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# Large scale *in vitro* propagation of *Stevia rebaudiana* (bert) for commercial application: Pharmaceutically important and antidiabetic medicinal herb

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ABSTRACT

*Stevia rebaudiana* is a valuable medicinal plant species and it is being used for the treatment of diabetes. Currently, there is a high demand for raw material of this medicinal herb due to ever increasing diabetes disorder among the population. In order to meet the increased demand an efficient *in vitro* propagation of *S. rebaudiana* was established. Nodal explants collected from the field were cultured on MS basal medium fortified with different concentrations of BAP (0.5–3.0 mg/l) and KIN (0.5–3.0 mg/l) individually for shoot bud induction. *In vitro* derived nodal buds were cultured on MS medium supplemented with different concentrations (0.5–3.0 mg/l) of BAP and KIN for multiple shoot bud regeneration. In the second experiment, *in vitro* derived buds were placed on MS medium supplemented with different concentrations of BAP (0.5–3.0 mg/l) in combination with 0.5 mg/l IAA or IBA or NAA for shoot bud multiplication. The highest frequency (94.50%) of multiple shoot regeneration with maximum number of shoots (15.69 shoots/explant) was noticed on MS medium supplemented with 1.0 mg/l BAP. For large scale plant production, *in vitro* derived nodal bud explants were cultured on MS medium fortified with 1.0 mg/l BAP, in which about 123 shoots/explant were obtained after three subcultures on the same media composition. Elongated shoots (>2 cm) dissected out from the *in vitro* proliferated shoot clumps were cultured on half-strength MS medium containing different concentrations of NAA (0.1–0.5 mg/l) and/or MS medium fortified with various concentrations (0.5–2.0 mg/l) of auxins (NAA, IAA and IBA) for root induction. Highest frequency of rooting (96%) was noticed on half-strength MS medium augmented with 0.4 mg/l NAA. The rooted plantlets were successfully transferred into plastic cups containing sand and soil in the ratio of 1:2 and subsequently established in the greenhouse. The present *in vitro* propagation protocol would facilitate an alternative method for rapid and large-scale production of this important antidiabetic medicinal plant.



## 1. Introduction

Medicinal plants are of great interest to researchers in the field of biotechnology as most of the drug industries depend, in part, on plants for the production of pharmaceutical compounds (Chand et al., 1997). Diabetes mellitus is a complex and a multifarious group of disorders that disturbs the metabolism of carbohydrates, fat and protein. It results from shortage or lack of insulin secretion or reduced sensitivity of the tissue to insulin. Diabetes mellitus is a common disorder among the Indian population. It is estimated that diabetes would affect approximately 57 million people by the year 2025. The management of diabetes is a global problem until now and successful treatment is not yet discovered. There are many synthetic medicines/drugs developed for patients, but it is the fact that it has never been reported that someone had recovered completely from diabetes (Li et al., 2004). The modern oral hypoglycemic agents produce undesirable and side effects. Thus, alternative therapy is required to shift towards the different indigenous plant and herbal formulations (Satyanarayana et al., 2007). Plant drugs are frequently considered to be less toxic and free from side effects than synthetic one. With the worldwide increasing demand for plant derived medicines, there has been a concomitant increase in the demand for raw material. However, the increasing human and livestock populations affected the status of wild plants, particularly those used in herbal medicine.

*Stevia rebaudiana* (Bert.) is a perennial sweet herb, belonging to the family Asteraceae. It is a natural, non-caloric sweet-tasting plant used around the world for its intense sweet taste. *Stevia* plant produces zero-calorie diterpene glycoside (Stevioside and Rebaudiooside) in its leaves as natural non-nutritive sweetener which is being used as substitute to sucrose (Chalapathi and Thimmegowda, 1997). The eight types of glycosides viz. rebaudioside (A–F), steviolbioside A and dulcoside A were identified (Starratt and Gijzen, 2004). *Stevia* making strides has the best alternative of the table

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sugar in the years to come. It is being commercially cultivated in China, Taiwan, Thailand, Korea, Japan, India and Malaysia.

In addition, *S. rebaudiana* possesses hypoglycemic, hypotensive, vasodilating, taste improving, sweetening, antimicrobial properties and increases urination function of the body. It has been found to be non-toxic, non-carcinogenic, non-mutagenic and is devoid of genotoxic effect. It does not affect blood sugar level hence safe for diabetics. The key benefit of *Stevia* is it stimulates the release of insulin and normalizes blood glucose levels. It is recommended for diabetes and has been extensively tested on animals and has been used by humans with no side effects. *Stevia* extract and stevioside are officially approved as food additives in Brazil, Korea and Japan. *Stevia* could be used as a major source of high potency sweetener (alternate to sucrose) in the near future (Starratt and Gijzen, 2004).

Currently, *S. rebaudiana* is being propagated by stem cuttings. Low seed germination percentage is a major limiting factor for large scale cultivation of *Stevia* plant species for commercial usage. Further vegetative propagation is also limited by the less number of individuals obtained from single plant. Therefore, a suitable alternative method for large scale plant production within a short period is the use of *in vitro* culture technology. The micropropagation of plants through shoot tip or axillary bud culture allows recovery of genetically stable and true to type progeny. There are few reports on *in vitro* clonal propagation of *Stevia* plants using leaf, nodal, internodal segment and shoot tip explants. Bespalhok and Hattori (1997) obtained only embryogenic callus from floret explants of *S. rebaudiana*. *In vitro* plant regeneration from shoot tip explants of *S. rebaudiana* was reported by Patil et al. (1996), Uddin et al. (2006) and Debnath (2008). Sivaram and Mukundan (2003) obtained maximum number of shoot buds (7.9 shoots/explant) from nodal explants on MS medium supplemented with 8.87 μM BAP and 5.71 μM IAA. Although few tissue culture protocols have been reported in the recent past, there is no efficient regeneration protocol available for commercial scale production of this important medicinal plant. The major goal of this project was to develop an efficient protocol for large-scale production of *Stevia* plants from an elite germplasm.

## 2. Materials and methods

### 2.1. Preparation of explants

*S. rebaudiana* plants were collected from Horticulture Research Station, Tamil Nadu Agricultural University (TNAU), Yercaud, Tamil Nadu and maintained in the greenhouse, Department of Biotechnology, Periyar University, Salem-11. For shoot bud induction, nodal explants were collected from 3 months old plants and were washed in running tap water. Explants were washed with few drops of Tween-20 to remove the superficial dust particles including microbes. Then, they were surface sterilized with 0.1% (w/v) mercuric chloride for 8 min followed by rinsing them for five times with sterile distilled water. Sterilized nodal explants were used for *in vitro* studies as described below.

### 2.2. Culture media and growth conditions

The culture medium consisted of MS (Murashige and Skoog's, 1962) salts, vitamins, 3% (w/v) sucrose and the pH of the media was adjusted to 5.6 with 0.1 N NaOH or HCl before adding of 0.7% (w/v) agar. Media (15 ml) were poured into 25 mm × 150 mm culture tubes (Borosil, Mumbai) and autoclaved at 121 °C for 15 min. The cultures were incubated at 24 ± 2 °C under 16/8 h (light/dark cycle) photoperiod (60 μE $m^{-2}$ $s^{-1}$) and irradiance provided by cool-white fluorescent tubes (Philips, India).

### 2.3. Shoot bud initiation

Surface sterilized nodal explants were cultured on MS medium supplemented with different concentrations of BAP (0.5–3.0 mg/l) and/or KIN (0.5–3.0 mg/l) for shoot bud induction. After two weeks of culture, direct shoot bud initiation from the nodal explants was noticed.

### 2.4. Induction of multiple shoots

In order to achieve multiple shoot bud regeneration, two different experiments were performed as described below.

#### 2.4.1. Experiment: I

In the first experiment, the effect of different concentrations of two cytokinins on multiple shoot regeneration was examined. Nodal explant derived *in vitro* regenerated shoot buds as explant source were cultured on MS medium fortified with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) of BAP and KIN individually for multiple shoot bud development.

#### 2.4.2. Experiment: II

In the second experiment, the influence of different concentrations of BAP in combination with three auxins on induction of multiple shoots was evaluated. To identify the best auxin for shoot bud multiplication, nodal explant derived *in vitro* developed shoot buds as explant source were cultured on MS medium containing different concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) in combination with 0.5 mg/l IAA or IBA or NAA.

### 2.5. Large scale production of shoot buds

For large scale plant production, *in vitro* regenerated shoot buds were cultured on MS medium supplemented with 1.0 mg/l BAP using 250 ml flasks. The cultures were subcultured onto the fresh same media composition once in 3 weeks interval. This process was repeated for another three subcultures (each 21 days) to examine the effect of subculture on production of large scale shoot buds. After 65 days of culture multiple shoots were counted for analysis of total number of regenerated shoot buds.

### 2.6. Rooting of elongated shoots and acclimatization

The elongated shoots (>2.0 cm height) were transferred onto half-strength MS medium fortified with different concentrations of NAA (0.1–0.5 mg/l) and full-strength MS medium with various concentrations of IAA or IBA or NAA (0.5–2.0 mg/l) for root induction. Plantlets with well-developed roots were removed from the culture tubes and gently washed under running tap water to remove adhering medium. Subsequently, they were transferred to plastic cups containing sterile sand and soil mixture in 1:2 ratio. The potted plantlets were initially maintained in the controlled environment for two weeks and subsequently they were shifted to the greenhouse. After twenty days, the plantlets were successfully established in the field.

### 2.7. Statistical analysis

Experiments were set up in a completely randomized block (CRB) design and each experiment had three replicates. The cultures were observed periodically and percent of response for shoot bud regeneration, multiple shoots development and rooting. A total number of shoots as well as roots were also recorded by visual observations. The analysis of variance (ANOVA) was performed