

MICROPROPAGATION OF *Stevia rebaudiana* Bertoni THROUGH TEMPORARY IMMERSION BIOREACTOR SYSTEM.

Norazlina Noordin¹, Rusli Ibrahim¹, Nur Hidayah Sajahan¹, Siti Maryam Mohd Nahar¹, Siti Hajar Mohd Nahar¹ and Nur Raifana Abdul Rashid²

¹Agrotechnology and Biosciences Division, Malaysian Nuclear Agency, Ministry of Science, Technology and Innovation Malaysia (MOSTI), Bangi, 43000 Kajang, Selangor and ²Faculty of Applied Sciences, UiTM Shah Alam, 40000 Shah Alam, Selangor.
azlina@nuclearmalaysia.gov.my

Abstract

Stevia rebaudiana Bertoni is a perennial herb that belongs to the family of Asteraceae. It is a natural sweetener plant known as sweet leaf, which is estimated to be 300 times sweeter than cane sugar. In this study, micro propagation of this natural herb via temporary immersion bioreactor system was successfully conducted. Shoot tips and nodal segment were used as explants to induce multiply shoots. It was found that shoot tips on MS medium supplemented with 1 mg/l Kinetin showed the highest shoot multiplication after 3 weeks of culture. Shoot elongation and rooting was successfully optimized in MS basal medium 2 weeks later. Mass propagation of stevia shoots were carried out in temporary immersion bioreactor and this system showed promising potential as an alternative approach for rapid and continuous production of *in vitro* stevia plantlets.

Keywords: *Stevia rebaudiana*, shoot tip, node, micropropagation, TIS

INTRODUCTION

Stevia is herbaceous perennial shrub originated from the highlands of Paraguay and sections of Argentina and Brazil. Stevia was discovered by Antonio Bertoni, a South American Natural Scientist, in 1887. This plant is a natural sweetener and famously known as “Sweet Weed”, “Sweet Leaf”, “Sweet Herbs” and “Honey Leaf”, which is estimated to be 300 times sweeter than cane sugar. *Stevia rebaudiana* Bert belongs to the family Asteraceae, one of 154 members of the genus Stevia, which produces sweet steviol glycosides (Robinson, 1930; Soejarto *et al.*, 1982). The leaves of stevia are the source of diterpene glycosides, stevioside and rebaudioside (Yoshida, 1986). Stevioside is regarded as a valuable natural sweetening agent because of its relatively good taste and chemical stability (Yamada *et al.*, 1997). Stevioside is of special interest to diabetic persons with hyperglycemia and the diet conscious (Arpita *et al.*, 2011). In Japan alone, an estimated 50 tons of stevioside is used annually with sales valued in order of \$220 million Canadian (Brandle and Rosa, 1992). Now, stevia has been introduced as a crop in a number of countries including Brazil, Korea, Japan, Mexico, United States, Indonesia, Tanzania and Canada (Shock, 1982; Saxena and Ming, 1988; Brandle and Rosa, 1992; Fors, 1995) for food and pharmaceuticals products. Currently *S. rebaudiana* production is centered in China with major market in Japan (Kinghorn and Soejarto, 1985). The product also can be added to tea and coffee, cooked or baked goods, processed foods, pickles, fruit juices, tobacco products, confectionary goods, jams and jellies, candies, yogurts, pastries, chewing gum and sherbets beverages (Arpita *et al.*, 2011).

Seeds of stevia show a very low germination percentage and vegetative propagation is limited by lower number of individuals (Sakaguchi *et al.*, 1982). Tissue culture is the only rapid process for the mass propagation of stevia and there have been few reports of *in vitro* growth of stevia (Miyagaya *et al.*, 1986), *in vitro* micropropagation from shoot tip and leaf (Uddin *et al.*, 2006). The present study was carried out in order to optimize and to establish a suitable protocol for *in*

in vitro propagation of *S. rebaudiana* Bertoni and also to evaluate the potential of temporary immersion bioreactor as a promising alternative for rapid and continuous mass propagation of stevia plantlets.

MATERIALS AND METHODS

Collection of explants and surface sterilization:

Young, actively growing shoot tips and nodal segments were collected from stevia seedlings that were maintained in the glasshouse of Malaysian Nuclear Agency. The collected explants of 2-3 cm were put under running tap water to remove any traces of soil and dirt and later shake with systemic fungicide 5% (w/v). In laminar air flow which provided a strict aseptic condition, the cleaned explants were surface sterilized using 5% (v/v) commercial sodium hypochlorite with a few drops of Tween 20 for 15 min. This step was repeated twice and then rinsed 4 times with sterile distilled water. Excess water adhering to the explants was air-dried then the explants were ready to be inoculated into culture media.

Shoot induction and multiplication

Sterilized shoot tips and nodal segment (with a single axillary bud) were cultured onto semi-solid MS Medium (Murashige and Skoog, 1962) supplemented with 6- benzylamino purine (BAP) or 6-furfurylaminopurine (kinetin) at concentrations ranging from 0, 0.5, 1.0, 2.0 and 4.0 mg/l. These plant growth regulators (PGRs) were added singly into the MS medium together with 3% (w/v) sucrose and 2.4 g/l gelrite. The pH of the medium was adjusted at 5.8 with 0.1M NaOH before autoclaving at 121°C for 15 min. The cultures were incubated in the incubation room at 24± 2°C with 16 h photoperiod. Observation on new shoots induction and shoot multiplication was done weekly.

Subcultures were done every 21 days interval. Nodal segments from the proliferated shoots were subcultured for further multiple shoot induction. The regenerated multiple shoots were cut and individual shoots were placed in semi-solid MS medium supplemented with different concentrations of Indole-3-butyric acid (IBA) for root induction.

Root induction

The *in vitro* grown and healthy looking stevia shoots were transferred into rooting medium for root induction. In this study, MS basal media was supplemented with different concentrations of IBA ranging from 0, 0.25, 0.5, 1.0 and 1.25 mg/l. The proliferated multiple shoots were separated and transferred subsequently to the rooting media. The efficiency of IBA over the control; MS basal medium was assessed in terms of days to root induction, number, length and the health (vigor) of the roots.

Temporary Immersion Bioreactor System

The aim of using temporary immersion bioreactor system (TIS) is to investigate the efficiency of this culture system in enhancing the mass propagation of stevia shoots in comparison to semi-solid media. 3 clumps of newly developed shoot buds were cultured into the TIS vessels containing 250 ml of liquid MS media supplemented with 1 mg/l kinetin. The immersion regime for this experiment was set at 6 hours cycle with 15 min immersion interval daily. Observation on shoot multiplication was done after 3 weeks of culture.

RESULTS AND DISCUSSION

Micropropagation of *S. rebaudiana*

1.0 Shoot induction and multiplication

For assessment of the PGRs efficiency on shoot induction and multiplication, nodal segments and shoot tips were inoculated in MS medium supplemented with different cytokinin sources. The performance on number of shoots formed and the length of shoots was monitored after 3 weeks of culture.

For BAP alone, the best concentration was at 0.5 mg/L. After 3 weeks, the average number of new shoots emerged from the shoot tips was 15 shoots with the average length of 5.5 cm. From the observation, stevia shoots produced in BAP demonstrated larger, greener, curly and abnormal looking phenotype. Callus growth was also observed at the base of the shoots

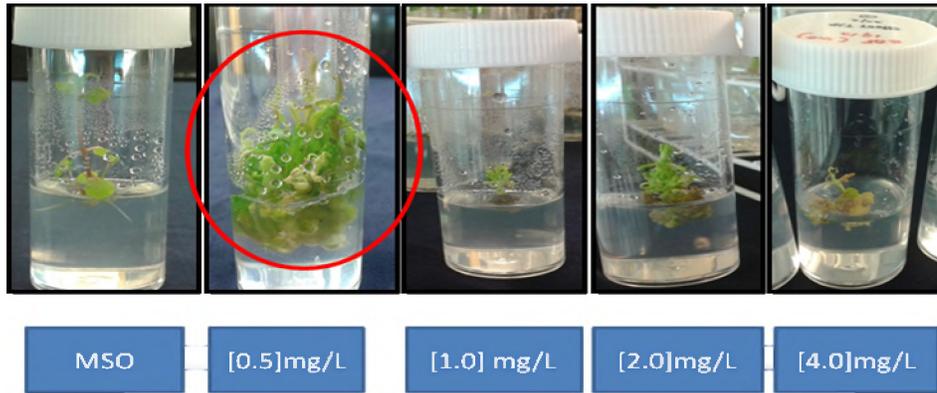


Fig. 1: Induction of shoots in different concentrations of BAP

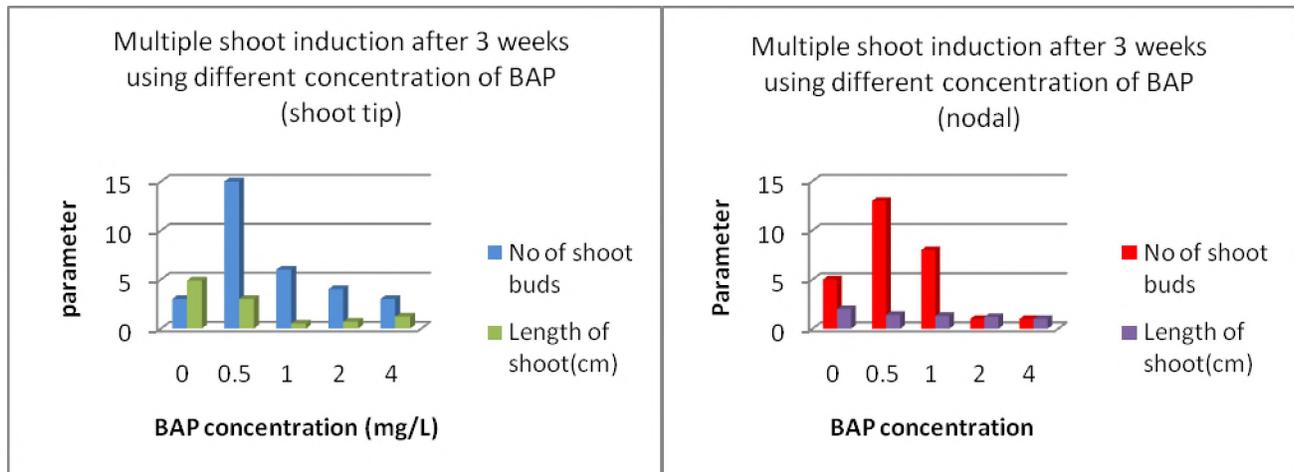


Fig. 2: Multiple shoot induction from shoot tips and nodal segments in BAP

Meanwhile, 1.0 mg/l Kinetin showed the highest number of shoot multiplication. The average number of new shoots produced was 16 shoots with the average length of 7.5 cm from single inoculants. After 3 weeks, it can be seen that multiple shoots formed into complete plantlets with vigor stem and normal looking leaves, no callus formation was observed too.

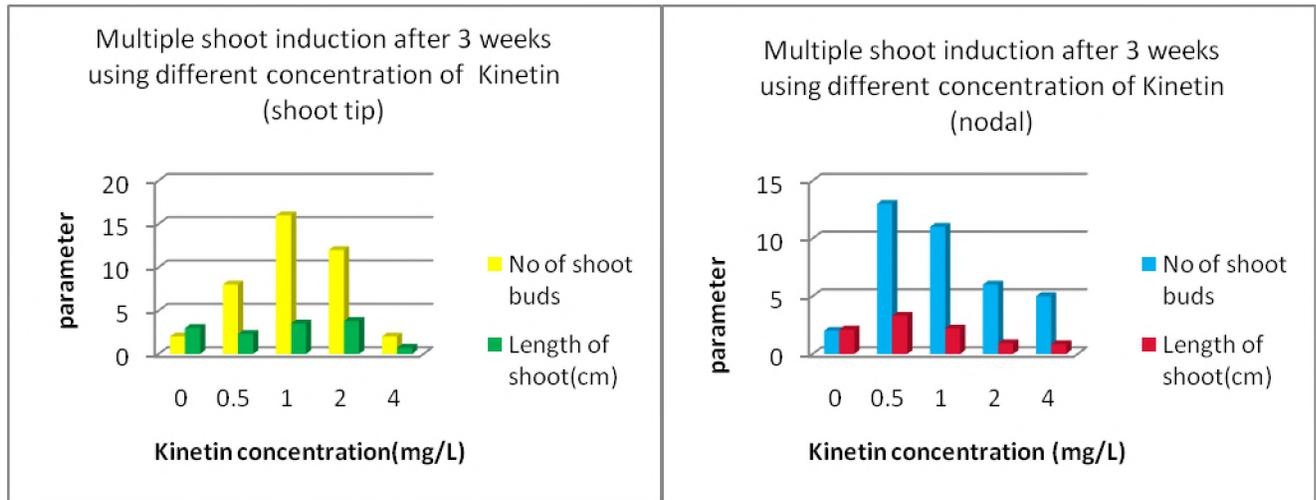


Fig. 3: Multiple shoot induction from shoot tips and nodal segments in Kinetin

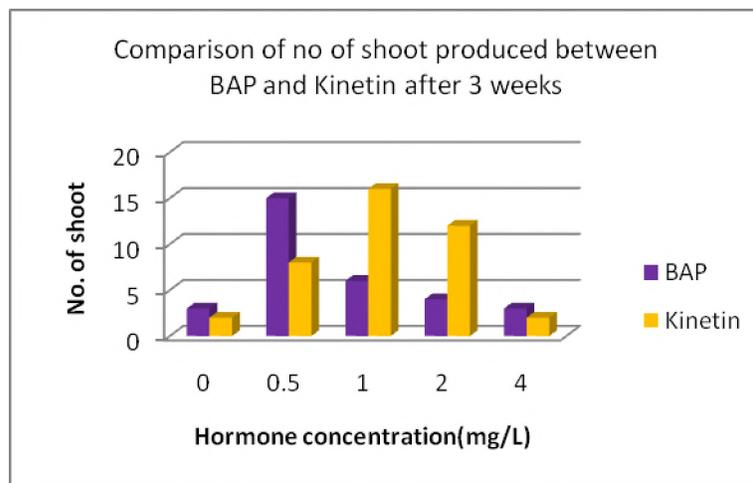


Fig. 4: Comparison on number of shoots produced in Kinetin and BAP

Comparatively, as shown in Fig. 4, Kinetin at 1 mg/l demonstrated the highest number of shoots produced and more importantly, the shoot produced are normal and healthy looking as to compare with 0.5 mg/l BAP. In BAP, the new shoots formed were abnormal looking, vitrified and stunted and this led to the off-type of stevia plantlets. From many studies on the micropropagation of plants, it has been found that kinetin is much more effective than BAP and this supports the results obtained from this present study (Evaldsson et al., 1885; Wang, 1986; Gantait et al., 2009). The large number of adventitious shoot buds developed in presence of kinetin is attributed to the fact that Kinetin triumphs over apical dominance, releases lateral buds from dormancy and upholds shoot formation (George and Sherrington, 1984)

2.0 Root induction

It was observed that after 3 weeks of culture, the root induction gradually decreased with the increasing concentration of IBA. Comparatively, 0.25 mg/l IBA showed the best effect in promoting root formation, the roots induced were short, hard and hairy. However, callus induction was also observed from the cut portion of the shoot and this condition was obviously significant in higher IBA concentrations. It was interestingly and noteworthy to observe that, in PGR free medium; MS basal medium performed best root induction and also promotes the elongation of the shoots. In MS basal medium, root formation was observed after 2 weeks of culture and the roots formed were long, healthy looking, abundance and vigor. From this study, auxin (IBA) had shown adverse effect in rooting by promoting callus growth. The competence of a PGR free MS basal medium for promoting *in vitro* rooting of stevia in this present study supports the earlier studies conducted in other stevia species (Arpita Das et al., 2011; Bespalhokk et al., 1997).

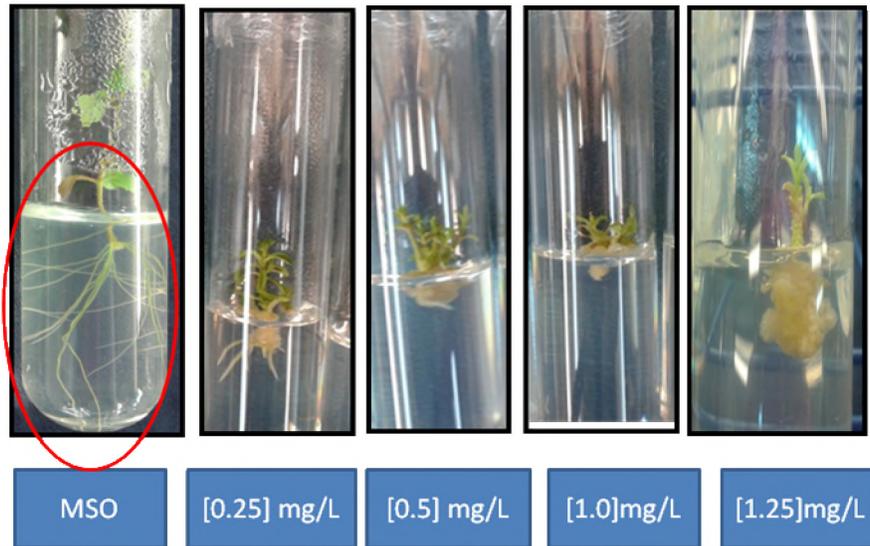
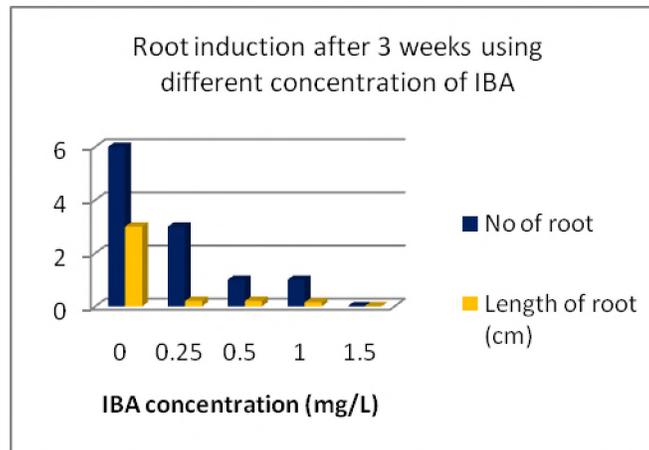


Fig. 5: Root induction in different IBA concentrations



Temporary Immersion Bioreactor System

From this preliminary experiment, it was found that TIS has shown great potential to be developed and established for enhancing the mass propagation of stevia plantlets. After 3 weeks of culture, shoot multiplication was greatly increased, with 30 new healthy shoots produced from each bud clumps, approximately 90 new stevia shoots per vessel. In comparison with semi-solid media with the same supplemented PGR, TIS gave 2.00 times more biomass production with the average shoot length of 9.3 cm. However, this result is still preliminary. Currently, work is conducted in order to optimize the immersion regime with different cycles and immersion periods for the multiplication of stevia plantlets in TIS.

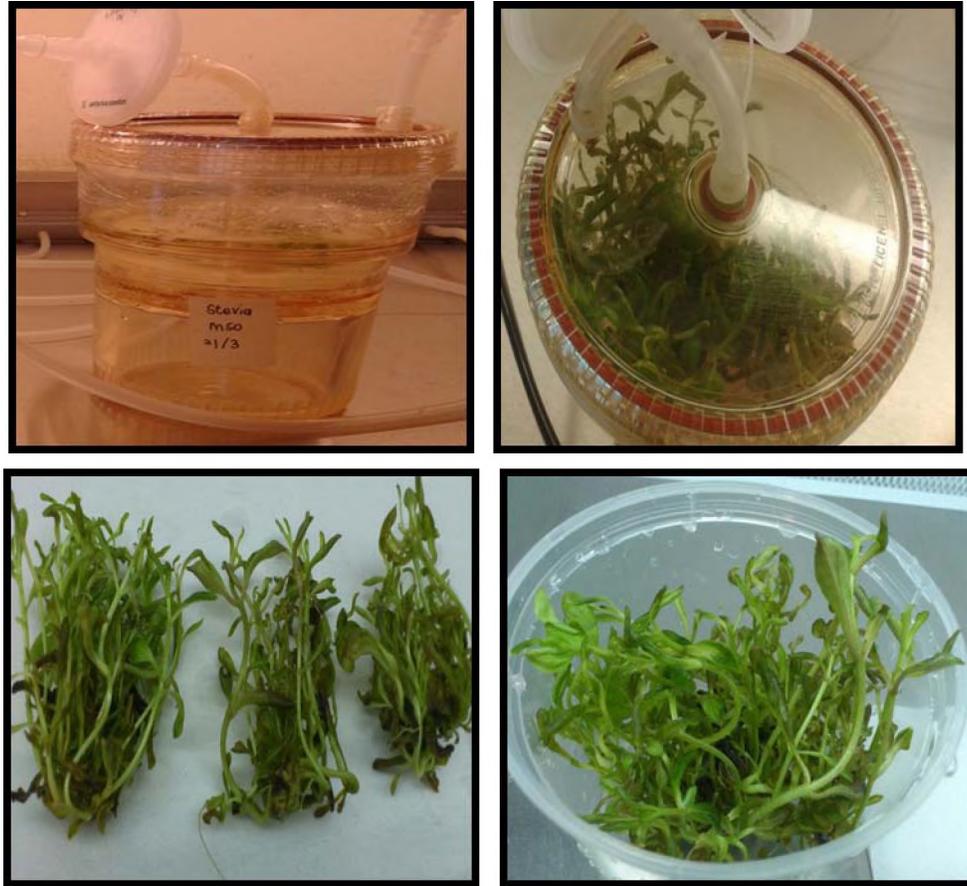


Fig. 6: Multiplication of stevia shoots in TIS

CONCLUSION

The micropropagation protocol of *Stevia rebaudiana* Bertoni has been successfully optimized and established. This study suggested that 1.0mg/L Kinetin is the optimal PGR for stevia shoot multiplication and MS basal medium played a good role in promoting the elongation and root induction of stevia plantlets. The preliminary work on multiplication of the *in vitro* shoots using temporary immersion bioreactor has shown great potential to be used for mass propagation in comparison with the semi-solid media.

ACKNOWLEDGMENT

The authors would like to thank the management of Malaysian Nuclear Agency for their continuous assistance, advices and supports on this project. Special thanks is also extended to the Ministry of Science, Technology and Innovation (MOSTI) for providing fund for this project under the Science Fund grant 02-03-01-SF0163: *Effects of chronic irradiation on growth and multiplication rate with reference to enhanced production of steviol glycoside in Stevia rebaudiana Bertoni.*

REFERENCES

- Arpita D., Gantait S. and Mandal N. (2011) Micropropagation of an elite medicinal plant: *Stevia rebaudiana* Bert. Int. J Agric. Reseach. 6(1):40-48
- Bespalhokk, J.C., L.G.E. Vicira and J.M. Hashimoto (1997). Embryogenic callus formation and histological studies from *Stevia rebaudiana* bert floret explants. Rev. Bras. Fisiol. 5:51-53
- Brandle JE, Rosa N (1992). Heritability for yield, leaf- stem ratio and stevioside content estimated from a landrace cultivar of *Stevia rebaudiana*. Canadian Journal of Plant Science. 72: 1263-1266.
- Brandle, J. E., A. N. Starratt and M. Gijzen (1998). *Stevia rebaudiana*: Its agricultural, biological and chemical properties. Canad. J. Plant Sci. 78:527-536
- Evaldsson, I.E and N.T. Welander (1985). The effects of medium composition on in vitro propagation and *in vitro* growth of *Cordyline terminalis* cv. Atom. J. Hortic. Sci. 60:525-530
- Fors A (1995). A new character in the sweetener scenario. Sugar J,58:30.
- Gantait, S., N. mandal, S. Bhattacharyya and P.K. Das (2009). *In vitro* mass propagation with genetic clonality in elephant garlic (*Allium ampeloprasum* L.). J. Crop Weed. 5:100-104
- George, S. and S. L. Sherrington (1984). Tissue culture in forest trees: Clonal propagation of *tectona grandis* L., (teak) by tissue culture. Plant Sci. Lett. 17:259-268
- Kinghorn AD, Soejarto DD (1985). Current status of stevioside as asweetening agent for human use. In Wagner, Hikino H, Fransworth NR. (eds).Economic and medical plant research. Academic Press,London.
- Miyagawa H. and N. Fujioka (1986). Studies on the tissue culture of *Stevia rebaudiana* and its components: II. Induction of shoot primordia. Planta Medica. 4:321-323
- Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15:473-497
- Robinson BL (1930). Contributions from the Grey Herbarium of Harvard University. *The Grey Herbarium of Harvard University, Cambridge.*
- Saxena NC, Ming IS (1988). Preliminary harvesting characteristics of Stevia. *Phys. Prop. Agric. Mat. Prod.* 3: 299-303.
- Soejarto DD, Kinghorn AD, Fransworth NR (1982). Potential sweetening agents of plant origin. *J. Nat. Prod.* 45: 590-599.
- Shock CC (1982). Experimental cultivation of Rebaudia's stevia in California. University of California Agronomy Progress Report No.122

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

Toyoda, K. and H. Matsui (1997). Assessment of the carcinogenicity of stevioside in F344 rats. Food Chem. Toxicol. 35(6):597-603

Uddin M.S., M.S.H. Chowdhury, M.M.M.H. Khan, M.B. Uddin, R. Ahmed and M.A. Baten (2006). *In vitro* propagation of *Stevia rebaudiana* Bert in bangladesh. Afr. J. Biotechnol. 5(13):1238-1240

Wang, W.C., 1986. Clonal propagation of *Stevia rebaudiana* bert through axillary shoot proliferation *in vitro*. Plant Cell Tissue Org. Cult. 6:159-166

Yoshida, S. (1986). Studies on the production of sweet substances in *Stevia rebaudiana*: I. Simple determination of sweet glucoside in Stevia plant by thin layer chromate-scanner and their accumulation patterns with plant growth. Jap. J. Crop. Sci. 55(2):189-195