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In vitro production of *Stevia rebaudiana* Bertoni

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Abstract: Experiments were conducted for the standardization of *in vitro* culture technique for the mass propagation of *Stevia rebaudiana,* a medicinally important, zero-calorie value, sweet tasted and an antidiabetic herb. Shoot tip, nodal segment and leaf were used as explants and they were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of BA, Kn and IAA both in individual and in combined form for shoot inductions and the best results were obtained from MS medium supplemented with BA+ IAA at the concentrations of 1.0 mg/l and 0.5 mg/l respectively. Among the explants used, shoot tip stood first in inducing shoot development. Best root formation of *in vitro* developed shoots could be achieved on half-strength Nitsch (N_6) medium supplemented with IAA at concentration 1.0 mg/l. The *in vitro* developed plantlets were transferred to pot and they were grown in greenhouse for hardening and finally they were planted in the open filed. Around 82% of plants were successfully established in natural field condition.

Key words: *in-vitro, Stevia rebaudiana***,** zero-calorie, MS and N_6 medium.

الإنتاج المختبرى لنبات ساتفيا ربودينا بارتونى

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الملخص: أجريت التجارب لتوحيد المقاييس والتقنيات بالمزرعة بالمختبر بكميات نوعية من نبات شيتدمبردام والذي يتميز بأهمية طبية و ذو قيمة صفرية للسعرات الحرارية وذو مذاق حلو وعشب مضاد لمرض السكري. وقد تم استخدام القمم النباتية النامية والغضة والأجزاء العقدية والأوراق كعينات حية ثم تم إكثارها بالوسط الغذائي موراشك واسكوك وبتراكيز مختلفة من بي ا + اى ا ا بشكل مفرد أو مركب لتخليق البادرات النباتية وأيضا الحصول على أفضل نتائج من الوسط الغذائي ام اس مع بي ا + هى ا ا وذلك بتركيز 1.0 ملجم/لتر و 0.5 ملجم/ لتر علي التوالي ومن خلال العينات الحية التي استخدمت اتضحت النتائج أن القمم النامية كانت أولا في تحفيز نمو وتطور البادرات واتضح أيضا أن الوسط الغذائي نيتش كان أفضل الأوساط في عمليات تشكيل القطاع الجزري بالزراعة بالمختبر مع وجود هرمون اى ا ا وذلك مع تركيز 1.5 ملجم وقد تم نقل الكثير من هذه النباتات المستزرعة بالمختبر واستزراعها بالأصص ومن بعد ذلك نقلها إلى البيوت المحمية لمعلية التقسية ثم زراعتها بالحقول المفتوحة وثبت أن 82% من هذه النباتات ذات الإكثار المختبرى قد نجحت في النمو علي مستوى البيئة الخارجية علي المستوى الحقلي

Introduction

The plant *Stevia rebaudiana* Bertoni is a perennial herb and belongs to the family Asteraceae. It is a native of certain regions of South America particularly in Paraguay and Brazil. *S. rebaudiana* is otherwise popularly known as stevia, sugar substitute, sweet weed, honey leaf and sweet herb of Paraguay. It is a natural alternative source to traditional sugar (sucrose) obtained from sugarcane, sugar beet etc., and a zero-calorie value one. In recent period, *S. rebaudiana* has received a greater attention due to its high range of sweet content (75-500 times more than cane sugar) and its therapeutic values for inhibiting fat accumulation and lowering blood pressure in human being (Soejarto et al., 1982; Chalapathi and Thimmegowda, 1997). The leaves of *S. rebaudiana* are the sources of diterpene glycosides, such as steviolbioside, rubsoside, rebaudioside A, B, C, D**,** E and F, dulcoside and stevioside (Starratt et al., 2002). Among them, stevioside stands first and use of this sweetening compound has increased dramatically due to health concerns related to sucrose usage, such as dental caries, obesity and diabetes. In addition,

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stevioside and other diterpene glycosides used in varity of foods and products, such as pickled vegetables, dried seafood, soy sauce, beverages, candies, chewing gum, yogurt and ice cream, as well as in tooth paste and mouth wash. Stevia extract and stevioside are officially approved as food additives in Brazil, Korea and Japan (Choi et. al., 2002; Mizutani and Tanaka, 2002) and in the United States. 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. It has also been successfully grown in United States of America, Canada, and Europe (Brandle et al., 2000). By all the means, now a day, *S. rebaudiana* has attracted economic and scientific interests. Further, as the leaves of *S. rebaudiana* are the prime sources of the above said quality, they are treated as potential source in the field of pharmacognosy and pharmacology.

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 (Miyazaki and Wanteable, 1974). The seeds of stevia show very less vigour and propagation and do not allow the production of homogenous population which leads to variability in sweetening level and composition (Felippe and Lucas, 1971; Miyagawa et al., 1986). Poor seed germination percentage is the limiting factor to large scale cultivation of this species. Vegetative propagation is also limited by the low number of individuals obtained from single plant. Hence, to overcome all these obstacles, micropropagation or *in vitro* culture technique can play a vital role for mass propagation and the production of genetically identical plants of *S. rebaudiana.* In this way, the present study was aimed at the findings of efficient protocol for *in vitro* mass propagation of *S. rebaudiana* and there are few earlier reports of micropropagation on the same species (Tamura et al., 1984; Ferreira and Handro, 1988; Patil et al., 1996; Sivaram and Mukundan, 2003; Mitra and Pal, 2007; Pourvi Jain et al., 2009).

Materials and Methods

Sources of Explant

For this study, the pot grown *S. rebaudiana* plants were collected from the Growmore Biotech Pvt. Ltd., Oosur, South India and they were grown and maintained in the greenhouse of department of Botany, Annamalai University, Chidambarm, South India. Explants were collected from shoot tip, nodal segment and leaf of 1-2 months old plants for shoot initiation. Among the explant sources, shoot tip and nodal segment were found best.

Surface sterilization

After excision, for surface sterilization, the explants were primarily rinsed in tap water for 10 min followed by gentle rinsed with 70% ethanol for few seconds and finally with 5% sodium hypochlorite solution for 5 min. After each step of sterilization, the explants were washed with sterile double distilled water for three times. Further sterilization procedures were carried out in laminar air flow chamber by using 0.1% (w/v) $HgCl_2$ for 3 min. The explants were then rinsed five times with sterile distilled water. Finally, in the laminar air flow chamber, the explants were cut into small pieces ranging in size from 0.5 to 1.0 cm long.

Inoculation

After complete sterilization and slicing, the explants were inoculated in MS medium (Murashige and Skoog, 1962), supplemented with cytokinins and auxins used either singly or in combination. The pH of the medium was adjusted to 5.8 and prior to autoclaving, 0.8 % agar (Himedia,

Mumbai) was added to the medium. Then, autoclaving was done at 121°C and 15 psi for 20 min.

Establishment of culture

After inoculation, the cultures were maintained at a temperature of 25±2°C with a photoperiod of 16 h per day. Lighting of 80μEm^{-2} s^{-1} was supplied by using cool and white fluorescent tubes (Philips India Ltd., Mumbai). Various types of growth regulator, viz. BA, Kn and IAA were added with MS medium either alone or in combination for better shoot induction (Table 1). The MS medium without adding of growth regulators was served as control. After 30-35 days of culturing, the multiple shoots were separated into pieces and the separation was done at the base of multiple shoots and they were transferred to 500 ml culture bottle containing 50 ml of the same kind of medium to get a more number of new shoots. For root initiation, the shoots were transferred to half-strength N_6 (Nitsch, 1951) medium supplemented with IAA, IBA and NAA. Then, the rooted plantlets were taken out from the culture tubes and washed with water carefully to remove the traces of agar and they were transplanted in a pot containing a mixture of sand, soil and vermicompost (1:1:1) and they were placed in the greenhouse for hardening. In the greenhouse situation, the potted regenerated plants were supplied with dilute MS basal medium devoiding of vitamins and sucrose, once in a week for a total period of four weeks. Similarly, sufficient irrigation was also done. All the studies were made with five replications and data were taken for calculating mean and standard errors of the same by using SPSS software (SPSS, 1999).

Table 1. Effect of growth regulators on shoot induction.

Sl. No.	Plant Growth Regulators (mg/l)	Types of explant		
		Shoot tip (Mean ± SE)	Nodal segment (Mean ± SE)	Leaf (Mean ± SE)
1	Nil	0.20 ± 0.19	0.40 ± 0.24	0
2	BA 0.2	2.40 ± 0.22	2.60 ± 0.22	0
3	BA 0.5	2.60 ± 0.24	3.00 ± 0.31	0.20 ± 0.16
4	BA 1.0	3.60 ± 0.40	4.00 ± 0.31	0.60 ± 0.24
5	BA 1.5	5.00 ± 0.31	5.40 ± 0.31	1.20 ± 0.37
6	BA 2.0	9.20 ± 0.37	8.20 ± 0.40	1.60 ± 0.24
7	BA 2.5	7.20 ± 0.37	5.60 ± 0.37	2.40 ± 0.32
8	BA 3.0	5.80 ± 0.37	4.40 ± 0.22	1.60 ± 0.22
9	Kn 0.5	1.40 ± 0.24	1.60 ± 0.24	0
10	Kn 1.0	3.20 ± 0.37	2.20 ± 0.24	0
11	Kn 2.0	4.40 ± 0.24	2.60 ± 0.20	0
12	Kn 3.0	4.80 ± 0.37	4.20 ± 0.24	0
13	BA 1.0 + Kn 0.2	3.60 ± 0.24	2.60 ± 0.37	0
14	BA 1.0 + Kn 0.5	5.40 ± 0.24	3.60 ± 0.24	0
15	BA 2.0 + Kn 0.2	6.80 ± 0.37	5.40 ± 0.24	0
16	BA 2.0 + Kn 0.5	10.80 ± 0.32	7.80 ± 0.24	0
17	BA 1.0 + IAA 0.2	8.40 ± 0.29	8.20 ± 0.28	0
18	BA 1.0 + IAA 0.5	16.20 ± 0.37	14.00 ± 0.31	0
19	BA 2.0 + IAA 0.2	11.20 ± 0.32	9.80 ± 0.35	0
20	BA 2.0 + IAA 0.5	9.20 ± 0.32	8.20 ± 0.37	2.20 ± 0.16

Results and Discussion

Explant selection and shoot induction

In this study, shoot tip, nodal segment and leaf were excised from the plant species of *Stevia rebaudiana* as explant sources, and the shoot tip and nodal segment were found best explants for shoot induction on MS medium supplemented with different concentrations of BA like 0.2, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l or Kn like 0.5, 1.0, 2.0, 3.0 mg/l individually. In another way, the MS medium supplemented with the combined form of BA + Kn and BA + IAA. In individual form, the BA concentrations above 2.0 mg/l level caused poor shoot induction in all the explants studied. The more number of shoot induction was observed in all the three explants studied on MS medium supplemented with BA 1.0mg/l + IAA 0.5 mg/l (combined form). When observations were made on the cultures of 40 days period, development of 16 (16.20±0.37, Table 1) shoots were observed from a single shoot tip explant, while 14 (14.00±0.31, Table 1) and 8 (8.2±0.37) shoot inductions were observed from a single nodal and leaf explants respectively. The combination of BA and Kn failed to induce shoot in leaf explant, but induce low number of shoot in shoot tip and nodal explants.

This *in vitro* propagation studies confirmed the importance of plant growth regulators in the initiations of callus, shoot, root and on the whole the regeneration of plant. In this way, the two cytokinins namely, BA and Kn used in this study, the BA proved a better one than Kn in shoot induction from all explants used i.e. shoot tip, nodal segment and leaf. Where as Kn could initiate shoot tip and nodal explants only and this is documented by early studies also (Murashige, 1974; Benne and Davies, 1986; Rogers et al., 1998). On the other hand, mineral nutrients are being as the basic component of culture media play a vital role in rapid growth of tissue and the extent and the quality of morphogenesis of tissue (Niedz and Evens, 2007). In this study, the synergistic effect of both BA + IAA at concentrations 1.0 mg/l and 0.5 mg/l respectively, were found best in regenerating shoot from shoot tip and nodal explants. Similarly the combination of BA (1.0 mg/l) and Kn (0.5 mg/l) worked well for the shoot proliferation and elongation from the same explants noted above. Similar type of results have also been got by early workers in the same species, i.e. *S. rebaudiana* (Tamura et al., 1984; Ferreira and Handro, 1988; Patil et al., 1996; Sivaram and Mukundan, 2003; Ahmed et al., 2007; Mitra and Pal, 2007; Pourvi Jain, 2009). This is also in consistent with early findings in papaya as well as in *Eucalyptus grandis* (Cononer and Litz, 1978; Teixetra and Da Silva, 1990).

Root induction

The micro-cuttings of *in vitro* proliferated shoots were implanted on half-strength N_6 medium supplemented individually with IAA, IBA and NAA at concentrations of 0.2, 0.5, 1.0 and 2.0 mg/l for root initiation. Among them, maximum number of root formation (93%) was observed in medium supplemented with (1.0 mg/l) IAA (11.80± 0.80, Table 2) and IBA at concentration 1.0 mg/l stood second (86%) in the order (10.40± 0.74, Table 2). The root induction was gradually decreased with increasing concentrations of auxin types. No root formation was observed on auxin free basal medium. Similar types of results were found by earlier workers in the same species (Sivaram and Mukundan, 2003; Ahmed et al., 2007; Mitra and Pal, 2007).

Table 2. Effect of different concentrations of IAA, IBA and NAA on root formation.

Concentrations of auxins (mg/l)	Percentage of root per shoot (%)	No. of roots per shoot (mean ± SE)	Average length of root per shoot (cm, mean± SE)
IAA			
0.2	72	5.20±0.37	3.00±0.17
0.5	87	6.20±0.37	3.78±0.26
1.0	93	11.80±0.80	4.80±0.31
2.0	70	4.20±0.37	3.06±0.13
IBA			
0.2	68	5.00±0.31	2.40±0.18
0.5	83	6.00±0.44	2.94±0.16
1.0	86	10.40±0.74	4.00±0.31
2.0	65	4.60±0.24	2.32±0.20
NAA			
0.2	57	3.40±0.50	1.96±0.16
0.5	64	4.80±0.37	2.40±0.15
1.0	70	6.40±0.37	3.60±0.20
2.0	49	4.00±0.54	2.16±0.14

Figure 1. The successive stages of *in vitro* propagation of *Stevia rebaudiana.* (a) Direct regeneration of shoot from explant, (b) Initiation of multiple shoot formation, (c) Development of more number of multiple shoot, (d) Root formation from regenerated shoot (e & f) Hardening and establishment regenerated plants.

Acclimatization of regenerated plants

For acclimatization, the plantlets were taken out from the culture tubes when the roots were partially brown in colour and the root portion was washed in the tap water to remove the attached medium. Then they were transferred to pots containing sand, soil and vermicompost in the ratio 1:1:1 and they were placed in greenhouse for hardening for the period four weeks. The use of sufficiently porous substratum that allows adequate drainage and aeration has been recommended for fast acclimatization of *in vitro* regenerated plants (Dunstan and Turner, 1984) and this study also it has been followed. After four weeks under greenhouse, the potted plants were transferred to natural field conditions for better establishment and at the end of this study, around 82% of plants were thrived well in natural situation.

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