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Research article

IN-VITRO MICROPROPAGATION AND MEDIA OPTIMIZATION OF *STEVIA REBOUDIANA* AN IMPORTANT BIO-SWEETENER AND MEDICINAL PLANT"

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ABSTRACT: The technique of Plant Biotechnology has an important role to play in the production of agriculture, horticulture and ornamental plants and in the manipulation of plants for improved agronomic performance. In the present study initiation of callus has been done by using explants from auxiliary and apical meristems of *Stevia rebaudiana*. Explants were inoculated on MS basal medium having Vitamin supplement with Auxin like 2-4, D and NAA (0.5mg/lt. -2.0mg/lt.) alone or in combination with cytokinis like BAP & Kinetin (0.52 mg/lt. -1.0 mg/lt.). Sucrose 30 gm. and agar 4.5 gm./lt. After the formation of explants, they were transferred into shoot induction medium containing different concentration of cytokine like BAP & Kinetin (0.5 mg/lt. to 30 mg/lt.) with additional vitamins. After few days numbers of multiple shoots were formed. Strong and elongated shoots were treated with root initiating medium i.e MS containing Auxin like IBA, NAA & activated charcoal. Maximum 60-70% callusing in 10 to 15 days was initiated on 2-4D (1-2mg/lt.) and 40-50% callusing were reported in medium containing (1.0 NAA & 0.5 mg/lt. BAP) in 25-30 days. Nodular compact cells were formed in 2-4D. The medium containing BAP alone showed shoot formation 70-80% with 40% coconut water. Second rooting medium used was containing NAA and activated charcoal and 85% rooting were observed within 7-10 days after transferring. Finally the cultured plants were transferred for hardening.
Key words: MS Media**,** *Stevia rebaudiana,* Auxin, cytokine, Kinetin

INTRODUCTION

Substances derived from the plants remain the basis for a large proportion of the commercial medications used today for the treatment of heart disease, Diabetes, high blood pressure, asthma etc. Stevia is a wonder herb from Paraguay. The leaves of stevia are 30 times sweeter than sugar with zero calories where as pure extract is 300 times sweeter than sugar. The sweet steviol glycosides have functional and sensory properties superior to those of many other high potency sweeteners. Stevia is likely to become a major source of high potency sweetener for the growing natural food market in the future. The task at hand is to convert stevia from a wild plant to a modern crop well suited to efficient mechanized production. Three most developed varieties of *Stevia rebaudiana* differ in their glucoside concentrations.. SRB – 123 Total Glucoside content varies between 9-12 percent. SRB – 512 Total glucoside content varies between 9-12 %. SRB – 128 – This unique variety has very high total glucoside content of 21 % .

TAXANOMIC POSITION

Kingdom : Plantae
Division : Mannoliophyta
Class : Magnoliopsida
Order : Asterales
Family : Asteraceae (Compositae).
Genus : *Stevia*
Specie : *rebaudiana*

Stevia rebaudiana Bertoni is one of 154 members of the genus Stevia. It is a member of the Compositae family Stevia,a perennial herb, has an alternate leaf arrangement, mid green and herbaceous growth habit with flowers arranged in indeterminate heads.

CHEMISTRY OF STEVIA

The sweet compounds found in stevia leaves are diterpene glycosides (steviol glycosides) and is synthesized in the initial stages, using much the same pathway as gibberellic acid, an important plant hormone. The steviol glycoside and gibberellin pathways diverge at kaurene. In stevia, laurene is converted to steviol, the "backbone" of the sweet glycosides, then glucosylated or rhaminosylated to form the principle sweeteners. Steviol glycosides are derived from the mevalonic acid pathway. This is a fundamental metabolic route that provides the two C_5 building block molecules, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), that are required for synthesis of all isoprenoid compounds (Ahmad et.al.1982, Tan et.al. 1994).

MATERIALS AND METHODS

The technique of micro propagation include isolation, inoculation and regeneration of plant cells.

Sterilization of room: The sterilization of the room is done by washing with disinfectant, wiping with 2% sodium hypochlorite solution or 95% ethyl alcohol. Lastly decontamination is done by UV radiations, which gives maximum sterilized conditions in working area. (Shen**1**995)

Preparation, sterilization and storage of nutrient medium: Murashige and skoog's (1962) nutrient medium was used throughout the experiment. In addition, media were supplemented with growth regulators, other additional vitamins, organic supplements and carbon.

Growth Hormones: Growth hormones used in plant tissue culture medium are ;

AUXINS : Indole 3- acetic acid (IAA), Napthalene acetic acid (NAA) and 2– 4-Dichloro phenoxy acetic acid (2-4-D) being thermostable were added to the medium prior to autoclaving while IAA being thermostable had to be filter sterilized.

CYTOKININS : 6-benzyl amino purine (BAP), kinetin (KN) and Thidiazuron (TDZ) were used.

*ORGANIC SUPPLEMENTS :*Casein hydrolysate (CH) and Coconut water (CW) were used.

CARBON SOURCES :

Varying concentration of sucrose, glucose, maltoses were used as carbon source. The composition of MS and Preparation of stock solution of nutrients was in accordance with Murashige and skoog's (1962). P^H was adjusted by adding 1N HCL or 1N NaOH.

Selection and Isolation of Explants: The explants like seeds, stems, apical and axillary nodes, leaves, etc. were selected from healthy, identified elite variety of required plant species from field. The seeds were isolated from fruits, apical and axillary meristem from the nodes of stem of the required plants.

Sterilization and Inoculation of Explants: The explants (apical and axillary nodes), of *Stevia rebaudiana* were excised and washed with tap water. For surface sterilization explants were treated with 70% alcohol and followed by rinsing with sterilized distilled water for 3 times. Further it was treated with 0.1 % mercuric chloride solution and finally rinsed with sterilized distilled water for 4 to 5 times to remove all the traces of surfactant. The sterilized explants were than inoculated on the prepared medium in the laminar air flow chamber. Inoculated cultures were incubated in the culture or growth room at controlled environment of the temperature of 25 +/- 4 ^{o}c of 16 hours photoperiod of 2000 lux and dark condition.

Shoot induction experiment: For these studies the sterile epical and axillary buds were inoculated on Murashige and Skoog (1962) basal medium supplemented with cytokinins like BAP and kinetin KN, in the concentration of (0.5-2.0) mg/l alone or in combination with other cytokinins of each, containing sucrose 30 g and gelled with agar 4 g/l. In addition auxins like IAA or NAA (0.1-1.0 mg/l) were used for promoting the shoot induction.

MEDIUM USED IN SHOOT INITIATION

Medium 1	MS + 0.5 mg/l BAP
Medium 2	MS + 1.0 mg/l BAP
Medium 3	MS + 1.5 mg/l BAP
Medium 4	MS + 2.0 mg/l BAP
Medium 5	MS + 0.5 mg/l KN
Medium 6	MS + 1.0 mg/l KN
Medium 7	MS + 0.5 mg/l BAP + 0.5 mg/l KN
Medium 8	MS + 1.0 mg/l BAP + 0.5 mg/l KN

The measurement of growth was taken by the percentage of buds showing response, number of shoots initiated per explants, shoot length and callus formation.

Multiplication of Shoots: Within one week the bud start responding by bud break and shoot appears after 6 to 10 days of culture, shoots were separated and transferred to different multiplication medium. Number of experiments was carried out to maximize the rapid multiplication of shoots. These include use of high concentration of cytokinin, BAP (1.0-3.0 mg/l), KN (1.0-2.0 mg/l) as compared to induction medium.

Different medium used for multiplication:

Medium 1 MS + 1.0 mg/l BAP
Medium 2 MS + 2.0 mg/l BAP
Medium 3 MS + 2.0 mg/l BAP +0.5 mg/l KN
Medium 4 MS + 3.0 mg/l BAP
Medium 5 MS + 1.0 BAP +1.0 KN
Medium 6 MS + 1.0 BAP + 2.0 KN
Medium 7 MS + 2.0 KN mg/l

The measurement was taken on the basis of percentage of shoot response, number of multiple shoot developed, shoot length and callus formed from each ten replicates.

Experiment for root induction: Regeneration multiple shoots were separated in different rooting medium.

Different media for root induction

MS + 10 g/l sucrose
MS + 1 mg/l NAA + 20g sucrose
MS + 200 mg activated charcoal
MS + 20g/l sucrose

RESULTS AND OBSERVATIONS

In the initial experiment Maximum 80% bud break and shoot initiation was reported in BAP (0.5-1.0mg). On an average 1-2 shoots were developed. The combination of BAP and Kinetin contained media, shows 40-45% of initiation was found with the formation of only one shoot with lots of callus formation. Maximum length of shoots was observed 1-2cm in the medium containing BAP alone in comparison to medium supplemented with BAP and Kinetin. Higher concentration of BAP gives short and weak shoots with the formation of non fragile callus (Table 1).

Table-1- Effect of Growth Regulators on shoot Induction in *Stevia Rebaudiana*.

S.No	Medium + Growth hormones mg/l	%age of shoot induction	No. of shoots per culture	Average shoot length in cm.	Callusing
1	MS+0.5 BAP	70%	1-2	1-2	-
2	MS+1.0 BAP	75%	1-3	2 cm	-
3	MS+1.5 BAP	70%	1-2	2cm	-
4	MS+2.0 BAP	60%	1-2	1-2	-
5	MS+0.5 KN	40%	1	1-2	+
6	MS+1.0 KN	42%	1	1-2	-++
7	MS+0.5 BAP+0.5 KN	45%	1	1	++
8	MS+1.0 BAP+0.5 KN	45%	1-2	1	+

The highest number of shoots was observed in the medium containing higher concentration of BAP (1.0-2.0 mg/l). These media show about 15-20 number of shoots per culture, when subculture in the same fresh medium after 15 days duration. The length of shoot elongated 4-5 cm and increases with culture duration. About 5-9 shoots with 2-3 cm were achieved in the medium containing Kinetin and BAP, and produces callus (Table 2). For further improvement in proliferation, shoots were subculture in the medium containing same BAP concentration. Cultures showed better response in shoot number (20-25) and shoot length (3-5cm) in the medium. Thus the medium containing BAP (1.0-2.0 mg/l) were standardized as the best media for mass production and elongation of *Stevia rebaudiana* (Table 3). The shoots of 2-3cm showed 90% rooting in the medium containing activated charcoal and NAA. About 2-4 thin long roots were developed which increase with the age of culture. Root length was 2-8cm.

Table-2- Effect of Growth Regulations on Multiplication of Shoot in *Stevai Rebaudiana*.

S.No	Medium + Growth hormones mg/l	%age of response	Average no. of shoots	Average shoot length in cm.	Callusing
1	MS+1.0 BAP		15	2-3	-
2	MS+2.0 BAP		15-20	2 -3	-
3	MS+2.0BAP+0.5KN		8-10	2-5	+
4	MS+3.0 BAP		4-5	3-5	-
5	MS+1.0 BAP+1.0KN		5-8	1-2	+
6	MS+1.0 BAP+2.0 KN		4-9	1-2	++

Table-3-Effect of Different Media on Root Induction in *Stevia Rebaudiana.*

S.No	Medium	Percentage of shoots rooting	Root length in cm	Root Morphology
1	MS+ 10g/l sucrose	50-60	2-3	Thin, short
2	MS+ 1.0 mg/l NAA+20g Sucrose	70-75	2-5	Thin, short
3	MS+200mg activated charcoal	85-90	3-5	Thin, long

CONCLUSION

The demand for high potency sweeteners is expected to increase Worldwide. The increasing in the number of diabetic patients and health conscious individuals would push forward the need for alternatives to sugar. Stevia is a potential alternative source for replacing artificial sweeteners like saccharin, aspartame, asulfam, etc. Unlike many low calorie sweeteners, stevioside is stable at high temperature and over a pH range of 3-9. In India several important and necessary steps have to be taken up for its propagation. Development of seedlings suitable to India would of the first requirement. A crop production system, providing information on optimized crop inputs, weed and disease control, harvesting and handling methods would have to be detailed out. Micropropagation technology of *Stevia rebaudiana* has been briefly analyzed. During propagation the protocol efficiency has revealed about 80% plant recovery. Pilot plant propagation system however need to be launched for the scaling up of production of million available superior planting material to make the cultivation of Stevia in India as economically viable venture, and contribute to sustainability of economy and to establish it as a natural, healthy sweetener.

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