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FORMULATION OF MEDIUM WITH LOW COST OPTIONS FOR *IN VITRO* CAULOGENESIS IN ETHNOMEDICINAL HERB *STEVIA REBAUDIANA*

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ABSTRACT
Tissue culture has become a routine method for propagation of plants. However, application of tissue culture technology is constrained by its high costs. In view of this, the aim of this research was to develop the tissue culture protocol with low cost media options by standardization of various media compositions for best growth response of the plant. There was 85% reduction in cost of media for plant caulogenesis by using inexpensive carbon source, water source and gelling agent. Laboratory reagent grade sucrose was replaced by locally available commercial sugar (market sugar and sugar cubes) as carbon source, bacteriological grade agar by isabgol (Plantago ovata), sodium alginate, starch as gelling agent and distilled water, tap water and packaged drinking water as a water source. Based upon cost analysis the use of isabgoal as a gelling agent, use of sugar cubes and packaged drinking water for preparation of media can be used to boost the caulogenesis in *Stevia rebaudiana.*

KEY WORDS: Caulogenesis, Cost effective, Micropropagation, Standardization, *Stevia rebaudiana*

INTRODUCTION

Tissue culture has become a routine method for *in vitro* propagation of the plants. Apart from propagation, the major application of plant tissue culture lies in the production of true-to-type high quality planting material that can be multiplied under aseptic conditions irrespective of the season and weather, on a year round basis. However, application of tissue culture technology is constrained due to overhead costs involved in it than the conventional methods of plant propagation. The cost of medium preparation can account 30-35% of the cost of micro propagation of the plants (Brink *et al.*, 1998). On the other hand, the gelling agents such as agar contribute 70% of the total cost of the media (Prakash, 1993).This situation calls for low cost alternatives to reduce the cost of production of tissue-cultured plants.

Stevia is nature's sweet secret. *Stevia rebaudiana* is a plant belonging to the Asteraceae family and contains sweet steviol glycosides, of which stevioside and rebaudioside A are the most abundant. The genus *Stevia* consists of 240 species of plants which are native to South America, Central America, Mexico and the Orient; several species have been found as far north as Arizona, New Mexico and Texas. *Stevia* (*Stevia rebaudiana* Bert.) is a semi bushy herb with natural sweetening compounds that are non-calorific, and is 230 times sweeter than sucrose (Hossain *et al.*, 2008). Leaves are sessile, 3-4 cm long. The stem is weak and woody. The rhizome has slightly branching roots (Madan *et al.*, 2010). *Stevia* has various properties such as antibacterial, antifungal, antiviral, cardio tonic (tones, balances, strengthens the heart), diuretic, hypoglycemic, vasodilator (Mehta *et al.*, 2012). *Stevia* grown best in well drained red soil and sandy loam soil. Saline soils should be avoided for the cultivation of the plant. In India, *Stevia* has been successfully cultivated in Rajasthan, Maharashtra, Kerala, and Orissa (Goyal *et al.*, 2010). In recent times, *Stevia* is very popular in many countries but large scale propagation techniques are yet to be standardized. Propagation through seeds is very difficult due to self incompatibility which results in sterile seeds. Hence *in vitro* propagation appears as an alternative technique for rapid multiplication of *Stevia* within a short span of time (Jagatheeswari and Ranganathan, 2012).There are fragmentary reports on micropropagation of *Stevia* (Das *et al.*, 2011).However, none of these studies were aimed at developing a low- cost micro propagation technology for *Stevia*. The current study has expored the feasibility of reducing the cost of *in vitro* multiplication of *S.rebaudiana* without compromising the quality of the micropropagules and plants so that the technology can be up- scaled with ease to a commercial scale.

MATERIALS AND METHODS

Selection of Plant Material and Establishment of Aseptic Cultures

Various geographical regions in Himachal Pradesh were screened for the collection of plant material. Elite plants are collected from Distt Kangra. Shoots were trimmed to size 2-3 cm and washed in liquid detergent solution 2% Teepol (Himedia Laboratories, India) and surface sterilized with (0.1-1% w/v) aqueos solution of Mercuric chloride (Merck, India) followed by 4-5 washings in autoclaved distilled water to remove the traces of Mercuric chloride. The sterile shoot tips were cultured on MS medium supplemented with different concentrations and combinations of auxins and cytokinins.

Preparation of Media and Incubation of Cultures

The basal MS medium (Murashige and Skoog, 1962) supplemented with different concentrations and combinations of phytoharmones were prepared, pH adjusted 5.6-5.8 using 0.1N HCl and 0.1N NaOH, autoclaved at 121^0C and 15lb in $^{-2}$

pressure for 15-20 minutes in 250ml Erlenmeyer flasks (Borosil, India) by dispensing 40ml molten media in each flask plugged with non- absorbent cotton wrapped in one layer of muslin cloth. The cultures were incubated at 25 ± 1^{0C} in plant tissue culture chamber under 16h photoperiod provided by cool fluorescent light .Data was collected on days to multiple shoot formation, % shoot apices with multiple shoots and number of shoots per explant.

Cost effective Media for Micropropagation

Once a MS medium supplemented with auxins and cytokinins was found suitable for *in vitro* multiplication of *Stevia rebaudiana*, modifications in replacing sucrose (Fisher Scientific) with table sugar, sugar cubes; agar with sodium alginate, isabgol, starch; and distilled water with tap water, packaged drinking water were tried to see the effect on shoot proliferation and growth. Data recorded for all parameters was analyzed statistically.

Cost Analysis

The cost of MS medium per liter calculated with and without alternatives. The cost of sucrose used in the standard medium was compared with the cost of table sugar and sugar cubes; agar with isabgol, starch, sodium alginate; distilled water with tap water and packaged drinking water (Table 1). Differences in cost between the conventional and alternatives substituents were determined.

RESULTS

Sterilization:

Among all the tested concentrations of mercuric chloride, the best response was seen with 0.1% (w/v) of mercuric chloride for five minutes which resulted in 100% healthy shoots.

Shoot proliferation:

Explants started to show signs of proliferation after two weeks of culturing. New buds appeared from the axil of leaves of shoot explants and buds developed into shoots by 4 weeks of culture. Micro shoots were inoculated on MS basal medium with different concentrations of BAP (0.5-2 mgL^{-1}) and IAA (0.1-0.5 mg L^{-1}), and combinations of BAP & IAA (in combination of 1mg/L+.1mg/l) for shoot proliferation. In medium containing BAP in different concentration, hundred percent cultures showed shoot proliferation and it was found that BAP gave better shoot proliferation in 1mg/l concentration with on an average of 18 average numbers of shoots and having a average length of 6.0 cm(Table 2). Out of fifteen different combinations tested for shoot proliferation, MS medium containing BAP(1 mg/l) + IAA(0.1mg/l)+ sucrose 3%(w/v)+ agar-agar (0.8%) was found to be best with 20 average number of shoots and having an average length of 6.5cm (Table 3).

Optimization of Low cost medium

After standardization of a suitable MS medium for *in vitro* shoot multiplication, the major components of nutrient medium such as carbon source, solidifying agents and water source were substituted with low cost substitutes. Seven different modifications of conventionally used MS medium were compared with the standardized media, which was found suitable for *in vitro* shoot multiplication. The substitution of conventionally used solidifying agent agar (0.8%) with alternative isabgol (1.5%) evoked almost similar response with average number of shoots 18 and 15 respectively (Table-4) (Figure 1). Replacing Sucrose with table sugar at same concentration did not cause a markable change in the shoot multiplication. MS medium containing sucrose yielded 18 average numbers of shoots, having a length number 6.3 cm and sugar cubes shows 17 average number of shoots, having a length number 6.0cm (Table -4) (Figure 2).

Similarly, the number of shoots produced during shoot multiplication was not affected significantly by different sources of water; distilled water showed 19 average numbers of shoots and packaged drinking water 18 average number of shoots (Table-4) (Figure 2). Water is one of the major components used in preparation of the culture media; however, distilled water is expensive in developing countries. Thus, the use of alternative sources of water such as rain or tap water can help to reduce the cost of propagation of *Stevia rebaudiana*.

DISCUSSION

Tissue culture is indispensable tool for the production of disease free planting material. The technology is however, costly. This study has shown that it is possible to use alternatives as low cost sources of tissue culture. The low cost micropropagation technology has certain distinctive features. This analysis would be of great practical use in setting up an industrial plant tissue culture units not only for micropropagation of *Stevia rebaudiana* and other plant species.BAP has proved better for proliferation of shoots. Franca *et al.* (1995) reported the importance of BAP in stimulating multiple shoot formation of *E.alba.* The role of BAP in bud breaking has been recorded for other medicinal plants such as Artemisia annua (Usha and Swamy, 1998), Tagetes erecta L.(Misra and Dutta,1999), Wedelia Calendulacea (Emmanuel *et al.*,2000), Adhatoda beddomei (Charantharayil and Sooriamuthu, 1994).

The substitution of conventional MS media components with alternatives reduces the cost of initiation and multiplication of *Stevia rebaudiana.* The carbon sources such as grade sucrose that is often used in the micropropagation of plants at laboratory contribute 34% of the production cost (Demo *et al.*, 2008). Our results show that the substitution of sucrose used in conventional tissue culture with table sugar reduced the cost by 94% (Table1). The use of market sugar instead of sucrose has been reported to reduce the cost of *in vitro* conservation of banana, with no significant effect on regeneration compared to sucrose (Agrawal *et al.*, 2010).The purpose of using low cost substitute for sucrose is to reduce the overall cost of micropropagation of *S.rebaudiana.* The substitution of conventionally used solidifying agent agar with alternatives reduces the cost by 81%. Multiple shoots are produced when distilled water is replaced with packaged drinking water with reduction in cost of propagation. This analysis shows that it is possible to develop a low cost culture protocol for production of *S.rebaudiana* plantlets with in short periods.

Table 1. Comparisons of the costs of the developed low cost medium and the conventional MS medium

MS medium	Low cost substitute	Cost in 1L of the medium in INR		Cost reduction %
Gelling agent		**Conventional**	**Low cost**	
Agar		39.6		
	Isabgol		7.5	81
	Sodium alginate		20.52	48.2
	Starch		57.6	-
Carbon source				
Sucrose		17.28		
	Sugar cubes		3	82.63
	Table sugar		1.05	93.92
Water source				
Distilled water		200		
	Tap water		-	
	Packaged Drinking water		20	90

Table 2. Effect of different concentrations of BAP on *in vitro* shooting of *Stevia redaudiana*

Treatments (mg/l)	Average no. of shoots Shoot no.± S.E	Average length of shoot length (cm)±S.E
0.5	15±0.332	5.7±0.01
1.0	**18±1**	**6.0±0.06**
1.5	14±0.332	5.5±0.003
2.0	14±0.33	5.6±0.012
control	6±0.33	3.0±0.01

Table 3. Effect of 1 BAP and IAA on *in vitro* shoot proliferation and multiplication

Treatments IAA (mg/l)	Average no. of shoot Shoot no.±S.E	Average length of shoot Length (cm)±S.E
.1	**20±0.33**	**6.5±0.012**
.2	17±0.33	6.0±0.02
.3	15±0.33	5.5±0.012
.4	14±0.33	5.2±0.04
.5	13±0.33	5.3±0.09
Control	16±0.33	5.8±0.02

Table: - 4. Effect of low cost media substituents on *in vitro* shoot multiplication of *Stevia rebaudiana* in MS media fortified with phytohormones (1BAP+0.1 IAA)

Concentration of gelling agents	Average no. of shoot Shoot no.±S.E	Average length of shoot Length (cm)±S.E
Isabgol (plantago ovate)0.5	12±2.33	4.3±0.02
0.8	13±2.33	5.0±0.02
1.0	14±0.33	5.3±0.01
1.5	**15±1**	**6.0±0.04**
Starch 3.0	11±1	4.3±0.389
Sodium alginate 1.0	11±0.33	4.3±0.029
1.5	13±1	5.5±0.09
Agar 0.8	**18±1**	**6.5±0.02**
Control	7±0.33	4.0±0.01
Carbon source		
Sucrose	**18±1**	**6.3±0.02**
Sugar cubes	17±1	6.0±0.04
Simple sugar	14±0.332	5.2±0.129
Without sugar Control	8±0.33	4.5±0.02
Source of water		
Distilled water	**19±1**	**6.5±0.02**
Packaged drinking water	18±1	6.3±0.02
Tap water	15±1	5.3±0.129

Figure 1. Effect of Different gelling agents on the *in vitro* shoot multiplication

Figure: 2 Effect of Different carbon and water sources on *in vitro* shoot multiplication

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