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# *In vitro* Propagation of *Stevia rebaudiana* (Bertoni): An Overview

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**A B S T R A C T**

*Stevia rebaudiana* is herbaceous perennial plant of Asteraceae family and an excellent substitute of sugar with medicinal importance. The potential uses of Stevia which produces stevioside, a non-caloric sweetener that does not metabolize in the human body, hence control blood sugar level. Conventional propagation methods are not produce adequate planting material. Plant tissue culture techniques are only technique to produce quality planting material. In present review, micro propagation methods and protocols are compile and generate the information to researchers' for further exploration for improvement of this valuable medicinal plant.

## Introduction

*Stevia rebaudiana* (Bertoni) is a medicinal plant belongs to Asteraceae family and known as stevia, sweet leaf, honey leaf, and candy leaf. Stevia is native to Paraguay (South America) and also called sweet herb of Paraguay. The leaves of this plant has a pleasantly sweet and refreshing taste which is educe by diterpene glycosides (stevioside and rebaudiosides), a high-potency sweeteners and substitute to sugar, being 300 times sweeter than sucrose (Madan *et al.*, 2010; Megeji *et al.*, 2005; Singh and Rao, 2005; Soejarto *et al.*, 1983; Soejarto DD, Kinghorn AD, 1982; Yadav *et al.*, 2011). Stevia is a well-known therapeutic agent serve as an efficient medication for diabetes, hypertension, myocardial and antimicrobial infections, dental troubles, and tumors (Chan *et al.*, 1998; Gregersen *et al.*, 2004; Jayaraman *et al.*, 2008; Jeppesen *et al.*, 2003, 2002; Kujur *et al.*, 2010; Marcinek and Krejpcio, 2016; Muanda *et al.*, 2011; Philippe *et al.*, 2014; Planas and Kucacute, 1968; Shivanna *et al.*, 2013; Šic Žlabur *et al.*, 2013; Singh *et al.*, 2015). In present time people are very calorie conscious which increases use of stevia in preparation of non-calorie food stuffs and become a major sweetening agent in food products in South-east Asia (Ashwell, 2015; Durán A. *et al.*, 2013; Marcinek and Krejpcio, 2016; Panpatil and Polasa, 2008; Salunkhe and Bhise, 2010; Savita *et al.*, 2010). Recently FDA of United States of America issued GRAS status to stevia product. The market grows at a rate of 4% per annum and has a business of around 1.3

billion US dollar. Two international business groups, Wilmar and Olam, have started a joint venture to invest 106.2 million US dollar to globally enhance the production and consumption of Stevia. India is supposed to have suitable conditions for the cultivation of stevia. It has been found that Indian stevia plant gives a higher stevioside yield of 10–18 percent in comparison to the reported 8–12 percent from other countries (Yadav *et al.*, 2011).

An International stevia supplier called GLG Life Tech has taken the initiative of introducing stevia to the Indian market and to facilitate its production and extraction in India. India itself is also stepping forward to compete in the stevia sweetener international market (Savita *et al.*, 2010; Yadav *et al.*, 2011). This will paved the path for commercial cultivation of stevia in developed as well as in developing countries, ahead to replace the cane sugar with a global production estimated to be around 40,000 million tons and for India it may be around 600 tons (Ahmed B., Hossain M., Islam R., Kumar Saha A., 2011; Gantait *et al.*, 2015; Yadav *et al.*, 2011). But still the cultivation and commercialization of stevia has not achieved the expected heights as the conventional propagation methods lack in providing rapid quality planting material to the farmers.

Today demand will need to be supported by high biomass yield varieties with improved agronomical traits as well as higher quantities and quality of diterpene glycosides production. This generates the need of stevia development *in vitro.*

The purpose of this overview is to summarize the existing literature for the *in vitro* culture of stevia that may help to recognize from beginning to end tissue culture technology in stevia and provide baseline for further improvement.

## Conventional propagation methods in stevia

In nature, seed germination in stevia is poor and unsuccessful commonly due to infertile seed (Goettemoeller and Ching, 1999; Kumar, 2013) and small endosperm (Yadav *et al.*, 2011). Even some plant selections produce virtually no viable seed due to their self-incompatibility (Raina *et al.*, 2013; Ramesh *et al.*, 2006; Yadav *et al.*, 2011). Plant raised from seed does not allow the production of homogenous plant population resulting in great variability in important features like sweetening levels and compositions(Brandle and Telmer, 2007; Kovylyaeva *et al.*, 2007).

Numbers of reports are available on successful propagation of Stevia via stem cuttings (Ramesh *et al.*, 2006; Shock, 1982; Smitha and Umesha, 2011). But the direct planting of stem cuttings in the field has limited success due to poor rooting (Khalil *et al.*, 2014; Pande and Gupta, 2013; Smitha and Umesha, 2011). Further, location from where cuttings are taken, the pair of leaves, length of the cuttings as well as the season effect rooting percentage and growth (Brandle *et al.*, 1998; Carneiro *et al.*, 1997; Ceunen and Geuns, 2013; Khalil *et al.*, 2014; Rajasekaran *et al.*, 2007). Published work indicated the necessity of higher quantities of starch, carbohydrates, sugar and phenolic compounds for root initiation (Brandle *et al.*, 1998; Ibrahim *et al.*, 2008).

Rooting of cuttings can sometimes be stimulated by the use of growth regulators (Brandle *et al.*, 1998; Ibrahim *et al.*, 2008). Moreover, a huge number of stem cuttings are required for mass propagation of plants which is an obstacle as number of mother plant need to be vanish. Although, vegetative propagation has been found next considerable option for the mass propagation of stevia. But ample supply of planting material is still not adequate for farmers because vegetative

propagation is limited by production of less number of individuals multiply from a single explant (Gantait *et al.,* 2015; Pande and Gupta, 2013; Philippe *et al.,* 2014; Yadav *et al.,* 2011).

## *In vitro* tissue culture of stevia

Plant regeneration from *in vitro* culture can be achieved by either organogenesis or embryogenesis. Administration of different phytohormone enhances and accelerates the production of *in vitro* plants with good agronomical traits and steviosides content in leaves (Brandle *et al.,* 1998; Durán A. *et al.,* 2013; Jain *et al.,* 2014). However, there are some fundamental factors like growth condition of the source material, medium composition, culture conditions and genotypes of donor plants whose role cannot be avoided for the successful *in vitro* culture plants (Gantait *et al.,* 2015; Ibrahim *et al.,* 2008; Pande and Gupta, 2013; Philippe *et al.,* 2014).

## Establishment of in vitro culture of stevia through nodal explant

Nodal part is the choicest explant for *in vitro* culture of stevia for researchers. Various reports on *in vitro* use of nodal explant had been well documented (Ahmed *et al.,* 2007; Atalay *et al.,* 2011; Debnath, 2008; Laribi *et al.,* 2012; Mitra and Pal, 2007; Nepovim and Vanek, 1998; Sivaram and Mukundan, 2003; Sung, 2006; Tamura *et al.,* 1984; Thiyagarajan and Venkatachalam, 2012). Initial work on nodal explant was stated by Yang and his co-workers with the achievement of highest axillary shoot proliferation. They concluded that the type of cytokinin was the most important factor affecting shoot multiplication (Table 1). Also in our lab we have obtain similar results, using TDZ for shoot induction and kin for shoot multiplication (Fig 1). Increasing BA concentration promoted shoot multiplication. Similar results were obtained by Sivram and Mukundan with shoot apex and leaf explants (Sivaram and Mukundan, 2003). However, medium supplemented with kin resulted in elongated shoots. For root induction, different concentrations of IBA and NAA were assayed. IBA showed to be more significant and effective for rooting than NAA in all concentrations used. The maximum root induction (100%) was observed on medium supplemented with 1.0 or 2.0 IBA (mg/l) and in our lab we observed root induction on MS basal medium without PGR/low concentration of IBA i.e. 0.5 mg/l (Fig 2). Similar result was obtained by medium fortified with low concentration of IAA (0.1 mg/l) and it was found that root induction gradually decreased with increasing concentration of auxin (Ahmed *et al.,* 2007; Atalay *et al.,* 2011). Venkatachalam also studied the effect of different concentrations of BAP in combinations with various auxins on multiple shoot bud regeneration. Of the three auxins combinations tested (IAA/IBA/NAA), BAP with IAA combination was found to be superior for induction of highest percent (92%) of multiple shoot bud development, followed by BAP and NAA (83%) and BAP along with IBA (75%) combinations. However, both last combinations (BAP with NAA and BAP with IBA) produced more callus with low percent (50%) of multiple shoot bud regenerations (Thiyagarajan and Venkatachalam, 2012). The lower percent of multiple shoot bud regenerations may be due to the profuse callusing at the basal part of differentiated shoot buds.

## Establishment of in vitro culture of stevia through stem tip explant

*In vitro* clonal propagation by stem-tips culture with a few leaf primordia was reported by Tamura and his group (Tamura *et al.,* 1984). They found that neither roots *nor*

callus developed when stem-tips were grown on a medium supplemented with cytokinin only whereas, the similar dose of kinetin proved effective in vegetative propagation, yielding 50-100 shoots from a single stem-tip in 80 days. Addition of auxin (NAA) along with kincould not enhances shoot formation, but did induce callus formation. The study suggested that the ability to form multiple shoots is dependent on the size of excised stem tip and / or the number of leaf primordia. Similar studies on shoot proliferation had been performed (Akita *et al.,* 1994; Ferreira and Handro, 1988; Sivaram and Mukundan, 2003) (Table 1).

They also reported that plant hormone is necessary for shooting, elongation and rooting. Recently similar results with MS medium in place of previously used LS medium nourished with kin were obtained by Das and coworkers (Das *et al.,* 2011). For root induction, they found that MS media without growth regulators worked dynamically whereas when it was supplemented with auxins (IAA and BA), it had an adverse effect on root induction. They also performed peroxidase assay along with Inter-simple sequence repeat (ISSR) fingerprinting to confirm the genetic fidelity of *in vitro* generated propagules.

## Establishment of in vitro culture of stevia through leaf explant

It is well established that a precise level of cytokinin would play a critical role in shoot organogenesis in various plants. Sreedhar and co-workers had tested various combinations of growth regulators and found that BA and kin could successfully induced adventitious bud from the midrib of leaf explant.

It was the first report of direct shoot induction from leaf explant. Initially, the shoot buds appeared as white knob like structures which later turned green leading to the formation of a pair of green leaves (Sreedhar *et al.,* 2008). In an earlier study, kin in combination with NAA or IAA failed to induce shoot formation on leaf explants of stevia whereas BA was found to be more effective (Sivaram and Mukundan, 2003). Contrarily, Tamura *et al.,* showed the formation of shoots only from the margins of stevia leaves in shoots cultured on a very high concentration of kin for 40 days (Tamura *et al.,* 1984).

This was probably because of the accumulation of high amount of cytokinin that is known to induce adventitious shoot formation. Effect of copper on *in vitro* culture of stevia leaf explants was also studied. It was found that an optimum level of copper helps to achieve maximum shoot bud induction and elongation along with BAP and IAA PGRs. This has also gave a positive effect on chlorophyll and biomass production(Jain *et al.,* 2009). A promising method of micro propagation of stevia has been developed with an aim to increase the biomass, survivability of the plantlets and stevioside production, using chlorocholine chloride, a plant growth retardant. The application of chlorocholine chloride along with IBA on *in vitro* generated microshoots from cotyledonary leaves explants found to be effective for inducing certain beneficial changes like desirable reduction in stem, elongation, profuse rooting, bigger leaf size, increase fresh weight of the plantlets, longer chlorophyll retaining capacity and higher stevioside production (Dey *et al.,* 2013).

Recently, Ramírez-Mosqueda and Iglesias-Andreu, 2016 made a recipe for in vitro plantlet regeneration through thin cell layer method. Different concentration and combination of exogenous growth regulators (BA and 2, 4-D) were tested with transverse thin cell layers and 2, 4-D showed best organogenesis.

**Table.1** *In vitro* culture of stevia

| Type of explants | Shooting media | Rooting media | References |
|---|---|---|---|
| Leaflets | 2-10 mg/1 BA | | Chen and Li, 1993 |
| Shoot primordial | 1.0 mg/1 BA | 0.1 mg/l NAA | Akita *et al.*, 1994 |
| Shoot apex, nodal, and leaf explants | 2.0 mg/l BA and 1.0 mg/l IAA | 1.0 mg/l IBA | Sivaram and Mukundan, 2003 |
| Nodal | 2.0 mg/l IAA and 0.5 mg/ l kin | 2.0 mg/l IBA | Hwang, 2006 |
| Nodal | 1.5 mg/l BA + 0.5 mg/l kin | 0.1 mg /l IAA | Ahmed *et al.*, 2007 |
| Nodal | 2.0 mg/l BA and 1.13 mg/l IAA | 2.0 mg/l IBA | Debnath, 2007 |
| Midrib | 2.0 mg/l BA and 1.0 mg/l kin | 1.0 mg/l IBA | Sreedhar, 2008 |
| Leaf and Nodal | 0.5mg/l BA and 0.5 mg/l IAA | | Jain *et al.*, 2009 |
| Nodal | 0.5 mg/l BA + 0.5 mg/l kin | 1.0 or 2.0 mg/l IBA | Alhady, 2011 |
| Nodal | 1.0 mg/l BA | 0.4 mg/l NAA | Venkatachalam, and Thiyagarajan, 2012 |
| Nodal | 1.0 mg/l BA | 0.5 mg/l IBA or IAA | Labiri, 2012 |
| Stem-tips with a few leaf primordial | 10 mg/l kin | 0.1 mg/l NAA | Labiri, 2012 |
| Nodal segment | MS + 0.5 BAP mg/l +2.0 Kin mg/l | MS +1.0 IBA mg/l | Mehta *et al.*, 2012 |
| Nodal segment | MS + 0.5 BAP mg/l + 2.0 Kin mg/l | MS +0.1 IBA mg/l +100 ppm Charcoal | Modi *et al.*, 2012 |
| Shoot tip, nodal segment | MS + 1 BAP mg/l + 2 Kin mg/l | MS + 0.5 IBA mg/l | El-Motaleb *et al.*, 2013 |
| Shoot tip | MS + 1.5 BAP mg/l + 10 Spermine mg/l | MS + 1.5 IAA mg/l | Guruchandran and Sasikumar, 2013 |
| Shoot tip, nodal segment | MS + 2.0 BAP mg/l | MS+0.5 IBA mg/l | Hassanen and Khalil, 2013 |
| Shoot tip | MS + 1.0 BAP mg/l | MS + 0.4 IBAmg/l | Javad *et al.*, 2013 |
| Nodal segment MultSht | MS + 1.0 lM TDZ | ½ MS | Lata *et al.*, 2013 |
| Nodal explant | ½ MS + 0.01 TDZ mg/l | ½ MS + 1.0 IBA mg/l | Singh and Dwivedi, 2013 |
| Nodal segment | MS + 1.0 BAP mg/l + 0.05 NAA mg/l | MS+0.5 IAA mg/l | Soliman *et al.*, 2013 |
| Shoot tip | MS + 1.0 IAA mg/l +1.0 BA mg/l | | Taleie *et al.*, 2013 |
| Nodal segment | MS + 1.0 BA mg/l | MS + 0.2 IAA mg/l | Nower, 2014 |
| Nodal segment MultSht | MS + 0.5 Kin + 1.0 IBA | | Singh *et al.*, (2014) |
| Thin cell layer | 6.78 µM 2, 4-D | Without PGR | Ramírez-Mosqueda and Iglesias-Andreu, 2016 |

**Table.2** Somatic embryogenesis studies in stevia plants

| **Explants** | **media** | **References** |
|---|---|---|
| Cell suspensions | BA (0.5 mg/l)+2,4D (0.5 mg/l) | Ferreira and Handro, 1988 |
| Leaf | NAA, 0.5 mg/l+ BAP, 0.5 mg/l | Swanson *et al.*, 1992 |
| Anther | BAP, 0.1 to 1.0 mg/l | Flachsland *et al.*, 1996 |
| Floret | 4.0 mg/l 2,4-D<br>0.5 mg/l kin | Bespalhok-Filho and Hattori, 1997 |
| Leaf | 2,4-D (2.0 mg/l)+ kin (0.2 mg/l) | Das *et al.*, 2006 |
| nodal, leaf and root | NAA (1.0 mg/l)<br>NAA (2.0 mg/l)<br>IBA (0.5 mg/l) | Gupta *et al.*, 2010 |
| Leaf disc | MS + 1.5 NAA mg/l or<br>5.0 Cyanobacterial media | Banerjee and Sarkar, 2008 |
| Nodal and leaf segment | MS + 1.0 2,4-D mg/l + 0.2 BAP mg/l + 0.2 TDZ mg/l | Banerjee and Sarkar, 2009 |

**Fig.1** Shoot multiplication through nodal explants

**Fig.2** Root induction in stevia


## Establishment of callus culture

Callus culture in stevia has been studied for the production of organs or somatic embryos. Regeneration from callus derived from different explants, including cell suspension (Ferreira and Handro, 1988), leaves (Gupta *et al.,* 2010; Swanson *et al.,* 1992), nodal (Gupta *et al.,* 2010), flower (Ahmed *et al.,* 2007), root (Gupta *et al.,* 2010), anthers (Flachsland *et al.,* 1996) has been achieved (Table 2). Callus is important for rapid mass multiplication, generation of variability, cell suspension culture, preservation of cell line culture and production of secondary metabolites. Uddin and workers reported the establishment of callus culture in stevia through Leaf, nodal and inter-nodal segments. They observed that inter-nodal segments initiated callus earlier than node and leaf. It was also reported that the highest amount of callus was found in MS medium with 2, 4-D at 3.0 mg/l but poorest callus was resulted with the increase in 2, 4-D concentration (Uddin *et al.,* 2006). In a similar study 100% callusing was obtain from leaf explants cultured on combination of NAA and 2,4-D (Gupta *et al.,* 2010). Studies suggested that leaf explants could serve as a best explant for callus production. The calli obtained from leaf

and root explants were shiny green while with nodal explants it was hard and brown. Though the role of 2, 4-D was well established in callus production but the study of Das and group reported that 2, 4-D in combination with kin is best for callus induction whereas, NAA and BAP are superior for callus maintenance (Das *et al.,* 2014). Recently, it is found that leaf explants of stevia when subjected to varying concentrations of sodium azide and colchicine (0- 0.250%) solution for varying period (12- 24 h), this influence the callus induction and growth but same will be delay when the concentration of mutagen increases (Pande and Khetmalas, 2012).

## Somatic embryogenesis in stevia

Bespalhok-Filho and coworkers reported the somatic embryogenesis in stevia from leaf explant to investigate the influence of growth regulators on the induction of somatic embryogenesis. They concluded that, combination of 10 or 25 mM 2, 4-D and 1.0 mM BA were found to be effective for somatic embryogenesis (Bespalhok-Filho *et al.,* 1993). In another experiment they used floret as explants and employed 2, 4-D and kin and observed a light green or light yellow color embryogenic callus, which was characterized by compact structure and presence of globular somatic embryos on its surface (Bespalhok-Filho and Hattori, 1997).

In conclusion, *Stevia rebaudiana* is a new emerging alternative source of calorie free sweetener gaining popularity worldwide. Lack of quality planting material is a bottle neck in large-scale cultivation of stevia. Tissue culture technique proved to be boon for the production of high quantity and quality planting material for farmers. At present, direct regeneration of plantlets via adventitious shoot bud induction from nodal explants is considered as preferred method for stevia plant regeneration. Future research emphasised on the development of protocols for direct regeneration of shoot buds from leaf explants, protocols for regeneration through somatic embryo-genesis need to be developed as it can help in producing true to type and homozygous plants with improved quality and development of improved genotypes with a high content of rebaudioside-A, higher biomass production, wider adaptability, better germination, viable seed production. Presently, the research is going on isolation, selection and multiplication of variants with high stevioside content to sustainably meet the worldwide demand of Stevia to the food processing and pharmaceutical industry.

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