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DEVELOPMENT OF TISSUE CULTURE PROTOCOL FOR MASS PRODUCTION OF S*TEVIA REBAUDIANA* (BERTONI) BERTONI

M. Munir[1], I. Fatima[1*], B. Aftab[1], A. A. Sheikh[2], A. Shabir[1], J. Ahmad[3] and M. S. Ahmed[3]

[1]Department of Biological Sciences, University of Veterinary and Animal Sciences, Ravi Campus Pattoki, Pakistan.
[2]Institute of Microbiology, University of Veterinary and Animal Sciences, Ravi Campus Pattoki, Pakistan.
[3]Agriculture Farms, University of Veterinary and Animal Sciences, Ravi Campus Pattoki.
Correspondence: Ishrat Fatima, fatimaishrat196@gmail.com

ABSTRACT

Stevia rebaundiana is containing sweet phytochemicals and is a zero-caloric natural alternative to artificial sweeteners. The direct organogenesis of *S. rebaundiana* was carried out using apical bud explant *in vitro*. The Murashige and Skoog medium was used and the culture was set up under optimized conditions of 24±2°C temperature, and 16 hours of light exposure with cool, white fluorescent light. The seven different concentrations of benzylaminopurine (BAP) and IAA (Indole-3-acetic acid) (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 and 0.07mg/L) were employed for shoot development and root development respectively. The results revealed that of all the tested concentrations of BAP used for shoot development, 0.07mg/L proved to be best for shoot initiation after 6 days of culture. On the other hand, the suitable concentration of IAA at which root initiation was observed after 7 days of subculture, was 0.06mg/L. The study can be beneficial for the researchers, food industry and growers for commercial production of the *S.rebaudiana* nursery.

Keywords: Direct organogenesis, Mass production, Medicinal plant, *Stevia rebaudiana*, Tissue culture.

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INTRODUCTION

The *Stevia rebaudiana* (Bertoni) Bertoni is a perennial, nutrient-rich, natural sweetest herb of the *Asteraceae* family with many pharmacological and therapeutic applications. *Stevia* is popularly known as honey yerba, honey leaf, meethi tulsi, sweet weed, sugar leaf, phawophul and candy leaf. The habitat of *Stevia* ranges from grassland, scrub forests, forested mountain slopes, and conifer to subalpine forests with a favourable temperature of 21-43 °C (Gupta *et al*., 2013). It is native to Paraguay and South America and is also cultivated in various other countries including Japan, Australia, the US, Indonesia, Thailand, Canada, Northern Russia, Malaysia, the Philippines and California etc (Libik-Konieczny *et al.*, 2018).

The suitable climate for *Stevia* growth includes semi-humid and subtropical regions with annual rainfall between 1500 mm to 1800mm. Preferable soil types are sandy or loam with pH from 6.5 to 7.5 and 12 hours of exposure to daylight (Ijaz *et al.*, 2015). The plant grows up to 2-3m in length with cylindrical roots. The stem is brittle, weak-pubescent, semi-woody and fragile while the leaves are sessile, elliptical and oppositely arranged. The flowers are small (15-17mm), pentamerous, white, hermaphrodite with sympodial cyme inflorescence whereas the fruit is spindle-shaped, five-ribbed achene. The flowering season of plants varies from mid of summer to late fall. Harvesting of the plant before the start of flowering contributes to increased stevioside contents in the leaves (Šic Žlabur *et al.,* 2013; Reis *et al.,* 2017).

Traditionally *Stevia* is being used as a non-caloric, natural sweetener and antioxidant agent in several bakery items, processed food and beverages, cosmetics, weight loss products and in various medical and household preparations (Treciokiene and Sostakiene, 2020). The variously preserved products of *Stevia* exhibit anti-inflammatory, anti-hypertensive, anti-diabetic, anti-oxidant, anti-carcinogenic, chemo-preventive activities and other health-promoting effects (Kurek and Krejpcio, 2019). These medicinal properties and natural sweetening ability of *Stevia* leaves are accredited to the presence of phytochemicals specifically stevioside, isosteviol and rebaudioside including rebaudioside A, B, C, D, E and F which are 250-300 times more sweetness than sucrose, thus can be used as a natural alternative to artificial sweeteners (Salehi *et al.*, 2019).

Although *Stevia* is an emerging sugar alternative and anti-diabetic plant in Pakistan the exact details of propagation around the year are not known to many people (Khalil *et al.,* 2014). The plant showed negligible growth in October and November and flowered at a rate of 10% but died in December because the low-temperature ranges resisted growth. The seeds start

germination in 7 to 10 days but propagation through seeds is not popular because of weak fertility, small size and low germination rate (Al-Taweel *et al.,* 2021). *In vitro* propagation could be an important substitute for traditional propagation of *Stevia* as the seeds have a negligible germination rate and the production of less number of individuals is a drawback of propagation by stem cuttings (Gantait *et al.*, 2015). Through plant tissue, culture whole plant can be regenerated using cells, tissues or any part of the plant organ as an explant under aseptic controlled conditions. The required growth conditions such as light, temperature, humidity and nutrient availability for inoculated explants are optimized up to the extent to which the explant can proliferate rigorously (Chandran *et al.,* 2020).

The tissue culture of *Stevia* using leaf (Kariena *et al.,* 2020), stem, nodal segments (Castro-González *et al.,* 2019), root (Reis *et al.,* 2011) and seed (Ahmad *et al.,* 2020) explants carried out in different experimental settings have been proved successful under given controlled and optimized conditions which depicted a healthy growth of regenerates and also had a positive impact on its phytochemistry. Hence the tissue culture is the most effective alternate process for the mass production of *Stevia* and also to observe the impact of stress factors on the *in vitro* regenerated plants (Kahrizi *et al*., 2017). Therefore, the present study was undertaken to analyze the effects of various given concentrations of cytokinin (BAP) and auxin (IAA) on the *Stevia* shoot formation from bud explant and on rooting of *in vitro* developed shoots respectively.

MATERIALS AND METHODS

The micropropagation of *Stevia rebaudiana* was performed in the tissue culture laboratory of the Department of Biological Sciences. The *Stevia rebaudiana* plants were purchased from National Agriculture Research Centre (NARC), Islamabad and were grown (Fig.1) in the Botanical Garden of the Department of Biological Sciences, University of Veterinary and Animal Sciences Lahore, Ravi Campus Pattoki through cuttings (5cm). The cuttings were potted in such a way that the 3cm length was in loam soil and were kept in the greenhouse to protect from winter from November 2020-January 2021. At the arrival of the spring season, the plants were shifted to direct sunlight for healthy growth.

Preparation of culture medium: MS medium (Murashige and Skoog, 1962) was used for the explants culture under laboratory conditions. Before starting the experiment, the working area, all the glassware, surgical tools and working medium were sterilized properly. Two litres of the working medium was prepared by mixing all the stock solutions and 30g/L of sucrose was added. This solution was then divided into 8 beakers of 250mL. The required concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, and 0.07mg/L of cytokinin (BAP) was added in beaker 1, 2, 3, 4, 5, 6 and 7 respectively while 8th beaker was used as control without hormone. The pH of each solution was then adjusted to 5.8. 1.2% agar (Oxoid LP0012) was added as a gelling agent in each beaker and media was boiled on a magnetic stirrer to dissolve the agar. 83mL of each of the prepared working media was poured into glass bottles. The glass bottles were tightly caped and the media was autoclaved at 120°C temperature and 15 psi pressure for 20 minutes to be used further for inoculation (Golkar *et al.,* 2019). The culture medium without a growth regulator was used as a control. The experiment was conducted in triplicate.

Fig.1 *S. rebaudiana* grown in the Botanical Garden

Collection and surface sterilization of explants: The apical buds were plucked to be used as explant from one-month-old mother plants. The dust was cleaned from the explants with tap water followed by rinsing with distilled water twice. The explants were exposed to surface sterilization in a solution containing 5% (v/v) New Robin bleaching liquid (Karachi, Pakistan) for 2 minutes. The final surface sterilization was performed by dipping the explants in 0.1% (w/v) $HgCl_2$ solution containing a few drops of Tween-20 for 2 minutes under the aseptic condition in a laminar airflow chamber followed by 4-5 times rinsing with autoclaved distilled water (Solanki *et al.,* 2018).

Inoculation of explants: All the material placed in the laminar hood was exposed to UV light for 20 minutes before inoculation. The sterilized explant was excised into 1-2mm pieces using a sterilized scalpel and inoculated in culture jars with the help of pointed forceps.

The culture bottles containing explants were tightly sealed and the date of inoculation, type and concentration of plant growth regulator was mentioned.

Optimization of culture conditions for shoot formation: The inoculated culture bottles were placed under 24 ± 2°C temperature, and 16 hours of light exposure with cool, white fluorescent light with 2000 Lux intensity. The experiment was conducted in triplicates and MS medium without hormone was run parallel as a control. The different given concentrations of cytokinin (6-Benzylaminopurine BAP) for shoot formation were checked. The shoot formation at all concentrations was recorded after every 24 hours.

Optimization of culture conditions for root formation: After 25 days of culture the *in vitro* regenerated shoots were shifted to half strength MS medium supplemented with seven different concentrations of Indole-3-acetic acid (IAA) (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07mg/L). IAA solution was strained using a syringe filter and added to the culture bottles while working in the safety cabinet to avoid contamination. The experiment was conducted in triplicates and MS medium without hormone was run parallel as a control. The observations were recorded after every 24 hours.

Statistical Analysis: The growth parameters such as the mean number of shoots that emerged, mean length of shoots after 25 days and the mean number of nodes in all concentrations of BAP while the mean number of roots that emerged and mean root length after 25 days in all concentrations of IAA were analyzed by applying one-way analysis of variance (ANOVA) of compare means method and the significant difference was calculated according to Fisher's Least Significant Difference test at the 5% significance level (Lemus-Mondaca *et al*., 2016).

RESULTS

Shoot formation: The results of *in vitro* micropropagation of *S. rebaudiana* employing seven different concentrations of BAP (0.03, 0.04, 0.05, 0.06 and 0.07 mg/L) for shoot formation from bud explant are provided in Table 1. The shoot initiation was started after 6 days of inoculation at 0.07mg/L BAP. The maximum number of shoots (1.3 shoots per explant) with an average shoot length of 1cm and an average number of nodes (01) was observed in MS medium containing 0.07mg/L BAP after 25 days. The medium supplied with 0.03, 0.04, 0.05 and 0.06 mg/L BAP concentrations also showed shoot induction with the mean number of shoot 1.0 per explant and the mean shoot length of each concentration was 0.5, 0.36, 0.36 and 0.43 cm respectively after 25 days. Shoot induction was also observed in the control treatment after 16 days of inoculation with a mean number of shoots of 0.33, a mean shoot length of 0.33 and a mean number of nodes of 0.33. The shoot induction frequency of T3, T4, T5, T6 and T7 was 100% followed by the control which was 33%. The most suitable concentration for rapid shoot induction from the apical bud of *Stevia* was found to be 0.07mg/L (Fig.2). Statistical analysis of data described that the mean difference of groups is significant ($p<0.05$).

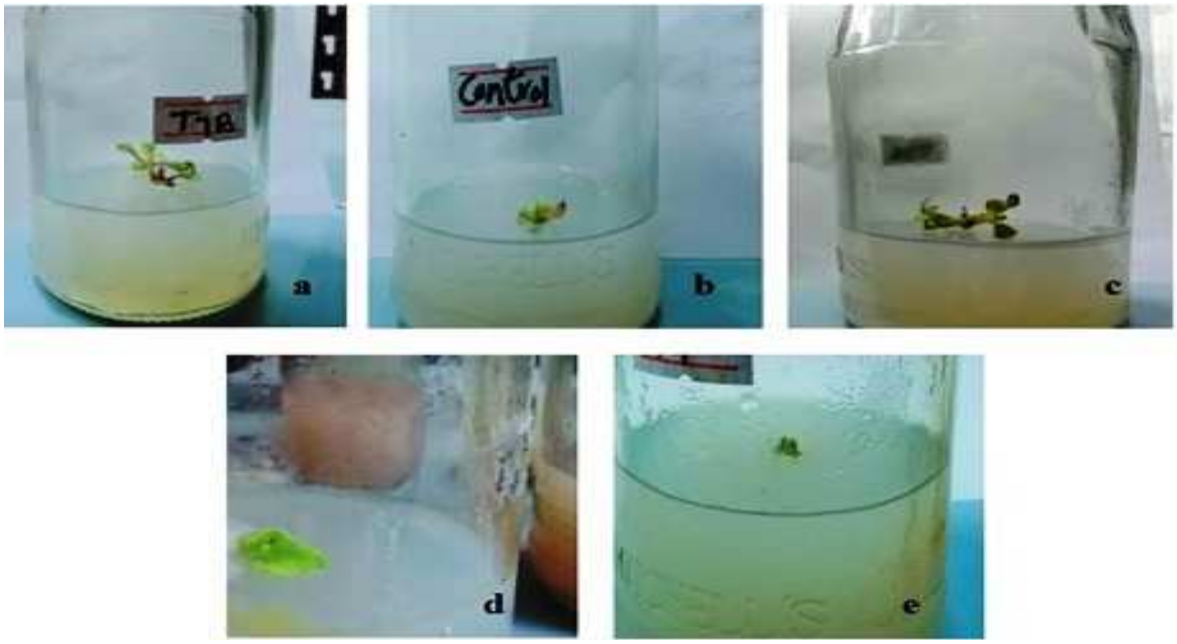

Fig.2. Development of shoot from apical bud explant of *Stevia* (a) 0.07mg/L (b) control (c) 0.03mg/L (d) 0.05mg/L (e) 0.06mg/L

Table.1 Shoot initiation from bud explant of *S. rebaudiana* at different concentrations of BAP.

The concentration of hormone mg/L	Growth started after inoculation (days)	Mean number of shoots	Shoot induction frequency (%)	Average Shoot length after 25 days (cm)	Mean number of nodes
0.01	0	0.00±0.00^{b}	0	0.00±0.00^{b}	0.00±0.00^{b}
0.02	0	0.00±0.00^{b}	0	0.00±0.00^{b}	0.00±0.00^{b}
0.03	7	1.00±0.00^{a}	100	0.5±0.00^{ab}	0.00±0.00^{b}
0.04	6	1.00±0.00^{a}	100	0.36±0.03^{ab}	0.00±0.00^{b}
0.05	6	1.00±0.00^{a}	100	0.36±0.06^{ab}	0.00±0.00^{b}
0.06	6	1.00±0.00^{a}	100	0.43±0.03^{ab}	0.00±0.00^{b}
0.07	6	1.33±0.33^{a}	100	1.00±0.50^{a}	1.00±0.50^{b}
Control	16	0.33±0.33^{b}	33	0.33±0.33^{ab}	0.33±0.33^{b}

The data is presented as means per treatment ± standard error. Different letters designate the significant differences assessed by Fisher's Least Significant Difference (LSD) test after performing a one-way analysis of variance (ANOVA). $p<0.05$.

Root formation: The 25 days old *in vitro* regenerated shoots were transferred to half-strength MS medium accompanied with IAA (0.03, 0.04, 0.05, 0.06 and 0.07 mg/L) concentrations for root formation. Root initiation under the influence of IAA concentrations was recorded in Table 2. The medium having 0.06mg/L IAA showed root formation after 7 days of shoot transplantation. The average number of roots was 0.33 and the average length was 0.1cm after 25 days. It was followed by root induction at the concentration of 0.02 mg/L after 9 days of a subculture where the mean number of roots and mean length after 25 days were recorded as 0.3 and 0.06cm respectively. The root induction frequency of both treatments (T2- 0.02 mg/l and T6- 0.06 mg/l) was 33%.

Table.2 Root initiation from *in vitro* regenerated shoots of *S. rebaudiana* at different concentrations of IAA.

The concentration of hormone mg/L	Growth started after sub-culture (days)	Mean number of roots	Root induction frequency (%)	Average root length after 25 days (cm)
0.01	0	0.00±0.00	0	0.00±0.00
0.02	9	0.33±0.33	33	0.06±0.06
0.03	0	0.00±0.00	0	0.00±0.00
0.04	0	0.00±0.00	0	0.00±0.00
0.05	0	0.00±0.00	0	0.00±0.00
0.06	7	0.33±0.33	33	0.10±0.10
0.07	0	0.00±0.00	0	0.00±0.00
Control	0	0.00±0.00	0	0.00±0.00

The data is presented as means per treatment ± standard error after applying One Way Analysis of Variance (ANOVA).

DISCUSSION

The main challenge in the commercial cultivation of *Stevia rebaudiana* is the incapability of its seeds to germinate vigorously which also resists the homogenate variety of *S. rebaudiana* with the same potential for sweetening and phytochemistry. So propagation through stem cuttings is a common practice by the farmers in the suitable season. But micropropagation via tissue culture technique is most suggested for rapid multiplication of *S. rebaudiana* in less time around the year producing healthy regenerates (Azzam *et al.,* 2021). In the current investigation the MS medium was prepared by mixing all the nutrients required for a complete culture medium and the growth conditions were maintained strictly according to the explant requirements such as 24±2°C, 16 hours light with 2500 lux intensity (Ghorbani *et al*., 2017). Earlier studies carried out on tissue culture of *S. rebaudiana* have used BAP either sole or in combination with other growth regulators such as kinetin+BAP in various concentrations for shoot organogenesis or callus formation from different explants such as the leaf, nodal segments and stem (Blinstrubienė *et al.*, 2020; Rasouli *et al.,* 2021; Tang *et al.,* 2021; Zayova *et al.,* 2022). Similarly, literature published on *S. rebaudiana* micropropagation has employed IAA either singly or combined with other auxins such as IBA and NAA for root development from *in vitro* regenerated shoots (Anbazhagan *et al.,* 2010; Aman *et al.,* 2013; Yücesan *et al.,* 2016). In the present study, BAP and IAA were also used for the direct shoot and root induction but with concentrations different from those previously employed.

In the first step, the bud explant was analyzed for shoot development with various concentrations of BAP. Results revealed that the bud explant cultured in the medium containing 0.07mg/L BAP (T7) started growth after 6 days of inoculation in all the three replicates of T7 treatment and produced the maximum mean number of shoot 1.33 ± 0.33 as compared to other treatments. On the other hand, the treatments T4, T5 and T6 also showed explant proliferation after 6 days of culturing except for T3 which started growth after 7 days of culture. These four treatments depicted the same mean number of shoots which was 1.00 ± 0.00 but they all differ in the mean length of shoots recorded after 25 days of inoculation which was 0.5 ± 0.0, 0.36 ± 0.03, 0.36 ± 0.03 and 0.43 ± 0.03 for T3, T4, T5 and T6 respectively. The control culture without hormone also expressed explant growth after 16 days of culturing but showed relatively minimum growth with 0.33 ± 0.33 mean shoot and 0.33 ± 0.33 cm of mean shoot length. The LSD test described that groups T3, T4, T5, T6 and T7 have no difference in the mean number of shoots but they significantly differ from T1, T2 and control. While for mean shoot length significant result was shown by T7 followed by T3, T4, T5, T6 and control. In the case of the mean number of nodes, the maximum growth was showed by T7 (0.07mg/L) 1.00 ± 0.50 followed by control 0.33 ± 0.33. This early shoot initiation can be attributed to the potential of BAP to accelerate the meristematic activity of apical cells ultimately enhancing the process of the shoot regeneration (Aitken-Christie *et al*., 1988). In another investigation, BAP 1.0mg/L concentration resulted in the formation of an 8.6 mean number of shoots, and 6 cm mean shoot length after 20 days of culture from shoot apical and nodal explants (Ali *et al.,* 2010). In an approach to standardize the procedure for indirect shoot development from leaf and stem explants, the medium was provided with two various concentrations of BAP 1.0 mg/L and 2.0 mg/L. The highest mean number of shoots 8.2 with a significant length of 1.8cm was obtained at 2.0 mg/L BAP from leaf explant (Zayova *et al.*, 2022). The experiment conducted by Hassanen and Khalil (2013) has also shown that BAP supplementation at 0.5 mg/L formed a maximum shoot length of 3.8cm in apical bud explant while BAP at 1.0 mg/L produced a 3.1cm length. The highest shoot multiplication (43.9 shoots/explant) was recorded at 2.0 mg/L BAP. All these previous studies are in contradiction with the current finding where 0.07mg/L of BAP provided good results for shoot development.

In the present investigation i*n vitro,* developed shoots were sub-cultured to half-strength MS medium after 25 days of shoot formation to observe their potential to develop root with the concentrations of IAA (0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07mg/L). The treatments T6 (0.06mg/L) and T2 (0.02mg/L) showed root initiation after 7 and 9 days of sub-culture respectively with the same mean number of roots 0.33 ± 0.33. The mean root length after 25 days for T2 and T6 was 0.06 ± 0.06 and 0.10 ± 0.10 cm respectively. Statistical evaluation of data presented that there is no significant difference between groups. A recent finding also concluded that IAA was good at 0.2 ppm to produce a 1.79 mean number of roots and 1.1cm mean root length after 30 days (Asmono and Sjamsijah, 2021). The study performed by Shulgina *et al.* (2021) reported that a combination of 0.5mg/L IAA + 1.0mg/L BAP with red monochrome light improved root multiplication coefficient by 30% *in vitro* generated shoots of *Stevia*. Thiyagarajan and Venkatachalam (2012) documented that IAA at 1.0mg/L resulted in the highest rooting frequency (80%) with a 4.6 average number of roots per shoot and 5.4 cm average root length.

Conclusion: The application of various concentrations of BAP in this study revealed that 0.07mg/L of BAP was best for the shoot induction in MS medium *in vitro* using apical bud explant. While for root induction, the best concentration of IAA proved to be 0.06mg/L initiated root after 7 days of incubation. The study can be beneficial for the researchers and growers for commercialization of the plant nursery and food industry. The present study suggested that other cytokinins can also be tested for shoot induction in *Stevia* sole or combination with BAP.

Conflict of Interest: Authors declare no conflict of interest.

Author Contribution: All the authors contributed equally to conceiving the idea, designing the study and data analyses.

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