MS medium supplemented individually with 0, 1.0, 6.0 mg/l IBA in each of 16 treatments for root initiation. No root formation was observed while IBA concentration was 0 and 1.0 mg/l. Among the treatments used for root initiation the highest percentage (75 %) of root formation was observed with MS medium + 6 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l NAA followed by 71.43 % when using MS medium + 6 mg/l IBA + 3.0 mg/l BAP + 3.0 mg/l NAA. In contrast, the lowest percentage (14.28 %) of root formation was obtained in MS medium + 6 mg/l IBA + 0 mg/l BAP + 1.0 mg/l NAA (Table 3 and Figure 4).Uddin *et al.* (2006) recorded more than 90% rooting in modified MS medium with 1.5 mg/l IBA. The lowest number of days (9.30) were required for root induction with MS medium + 6 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l NAA. This may be due to selection of appropriate growth regulator with suitable concentration.

Table 3. Effect of different concentrations of BAP, NAA and IBA on root initiation of *Stevia rebaudiana*

Concentration of BAP, NAA and IBA (mg L^{-1})	Root formation (%)	No. of roots $plantlet^{-1}$	Days for root induction
0 + 0 + 0	0.000 l	0.000 m	0.000 m
0 + 1.0 + 6.0	**14.28 k**	**0.710 l**	21.20 a
0 + 2.0 + 6.0	16.67 j	0.830 k	20.10 b
0 + 3.0 + 6.0	20.00 i	1.000 j	19.50 b
1.0 + 0 + 6.0	33.33 h	1.670 i	17.50 cd
1.0 + 1.0 + 6.0	50.00 e	2.500 f	15.20 fgh
1.0 + 2.0 + 6.0	**75.00 a**	**3.750 a**	9.300 l
1.0 + 3.0 + 6.0	58.34 d	3.000 d	14.20 hi
2.0 + 0 + 6.0	45.72 f	2.370 g	16.20 ef
2.0 + 1.0 + 6.0	57.14 d	2.860 e	15.10 gh
2.0 + 2.0 + 6.0	58.57 d	2.920 de	14.00 ij
2.0 + 3.0 + 6.0	48.57 e	2.500 f	16.50 de
3.0 + 0 + 6.0	42.86 g	2.140 h	17.90 c
3.0 + 1.0 + 6.0	50.00 e	2.500 f	16.00 efg
3.0 + 2.0 + 6.0	66.67 c	3.330 c	13.00 j
3.0 + 3.0 + 6.0	**71.43 b**	**3.570 b**	11.30 k
LSD (0.05)	**1.374**	**0.1179**	**1.01**
CV (%)	1.86	3.14	4.09
Level of significance	**	**	**

****=** Significant at 1**%** level of probability, ($p \leq 0.01$)

Figure 4: Plate 3- 4: Root formation in proliferated shoots of *Stevia rebaudiana* using MS medium + 6 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l NAA

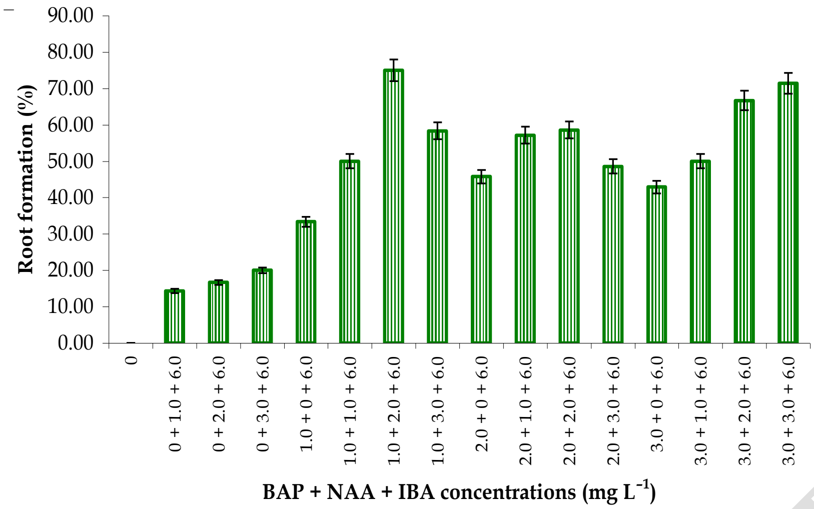

Figure 5: Percentage of root formation in regenerated shootsof *Stevia rebaudiana*using different concentrations and combinations of BAP + NAA + IBA (mg L^{-1}).

In contrast, the maximum number of days (21.20) were required for root induction when using MS medium + 6 mg/l IBA + 0 mg/l BAP + 1.0 mg/l NAA. No root formation was observed in MS medium without growth regulator.
Similar types of results were found by earlier workers in the same species (Sivaram and Mukundan, 2003; Ahmed *et al.*, 2007; Mitra and Pal, 2007). However Tadhani and Subhash(2006) reported 0.1 mg/L IBA was the best concentration for rooting.
Amini et al. (2024) investigated on concentration and composition of various phytohormones in the efficiency of micropropagation of Stevia rebaudianaBertoni and the highest number of roots was found in the treatment with 1 mg/L IBA.
Alani et al. (2023) stated that in their experiment, best combination for the highest rooted percentage of shootlets (93%) was recoded at 2.0 mgl^{-1} IBA.
Singh et al. (2020) stated that for root initiation, ½ MS media is best along with 1 mg/l NAA which resulted in highest (11.08) number of roots per shoot.
Munir et al. (2023) also attempted to generate Stevia through micropropagation and showed that IAA at 0.06mg/L concentration performed better for root generation.
Razak et al. (2014) reported that maximum number of roots (30.12 ± 2.1 roots per explants) was obtained on a MS medium containing 1.0 mg L-1 IBA. Our experiment used combination of IBA, BAP and NAA that ensured best root regeneration under existing circumstances.Kumari and Chandra (2015)found in their experiment that among different concentrations of IBA, IAA and NAA, 1 mg L^{-1} IBA was proved most effective with maximum numbers (7.6 ± 0.3) of roots which supports findings of our experiment.

CONCLUSION

The use of phytohormones in tissue culture is a crucial step in the process. In our experiment, we used several combinations of NAA, BAP and IBA for regeneration of stevia from shoot tips. We had divided the experiment in two phases; shoot elongation from explants and root initiation in shootlets. 16 combinations of treatments for shoot initiation and 16 combinations of treatments for root initiation was used. Among this, MS medium supplemented with 1.0 mgL^{-1} BAP + 2.0 mgL^{-1} NAA could be recommended for multiple proliferation of shoot from shoot tips. MS medium supplemented with 6.0 mgL^{-1} IBA+ 1.0 mg/l BAP + 2.0 mg/l NAA could be used for root formation.
The result of the experiment clearly support the possibility of mass propagation *Steviarebaudiana*Bertoni by adopting above mentioned *in vitro* technique. Thus, *in vitro* propagation can become an important alternative to conventional breeding procedures for *Stevia rebaudiana*Bertoni.

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REFERENCES

Ahmed, M. B., Salahin, M., Karim, R., Razvy, M. A., Hannan, M. M., Sultana, R., Hossain, M. and Islam, R., 2007. An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana*) in Bangladesh. *American-Eurasian J. Sci. Res.*, 2(2): 121-125.

Alani, M. A., Elkaaby, E. A., Alani, J. A., Alaubaidy, A. A., &Alshimmary, L. A. (2023, August). Employment of In Vitro Technique to Propagate Stevia (Stevia rebaudiana) Plant. In *IOP Conference Series: Earth and Environmental Science* (Vol. 1225, No. 1, p. 012025). IOP Publishing.

Ali, A., Gull, I., Naz, S. and Afghan, S., 2010.Biochemical investigation during different stages of *in vitro* propagation of *Stevia rebaudiana*. *Pak. J. Bot*., 42(4): 2827-2837.

AminiNeisiani, A., Saidi, A., &Tohidfar, M. (2024). Investigation of the concentration and composition of various phytohormones in the efficiency of micropropagation of Stevia rebaudianaBertoni. *Plant Productions*.

Chang, L., Wang, Z. and Yang, D., 2007. Research Summary of Method for Chemical Analysis for Steviosides. *Foreign Medical Sciences, Section Hygiene*, 197-200.

Darabpour, E., Motamedi H. and Nejad, S.M.S., 2010. Antimicrobial properties of Teucriumpolium against some clinical pathogens. *Asian Pac. J. Trop. Med.*, 3(2): 124-127.

Felippe, G.M. and Lucas, N.M.C., 1971. Estudo da viabilidade dos frutos de *Stevia rebaudiana* Bert. *Hoehnea*1:95-105 [in Portuguese, English abstract].

Galbis, B.C.M., Pérez, P.M.C., Espinosa, J. A., Celdrán, M. A., Martínez and Rodrigo, D., 2014. Use of the modified Gompertz equation to assess the *Stevia Rebaudiana*Bertoniantilisterial kinetics. *Food Microbiol*., 38: 56–61.

Gupta, E., Purwar, S., Sundaram, S. and Rai, G.K., 2013. Nutritional and therapeutic values of *Stevia rebaudiana*: A review. *J. Med. Plants Res.*, 7(46): 3343-3353.

Jeppesen, P. B., Gregersen, S., Rolfsen, S. E. D., Jepsen, M., Colombo, M., Agger, A., Xiao, J., Kruhoffer, M., Orntoft, T. and Hermansen, K., 2003. Antihyperglycemic and blood pressure reducing effects of stevioside in the diabetic gotokakizaki rat. *Metabolism*, 52:372-378.

Jitendra, M., Monika, S., Ratan, S. D., Priyanka, G., Priyanka, S. and Kiran, D. J., 2012. Micropropagation of an anti -diabetic plant - *Stevia rebaudiana*Bertoni, (natural sweetener) in Hadoti region of South-East Rajasthan, India. *ISCA J. Biological Sci*. 1(3):37-42.

Kumari, M., & Chandra, S. (2015). Stevioside glycosides from in vitro cultures of Stevia rebaudiana and antimicrobial assay. *Brazilian Journal of Botany*, *38*, 761-770.

Laribi, B., Rouatbi, N., Kouki, K., &Bettaieb, T. (2012). In vitro propagation of Stevia rebaudiana (Bert.)-a non caloric sweetener and antidiabetic medicinal plant.

Lee, C. N., Wong, K., Liu, J., Chen, Y., Chen, J. and Chan, P., 2001. Inhibitory effect of stevioside on calcium influx to produce anti-hypertension. *Planta Med.*, 67:796–799.

Midmore, D. J. and Rank, A.H., 2002. A new rural industry – stevia – to replace important chemical sweeteners. Report for the Rural Industries, Research and Development Corporation Boston, Massachusetts (USA). 13 p.

Mitra, A. and Pal, A., 2007. *In vitro* regeneration of *Stevia rebaudiana* (Bert) from the nodal explant. *J. Plant Biochem. Biotechnol.*,16: 59-62.

Mondaca, L. R., Gálvez, V. L. A., Bravo, Z. and Hen, K. A., 2012. *Stevia Rebaudiana*Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chem.*, 132(1): 1121-1131.

Monteiro, R., 1980. Taxonomia e biologia da reproducao da *Stevia rebaudiana* Bert. Ph.D. thesis, Univ. Estadual de Campinas, Brazil.

Muanda, F. N., Soulimani, R., Diop, B. and Dicko, A., 2011. Study on chemical composition and biological activities of essential oil and extracts from *Stevia Rebaudiana*Bertoni leaves. *LWT–Food Sci. & Technol.*, 44(9): 1865–1872.

Munir, M., Fatima, I., Aftab, B., Sheikh, A. A., Shabir, A., Ahmad, J., & Ahmed, M. S. (2023). DEVELOPMENT OF TISSUE CULTURE PROTOCOL FOR MASS PRODUCTION OF STEVIA REBAUDIANA (BERTONI) BERTONI. *Journal of Animal & Plant Sciences, 33*(5), 1126-1132.

Nakamura, S. and Tamura, Y., 1985. Variation in the main glycosides of stevia. *Jpn J. Trop. Agric.*, 29: 109-116.

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

Rezvankhah, M., Askari, H., Tohidfar, M., &Rezadoost, H. (2022). Economic micropropagation of Stevia rebaudianaBertoni and evaluation of in vitro cultures in order to improve steviol glycosides. *ScientiaHorticulturae*, *305*, 111372.

Rieck, W. U., 2012. The leaves of *Stevia Rebaudiana* (Bertoni), their constituents and the analyses thereof: a review. *J. Agri. Food Chem*., 60: 886–895.

Rodriguéz-Páez, L. A., Pineda-Rodriguez, Y. Y., Pompelli, M. F., Jimenez-Ramirez, A. M., Genes-Avilez, O. J., Jaraba-Navas, J. D. D., ... & Rodríguez, N. V. (2024). Micropropagation Protocols for Three Elite Genotypes of Stevia rebaudianaBertoni. *Horticulturae*, *10*(4), 404.

Sakaguchi, M. and Kan, T., 1982. Japanese researches on *Stevia rebaudiana* (Bert.) Bertoni and stevioside. *CiCult.*, 34: 235-248.

Silvia, T. and Luciana, G. A., 2013. *Stevia Rebaudiana*Bertoni as a source of bioactive compounds: the effect of harvest time, experimental site and crop age on steviol glycoside content and antioxidant properties. *J. Sci. Food Agric.*, 93: 2121–2129.

Singh, B., Gajera, B., Desai, P., Modi, A., Patil, G., & Narayanan, S. (2020). Micropropagation protocol for Stevia rebaudiana through axillary shoot proliferation. *The Indian Journal of Agricultural Sciences*, *90*(3), 483-488.

Sivaram, L. and Mukundan, U., 2003. *In vitro* culture studies on *Stevia rebaudiana*. *In vitro cell. and dev. biol. Plant.*, 39: 520-523.

Sridhar, T. M. and Aswath, C. R., 2014. Influence of additives on enhanced *in vitro* shoot multiplication of *Stevia rebaudiana* (Bert.)—an important anti diabetic medicinal plant. *American J. Plant Sci.*, 5(1): 8.

Sritonkum, P., 1995 Micropropagation of *Stevia rebaudiana*Bertoni M.Sc. Thesis. Chulalongkorn University. *Stevia rebaudiana*Bertoni floret explants. *R. Bras. Fisiol. Veg.*, 9: 185-188.

Tadhani, M. B. and Subhash, R., 2006. *In vitro* antimicrobial activity of *Stevia Rebaudiana*Bertoni leaves. *Trop. J. Pharm. Res.*, 5(1): 557–560.

Thiyagarajan, M., &Venkatachalam, P. (2012). Large scale in vitro propagation of Stevia rebaudiana (bert) for commercial application: Pharmaceutically important and antidiabetic medicinal herb. *Industrial Crops and Products*, *37*(1), 111-117.

Toffler, F. and Orio, O. A., 1981. Accenisulla pin atatropicale 'Kaa-he-e' ou 'erba dolce'. *Rev. Soc. Sci. Aliment.*, 4: 225-230. [English summary.]

Uddin, S. M., Chowdhury, S. H. M., Khan, M. M., Uddin, B. M., Ahmed, R. and Baten, A. M., 2006. *In vitro* propagation of *Stevia Rebaudiana* Bert in Bangladesh. *Afr. J. Biotechnol.*, 5: 1238-1240.

Yu, C. and Shi, Y., 2009. Determination methods of stevioside. *Sugar Crops China.* 1: 65-67.