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*Short Communication*

# An efficient protocol for *in vitro* clonal propagation of natural sweetener plant (*Stevia rebaudiana* Bertoni)

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**Leaf segments of *Stevia* were cultured on MS medium supplemented with varying concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mg/L$^{-1}$) of growth regulators (BAP and 2, 4-D). Ninety one percent aseptic cultures were obtained when sterilized with 0.1% $HgCl_2$ for 10 min. The highest amount of callus was obtained in MS medium supplemented with1.0 mg/L$^{-1}$ BAP+0.5 mg/L$^{-1}$ 2, 4-D, respectively. On the other hand, 2.5 mg/L$^{-1}$ BAP + 0.5 2,4-D showed lowest performance. Highest shoot was obtained in 2.0 mg/L$^{-1}$ BAP. These shoots was transfer into different concentration of IBA, NAA and IAA (0.5. 1.0, 1.5, and 2.0) used. Highest rooting percentage was recorded on MS medium with 0.5 mg/L$^{-1}$ IAA. The rooted plantlets were transfer into mist chamber with relative humidity for 2 - 4 week after these plants were hardened and successfully established in soil.**

**Key words:** *Stevia* rebaudiana, Sweetener, Callus, leaf.

## INTRODUCTION

*Stevia* (*Stevia rebaudiana* Bert.) is a small, herbaceous, semi-bushy, perennial shrub of Compositae family originated from Paraguay. It grows well at the temperature ranging between 15 - 30°C. It is one of 154 members of the genus Stevia, which produces stevioside, a diterpinoid glycoside isolated from plant leaves (Robinson, 1930). Stevioside of special intrestto diabetics, persons with hyperglycemia and the diet conscious. A sugar-free, no calorie natural sweetener is especially helpful for people who are diabetic, prone to yeast infections, or trying to lose a few extra pounds by controlling calories. The product can be added to tea and coffee, cooked or baked goods, processed foods and beverages. Dry leaves of this plant are 30 times sweeter than sugar with Zero calories. The first report of com-mercial cultivation in Paraguay were in 1964 (Sumida, 1968) began a large effort aimed at establishment. Stevia as a crop in Japan, since then Stevia has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, U.S. and Canada (Lee et al., 1979). The herbal drug industry is considered to be a high growth industry of the late 90s and seeing the growing demand, it is all set to flourish in the next century in ancient Indian traditional Ayurvedic system of medicine.

*Stevia* has various properties such as antibacterial, anticandidal, antifungal, antiviral, cardio tonic (tones, balances, strengthens the heart), diuretic, hypoglycemic, vasodilator. Seed germination of *Stevia* is often poor or very low (Miyazaki and Watenabe, 1974).

Therefore, there are basically two options for multiplication. The first is tissue culture and second is vegetative propogation.

## MATERIALS AND METHODS

### Plant materials

The explants; leaf segments were used as experimental material. The explants were collected from 5 - 6 months old field grown plants at FRI (Forest Research Institute, Dehradun) experimental field. The explants were treated with Tween 20 (fungicide) 5 - 6 drops for 5 min with constant shaking for clean fungus content from leaf surface then washed thoroughly with distilled water. Then the materials were taken under laminar air flow cabinet and surface sterilization was done with (0.1% $HgCl_2$) mercuric chloride for 10 min followed by 4 - 5 times rinse with sterile distilled water to remove traces of $HgCl_2$ from material.

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**Table 1.** Effects of cytokinin and auxin in MS medium on *in vitro* shooting and callus formation from leaf segments of *Stevia*.

| Growth regulators (mg/L$^{-1}$) | % of shoot | Shoots /Cultures | Average shoot length (cm) | Texture |
|---|---|---|---|---|
| BAP (0.5) | 45.67 | 3.13 ± 0.11 | 2.15 ± 0.28 | Green |
| BAP (1.0) | 56.31 | 4.33 ± 0.29 | 2.32 ± 0.27 | Green |
| BAP (1.5) | 60.10 | 5.40 ± 0.18 | 2.43 ± 0.11 | Green |
| BAP (2.0) | 76.44 | 6.78 ± 0.23 | 3.34 ± 0.55 | Green |
| BAP (2.5) | 58.27 | 5.12 ± 0.41 | 2.00 ± 0.42 | Green |
| | | **Amount of callus** | | **Callus status** |
| BAP + 2-4-D (0.5)+ (0.5) | | ++++ | | Good |
| BAP + 2-4-D (1.0) + (0.5) | | +++++++ | | Best |
| BAP + 2-4-D (1.5) + (0.5) | | ++++ | | Good |
| BAP + 2-4-D (2.0) + (0.5) | | +++ | | Average |
| BAP + 2-4-D (2.5) + (0.5) | | + | | Dead |

**Preparation of culture media**

Stock solution of all components were prepared with appropriate amount of all the components, Appropriate amount of all stock solution were mixed following the standard media preparation procedure. The pH of the media was adjusted to 5.6 - 5.8 and growth regulators were added at different concentration (0.5, 1.0, 1. 5, 2.0, 2.5 mg/L$^{-1}$ ). In Each treatment 2 explants were inoculated in 50 test tubes, 10 test tubes each.

**Inoculation technique**

Leaf segments into 1 cm$^2$ square pieces and the aseptically inoculated onto callus induction media. All inoculation and aseptic manipulation were carried out in laminar air flow cabinet. Before use, the floor of cabinet was cleaned with 90% ethyl alcohol to reduce chance of contamination. The instruments like scalpels, forceps, needles etc, were sterilized by an alcoholic dip and flaming method inside the laminar air flow chamber. Before the inoculation hands were cleaned by spraying 70% ethyl alcohol. After the inoculation tubes were shelved in dark condition. The temperature of the culture room was maintained at 25 ± 1°C.

**Data collection**

From the seventh day of inoculation, regular visual observation was done up to five weeks to records the days of callus initiation and maturation. The explants showing embryogenic callus was recorded after 4 - 5 weeks, Histological sections and microscopic observation was done under microphotograph.

## RESULT AND DISCUSSION

### *In vitro* shoot proliferation and multiplication

The auxiliary buds in callus zones proliferated within 6 - 7 weeks of culture on cytokinin supplemented MS medium. When the explants were cultured on 2 mg/L$^{-1}$ BAP containing media (76.44%) of them produced shoots. the auxiliary shoots proliferated and elongated (3.34 ± 0.55 cm) within three weeks of culture. In the experiment MS medium containing 2 mg/L$^{-1}$ BAP showed the best response of multiplication (Uddin et al., 2006). Shoot formation with 6.78 ± 0.23 microshoots per explants (Table 1). Further more, MS medium containing 0.5 mg/L$^{-1}$ 2,4-D +1.0 mg/L$^{-1}$ BAP also showed the maximum callusing in leaf segment explants (Table 1).

### Rooting induction from microshoots

*In vitro* raised microshoots (3-4 cm) were excised from callus culture and grown on MS medium with growth regulators. Initiation of roots from microshoots was very slow in NAA. The percentage of shoots that formed roots and no of roots/ shoots significantly varied at different concentration of IBA, IAA and NAA.

The highest rooting 98.12% in microshoots was obtained as MS medium containing 0.5 mg/L$^{-1}$ IAA with 3% sucrose with in 2 weeks of microshoot transfer (Table 2). Further more the no of roots per microshoots was optimal (13.7 Roots/ Microshoots) on MS medium containing (0.5 mg/L$^{-1}$ IAA) but the average root length per shoot was higher (3.65 cm) on 1.5 mg/L$^{-1}$ IBA containing MS media (Murashige and Skoog, 1962).

The effect of IBA, IAA and NAA on root induction was positive; however, auxins concentrations had a positive effect on the number of roots/ shoots and it was higher in the case of IAA than IBA and NAA. Steven et al. (1992) reported that 1.0 mg/L$^{-1}$ NAA MS medium showed maximum rooting in propagated shoots of *Stevia*. The potential of IBA in root induction has been reported in many species (Epstein et al., 1993). In root induction experiment, 0.5 mg/L$^{-1}$ IAA was found better as maximum roots proliferated that result as better reported by Steven et al. (1992).

### Acclimatization and field establishment

Rooted plantlets were shifted to plastic pots containing

**Table 2.** Effects of IBA, NAA or IAA on adventitious root formation from micro cuttings in *S. rebaudiana*.

| Auxins types | Concentration (mg/l) | % of cuttings rooted | No. of roots per shoots | Average root length (cm) | Day of mergence of roots (days) |
|---|---|---|---|---|---|
| IAA | 0.5 | 98.12 | 13.71 | 2.10 | 6-12 |
| | 1.0 | 91.40 | 9.88 | 2.35 | 6-14 |
| | 1.5 | 74.99 | 7.31 | 2.17 | 6-12 |
| | 2.0 | 64.31 | 4.98 | 3.01 | 6-17 |
| NAA | 0.5 | 81.50 | 8.65 | 2.41 | 6-12 |
| | 1.0 | 70.20 | 7.57 | 2.93 | 6-12 |
| | 1.5 | 59.72 | 5.34 | 2.42 | 6-12 |
| | 2.0 | 54.43 | 4.45 | 2.78 | 6-12 |
| IBA | 0.5 | 68.72 | 7.88 | 3.07 | 6-14 |
| | 1.0 | 60.68 | 5.95 | 3.43 | 6-14 |
| | 1.5 | 51.85 | 5.02 | 3.65 | 6-14 |
| | 2.0 | 40.43 | 3.55 | 2.98 | 6-12 |

sterilized soil, sand and peat moss at 1:1:1 ratio covered with transparent polythene begs and placed in acclimatization room at 28°C with 70 - 90% humidity. After the 2 weeks and than completely removed of after 3 weeks for proper hardening. A total no of 71% plants of survived thoroughly this procedure. In many species the reduced the survival rate of the explants during acclimatization is affiliated to the utilization of high concentration of IBA and NAA in rooting medium (Al-maarrie et al. 1994).


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