IN VITRO PROPAGATION OF STEVIA REBAUDIANA (BERT.) A NATURAL, NON CALORIC SWEETENER HERB

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Abstract: Stevia rebaudiana is a medicinally important, zero-caloric value, sweet tasted and an antidiabetic herb. In vitro micropropagation of Stevia was has been done by using nodal segments as an explant that were cultured on Murashige and Skoog media supplemented with five different concentrations of benzylaminopurines (BAP) (0.0-2.0mg/l) in combination with kinetin (KN) (0.0-2.0 mg/l). After, five weeks of initiation, maximum numbers of shoots (5.16±0.76) were obtained on media supplemented with BAP (1.0 mg/l) + KN (1.0 mg/l). Best shoot length (7.00±0.64 cm) was observed on media containing BAP (0.5 mg/l) + KN (0.5 mg/l) with the highest 95% survival rate. Shootlets were regenerated through axillary shoot proliferation on MS media with same hormone concentrations those were used for establishment of explants. Maximum number of shoots (5.28±0.75) and shoots length (7.45 ± 0.24 cm) were recorded on MS media containing BAP (1.5mg/l) + KN (1.5 mg/l) and BAP (2.0mg/l) + KN (2.0mg/l) with a survival rate 85% and 95% respectively. The single shoots were transferred for rooting to half MS media containing indole 3-butyricacid (IBA) (0.0-2.5mg/l). Most efficient rooting response with average length 1.68 ± 0.3 cm and number of roots (3.60 ± 0.29) were observed on half MS media supplemented with 1.0mg/l IBA, where survival rate was 90%. The well-rooted Stevia plantlets transferred to green house condition for primary hardening that showed promising survival rate.

Key words: Stevia rebaudiana, Micropropagation

INTRODUCTION

Stevia rebaudiana Bertoni, is a small, herbaceous, semi-bushy and perennial shrub of the Compositae family originated from Paraguay [1]. Stevia belongs to the Asteraceae family and native to tropical and subtropical regions of North and South America. There are near about 240 species of Stevia Genus. It is grown widely in countries like Brazil, Colombia, Paraguay and Venezuela. In Venezuela it is being used over since 1500 years. It is a natural sweetener plant known as “Sweet Weed”, “Sweet Leaf”, “Sweet Herbs” and “Honey Leaf”, which is estimated to be 300 times sweeter than cane sugar [2]. There are two compounds in Stevia leaves, i.e. Stevioside (10%-20%) and Rebauidious-A (1-3%). The Stevioside is stable at 100 °C this is the main advantage of Stevioside over other sweetener. It is used as a sweetener in food products and soft drinks in Japan. Stevia shares at 40% sweetener market in the world [3].

As per WHO report, India has the highest diabetics in the world and the scene feared to be getting worse
each year. Therefore, there is an unlimited market potential in India itself for the Stevia, because of its nontoxic & zero calorie sweeteners. According to the American Diabetes Association, Stevia is a good substitute for sugar especially for diabetics. The leaf contains a compound called steviol glycoside that is not absorbed by the body and cannot be broken down, and is flushed out directly. It is especially good for diabetics, because, it stabilizes a patient’s blood sugar by increasing insulin resistance, inhibits the absorption of glucose in the body and promotes the health of the pancreas. Stevia can help people enjoy nature-origin sweetness while reducing calories as part of a healthful and balanced diet. Stevia has various properties such as antibacterial, antifungal, antiviral, cardio tonic (tones, balances, strengthens the heart), diuretic, hypoglycemic and vasodilator [1].

Use of Stevia, as a sweetener, approved by USFDA (US Food and Drug Administration), WHO (World Health Organization) and EFSA (European Food Safety Authority). Now, Stevia has been introduced as a crop in a number of countries, including Brazil, Korea, Japan, Mexico, United States, Indonesia, Tanzania and Canada for food and pharmaceutical products [4].

Generally, propagation of S. rebaudiana is done by stem cutting and the main problem involved in the cultivation of this species is its heterozygous and self-incompatibility natures which lead to the lack of fertilization [5]. The seeds of Steviashow very less vigor and propagation and do not allow the production of a homogeneous population, which leads to variability in sweetening level and composition [6,7]. Poor seed germination percentage is the limiting factor to large scale cultivation of this species.

As exotic plants such as Stevia are restricted to their natural environment, the main advantage of tissue culture technology lies in production of high quality and uniform planting material that can be multiplied on a year round basis. Biotechnological approaches such as in vitro plant tissue culture methods have been applied for the multiplication of Stevia all over the world via leaf, nodal and axillary shoot explants have been established for increasing the cultivation area and production [1,8-11]. Multiple shoots were regenerated from nodal segments and found highest regeneration rate in MS media supplemented with BA (1.5 mg/L) + KN (0.5 mg/L). MS media with 0.1 mg/L IAA showed 97.66% rooting response [12]. Successful, callus culture from leaf, nodal and inter-nodal segments were observed on MS media containing 2,4-Dichlorophenoxyacetic acid (2,4-D) (2.0-5.0 mg/L) [13]. It has a potential commercial value, because of that, private and public biotechnology companies are producing Stevia in huge quantity and marketing its products.

**MATERIAL AND METHODS**

**Plant material and surface sterilization:** Young buds (2-4 cm) of Stevia plants were collected form Sunrise Agro Services, Wakad, Pune and brought to the tissue culture laboratory of Department of Plant Biotechnology, College of Agril. Biotechnology, Loni. Under in vitro sterile conditions they were dissected to generate nodal segments of (1.5-2 cm) in length and were washed thoroughly under running tap water for 20 min. They were treated with 0.1% (w/v) bavistin for 5 min followed by washing with distilled water 5-6 times. The nodal segments were surface sterilized with 0.1% (v/v) savlon for 3 min, followed by treated with 0.1% streptocyclin for 3 min and finally 0.1% mercuric chloride treatment was given for 5 min and finally washed with sterile distilled water 5-6 times. Surface sterilized explants were inoculated onto 5 different MS media with different concentrations of BAP and KN.

**Establishment stage and culture conditions:** For establishment of culture the surface sterilized nodal segment were transferred to MS basal medium supplemented with different concentrations of BAP (0.0-2.0 mg/l) in combination with KN (0.0-2.0 mg/l) containing 3 % (w/v) (Figs. 1, 2). The pH of the medium was adjusted up to 5.7 with 1N HCl/NaOH before gelling with 0.8% (w/v) agar (Himedia, India). All the experiments were done using analytical grade chemicals. The explants initially were implanted vertically on the culture medium in sterile culture bottles. The inoculated culture bottles were transferred to the incubation room having sterile controlled environmental conditions such as temperature 25°C ± 2°C, relative humidity (RH) 50-80%, 16 hours photoperiod/day with 1500-2000 lux light intensity. Average shoot length, average number of shoots/explants and survival percentage were evaluated after 5 weeks from culture, on establishment media.
Table 1: Establishment of *Stevia rebaudiana* nodal explants using different combinations of BAP and KN after 5 weeks of culture

<table>
<thead>
<tr>
<th>No.</th>
<th>Media + Hormones</th>
<th>Average shoot length (in cm) (mean ± SD)</th>
<th>Average number of shoots/explants (mean±SD)</th>
<th>Survival%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>MS media</td>
<td>1.73±0.26</td>
<td>1.33±0.52</td>
<td>50</td>
</tr>
<tr>
<td>2.</td>
<td>MS + 0.5 mg/l BAP + 0.5 mg/l KN.</td>
<td>7.00±0.64</td>
<td>4.00±0.84</td>
<td>95</td>
</tr>
<tr>
<td>3.</td>
<td>MS + 1.0 mg/l BAP + 1.0 mg/l KN.</td>
<td>6.05±0.32</td>
<td>5.16±0.76</td>
<td>80</td>
</tr>
<tr>
<td>4.</td>
<td>MS + 1.5 mg/l BAP + 1.5 mg/l KN.</td>
<td>2.85±0.47</td>
<td>2.57±0.97</td>
<td>60</td>
</tr>
<tr>
<td>5.</td>
<td>MS + 2.0 mg/l BAP + 2.0 mg/l KN.</td>
<td>4.64±0.34</td>
<td>3.1±0.73</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 2: Effect of BAP and KN on *in vitro* shoot multiplication of *Stevia* after 5 weeks of culture

<table>
<thead>
<tr>
<th>No.</th>
<th>Media + Hormones</th>
<th>Average shoot length (in cm) (mean±SD)</th>
<th>Average number of shoots/explants (mean±SD)</th>
<th>Survival%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>MS media</td>
<td>3.08±0.47</td>
<td>3.14±0.68</td>
<td>60</td>
</tr>
<tr>
<td>2.</td>
<td>MS + 0.5 mg/l BAP + 0.5 mg/l KN.</td>
<td>2.01±0.41</td>
<td>2.33±0.81</td>
<td>55</td>
</tr>
<tr>
<td>3.</td>
<td>MS + 1.0 mg/l BAP + 1.0 mg/l KN.</td>
<td>4.32±0.56</td>
<td>5.28±0.75</td>
<td>85</td>
</tr>
<tr>
<td>4.</td>
<td>MS + 1.5 mg/l BAP + 1.5 mg/l KN.</td>
<td>6.42±0.45</td>
<td>4.1±0.36</td>
<td>95</td>
</tr>
<tr>
<td>5.</td>
<td>MS + 2.0 mg/l BAP + 2.0 mg/l KN.</td>
<td>7.45±0.24</td>
<td>4.10±0.36</td>
<td>95</td>
</tr>
</tbody>
</table>

Table 3: Effect of auxin (IBA) on root induction from isolated shoots of *Stevia rebaudiana* after 5 weeks

<table>
<thead>
<tr>
<th>No.</th>
<th>Media + Hormones</th>
<th>Average root length (in cm) (mean±SD)</th>
<th>Average number of roots/explants (mean±SD)</th>
<th>Survival%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>½ MS + 0.5 mg/l IBA</td>
<td>0.43±0.04</td>
<td>2.80±0.29</td>
<td>60</td>
</tr>
<tr>
<td>2.</td>
<td>½ MS + 1.0 mg/l IBA</td>
<td>1.68±0.3</td>
<td>3.60±0.29</td>
<td>90</td>
</tr>
<tr>
<td>3.</td>
<td>½ MS + 1.5 mg/l IBA</td>
<td>1.06±0.09</td>
<td>1.40±0.42</td>
<td>85</td>
</tr>
<tr>
<td>4.</td>
<td>½ MS + 2.0 mg/l IBA</td>
<td>0.92±0.14</td>
<td>1.38±0.35</td>
<td>70</td>
</tr>
<tr>
<td>5.</td>
<td>½ MS + 2.5 mg/l IBA</td>
<td>0.51±0.05</td>
<td>1.08±0.16</td>
<td>55</td>
</tr>
</tbody>
</table>

**Shoot Multiplication:** Shoot proliferation was carried out using most vigorous shoots excised from establishment stage. For shooting, the MS media fortified with different concentrations BAP (0.0-2.0 mg/l) in combination with KN (0.0-2.0 mg/l) were used. Shoot proliferation was determined after five weeks of culture. The data such as average number, length and survival percentage of shoots were recorded.

**Rooting and acclimatization:** For rooting, shoot segments excised from multiplication stage were cultured on half MS basal media with indole 3-butyric acid (IBA) (0.0-2.5 mg/l). The data such as average length, average number of roots per explants and survival percentage was recorded after 5 weeks of culture. Rooted micro shoots were excised from the culture and the roots were washed in sterile distilled water. The rooted plantlets then, transferred to plastic pots containing coco pits and sand (1:1) in the green house, maintaining proper temperature (28 ± 2°C) and relative humidity (70-80 %).

**Data analysis:** Various growth data were recorded, such as the number of multiple shoot formation, shoot length, number of roots, root length and survival percentage of the plant, during micropropagation. All the studies were made with 8 replicates and the mean and standard deviation (SD) were calculated by using IBM SPSS statistics software (IBM 2013).

**Figs. 1 to 8:** The successive stages of *in vitro* propagation of *Stevia rebaudiana.* (Fig. 1-2). Direct regeneration of shoots from nodal segments as explants of Stevia cultured on Murashige and Skoog media supplemented with five different concentrations of benzylaminopurines (BAP)'(0.0-2.0 mg/l) in combination with kinetin (KN) (0.0-2.0 mg/l). (Fig. 3-4). Initiation of the multiple shoot formation. (Fig. 5-7). Development of more number of multiple shoot on MS media supplemented with same hormone concentrations that were used for establishment of explants. (Fig. 8) Root formation from regenerated shoot with varying concentrations of indole 3-butyric acid (IBA) (0.0-2.5 mg/l). (Fig. 9) Primary Hardening and establishment of plants under controlled greenhouse conditions.
RESULTS AND DISCUSSION

Data was obtained after 5 weeks of initiation of culture showed that nodal segment effectively regenerated on all tested media. The highest survival rate 95% was observed on MS media supplemented with BAP (0.5 mg/l) + KN (0.5mg/l) compared with the control which gave the lowest survival rate as 50 % (Table 1). Maximum numbers of shoots (5.16 ± 0.76) were obtained on media supplemented with BAP (1.0mg/l) + KN (1.0mg/l) (Figs. 3,4). Effective shoot length (7.00 ± 0.64cm) was observed on media containing BAP (0.5mg/l) + KN(0.5mg/l). Similarly, when MS media supplemented with BAP (0.5mg/l) + KN (0.5mg/l) for the establishment of Stevia, showed 90% survival rate [9]. Promising axillary shoot proliferation was observed on MS media containing 2 mg/LBA, 10 mg/L KNand 10 mg/L N6-2-iP [14].

In shoot multiplication the highest shoot length with survival rate 95% was obtained onthe MS media supplemented with BAP (2.0 mg/l) + KN (2.0mg/l) (Table 2). Maximum numbers (5.28 ± 0.28) of shoots were observed in media supplemented with BAP (1.5mg/l) + KN (1.5mg/l) (Figs. 5-7). However, earlier studies observed that BAP at 1.5 mg/l was the most effective plant growth regulator with regard to Stevia shoot proliferation and media enriched with 1.0 to 2.0 mg/l KN, showed maximum response [15]. MS media supplemented with 2 mg/L KN was found to be more efficient for multiple shoot proliferation, resulting in more than 11 shoots from a single shoot tip explants within 35 days of culture [16].

IBA, with different concentrations, induced rooting on the half MS media (Table 3). Highest root length (1.68±0.3cm) and the highest number of roots (3.60±0.29) were recorded with survival rate 90% on half MS media supplemented with 1.0mg/l IBA (Fig. 8). Earlier studies observed that Micropropagation using stem node segment obtained from 2 years old plant resulted in maximum root induction (100%) on MS media supplemented with 1.0 and 2.0 mg/L IBA, whereas the application of NAA decreased the root induction [9]. IBA (0.4 to 2 mg/l) for rooting showed 100% rooting [15]. Regeneration of *S. rebaudiana* through callus culture showed better response in terms of rooting on one fourth MS media, supplemented with 0.1 mg/L IBA and they were hardened successfully in the tera care media with 63% survival rate [17]. The well-rooted Stevia plantlets shifted to greenhouse condition, later for further growth the acclimatized plants were shifted to field conditions. The growth characteristics of plants raised *in vitro* did not show any significant morphological variations from those of the natural habitat (Fig. 9).

CONCLUSION

Stevia, as a sweet herb, is an important medicinal plant. It is becoming an endangered species due to its infertile and small sized seed. The methods of vegetative propagation are not efficient to save this rare plant. Biotechnology companies are commercially producing Stevia through tissue culture and marketing Stevia in different form, such as, leaf powder, liquid and fresh leaves. To meet the market demand, the current study, paved the way for the propagation and cultivation of large numbers of Stevia plants by using the tissue culture technique.

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