Micropropagation and Acclimatization of Stevia rebaudiana Bertoni

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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 (11.8±3.44 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 rebaudioside. Stevioside is 300 times sweeter than sucrose and is regarded as a calorie-free, low-calorie sweetener (Bridel and Lavielle, 1931). Therefore, it is of special interest to the diet-conscious and diabetic persons who use hypoglycemics. 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 country, 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 Waterabe, 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
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 agar. 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
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. Adventitious 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.* (1991) 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 Marjusha and Sathyararayana (2010).

**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.

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