http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 16 (17): 887-890, 2013
ISSN 1028-8880 / DOI: 10.3923/pjbs.2013.887.890

Micropropagation and Acclimatization of *Stevia rebaudiana* Bertoni

[1]Kittisak Chotikadachanarong and [2]Srisuluk Dheeranupattana
[1]Biology Program, Faculty of Science and Technology,
Chiang Mai Rajabhat University, Chiang Mai, Thailand
[2]Department of Biology, Chiang Mai University, Chiang Mai, Thailand

Abstract: Multiple shoot induction of *Stevia rebaudiana* Bertoni was studied by node explants that were cultured on solidified MS media and supplemented with 0, 1, 2, 3 and 4 mg L^{-1} kinetin for 4 weeks. The results showed the maximum amount of multiple shoot induction (9.31±4.17 shoots/explant) when cultured on MS media supplemented with 3 mg L^{-1} kinetin. *In vitro* shoots were rooted on solidified MS media supplemented with 0, 0.1, 0.5 and 2 mg L^{-1} Naphthaleneacetic Acid (NAA) for 4 weeks. The highest number of roots (11.18±1.34 roots/shoot) was detected on a concentration of 0.1 mg L^{-1} NAA while the high survival rate (80%) was obtained when the rooted plantlets were transferred to greenhouse conditions.

Key words: Stevia, kinetin, naphthaleneacetic acid, *in vitro* culture

INTRODUCTION

Stevia rebaudiana Bertoni, commonly known as sweet leaf, sugar leaf, or stevia, is a perennial herb of the Asteraceae (Compositae) and is native to certain regions of South America. Leaves of *stevia* are a source of diterpene glycosides, stevioside and rebudioside. Stevioside is 300 times sweeter than sucroseand is regarded as a calorie free bio-sweetener (Bridel and Laviellem, 1931). Therefore, it is of special interest to the diet conscious and diabetic persons with hyperglycemia. Leaf extracts of this plant have been used as natural sweetening agents because of the leaf extract's relatively good taste and chemical stability. For example, stevia has been widely used for decades as a sweetener in the food industry of Japan (Andrew and David, 2006).

In Thailand, *S. rebaudiana* was cultivated in the northern region of the county, in Chiang Mai, Chiang Rai, Lamphun, Lampang and Phayao Provinces. Because this region is geographically characterized as being surrounded by multiple mountain ranges and as having cooler winters than other regions in Thailand, it is considered appropriate for the growing of this plant. *S. rebaudiana* propagation is usually done by stem cutting, but this process requires a significant amount of labour and it is limited by the number of parent plants available. However, the risk of working with, new plants is that these plants may be infected with microorganisms that cause diseases. On the other hand, propagation of these plants through seeds has so far been considered an unreliable method due to the relatively low germination percentage (Miyazaki and Watenabe, 1974).

The aim of this study was to find an efficient protocol for *S. rebaudiana* rapid clonal propagation and to produce a disease-free plant-by-plant tissue culture technique.

MATERIALS AND METHODS

Plant material: Seeds of *Stevia rebaudiana* Bertoni were collected from plants growing in Pa Sang District of Lamphun Province, Northern Thailand. Seeds were washed under running tap water for 15 min, then immersed in 2% Carbendazim (antifungal solution) for 30 min. They were then surface sterilized with 10% Clorox plus two drops of Tween 20 for 15 min before being washed 3 times with sterilized distilled water inside the laminar air flow chamber. Sterilized seeds were cultured on MS media (Murashige and Skoog, 1962) supplemented with 1 mg L^{-1} Benzyladenine (BA) (Sigma-Aldrich, Germany) (Dheeranupattana *et al*., 2007). Shoots were regenerated after being cultured for 4 weeks. Nodal segments, approximately 1 cm in length, were cut from sterilized shoots for explants in the experiment.

Multiple shoot induction: *In vitro* nodal explants were cultured on MS media supplemented with 0, 1, 2, 3 and 4 mg L^{-1} kinetin (Sigma-Aldrich, Germany) with 3% (w/v) sucrose and solidified with 0.7% agar. The pH of the

Corresponding Author: K. Chotikadachanarong, Biology Program, Faculty of Science and Technology,
Chiang Mai Rajabhat University, Chiang Mai, Thailand

medium was adjusted to 5.8 with 0.1 M KOH before the cultures were autoclaved at 121°C for 15 min. Cultures were incubated at 25±2°C under 16 h day^{-1} photoperiods (2000 lux, day light fluorescent tubes). The number of new shoots formed per explant and the average shoot length (cm) were recorded at the 4th week.

Root induction: *In vitro* shoots were rooted on MS media supplemented with 0, 0.1, 0.5 and 2 mg L^{-1} Naphthaleneacetic Acid (NAA) (Sigma-Aldrich, Germany) with 3% (w/v) sucrose, solidified with 0.7% (w/v) agar and the pH was adjusted to 5.8 at 25±2°C, under 16 h day^{-1} photoperiods (2000 lux, day light fluorescent tubes) for 4 weeks. The percentage of rooted explants, number of roots and root length were determined after being cultured for 4 weeks.

Acclimatization: Plantlets were removed from culture media and washed with water carefully to remove the ager. They were immersed in 2% Carbendazim for 15 min and transplanted to polythene bags containing a mixture of sand, soil and vermicompost (1:1:1) in the greenhouse. The potted plants were irrigated and initially covered with transparent plastic bags which was gradually eliminated within four weeks time for completing their acclimatization.

Research location: All experiments were conducted at the Plant Tissue Culture Laboratory, Faculty of Science and Technology, Chiang Mai Rajabhat University, Chiang Mai, Thailand for 1 year (January 2012-January 2013).

Statistical analysis: All treatments were repeated 3 times with 15 replicates per treatment and arranged in a completely Randomized Design (CRD). All the values are expressed as the Mean±Standard Deviation (SD). The data was analyzed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at a 5% level of significance.

RESULTS AND DISCUSSION

Multiple shoot induction: In this study, nodal segments were excised from the *in vitro* plants of *S. rebaudiana* as explants sources. *In vitro* nodal explants were cultured on MS medium supplemented with different concentrations of kinetin at 0, 1, 2, 3 and 4 mg L^{-1} for 4 weeks. Table 1 shows the number of shoots increased when the concentration of kinetin was increased from 0 to 3 mg L^{-1}. The highest number of shoots, as well as the greatest production of healthy shoots was observed with 3 mg L^{-1} kinetin at 9.31±4.17 shoots/explant which was significantly higher than the results recorded for other concentrations (Table 1, Fig. 1). However, at the highest concentration of kinetin (4 mg L^{-1}) the number of stevia shoots decreased with an average shoot number of 4.55±2.46 shoots/explant. The large number of shoots produced in the presence of kinetin was due to the fact that kinetin greatly reduced apical dominance and released lateral buds from dormancy and enhanced shoot formation (George and Sherrington, 1984). Similar reports regarding the efficiency of high and low concentrations of kinetin on shoot multiplication by nodal culturing of stevia plants were also obtained from the studies of Das *et al.* (2011), who obtained the highest number of shoots (11.33 shoots/explant) after culturing on MS medium plus 2.0 mg L^{-1} kinetin for 35 days. Pratibha *et al.* (2010) reported that the maximum number of shoots (35 shoots/explant) were recorded on MS medium plus 4.0 mg L^{-1} kinetin for 8 weeks and Yang *et al.* (1981) observed the maximum shoot induction (13.6 shoots/explant) on MS medium supplemented with kinetin 10 mg L^{-1} over 50 days.

On the other hand, the hormone free MS medium (control) gave the lowest shoot numbers with an average shoot number of 3.33±1.35 shoots/explant, whereas, it significantly increased shoot elongation over

Fig. 1: Shoots induced with 3 mg L^{-1} kinetin after being cultured for 4 weeks

Table 1: Effects of various concentrations of kinetin on multiple shoot induction and shoot length of *Stevia rebaudiana* after four weeks of culturing

Treatment (mg L^{-1})	Mean of shoot number±SD (shoot/explant)	Shoot length±SD (cm)
0 kinetin	3.33±1.35^{c}	6.13±2.69^{a}
1 kinetin	4.00±2.77^{c}	2.70±1.54^{b}
2 kinetin	6.36±2.13^{b}	2.72±1.22^{b}
3 kinetin	9.31±4.17^{a}	2.93±1.99^{b}
4 kinetin	4.55±2.46^{bc}	2.72±1.49^{b}

Values expressing the mean±standard deviation followed by similar letters in a column do not differ significantly at $p<0.05$

all other kinetin treatments (6.13±2.69 cm). The results showed the increasing effect of kinetin on shoot numbers but it decreased the shoot length of *S. rebaudiana* nodal cultures.

Root induction: Stevia shoots obtained from 3 mg L^{-1} of kinetin were subcultured in rooting medium (MS media supplemented with 0, 0.1, 0.5 and 2 mg L^{-1} NAA) for 4 weeks. Adventitial root production started after 1 week of culturing and it was observed in all treatments. Table 2 shows that the highest number of roots, at 11.18±1.34 root/explant, was obtained with 0.1 mg L^{-1} NAA. However, the average root length per shoot was higher 0.90±0.07 cm on the NAA free MS medium. The results revealed that there were no significant differences on the number of roots and the length of the roots between the treatments with and without NAA (Table 2). Meanwhile, the effect of NAA on stevia root induction has been investigated in many reports, such as that of Rafiq *et al.* (2007) who found that 0.5 mg L^{-1} NAA could produce maximum root induction with an average of 6.20±0.2 roots/explant. Similarly, Steven *et al.* (1992) and Anbajhagan *et al.* (2010) reported that 1.0 mg L^{-1} NAA MS medium showed maximum root induction. Verma *et al.* (2011) obtained 2.0 mg L^{-1} NAA generated the highest root number (8.9 roots/explants). While, Yang *et al.* (1981) reported the highest root number (8.0 roots/explant) on MS medium, plus a very high concentration at 10.0 mg L^{-1} NAA.

Acclimatization: Rooted plantlets obtained from MS media supplemented with 0, 0.1, 0.5 and 2 mg L^{-1} NAA were transferred to greenhouse conditions for 4 weeks. The acclimatization of plantlets by covered with transparent plastic bags to control humidity improved the survival rate of the plants during the hardening process. Table 3 demonstrated that plantlets obtained from the rooting medium without NAA and with 0.1 mg L^{-1} NAA showed the highest survival rate (80%) of *Stevia rebaudiana* plantlets, followed by 0.5, 1.0 and 2.0 mg L^{-1} NAA, respectively. The results suggested that high concentrations of NAA in the rooting medium reduced the survival rate of the plantlets during acclimatization, as it did for many species (Al-Maarri *et al.*, 1994). This acclimatization of stevia plantlets was successful, as it was shown in earlier studies, i.e., Laribi *et al.* (2012), Anbajhagan *et al.* (2010), Das *et al.* (2011), Verma *et al.* (2011) and Meera Manjusha and Sathyanarayana (2010).

Table 2: Effects of NAA on root induction and root length of *Stevia rebaudiana* nodal culturing for 4 weeks

Treatment (mg L^{-1})	Mean of root number±SD (root/explant)	Root length±SD (cm)
0 NAA	6.23±1.23^{b}	0.90±0.07^{a}
0.1 NAA	11.18±1.34^{a}	0.78±0.08^{ab}
0.5 NAA	7.22±1.48^{ab}	0.70±0.08^{ab}
1.0 NAA	9.25±1.57^{ab}	0.63±0.09^{b}
2.0 NAA	7.43±1.68^{ab}	0.70±0.10^{ab}

Values expressing the Mean±SD followed by similar letters in a column do not differ significantly at $p<0.05$

Table 3: Survival rate of *Stevia rebaudiana* plantlets rooted with different concentrations of NAA after being transferred to greenhouse conditions for 4 weeks

Treatment (mg L^{-1})	Survival rate (%)
0 NAA	80
0.1 NAA	80
0.5 NAA	70
1.0 NAA	60
2.0 NAA	60

CONCLUSION

This study investigated a new protocol for *in vitro* mass propagation of *Stevia rebaudiana* by nodal cultured on MS medium plus kinetin for shoot induction and identified a hormone-free medium for root induction. This protocol is a rapid technique for propagation and a potentially effective approach for *S. rebaudiana* large-scale production in Thailand.

ACKNOWLEDGMENT

Authors would like to thank the National Research Council of Thailand and Research and Development Institute, Chiang Mai Rajabhat University, Chiang Mai, Thailand for financial support of this study.

REFERENCES

Al-Maarri, K., Y. Arnaud and E. Miginiac, 1994. Micropropagation of *Pyrus communis* cultivar passe crassane seedlings and cultivar Williams: Factors affecting root formation *in vitro* and *Ex vitro*. Sci. Hortic, 58: 207-214.

Anbajhagan, M., M. Kalpana, R. Rajendran, V. Natarajan and D. Dhanavel, 2010. *In vitro* production of *Stevia rebaudiana* Bertoni. Emir. J. Food Agric., 22: 216-222.

Andrew, H.R. and J.M. David, 2006. Stevia: An Intense, Natural Sweetener: Laying the Groundwork for a New Rural Industry: A Report for the Rural Industries Research and Development Corporation. RIRDC, Australian, ISBN: 9781741512830, Pages: 20.

Bridel, M. and R. Laviellem, 1931. The sweet principle of Kaa-he-e (*Stevia rebaudiana*). J. Pharm. Chem., 14: 99-154.

Das, A., S. Gantait and N. Mandal, 2011. Micropropagation of elite medicinal plant: *Stevia rebaudiana* Bert. Int. J. Agric. Res., 6: 40-48.

Dheeranupattana, S., M. Wangprapa and A. Jatisatienr, 2007. Effect of sodium acetate on stevioside production of *Stevia rebaudiana*. Proceedings of the International Workshop on Medicinal and Aromatic Plants, January 15-18, 2007, Chiang Mai, Thailand, pp: 269-272.

George, S. and S.L. Sherrington, 1984. Tissue culture in forest trees: Clocal propagation of *Tectona grandis* L. (teak) by tissue culture. Plant Sci. Lett., 17: 259-268.

Laribi, B., N. Rouatbi, K. Kouki and T. Bettaieb, 2012. *In vitro* propagation of *Stevia rebaudiana* (Bert)-A non caloric sweetener and antidiabetic medicinal plant. Int. J. Med. Arom. Plants, 2: 333-339.

Meera Manjusha, A.V. and B.N. Sathyanarayana, 2010. Acclimatization studies in Stevia (*Stevia rebuadiana* Bert.). Acta Hortic., 865: 129-133.

Miyazaki, Y. and H. Watenabe, 1974. Studies on the cultivation of *Stevia rebaudiana* Bertoni; on the propagation of the plant (English abstr.). Jap. J. Trop. Agric., 17: 154-157.

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

Pratibha, G., S. Satyawati and S. Sanjay, 2010. Micropropagation of *Stevia Rebaudiana* (natural sweetener) using kinetin for steviol glycoside production. Res. J. Biotech., 5: 63-67.

Rafiq, M., M.U. Dahot, S.M. Mangrio, H.A. Naqvi and I.A. Qarshi, 2007. In vitro clonal propagation and biochemical analysis of field established stevia rebaudiana bertoni. Pak. J. Bot., 39: 2467-2474.

Steven, M.S., B.M. Gail and W.W.B. Christopher, 1992. Stevioside biosynthesis by callus, root, shoot and riited-shoot cultures *in-vitro*, Plant. Cell Tiss. Org., 28: 151-158.

Verma, S., K. Yadav and N. Singh, 2011. Optimization of the protocols for surface sterilization, regeneration and acclimatization of *Stevia rebaudiana* Bertoni. American-Eurasian J. Agric. Environ. Sci., 11: 221-227.

Yang, T.W., Y.I. Hsing and W.C. Cheang, 1981. Clonal propagation of *Stevia rebaudiana* Bertoni through axillary shoot proliferation *in vitro*. Bot. Bull. Academia Sin., 22: 57-62.