Some factors affecting the *in vitro* growth of *Stevia rebaudiana* Bertoni

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Abstract

Gelling agent plays an important role in the *in vitro* growth of *Stevia rebaudiana.* Agar solidified MS medium fortified with 1.5 mg/l BA + 0.5 mg/l Kin was found the best concentration for shoot induction from apical meristem and nodal explants, while gelrite solidified MS medium containing the same concentrations of hormones produced weak plants. Healthy and profuse growth of regenerated shoots along with roots were observed when shoots were again subcultered in agar solidified MS medium with 0.1 mg/l IBA, whereas gelrite solidified medium with 0.1 mg/L IBA again produced 1-2 average number of shoots and roots. The medium became liquid in case of gelrite medium during the growth period and the pH of the medium was also changed. Gelling effect was further confirmed by transferring the shoots from gelrite solidified medium to agar solidified medium with the same hormones concentration. Shoots resumed normal growth and proliferation after they were transferred to agar medium. This effect may be due to the change in concentration of the cations (Ca, Mg).

Keywords: Stevia rebaudiana; micropropagation; gelling agents; artificial sweetener

Abbreviation:
BA: Benzylaminopurin; Kin: Kinetin; IBA: Indole-3-butyric acid; MS: Murashige and Skoog

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Introduction

The *in vitro* growth and development of plant tissue is determined by a number of complex factors including genetic make-up of the plant, chemical factors (nutrient medium components: water, macro and micro elements, sugars and plant growth regulators), gelling agent, and also some physical growth factors (Pierik, 1988).

Studies performed by Debergh, (1983), Thentz and Moncousin (1984), Ibrahim (1994) and others showed that the recommended rates of gelling agents are dependent upon explant type and micropropagated plant/culture conditions.

Nutrients as well as gelling agents, e.g., agar or gellan gum supplied in the culture medium play important roles in *in vitro* growth of plant tissues during tissue culture studies. Agar is produced from a family of red seaweeds

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(*Rhodophycae*) primarily from two genera, *Gelidium* and *Gracilaria* which are a mixture of agarose and agaropectin. Agarose is a linear polymer with a molecular weight of 120,000. Agaropectin is a mixture of smaller molecules occurring in lesser amounts than agarose units whereas different gellan gums like gelrite or phytagel are produced by certain non-pathogenic bacteria which secrete a high molecular weight polysaccharide gum that, when purified , is what we know as gellan gum. Gellan gum is a high molecular weight polysaccharide composed of repeating tetrasacchride (4 simple sugars) unit, one glucuronic acid, and one rhamnose unit. Agar is most frequently used for solidification of plant culture media because of its desirable characteristics such as clarity, stability, resistance to metabolism during use and its inertness (Ibrahim, 1994). Gelrite, the alternate gelling agent, is increasingly used because it forms clear gels and contains no contaminants (Pierik, 1987) as well as little lot-to-lot variability than agar which is subject to the natural variability inherent in source algae harvested from the world's marine waters for agar production.

It is found in the literature that plants respond to exogenous cytokinin when grown on agar solidified nutrient media whereas it has been observed that in some instances growth regulator supplied in gelrite solidified media inhibited the growth of the plant tissues. These effects could not be mimicked by additional inorganic salts, or by the addition of glucose/ rhamnose to gelrite-gelled basal media. The gallant strongly influences cellular differentiation and cytokinin-sensitivity of *in vitro* plant cell cultures (Hadeler et al., 1995). It is also indicated that adding appropriate gelling agent decreases vitrification and insures healthy vigorous plantlets with higher chlorophyll content (Ebrahim and Ibrahim, 2000). Selection of proper gelling agent for successful *in vitro* growth is a pre-requisite.

Stevia rebaudiana (Asteraceae family) is originally a South American wild plant. It comprises about 150-300 species of perennial herbs and shrubs, growing mostly at altitudes of 500-3000 meters in semi-dry mountainous terrains. It is a natural sweetener plant known as "sweet weed", "sweet leaf", "sweet herbs" and "honey leaf". Its sweetening effect is estimated to be 300 times more than that of cane sugar (Chalapathi and Thimmegowda, 1997; Liu and Li, 1995). The plant has been used for sweetening beverages and foods since 1600 (Glinsukon et al., 1988). The sweetening compound was isolated from *Stevia* leaves by Crammer and Ikan (1986). It was named as "stevioside" which is the complex of three glucose molecules and one molecule of steviol aglycone, a diterpenic carboxylic alcohol (Bell, 1954; Wood et al., 1955). Stevioside has very high sweetening potency, 300 times that of sucrose but little caloric value (Kinghorn and Sojarto, 1985). Its sweetness is stable to heat and yeast fermentation and is used by those with obesity, diabetes mellitus, heart disease and dental caries (Fujita and Edahiro, 1979). Stevioside, the major component of *Stevia*, can also inhibit the growth of certain bacteria. Steviol and stevioside have also been reported to have therapeutic value as diuretic (Malis, 1997) and also as diabetic drugs. They have been proposed to have a potential role as antihyperglycemic agents by stimulating insulin secretion from pancreas (Jeppensen et al., 2000, 2002, and 2003). Continued consumption of stevioside extract for three months reduced blood pressure in hypertensive patients (Chan et al., 2000).

Both stevioside and steviol alter glucose metabolism and glucose absorption (Suanarunsawat and Chaiyabutr, 1997). Being a non-caloric natural source, they are used as an alternative to artificially produced sugar substitutes. The sweet compounds pass through the digestive process without chemically breaking down, making *Stevia* safe for those who need to control their blood sugar level (Strauss, 1995). The Guarani Indians of Paraguay, where *Stevia* originates, have used it for centuries as a sweetener for maté tea (Brandle and Rosa, 1992). Since the 1970s, *Stevia* extracts have been widely used in many countries as a sugar substitute. In Japan, for instance, *Stevia* extracts account for about 5.6% of the sweetener market (Strauss, 1995). The FDA now allows the sale of *Stevia*, as a nutritional supplement (Whitaker, 1995) which is an indicative of its demand as a crop. Seed material is the basic need for growing any crop. In case of *Stevia* seeds can be germinated in the glasshouse in spring and the plants are transplanted into the field (usually at 6-7 weeks

old). The whole plant is harvested once, just before flowering to obtain maximum glycoside content (Kinghorn and Sojarto, 1985). Due to poor seed germination of *Stevia* (Uddin et al., 2006), there are two options for multiplication, i.e., tissue culture or stem cutting. Propagation of *Stevia* is usually by stem cuttings which roots easily, but require high labor inputs. Poor seed germination is one of the factors limiting large-scale cultivation. Shock (1982), Duke (1993), and Carneiro (1997) all pointed to poor production of viable seeds. Therefore, propagation is a special concern for growers who want to grow *Stevia* as an annual crop. Plant tissue culture techniques are used to produce healthy plant material in bulk for certain crops like potato, sugarcane etc. Studies have been done for micropropagation of *Stevia rebaudiana* from shoot primodia, leaf, nodal and inter-nodal segments (Ahmad et al., 2007; Uddin et al., 2006; Akita et al., 1994; Ferreira and Handro, 1988; Debnath, 2008). Recently, Kalpana et al., 2010 improved the micropropagation method for the enhancement of *Stevia* rubadiana biomass by incorporation of a range of higher concentration of micronutrient in the medium. The plant extracts refer to a concentrated preparation of active constituents of the medicinal herbs (Sapna et al., 2008). Systematic screening of plant extracts may result in the discovery of novel effective antimicrobial compounds (Tomoko et al., 2002).

This study aimed to establish a protocol for propagation of *Stevia rebaudiana via* tissue culture techniques to ensure supply of healthy seed material of this exotic plant to the local grower to grow this potential cash crop for Pakistan agriculture and pharmaceutical industries. It is a new low calorie sweetening material to be used instead of sugar for patients suffering from diabetes and mellitus/obesity or for players drinks. During present study effect of MS salts and hormones as well as gelling agent on proliferation rate, organogenesis, and growth of plant *in vitro* was evaluated. The type and concentration of the gelling agent also its effects on the *in vitro* growth and development of *Stevia rebaudiana* were studied.

Materials and Methods

The explants (apical meristem and nodal segments) of the exotic plant *Stevia rebaudiana* were collected from field grown plants. The explants were washed with tap water to remove the superficial dust particles, gently soaked in a mild commercial detergent for few seconds to remove microbial spores, again washed with tap water, and then rinsed with distilled water. The explants were finally surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution for 3-5 minutes and then washed four times with sterilized distilled water under laminar air flow. These explants were then inoculated aseptically onto Murashige and Skoog's, medium (1962) fortified with different concentrations and combinations of growth regulators (BA, Kin and IBA 1.0 mg/l or 1.5 mg/l) adding 30 g/l sucrose. MS medium supplied with 0.8% agar or 0.2% gelrite were used in the present study with same hormonal combination/concentrations in each case. The pH of the medium was adjusted to 5.8 with 1N NaOH and HCl prior to the addition of gelling agents. After cooking of the media to dissolve the gelling agent completely, it was autoclaved for 20 minutes under 15 psi and 121 °C. After inoculation, the cultures were incubated in a controlled environment of 25±2 °C temperature with 16 h photoperiod by cool white fluorescent light (2000 Lux).

Statistical analysis

The results obtained in present study were statistically analyzed with one-way analysis of variance in a completely randomized design. The means were separated by Duncan multiple range test at 1% and 5% level of significance as described by Steel and Torrie (1980). Each treatment consisted of three replications.

Results

Apical meristem and nodal explants incubated on MS medium solidified with 0.8% agar fortified with different concentrations of BA (1.5 mg/l & 1.0 mg/l) in combination with Kinetin (1.0 mg/l & 0.5 mg/l) showed *in vitro* response after three

weeks. A single shoot emerged directly from both explants on each concentration and combination.

Apical meristem showed superior response in MS medium supplied with 1.5 mg/l of BA along with 0.5 mg/l of Kin. Soft, friable and dirty white calluse were produced at the base of induced shoots. A balanced ratio of BA and Kin (1.0 mg/l each) also induced shoots from apical meristem with dirty white callus at the base of the explants. It was observed during the study that the apical meristem induced shoot formation earlier than the nodal explants on matching hormonal concentrations and combinations used in the present study. Single shoot induction from nodal explants grown on media containing 1.5 mg/l of BA + 0.5 mg/l of Kin was observed with compact green callus at the base after three weeks. Similar observations were made for shoot induction from nodal explant on 1.0 mg/l BA + 1.0 mg/l Kin. Similarly, experiment was repeated using 0.2% gelrite in the growth medium for solidification without any change in hormonal supplements used in case of agar solidified medium. But growth response on the gelrite solidified medium was very slow and weak shoot induction was observed.

Explants from the *in vitro* induced shoots from both types of explants were transferred separately to media solidified with agar or gelrite containig respective hormonal concentrations and combinations as described above, results are shown in Table 1. After six weeks, $11^{a}\pm1.00$ and $8^{b}\pm1.53$ shoots were produced from apical meristem and nodal explants respectively on agar solidified MS medium containing 1.5 mg/l BA in combination with 0.5 mg/l Kin.

This combination/concentration was found to be the most favourable for high frequency multiple shoot induction. While $9^{b}\pm0.58$ and $8^{b}\pm1.53$ shoots from apical meristem and nodal explants respectively were observed on media supplemented with 1.0 mg/l each of BA and Kin (Table. 1). On the other hand explants grown on gelrite solidified media, irrespective of hormonal combination or explant origin, produced weak single shoot per explants.

These micro-propagated shoots of apical meristem and nodal explants were implanted in both agar and gelrite solidified MS medium without hormones as well as supplemented with 0.1 mg/l IBA and 0.1 mg/l IBA + 0.25 mg/l BA for rooting results are shown in Table 2. An interesting feature was noticed at this stage in case of agar solidified MS medium. That is no root production on hormone free medium was observed but $30^{b}\pm1.53$ healthy elongated shoots along with $22^{bc}\pm 1.04$ healthy roots were produced in medium containing 0.1 mg/l IBA alone after four weeks of growth. However, explants produced a cluster of $45^{a}\pm 1.00$ small weak shoots with small leaves on medium supplied with 0.1 mg/l IBA in the presence of 0.25 mg/l BA without any root induction (Table 2).

But in case of gelrite solidified MS medium without hormones as well as supplemented with 0.1 mg/l IBA and 0.1 mg/l IBA + 0.25 mg/l BA, only 1 or 2 weak elongated shoots with small leaves and 2-3 roots were observed. In case of gelrite solidified medium it was observed that after the elongation of *Stevia* shoot, the gelrite solidified medium became watery on third week and the further development of plant was checked accompanied by shoots survivals within a week, and about 80% of the shoots survived.

This gelling agent effect was further confirmed by transferring these plantlets from gelrite to agar solidified media without any other change. The transferred shoots started proliferating a few days after transfer to agar-containing media. This resulted in the resumption of normal growth and proliferation after they were transferred to agar containing nutrient medium.

Discussion

Some factors affecting the *in vitro* growth of important non-caloric sweetening herb, *Stevia rebaudiana* are observed in the present study. At the same time, efficient methods for *in vitro* clonal propagation were also devised to explore its potential for micro-propagation from apical meristem and nodal segments of *Stevia*.

Fig. I. Elongation and multiplication of regenerated shoots sub cultured on MS medium supplemented with 0.1 mg/l IBA

Fig. II. Cluster of small shoots produced from regenerated shoots sub cultured on MS medium supplemented with 0.1mg/l IBA + 0.25mg/l BA

Apical meristem showed earlier and superior response as compared to nodal segments on agar solidified MS medium fortified with 1.5 mg/l of BA + 0.5 mg/l Kin with soft, friable and dirty white callus at the base. Present results support the findings of Ahmad et al. (2007). Growth medium solidified with 0.2% gelrite without any change in hormonal concentrations as in case of agar solidified medium was not found favourable during present study. These results are contradictory to the findings by Ichi et al. (1986) and Huang and Chi (1988) who reported the superiority of gelrite over agar as a gelling agent for tobacco and banana respectively. Whereas Pasqualetto et al. (1986) reported the favourable effect of agar on apple (*Malus domestica*) which is in accordance with the present results. This gelling agent effect was further confirmed during this study by transferring the plants from gelrite to agar media with the same hormonal concentrations wherein transferred shoots began to recover in a few days and multiplied rapidly in a week.

Furthermore, it is reported in literature that the firmness of the media is affected by factors like pH, antioxidants, sugar or salt concentration. Liquefaction of the plant growth media may also be due to the change in divalent ions like Ca or Mg concentration in the medium. These two components of the MS salts affect the gelling and liquefaction of the gelrite medium (Phytoteknica, 2009). This effect may be due to the change in concentration of the said cations which is used by the plant part growing therein and subsequently affecting the firmness of the medium. It is further supported by ICP emission spectrometry which revealed that the gellan gum gelrite (K 9A40, Kelco, USA) of bacterial origin contained more cations helping in solidification than the polysaccharide agar of marine origin (purified quality M 1614 of Merck, FRG) (Scherer et al., 1988).

Table 1
Effects of cytokinins in agar solidified MS medium on *in vitro* shoot proliferation in apical meristem and nodal segments of *Stevia rebaudiana*

Hormonal Concentrations BA+Kin mg/L	Explant	Callus Texture & Color Produced at the Base of Shoots	Morphogenic Potential
1+1	Apical meristem	Soft, friable and dirty white	9^{b} ±0.58 healthy shoots
1.5+0.5	Apical meristem	Soft and dirty white	11^{a}±1.00 healthy shoots
1+1	Nodal segment	Compact and light green	6^{c}±0.87 healthy shoots
1.5+0.5	Nodal explant	Compact and dark green	8^{b}±1.53 healthy shoots

Mean separation in columns by Duncan's multiple range test, p = 0.05

Table 2.
Effect of cytokinin and auxin interaction on *in vitro* growth of regenerated *Stevia* shoot cuttings from apical meristem and nodal segment on agar solidified MS medium

Hormonal Concentrations mg/L	Callus Texture & Color	Total No. Shoots per Culture	Total No. Roots per Culture
0.1 IBA	Hard, compact and green callus at base of shoots	30^{b}±1.53 elongated healthy shoots with lush green leaves	22^{bc}± 1.04healthy roots
0.1 IBA + 0.25 BA	Hard, compact and light green callus at base of shoots	45^{a}± 1.00small weak shoots with small leaves	-
Hormones free MS medium	Hard and light green considerable callusing	10^{c}± 0.58small weak shoots	-

Mean separation in columns by Duncan's multiple range test, p = 0.05

Explants grow on gelrite solidified medium and use these cations for their growth, thus liquefying the gel. In case of agar, presence of cations does not affect the gelling that is why it never liquefies even after weeks of explants growth on the absence of cations (Phytotechnica, 2008). In addition to this, pH of the medium altered due to the change in salt concentrations in the medium which ultimately affect the solidification of the medium as well as the growth of the *Stevia* plant.

Present results are also in accordance with the results of Corchete et al. (1993) who reported the dependence of success in micro-propagation of *Siberian elm* on agar or germ-plasm used. Ebrahim and Ibrahim (2000) has also found that addition of gelling agent decreased culture vitrification and insured obtaining healthy vigorous plantlets with higher chlorophyll content. Ibrahim et.al (2008) also reported good effect of agar on *Stevia* when combined with gelrite. Therefore in the light of present study it is found that gelling agent as well as hormonal composition put pronounced effect on *in vitro* growth of *Stevia rebaudiana* and further studies are required to assess the effect of blending agar and gallan gum (gelrite), e.g., agargellan.

The micropropagated shoots of apical meristem and nodal explants implanted in both agar and gelrite, solidified MS medium without hormones as well as supplemented with hormones (Table 2) for rooting. No roots production were observed in hormones free mediums while 0.1mg/l IBA produced a large number of healthy shoots along with roots on affecting the solidification of medium due to decrease in the said cations concentration agar solidified MS medium. These findings are in contrast with the findings of Ahmad et al. (2007) who reported an average number of 2-3 roots per culture in the medium containing IAA, NAA and IBA. A rosette of shoots with small leaves was developed rather than elongated normal shoots on medium supplied with 0.1mg/l IBA + 0.25 mg/l BA without any root induction (Table 2). This response may be due to the addition of a cytokinin, i.e., BA in the medium which results in decreased cell size/number due to the cell division prior to the cytoplasm division.

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