



In vitro micropropagation of *Stevia rebaudiana* in Bihar

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ABSTRACT

The shoot apex, leaf, nodal stem and internodal stem of *Stevia rebaudiana* (Bert.) Bertoni were cultured on 21 different media, having different concentrations and combinations of auxins (2,4-D, IBA, NAA) and cytokinins (KIN, BA) supplemented to Murashige and Skoog (MS) basal medium. Besides these explants, the formed callus and developed shoots were also cultured. The tissue culture experiments resulted in establishment of cultures, swelling of explants, elongation of existing shoot, callus formation, caulogenesis, rhizogenesis and regeneration of plantlets, acclimatization of plantlets and ultimately development of micropropagation protocol. During acclimatization, the plantlets regained their photosynthetic efficiency and transpiration mechanism. The size of leaf also increased during acclimatization. In the present investigation a survival rate of 48% was found during hardening. Good callus formation and differentiation of shoots from formed callus showed the possibility of plant improvement through tissue culture.

Key words: Micropropagation, Rebaudioside, *Stevia rebaudiana*, Stevioside, Tissue culture

Stevia, a natural substitute for artificial sweetener having glycoside up to 400 times sweeter than sugar, it is known as golden crop of the future. The plant *Stevia rebaudiana* (Bertoni) Bertoni is a perennial herb and belongs to the family Asteraceae. It is a native of certain regions of South America particularly Paraguay and Brazil. It was rediscovered by Dr M S Bertoni in 1988 from Paraguay. The leaves of *S. rebaudiana* are the source of diterpene glycosides, such as steviolbioside, rubsocide, rebaudioside A, B, C, D, E, and F, dulcoside and stevioside (Starratt *et al.* 2002). *Stevia* creates an intense effect on the taste buds without raising blood sugar levels. The normal proportions of glycosides are stevioside (5-10%), rebaudioside A (2 - 4%), rebaudioside C (1-2%) and dulcoside A (0.5-1%), stevioside being the most abundantly produced glycoside. Chalapathi *et al.* (1997) described the future aspects of natural non-calorie sweetener extracted from the leaves of the South American herb *Stevia rebaudiana* which is about 250–300 times more sweet than sugar and scientific interests. Its sweet compound passes through the digestive process without chemically breaking down, making stevia safe for those who need to control their blood sugar level. There have been no reports to date of adverse effects from the use of stevia products by humans (Brandle and Rosa,

1992). *Stevia* extract and stevioside are officially approved as food additive in Brazil, Korea and Japan (Choi *et al.* 2002, Mizutani and Tanaka 2002) and in the United States. In Japan about 40% of the sweetener market is *Stevia* based. Japan was the first Country in Asia to market stevioside as a sweetener in food and drug industries. Since then, cultivation of this plant has expanded to several countries in Asia, including India, China, Malaysia, Singapore, South Korea, Taiwan and Thailand.

Today China grows 80% of the world's *Stevia* leaf. Cultivation of *stevia* is done mainly by stem cutting because of its heterozygous and self incompatible nature which leads to the lack of fertilization (Miyazaki and Wanteable 1974) requires high labour inputs. The seeds show very less vigour, and propagation through seeds does not allow production of homogenous population which leads to variability in sweetening level and composition (Felippe and Lucas 1971, Miyagawa *et al.* 1986). Poor seed germination and low success rate of its propagation through stem cutting are the limiting factor to large scale cultivation of this species. Propagation is the main concern for those who must grow *Stevia* as an annual. Once good varieties have been created, a tissue culture micropropagation technique will be more economic. The whole plant is harvested once, just before flowering to obtain the maximum glycoside content. Harvesting is easy. It is a perennial plant yielding harvests for more than 3–5 yrs. Leaf yield of 2850 kg/ha with a stevioside concentration of 105 mg/g has been achieved. This is equivalent to 66.2 t/ha of sugar. Since vegetative propagation depends on season and cannot be done round the year, micropropagation or *in-vitro* culture technique can

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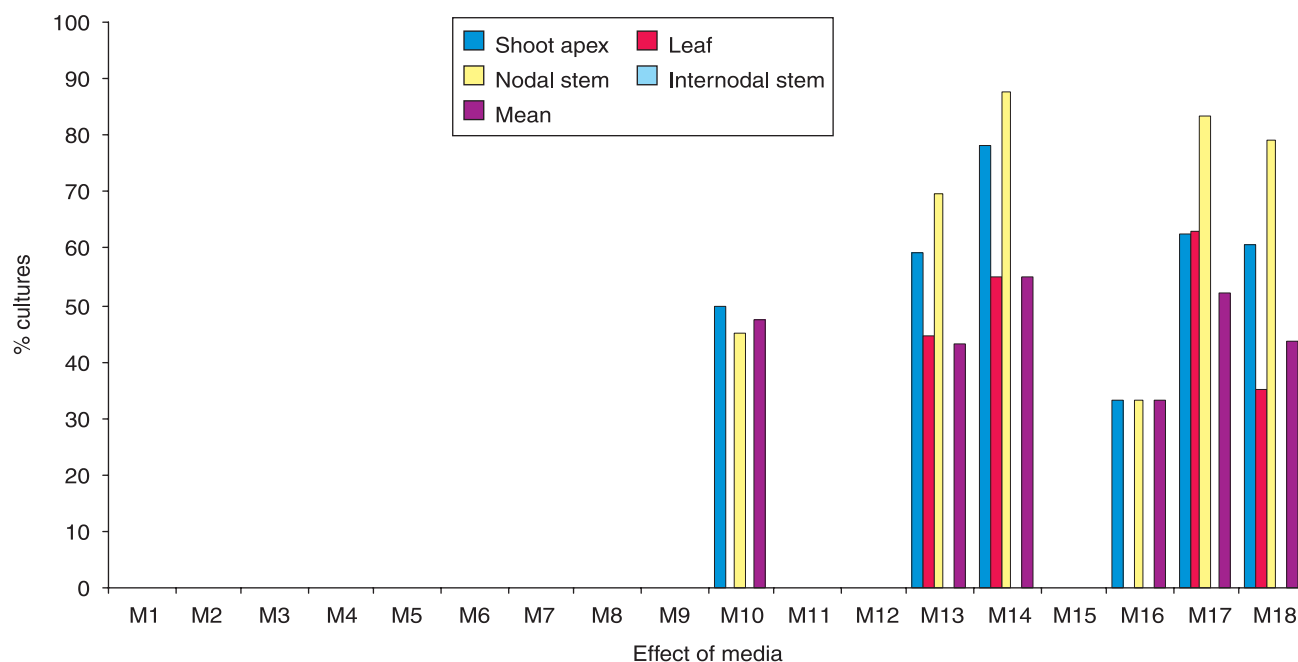


Fig 1 Caulogenesis: Effect of media.

play a vital role for mass multiplication and production of genetically identical (homogenous with even higher levels of sweetening compounds) plants of *S. rebaudiana* in order to meet the growing demand. There are few earlier reports of micropropagation in the same species by Tamura *et al.* (1984), Ferrira and Handro (1988), Patil *et al.* (1996), Sivram and Mukundan (2003), Mirta and Pal (2007), and Pourvi Jain *et al.* (2009). Keeping in view the importance of this natural sweetener plant and farmers' demand, the present study was aimed to develop efficient protocol for *in-vitro* mass propagation of *S. rebaudiana* and exploration of possibility of improvement of stevia through tissue culture.

MATERIALS AND METHODS

The present experiment was carried out at Department of Genetics and Molecular Biology, Rajendra Agricultural University, Pusa, Bihar. The explants were collected from shoot apices, nodal stem, internodal stem and leaf of 1-2 months old greenhouse grown *S. rebaudiana* plants for shoot initiation. After excision, surface sterilization of the explants was done by rinsing in tap water followed by dipping in 1% teepol detergent solution for 10 min and again washing with tap water. In another experiment, the explants were surface sterilized with 5% calcium hypochlorite solution for 15–25 min and surface sterilized with 0.1% mercuric chloride for 1–4 min. The surface sterilized explants were rinsed with sterile distilled water 3 times. The explants were cut into small pieces ranging in size from 0.3–0.5 cm, inside the laminar flow and kept for inoculation. After complete sterilization and slicing, the explants (shoot apex, leaf, nodal stem and internodal stem of *Stevia rebaudiana*) were inoculated in MS medium (Murashige and Skoog, 1962), supplemented with cytokinins (KIN, BA) and auxins (2,4-D, IBA, NAA) used on 21 different media having

different concentrations either singly or in combination. Besides these explants the formed callus and developed shoot were also cultured. The pH of medium was adjusted to 5.8 and prior to autoclaving, 0.8% agar was added to the medium. Then autoclaving was done at 121°C at 15 psi for 20 min. The cultures were inoculated and maintained at a temperature of 25±2°C with relative humidity (RH) 60–80%. The continuous light of about 2 kilo lux was provided through compact fluorescent lamps. Various types of growth regulations, viz. BA, KIN, IBA, NAA, and 2,4-D were added with medium either alone or in combination for better shoot induction. The differentiated shoots and formed callus were sub-cultured in a media after 30–45 days of culture. Sub culturing was essential to increase the frequency of regeneration from callus and to develop roots from the base of *in-vitro* grown shoots. The shoots were allowed for root initiation under MS medium supplemented with IBA and subsequently the healthy plantlets were transferred to pot. The healthy plantlets were pulled out of culture tubes

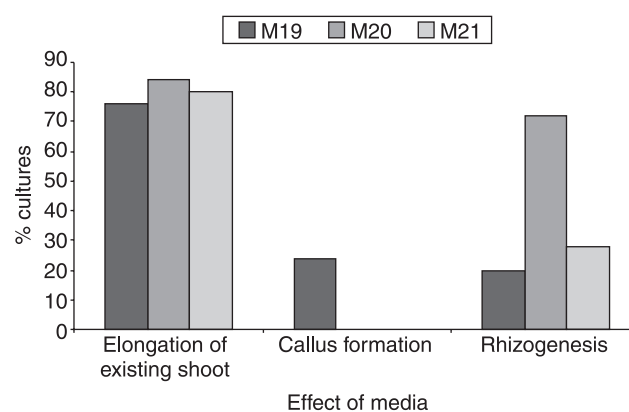


Fig 2 Rhizogenesis (Differentiated shoot culture): Effect of media

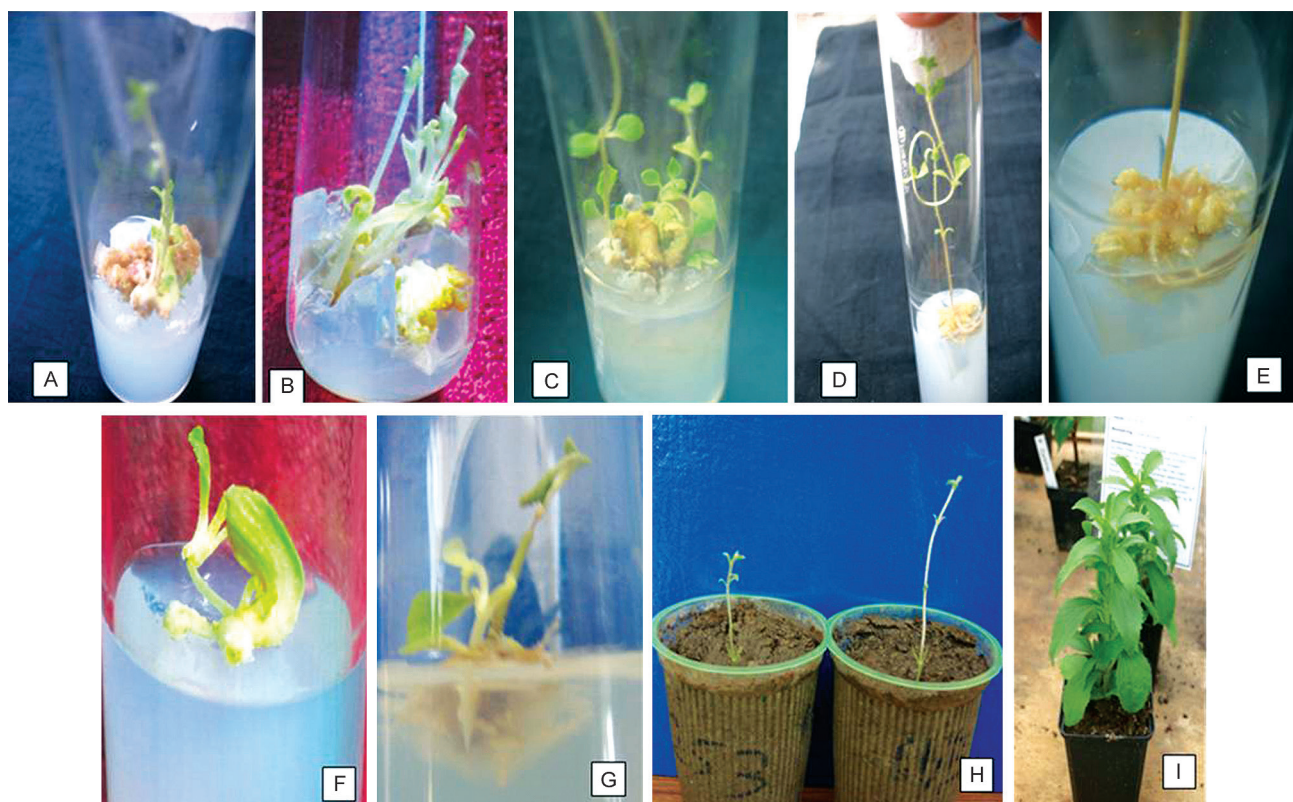


Fig 3 The successive stages of *in-vitro* propagation of *Stevia rebaudiana* (A): Regeneration of shoot from callus derived from explant, (B & C): Initiation of multiple formation, (D & E) Root formation from regenerated shoot, (F & G): Multiple shoot and root formation after subculturing, (H & I): Hardening and establishment of regenerated plants.

and the medium was removed gently by washing with luke warm water without harming the roots. The plantlets were transferred to plastic pots containing sterilized potting mixture of sand and farmyard manure (FYM) in 1:1 ratio. Each pot was watered properly. The pots were placed inside a plastic tub containing some water. A polythene bag was used to cover the complete set. Small holes were made in polythene bag. The plantlets were acclimatized progressively by decreasing the moisture of whole system. For this purpose the size of holes was gradually increased.

Finally, the polythene bag was removed after one and half month and the plants were transferred to the field. Tissue cultured plants of *Stevia* were evaluated morphologically for screening out the variant plants.

RESULTS AND DISCUSSION

Among the explant sources, the nodal stem followed by shoot apex and internode were found excellent for the establishment of culture on MS medium having composition M_{18} ($\frac{1}{2}$ MS+1.0 mg/l BA+0.05 mg/l KIN). The medium M_{14} (MS+0.1 mg/l NAA+1.0 mg/l BA) followed by M_{17} ($\frac{1}{2}$ MS+0.1 mg/l NAA+1.0 mg/l BA) and M_{18} ($\frac{1}{2}$ MS+1.0 mg/l BA+0.05 mg/l KIN) and the explant nodal stem followed by shoot apex and leaf showed the best caulogenesis (Fig 1). This *in vitro* propagation study confirmed the importance of plant growth regulators in the initiations of callus, shoot, root as well as whole regeneration of the plants. Among the cytokinin treatments in culture medium, BA proved to be

better than K in case of shoot induction from all explants used. i.e. shoot apex, nodal stem and leaf. Similar results were also found by earlier workers in the same species (Sairkar *et al.* 2009, Razak *et al.* 2014).

The micro-cutting of *in-vitro* proliferated shoots were implanted on MS medium supplemented with IBA at concentrations 0.5, 0.1, 1.5 mg/l for root initiation. Among them, maximum number of root formation (72%) was observed in medium M_{20} supplemented with (0.5 mg/l) IBA (Fig 2). The number of roots per plant ranged from 3–8 on all the media. The root induction was gradually decreased with increasing concentrations of auxin types. No root formation was observed on auxin free basal medium. Our results are in conformity the earlier findings with (Sivaram and Mukundan (2003), Ahmed *et al.* (2007), Mitra and Pal, (2007), Anbazhagan *et al.* (2010), Razak *et al.* (2014).

Forty eight percent (48%) of the tissue culture plants survived during acclimatization (Fig 3). The tissue culture plantlets that were evaluated for any morphological changes and abnormalities showed no variation. The tissue culture of *Stevia rebaudiana* led to the development of a micropropagation protocol.

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