International Scholars Journals

African Journal of Agriculture ISSN 2375-1134 Vol. 11 (7), pp. 001-003, July, 2024. Available online at https://internationalscholarsjournals.org/journal/ajaoas/table-of-contents © International Scholars Journals

Full Length Research Paper

Advancements in Tissue Culture Methods for *Stevia rebaudiana* Bert Cultivation in Bangladesh

Mohammed Salim Uddin[1], Mohammad Shaheed Hossain Chowdhury[1], M. Muoztaba Mahfuzul Haque Khan[1], Mohammad Belal Uddin[1], Romel Ahmed[1], Md. Azizul Baten[2]*

[1]Department of Forestry, Shah Jalal University of Science and Technology, Sylhet-3114, Bangladesh.
[2]Department of Statistics, Shah Jalal University of Science and Technology, Sylhet-3114, Bangladesh.

Accepted 28 March, 2024

An experiment was conducted on *in vitro* culture of *Stevia rebaudiana* Bert, an important non-caloric sweetening herb to explore its potential for micro-propagation. Leaf, nodal and inter-nodal segments of the selected herb as explant were cultured on MS medium containing 2,4- D at 2, 3, 4 and 5 mg/L for callus induction. Inter-nodal segments initiated callus earlier than node and leaf. The highest amount of callus was found in MS medium with 3.0 mg/L 2,4-D and MS medium with 5.0 mg/L 2,4-D gave the poorest callus.

Key words: *In vitro* propagation, 2,4-D, *Stevia rebaudiana*.

INTRODUCTION

Stevia rebaudiana Bert Belonging to the family Compositae, is one of the most valuable tropical medicinal plant. *S rebaudiana* is originally a South American wild plant (Katayma et al., 1976), but it could be found growing in semi-arid habitat ranging from grassland to scrub forest to mountain terrain. The plant has gained wide access to Pacific Rim countries, where in recent decades it is being cultivated domestically, used in its raw leaf form and is now commercially processed into sweetener. Seed germination of *Stevia* is often poor (Miyazaki and Wantenabe, 1974). Therefore, there are basically two options for multiplication; tissue culture and sstem cutting.

Stevia rebaudiana Bert is one of 154 members of the genus *Stevia*, which produces sweet steviol glycosides (Robinson, 1930; Soejarto et al., 1982). The first report of commercial cultivation in Paraguay was in 1964 (Katayama et al., 1976; Lewis, 1992). Sumida (1968) began a large effort aimed at establishing *Stevia* as a crop in Japan. Since then, it has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada (Lee et al., 1979; Goenadi, 1983; Shock, 1982; Saxena and Ming, 1988; Brandle and Rosa, 1992; Fors, 1995). In Brazil and Paraguay it grows wild. It is small shrubby perennial growing up to 65 cm tall, with sessile, oppositely arranged lanceolate to oblanceolate leaves, serrated above the middle. The property of the species that called attention to the plant was the intense sweet taste of the leaves and aqueous extracts. From the leaves of *Stevia*, stevioside, sweet crystalline diterpene glycosides are extracted. Pure extract stevioside is non-caloric and 30 times sweeter than sugar (Bhosle, 2004). Other attributes of this natural, high intensity sweetener include non-fermentable, non-discoloring, maintain heat-stability at 100 °C and features a lengthy shelf life. The product can be added to tea and coffee, cooked or baked goods, processed foods and beverages. In the Pacific Rim countries like China, Korea and Japan *Stevia* is regularly used in preparation of food and pharmaceutical products. In Japan alone, an estimated 50 tons of stevioside is used annually with sales valued in order of $220 million Canadian (Brandle and Rosa, 1992). Currently *S. rebaudiana* production is centered in China with major market in Japan (Kinghorn and Soejarto,

*Corresponding authors E-mail: batenatsust.edu, baten_mathatyahoo.com.

Table 1. Composition of MS media (1 liter).

Category	Chemicals	Amount
Macro salts	NH_4NO_3	1.65 g
	KNO_3	1.90 g
	$CaCl_2.2H_2O$	0.44 g
	$MgSO_4.7H_2O$	0.37 g
	KH_2PO_4	0.17 g
Micro salts		
	$FeSO_4.7H_2O$	27.80 mg
	$Na_2EDTA2H_2O$	33.60 mg
	KI	0.83 mg
	H_3BO_4	6.20 mg
	$MnSO_4.4H_2O$	22.30 mg
	$ZnSO_4.7H_2O$	8.60 mg
	$Na_2MoO_4.H_2O$	0.25 mg
	$CuSO_4.5\ H_2O$	0.025 mg
	$CoCl_2.6\ H_2O$	0.025 mg
Organic supplements		
	Myoinositol	100.00 mg
	Nicotinic acid	0.05 mg
	Pyridoxine HCl	0.05 mg
	Thiamine HCl	0.05 mg
	Glycine	0.02 mg
	Sucrose	30.00 g

1985). No negative clinical reports have appeared in any of these countries where *Stevia* is readily available. For more than a decade, stevioside has approved and is widely used in Brazil. It is used as a table top sweetener, in soft drinks, baked goods, pickles, fruit juices, tobacco products, confectionary goods, jams and jellies, candies, yogurts, pastries, chewing gum and sherbets. Stevioside is of special interest to diabetic persons with hyperglycemia and the diet conscious.

The present investigation was undertaken to find out suitable sources of explants and suitable concentration of 2,4-D for callus induction in micro propagation of *S. rebaudiana*.

MATERIALS AND METHODS

Plant materials

The explants (leaf, nodal and inter-nodal segments) were collected from several days old *in vitro* generated *Stevia rebaudiana* at the Biotechnology Laboratory of Bangladesh Sugarcane Research Institute, Ishurdi of Pabna district. The explants were cut into small pieces (about 0.1 m long) and then were treated with 1% savlon for 5-6 min with constant shaking and washed thoroughly with distilled water. Then the explants were surface sterilized with a 0.1% mercuric chloride solution containing two-three drops of tween-80 for 5 min under aseptic condition and then washed four times with sterilized distilled water. The explants were then inoculated aseptically into MS (Murashige and Skooge, 1962) medium with different concentrations and combinations of growth regulators (Table 1).

Preparation of stock solution

After mixing all salts and organic components the pH was adjusted to 5.7 by adding 1 M NaOH. Then 5.6 g agar was mixed to the media (by using microwave oven) for making it semi-solid. Total amount of media was then subdivided into four conical flasks each containing 250 ml. Then the hormone, 2,4-D, was added at 2, 3, 4 and 5 mg/L. Each 250 ml media was then poured into 18 test tubes. Thus a total of 72 test tubes were filled with media. In the same way other 36 test tubes were prepared and finally 108 test tubes were used in the experiment. The test tubes were autoclaved 121°C temperature and 15 psi for 20 min.

Callus induction medium

MS basal media supplemented with 2,4-D at varying concentrations (2-5 mg/l) were prepared for callus induction. After mixing all stock solutions and 3% sugar, pH of the medium was adjusted to 5.5- 5.8. The agar (0.6%) was dissolved and media were dispersed in the test tubes and capped with cotton plugs. Test tubes containing media were autoclaved at 121°C at 15 psi for 20 min.

Culture environment

Unless mentioned specially, all cultures were grown in an air-conditioned culture room illuminated by 40 w white florescent tube light with intensity varied from 2000-3000 lux. The temperature of the culture room was maintained around 25°C. The photoperiod was maintained as 16 h light and 8 h darkness. Visual observation of culture was made every week and data were recorded after 2 weeks of inoculation.

RESULTS AND DISCUSSION

Among the explants used for the culture inter-nodal segments were found to be able to produce profuse callus. The results of callus production and multiplication are presented in Tables 2 and 3. Inter-nodal segments initiated callus earlier than leaf and nodal explants. The highest amount of callus was found in the MS medium with 3.0 mg/L 2,4-D. Length of day under which *Stevia* is cultivated has an effect on stevioside concentration, which is supported by Kinghorn and Soejarto (1985). The authors observed that in subtropical regions of the world, stevioside concentration is high in *Stevia* plant when it is cultivated under long days.

The result of the experiment and other earlier research report clearly support the possibility of propagating *S. rebaudiana* by adopting *in vitro* techniques. The climatic requirements of this tropical elite medicinal plant indicate that it can be introduced in the hilly areas of Sylhet and Chittagong. The unique selling points of *Stevia* sweetener are very strong in Bangladesh due to the presence of diabetic and other metabolic disease including obesity.

Table 2. Production of *Stevia rebaudiana* callus in different concentrations of hormones.

2,4-D	Explant	Test tube								
		1	2	3	4	5	6	7	8	9
2 mg/L	Leaf	+	+	+++	+	++	+	+	+	++
	Inter-node	Failed	+	++	++	+	+	++	++	++
	Node	Failed	+	+	++	+	+	+	+	+
3 mg/L	Leaf	+	+	+	++	+	++	++	+	+
	Inter-node	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Node	Failed	+	+	++	++	+	+	+	+
4 mg/L	Leaf	+	+	+	++	+	+	+	+	+
	Inter-node	+	++	+	+	++	++	+	+	++
	Node	+	+	+	+	++	+	+	+	+
5 mg/L	Leaf	+	++	++	+	+	+	+	+	+
	Inter-node	+	+	+	+	+	+	+	+	+
	Node	+	+	+	+	+	+	+	+	+

+, Poor callus; ++, medium callus; and +++, profuse callus.

Table 3. Periods, colors and amounts of *Stevia rebaudiana* callus formation at different hormone concentrations.

Hormone(2,4-D)	Days of callus initiation	Color of callus	Amount of callus	Remarks
2 mg/L	10	Mainly brownish	Medium	
3 mg/L	11.5	Brownish to light green	Highest	Best callus
4 mg/L	11	Brownish	Poor	
5 mg/L	12	Brownish to blackish	Poorest	Very poor, often blackish color

Here *Stevia in vitro* propagation has been demonstrated with its overall potentiality and suitability. *In vitro* propagation can become an important alternative to conventional propagation and breeding procedures for wide range of plant species.

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