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# Plant regeneration of an anti-diabetic plant: *Stevia rebaudiana* L. Bertoni and evaluation of its antimicrobial activity using *in vivo* leaf extracts

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**Abstract**
A convenient and faster protocol established for clonal propagation of *Stevia rebaudiana* Bertoni through callus and shoot culture, with its antimicrobial screening. MS medium with (0.5-2.0mg/L) BAP was found to be optimal for shoot initiation. A high-frequency multiplication rate was obtained from nodal segments in MS medium supplemented with 0.5 mg/l BAP+2.0 mg/l Kn. Root induction occurred with IBA 1.0 mg/l then transferred to poly tray containing sand, vermiculite and soil (1:1:2) and acclimatized for a period of 3 weeks. *In vivo* grown leaf extracts in different solvent system exhibited a concentration dependent antibacterial and antifungal inhibition against standard by disc diffusion method. The methanolic extracts of *Stevia* leaf showed best antibacterial and antifungal activity against a number of microorganisms. This proved Stevia's potential as antimicrobial agent from non antibiotics sources. Therefore, commercial manufacture of active constituents from these improved elite lines would be useful and profitable.

**Keywords:** *Stevia rebaudiana*, Regeneration, Antimicrobial activity, Leaf extract

## 1. Introduction
In ancient Indian traditional Ayurvedic system of mediine, *Stevia rebaudiana* has a long history of use by tribal people. *Stevia rebaudiana* Bertoni, belonging to the family Asteraceae, is a perennial and endemic, medicinal shrub [23]. Medicinal plants constitute one of the most important groups of wild plants in terms of their contribution to the economy and wellbeing of farmers.

*Stevia* is herbaceous perennial shrub originated from the highlands of Paraguay and sections of Argentina and Brazil. This plant is a natural sweetener and famously known as "Sweet Weed", "Sweet Leaf", "Sweet Herbs" and "Honey Leaf". *Stevia rebaudiana* Bert belongs to the family Asteraceae, one of 154 members of the genus *Stevia*, which produces sweet steviol glycosides [18]. The leaves of *Stevia* are the source of diterpene glycosides, stevioside and rebaudioside [30]. Stevioside is regarded as a valuable natural sweetening agent because of its relatively good taste and chemical stability. In Japan alone, an estimated 50 tons of stevioside is used annually with sales valued in order of $220 million Canadian [3]. Now, *Stevia* has been introduced as a crop in a number of countries including Brazil, Korea, Japan, Mexico, United States, Indonesia, Tanzania and Canada [21, 20, 8, 3] for food and pharmaceuticals products. *Stevia* as a sweetener also have bitter after taste due to the presence of essential oils, tannins, and flavanoids. *Stevia* has many properties such as antifungal, anti-bacterial, anti-oxidant, antimicrobial, anti-inflammatory, anti-diabetic, hypertensive, and hyperglycaemic [9].

Seeds of *Stevia* show a very low germination percentage and vegetative propagation is limited by lower number of individuals [18]. 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* [12], *in vitro* micropropagation from shoot tip and leaf [26]. The present study was carried out in order to optimize and to establish a suitable protocol for *in vitro* propagation of *S. rebaudiana* and comparison of antimicrobial activity of dried leaf extract in different solvent system.

## 2. Material and Methods
### 2.1 *In vitro* studies
To carry out the experiment, Medicinal Plant was selected for the study namely *S. rebaudiana.* The plant collected from green house of Tectona Biotech Resource Centre (TBRC), Bhubaneshwar and brought to the laboratory. The elite plants selected were maintained under hygienic conditions. Young, actively growing leafs and nodal segments ranging in size from 1 to 1.2 cm were collected from *Stevia* seedlings. The explants were washed thoroughly under running tap water for 30 minute then treated with a few drop of liquid detergent (Rankleen, Ranbaxy India) and Tween 20 (Himedia Laboratories, India) for 10 minutes with lively shaking. After washing with detergent explants were again washed with running tap water to remove any traces of detergent and kept in 1% (w/v) bavistin (BASF India Limited) for 30 mint. Futher the explants was shifted to 0.1% sodium hypochlorite for 5 min. Subsequently the explants were transferred to laminar airflow cabinet and transferred to 500 ml sterilized glass bottle containing MS medium supplemented with various concentration of 2-4,D or NAA alone or in combination for callus induction. The obtained calli were transferred to MS medium containing BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/l)) for shoot proliferation. For multiplication the best responding explants were transferred to MS media containing 0.5 mg/l BAP with Kn (1.0, 2.0 mg/l) and 2.0 mg/l BAP with IAA (0.5, 1.0 mg/l) containing 3% sucrose. Elongated shoots were transferred to rooting medium, which consisted of MS supplemented with different concentrations of indole-3-butyric acid (IBA) at 0.5, 1.0, 1.5, 2.0, and 2.5 mg/l. All the cultures were grown at 25 ± 2 °C under 16 h photoperiod supplied by two with a photosynthetic photon

flux density provided by white fluorescent lamps. After 2 weeks, the rooted plantlets were thoroughly washed in distilled water to remove the agar and transferred to small pots containing Vermimiculite, soil, and sand (1:2:1). The pots placed under transparency box and maintained under 25±3 0C temperature and 70% relative humidity. After an adaptation period (4 weeks), acclimatized plantlets were transferred to greenhouse.

### 2.2 Aantimicrobial activity assay

#### 2.2.1 Preparation of leaf extracts of *S. rebaudiana*

The leaves of *Stevia* was air dried and pounded into fine powder using Mortar and Pestle. The powdered plant material was subjected for successive extraction using solvents such as chlorophyl and methanol, by using plant extracts. Leaves (2.0 g) were collected, dried and subjected to 100ml cold extraction at $4^0$C for 24 h. The paste was filtered using cheesecloth and was centrifuged (10,000rpm, 10 min, 250C). The extract was stored in the refrigerator for further use.

#### 2.2.2 Preparation of inoculum

Stock cultures were maintained at 4˚C on nutrient agar slants. Active cultures for experiments were prepared by transferring a loopful of culture to 10 mL of nutrient broth (Hi Media) and incubated at 37˚C for 24 hours for bacterial proliferation.

#### 2.2.3 Disc Preparation

The 6mm (diameter) discs were prepared from whatmann No. 1 filter Paper the discs were sterilized by autoclave at 12°C. After the sterilization the moisture discs were dried on hot air oven at 50°C.Then various solvent extract discs and control discs were prepared.

#### 2.2.4 Assay for antibacterial activity

The disc diffusion method for antibiotic susceptibility testing was the Kirby-Bauer method. Each extracts were made to a final concentration of 2mg/100mL. 24 hour old cultures of test organisms (50µl) were seeded onto Mueller Hinton agar plate and uniformly spread with a spreader. After solidified, the plant extract was introduced into the disc and the plates were incubated at 37˚C for 24 hours. The antibacterial activity of the plant extract was determined by measuring the diameter of the inhibition zone. Stock solutions of wide spectrum antibiotics like ampicillin were prepared as 1mg/ml (w/v) concentration in sterile distilled water and filter sterilized by using syringe filters.

#### 2.2.5 Assay for antifungal activity

Potato dextrose agar (Hi Media) was prepared and poured into the petriplates. After solidification a loopful of culture was placed in the centre of the plate. All the plates were incubated at 25˚C for 4 days18. The growth of the fungal cultures was measured and compared with the respective standard plates like fungicide (1.0mg/1ml). The antifungal assay for each of the extracts against all microorganisms tested was performed in triplicates.

## 3. Results and Discussion

*In vitro* propagation studies show that the plant hormones play an important role in the callogenesis and regeneration. The various combinations of the hormones used for callus induction are given in Table 1. Callus initiation was observed in cut surface of leaves after 15 to 18 days of culture initiation. Maximum amount of callusing (100%) was observed on the MS medium supplemented with combination of 2, 4-D and NAA (0.5 +1.5 mg/l) (Fig-1), while at higher concentrations of NAA, the callus induction response was only 41.3%. The best was calli formation in MS medium containing 10 µM NAA+8.8 µm BA was observed in many cases. Callus formation has also been observed by [28, 2, 14], from leaf and floret. In some cases of *S. rebaudiana* probably the callus observed may be embryogenic in nature [6].

Multiple shoots were found to be developing from nodal explants when cultured on MS medium with BAP in the range of 0.5-3.0 mg/l. However, initiation of multiple shoots in most of treatments was observed within two weeks of culture (Figure 1). BAP at its 0.5mg/l concentration evoked best response. Shoot tips showed a better response to shoot proliferation than nodal segments and BAP (1.0 mg/L) was superior to all other hormonal treatments for shoot proliferation [9]. Similar relationship between size of stem-tips and number of leaf primordial were also found and came to the same conclusion [26]. In some studies, they used 3-4 auxiliary bud as explants for maximum regeneration [16], whereas some used shoot apex, nodal and leaf explants ranging in size from 1 to 1.2cm [23].

Among combination of BAP with Kn and IAA, best response for shoot multiplication was observed on medium containing 0.5 mg/l BAP + 2.0 mg/l Kn (average number of shoots (3.40±0.37) and best shoot length was observed on medium containing 0.5 mg/l BAP+ 1.0 mg/l Kn (average shoot length 6.66±0.38cm). Shoot tips showed better response for shoot proliferation than nodal segments and BAP (1.0 mg/L) was superior to all other hormonal treatments for shoot proliferation [9]. Higher percentage of rooting were obtained in half strength MS basal medium supplemented with NAA (1.0 mg/L), were as in the early report full strength MS medium supplemented with NAA (1.5 mg/L) was the best medium for rooting [9]. The elongated shoots were transferred to the rooting medium in different concentration of IBA. The best rooting response, however, was observed on medium containing 1.0mg/l IBA, where roots measuring 1.68±0.32 cm (average) were formed (Table-3). Similar studies on shoot proliferation has been performed [16, 14, 22, 1, 5, 23]. They also reported that plant hormone is necessary for shooting, elongation and rooting. *In vitro* rooted plantlets were initially hardened in culture room conditions where leaves expanded. After 3 week, the plantlets were shifted to mist house. There was an increase in length of shoots and new leaves emerged which expanded quickly (figure-E).

The medicinal properties are attributed to the primary and secondary metabolites synthesized by the plants [4]. The results of antimicrobial activity of leaf extract of *Stevia* are shown in Graph-1. Different concentration of Methanol and chloroform solvents extracts (100, 150, 200 and 250µl) were tested against the pathogenic bacteria, namely, *Escherichia coli, Bacillus subtilius* A concentration of 150µl of methanol extract was found sufficient enough to inhibit the growth of test microorganism *E.coli* (25mm) and *Bacillus subtulis* (23mm) completely in Petri plates compare to chloroform extracts of E.coli (7mm) and *Bacillus subtulis* (17.6mm). The Antifungal activity of *Stevia* plant fractions were evaluated against *Aspergillus niger*, and *Penicillium chrysogenum.* The two solvents extracts i.e. methanol chloroform was tested on

increasing conc. (100-250µl). All the extracts inhibited the growth of *Aspergillus* and *Penicillium* species but as the incubation time was prolonged, a concentration of 150µl of methanol extract showed higher inhibitory activity (33.1mm) against *Penicillium than* the chloroform extracts (28.3mm). The antifungal activities of the solvent extracts of *Stevia rebaudiana* also varied significantly among the test organisms as shown in Graph-2.

The results of present study indicate that the *Stevia* leaf extracts have inhibitory activities against microorganisms, although their antibacterial activities are lower than that of the standard (Ampicillin). However, in the case of fungi, methanol extract had significantly higher activity than the standard (Bavistin).In some studies; they also recorded very low antibacterial activity for water extracts of *Stevia rebaudiana* leaves [25]. Previous reported shows that water extracts do not have much activity against bacteria [12, 28, 19]. However, it also noted that the growth media also seems to play an important role in the determination of antibacterial activity [9]. This may be possible that the secondary metabolite "stevioside" is responsible for the antimicrobial activity [26]. It may also be concluded that the secondary metabolite is most soluble and acts as antimicrobial substance when it is in methanolic solvent system.

**Table 1:** Callus induction using different kinds of auxins

| Medium | Concentration of growth regulators (mg/l) | | Percentage showing Callusing (mean±SD)* |
|---|---|---|---|
| | 2,4-D | NAA | |
| T1 | 2.0 | 0 | 0.45±0.15 |
| T2 | 0 | 0.5 | 0.33±0.22 |
| T3 | 1.0 | 1.0 | 0.66±0.69 |
| T4 | 1.0 | 2.0 | 0.77±0.29 |
| T5 | 0.5 | 1.5 | 0.46±0.33 |

*Means of 15 replicates/treatment; repeated thrice

**Table 2:** Effect of BAP on shoot proliferation from calli explant of *Stevia rebaudiana*

| Concentration of growth regulators (mg/l) | % of regeneration | Av. No. of Shoot bud/explants (mean±SD)* | Shoot hight in cm (mean±SD)* |
|---|---|---|---|
| BAP | | | |
| 0.5 | 80 | 3.42±0.58 | 6.56±0.84 |
| 1.0 | 40 | 2.85±0.51 | 3.31±0.22 |
| 1.5 | 40 | 1.57±0.40 | 5.70±0.41 |
| 2.0 | 65 | 2.71±0.56 | 7.65±0.54 |
| 2.5 | 45 | 1.85±0.27 | 6.15±0.24 |
| 3.0 | 70 | 2.28±0.71 | 6.56±0.51 |

*Means of 15 replicates/treatment; repeated thrice

**Table 3:** Interactive effect of cytokinin on shoot multiplication of shoot clumps of *Stevia rebaudiana*

| Concentration of growth regulators (mg/l) | % of Regeneration | Av. no. of shoots/ explants (Mean± SE)* | Av. length of shoots (in cm) (Mean±SE)* |
|---|---|---|---|
| 0.5BAP+1.0 Kn | 75 | 2.11±0.41 | 6.66±0.38 |
| 0.5 BAP+2.0Kn | 85 | 3.40±0.37 | 5.70±0.41 |
| 2.0 BAP+0.5 IAA | 76 | 2.18±0.33 | 4.12±0.29 |
| 2.0 BAP+ 1.0 IAA | 80 | 2.91±0.25 | 5.52±0.33 |

*Means of 15 replicates/treatment; repeated thrice

**Table 4:** Effect of IBA on root proliferation in elongated segments of *S.* rebaudiana

| Concentration of growth regulators (mg/l) | Rooting response (%) | Av. no. of roots explants (Mean±SE)* | Av. length of roots (in cm) (Mean±SE)* |
|---|---|---|---|
| 0.5 IBA | 85 | 2.75±0.70 | 0.44±0.35 |
| 1.0 IBA | 90 | 3.80±0.61 | 1.66±0.33 |
| 1.5 IBA | 80 | 1.39±0.50 | 1.05±0.05 |
| 2.0 IBA | 78 | 1.40±0.51 | 0.50±0.04 |

*Means of 15 replicates/treatment; repeated thrice

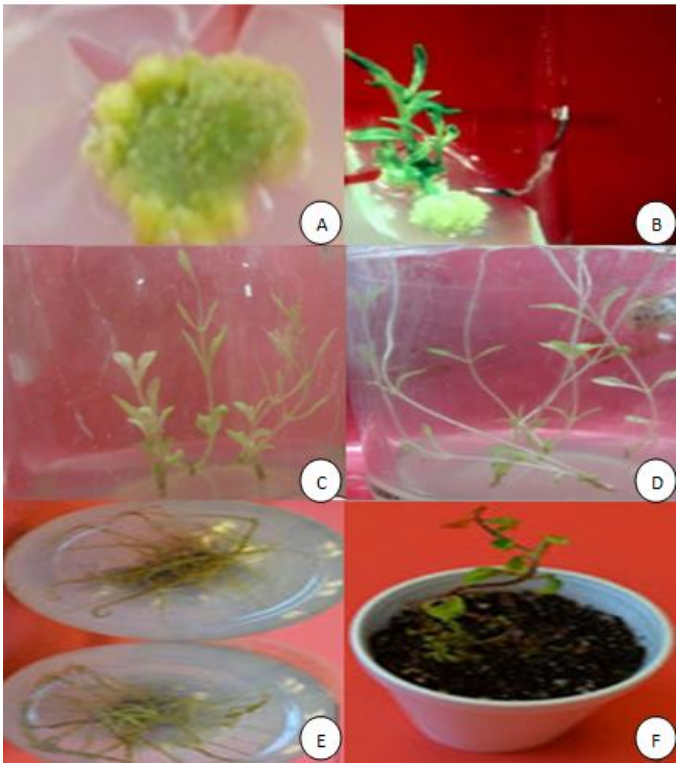


**Fig 1: (A-F) -** Micropropagation of *Stevia rebaudiana*

**A.** Callus induction in MS supplemented with 2, 4-D and NAA (0.5 +1.5 mg/l)
**B.** Bud regeneration from calli in MS supplemented with 0.5 mg/l BAP
**C.** Shoot multiplication in MS with 0.5 mg/l BAP + 2.0 mg/l Kn
**D.** Shoot Elongation in MS with 0.5 mg/l BAP + 2.0 mg/l Kn
**E.** Root formation on ½ MS with 1.0 mg/l IBA
**F.** Rooted plantlets transferred to pots for hardening under greenhouse conditions.

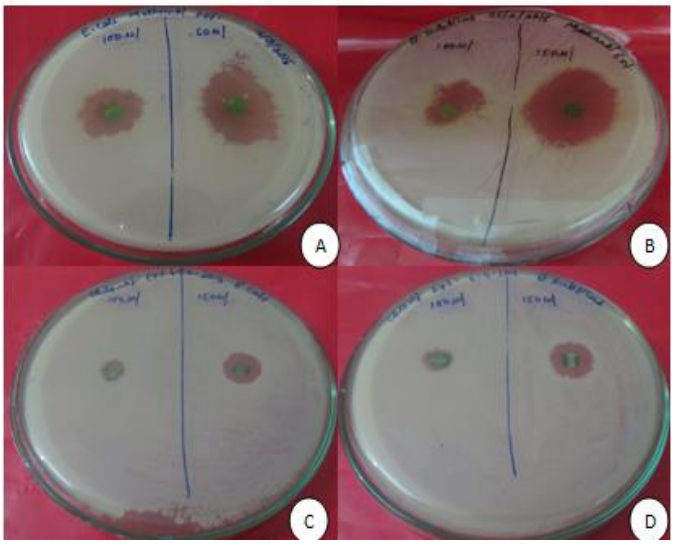


**Fig 2: (A-D) -** Antibacterial activity of leaf extract of Stevia against E. coli and B. subtilus.

**A** and **B-** Methanol extract againts E.coili and B. subtilus
**C** and **D-** Chloroform extract againts E.coili and B. subitlus

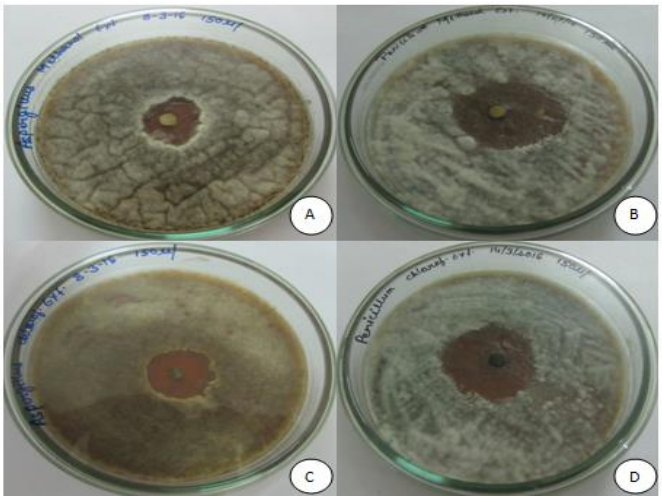


**Fig 3:** (A-D)-Antifungal activity of leaf extract of Stevia against Aspergillus and Penicillium.

A and B- Methanol extract againts Aspergilus and Penicillium
C and D Chloroform extract againts Aspergilus and Penicillium

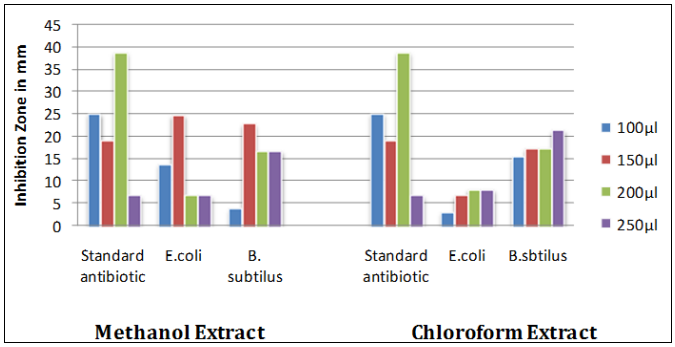


**Fig 4:** Effect of antibacterial activity of methanol and chloroform extracts of *Stevia rebaudiana* leaves *in vivo* (zone of inhibition in mm)

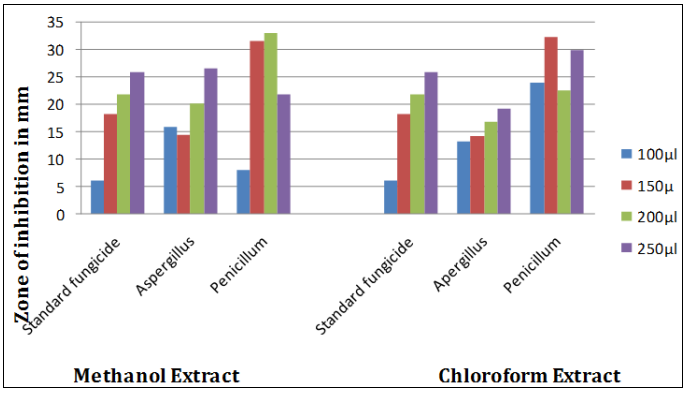


**Fig 5:** Effect of antifungal activity of methanol and chloroform extracts of *Stevia rebaudiana* leaves *in vivo* (zone of inhibition in mm)

## 4. Conclusion

The present study demonstrates a simple and efficient method for high frequency shoot regeneration from leaf explants of *S. rebaudiana*. A high-frequency multiplication rate was obtained from nodal segments in MS medium supplemented with 0.5 mg/l BAP+2.0 mg/l Kn. Root induction occurred with

IBA 1.0 mg/l. The possibility of the presence of some bioactive components in the methanol crude extracts of *S. rebaudiana* have grate antimicrobial potential against bacterial and fungal pathogens. This study would lead to the establishment of the some compounds that could be used to formulate new and more potent antimicrobial drugs of natural origin.

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