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IMPROVED MICROPROPAGATION METHOD FOR THE ENHANCEMENT OF BIOMASS IN *STEVIA REBAUDIANA* BERTONI

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Abstract
Incorporation of a range of higher concentrations of $CuSO_4$–$5H_2O$ in MS medium significantly enhanced direct shoot bud induction and proliferation from cultured leaf and nodal explants taken from mature plants of *Stevia rebaudiana* Bertoni. Shoot bud induction medium was supplemented with BAP (2.2 µM) and NAA (2.8 µM). When the concentration of $CuSO_4$–$5H_2O$ in the induction medium was raised to 0.5 µM (five times the MS level, i.e. 0.1 µM) there was significant increase in percentage response along with increase in shoot bud number per explant. The shoots were healthy, well developed with dark green broader leaves. There was remarkable increase in total biomass at increased (0.5 µM) copper level in the medium. During proliferation stage also presence of high copper levels in the medium favoured increase in shoots bud number per explant.

Key Words: Honey leaf; *S. rebaudiana*; Organogenesis; Copper; Micronutrient.

Introduction

Stevia rebaudiana Bertoni is a small perennial shrub with green leaves that belongs to the Asteraceae family. It grows primarily in the Amambay mountain range of Paraguay but over 150 various species of stevia have been identified around the world [1]**.** The leaves of stevia are source of diterpene glycosides, such as stevioside and rebaudiosides, which are estimated to be 100–300 times sweeter than sucrose [2-4]. *S. rebaudiana* is the only species at present which posses an inordinate ability to sweeten. Its common form is known as diterpene glycosides, a fine white powder extracted from the leaves of the plant [4].

Now a day it has been used as a natural sweetener substituting sugar, which has no side effects and available as concentrated liquid, crushed leaf or concentrated white powder[5].The sweet compound passes through the digestive process without chemically breaking down, making Stevia safe for consumption for those who need to control their blood sugar level. The first report of commercial cultivation in Paraguay was in 1964 [6, 7]. Since then, it has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada [8-12]. In Brazil and Paraguay it grows wild. The property of the species that called attention to the plant was the intense sweet taste of the leaves and aqueous extracts. 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 [13]. 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.

Its medicinal and commercial value lead to the world wide demand for large-scale production of stevia plants from elite germplasm. The plant is propagated by seed or stem cutting. Although seed propagation is very common method, seed is not efficient because of low fertility and self incompatibility of the flowers [14, 15]. The propagation by seeds does not allow the production of homogenous population resulting in variability in sweetener level and composition [16, 17]. Vegetative

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propagation by stem cuttings is also limited by the low number of individuals that can be obtained simultaneously from single plant. Micropropagation can provide genetically uniform plants in large numbers. There are few reports of micropropagation from shoot tip, leaf and nodal cultures [18-22].

In the last decade improved regeneration has been reported in many plants as a result of higher levels of heavy metal copper in the medium [23-28]. The objective of the present study was to evaluate the effect of different concentrations of the heavy metal micronutrient copper on direct induction of shoot buds from leaf and nodal explants of *S. rebaudiana*. The protocol advocates the use of higher copper level in MS medium at different stages of micropropagation in order to enhance percentage response and number of shoots produced at induction and proliferation stages.

Materials and Methods

Plant material and explants sterilization

The pot grown *S. rebaudiana* plants were collected from the greenhouse of department of Botany, Annamalai University, Chidambarm, Tamilnadu, India. For shoot induction, explants were collected from nodal segments and leaves of 3 to 6 months old pot grown plants. Then the explants were cut into small pieces ranging in size from 1 to 1.5 cm long and primarily washed with 1% savlon. After excision, for surface sterilization, the explants were rinsed in running tap water for 20 min followed by gently rinsed with 70% ethanol for 30 seconds and treated with 1.0% sodium hypochlorite solution for 5 min. Finally sterilization was carried out in the laminar airflow chamber using 0.1% (w/v) $HgCl_2$ for 3 min. After each step of sterilization the explants were washed with sterile double distilled water for five times. Then, the inoculation of explants was done on MS medium. The MS medium [29] was prepared with 3% (w/v) sucrose and BAP+NAA and solidified with 0.8% agar (Himedia Mumbai). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C and 1.2–1.3 kg/cm^2 pressure for 20 min.

Culture establishment

Leaf explants were cut from petiolar end and placed on sterile medium with dorsal surface in contact with the medium supplemented with BAP and NAA. All the cultures were incubated in culture room, at a temperature of 25±2°C, 16 h photoperiod and light intensity of 25 µmol/(m^{-2} s^{-1}) provided by white fluorescent tubes (Philips India Ltd., Mumbai). After 4 weeks the shoot buds induced on leaf explants were excised from base along with some portion of mother explants and placed on proliferation medium supplemented with BAP (3.5µM) and Kn (1.8 µM). Cultures were established in different culture vessels and it was observed that shoots regenerated in flask shows normal morphology and well elongated. After 2–4 weeks there was significant increase in shoot number. The leaves and nodal explants from these regenerated shoots were used as explants for studying the effect of different concentrations of copper in MS medium on regeneration in stevia leaf and nodal explants cultures. The explants were placed on MS medium supplemented with BAP (2.2 µM) + NAA (2.8 µM) and sucrose 3% (w/v). This was considered as control induction medium containing a usual $CuSO_4$–$5H_2O$ level of 0.1 µM. Different levels of $CuSO_4$–$5H_2O$ (0.1, 0.5, 1, 2, 3 and 5 µM) were added in MS medium with BAP (2.2 µM) + NAA (2.8 µM) at the same levels as the control induction medium. The elongated shoots were subcultured in proliferation medium supplemented with BAP (3.5 µM) + Kn (1.8 µM) along with different levels of $CuSO_4$–$5H_2O$ (0.1, 0.5, 1, 2, 3 and 5 µM). The cultures were kept under controlled conditions in culture chamber and observed regularly for 4 weeks.

Rooting and transplantation

For root formation, these shoots that developed MS multiplication medium with different levels of $CuSO_4$–$5H_2O$ were transferred individually into half-strength MS agar (0.7%) medium supplemented with IAA (2.8 µM). The shoots were maintained for 4 weeks under the same culture condition as for development of roots. After this time, the percentage of rooted shoots was recorded. Rooted plantlets were taken out from the culture flasks and carefully washed with water to remove agar and they were transplanted to pots filled with sterilized mixture of sand, vermicompost and soil (1:1:1) and grown for 5 weeks in greenhouse conditions to determine the percentage of plants that survived. The potted plants were irrigated with water twice a day for 30 days. Established plantlets were then repotted in 30cm pots. Each experiment was repeated three times and 10 replications per treatment were taken into account. All the data were analyzed by using ANOVA and the means and the standard errors of data were calculated with SPSS software [30].

Results and Discussion

A number of treatments of cytokinin (BAP) ranging from 0.4 - 8.8 µM (viz., 0.4, 2.2, 4.4 and 8.8 µM) with auxin (NAA) ranging from 2.8, 5.6 and 9.2 µM were

employed for shoot formation. Mitra and pal [22] obtained higher proliferation of shoot by using nodal segment of *S. rebaudiana* in MS media supplemented with IAA + Kn. Sivaraman and Mukundan [21] regenerated shoots from leaf, nodal and shoot apex explants of *S. rebaudiana* in MS medium supplemented with BAP + IAA. From this experiment it was evident that best result obtained from (2.2 µM) of BAP with (2.8 µM) of NAA which produced 87% shoots formation and showed highest number of shoots (8.2±0.5) and highest average length of the shoot (6.8±0.3) per culture. During induction synergistic effect of BAP (2.2 µM) and NAA (2.8 µM) at lower concentration was found to be optimum for regenerating maximum number, i.e. four to eight shoot buds from nodal and leaf explants (Table 1).The initial BAP and Kn combination at BAP (3.5 µM) and Kn (1.8 µM) level worked well for the purpose of shoot proliferation and elongation from the explants. Effect of copper on shoot bud induction was observed that maximum shoot bud induction and elongation was present at optimum copper level (0.5µM) for leaf and (1 µM) for nodal explants (Table 2).

Table1. Effect of BAP, Kn and NAA on MS medium for regeneration of shoot from nodal explants of *S. rebaudiana*

Plant growth regulators (µM)			Shoot formation (%)	No of shoot bud induced per explants Mean ±S.E.	Average length of shoot (cm) Mean ±S.E.
BAP	Kn	NAA			
0.4	0	0	23	3.4±0.3	4.4±0.2
2.2	0	0	68	5.3±0.4	5.0±0.2
4.4	0	0	75	7.0±0.2	6.7±0.4
8.8	0	0	28	4.6±0.5	5.6±0.1
0	0.4	0	22	1.3±0.1	4.3±0.4
0	2.3	0	30	3.8±0.2	3.8±0.4
0	4.6	0	65	5.6±0.2	4.6±0.3
0	9.2	0	40	3.0±0.3	4.0±0.1
0.4	4.6	0	42	3.4±0.4	4.4±0.2
1.7	3.7	0	54	4.0±0.3	4.0±0.4
3.5	1.8	0	80	6.4±0.2	5.4±0.2
4.4	0.4	0	63	4.8±0.2	4.8±0.3
2.2	0	2.8	87	8.2±0.5	6.8±0.3
2.2	0	4.6	72	5.0±0.3	5.5±0.2
2.2	0	9.2	46	2.5±0.1	3.5±0.2

The effect of copper on biomass was significant increase in total biomass (shoot number, shoot length, leaf number and size) of regenerated shoots at optimum copper (0.5 µM) level (Tables 2 and 3). Absences of copper in the medium and higher levels (5 µM) were detrimental to leaves and resulted in browning, chlorosis and finally necrosis of explants. Effect of culture vessel on shoot bud proliferation in different type of culture vessel used had profound effect on the shoot bud quality at proliferation stage. Hyperhydricity leading to vitrified shoots occurred when cultures were raised in petriplates. When flask was used, the shoots were well elongated with normal morphology. The shoot buds from leaf explants differentiated directly without any intervening callus phase.

Table 2. Effect of $CuSO_4$ shoot buds formation of *S. rebaudiana*

$CuSO_4$ (µM)	Shoot bud formation (%)	No of shoot buds per explants (Mean±S.E.)	Average shoots length (cm) (Mean±S.E.)
Leaf explants cultured on MS+BAP (2.2 µM) + NAA (2.8 µM)			
0.1[a]	12	4.0± 0.2	5.4± 0.6
0.5	39	7.1±0.7	7.5±0.3
1	28	3.7 ±0.3	6.2±0.4
2	23	5.5±0.5	5.5±0.5
3	47	6.0±0.2	7.0±0.2
5	18	2.8±0.5	3.2 ±0.3
Nodal explants cultured on MS+BAP (2.2 µM) + NAA (2.8 µM)			
0.1[a]	81	4.8±0.4	3.5±0.4
0.5	85	9.6±0.3	4.8±0.6
1	96	14.0±0.3	7.5±0.2
2	68	6.0±0.2	4.8±0.7
3	77	3.2±0.4	3.5±0.8
5	46	1.5±0.5	2.7±0.3
Leaf explants cultured on MS+BAP (3.5 µM) + Kn (1.8 µM)			
0.1[a]	18	11.0± 0.3	5.8± 0.4
0.5	79	27.2±0.6	8.0±0.2
1	65	13.5 ±0.4	6.7±0.5
2	73	18.5±0.2	5.4±0.3
3	37	7.6±0.4	7.8±0.3
5	45	15.8±0.3	3.5 ±0.2
Nodal explants cultured on MS+BAP (3.5 µM) + Kn (2.8 µM)			
0.1[a]	71	4.5±0.2	4.3±0.3
0.5	76	7.2±0.6	5.2±0.2
1	92	16.4±0.5	8.5±0.4
2	57	10.3±0.3	5.4±0.3
3	37	3.8±0.3	4.0±0.3
5	26	1.2±0.2	3.6±0.2

The effect of copper on shoot proliferation, during proliferation also higher a level of CuSO4 (0.5 µM) was found to be favorable for regeneration of new shoots (Table 2). In the case of proliferation from nodal explants, CuSO4 at 1 µM concentration was found to be optimum, producing 9–16 shoots as compared to 4–8 shoots produced in control (Tables 1 and 2). Copper is one of the essential elements required for normal plant growth and development, although it is potentially toxic at higher levels. Both absence of it and excess inhibit plant growth and impairs important cellular processes like photosynthesis and mitochondrial electron transport. Therefore, optimum copper concentration at induction and proliferation stages of micropropagation favours in vitro morphogenesis and normal growth and development of plants. Mineral nutrients are the basic components of tissue culture media.

Table 3. Effect of copper for total biomass in *S. rebaudiana*

S. No.	Copper concentrations (µM)	Total biomass (mg - Fresh wt.)
1	0.1[a]	270
2	0.5	385
3	1	290
4	2	235
5	3	220
6	5	200

How rapidly a tissue grows and the extent and the quality of morphogenetic responses are strongly influenced by the type and concentrations of nutrients supplied [27]. Inorganic macronutrient and micronutrient

levels used in most plant tissue culture studies are based on levels established by Murashige and Skoog for tobacco tissue culture [29].

However, many plant species and varieties do not respond well to the classical approach, i.e. using the MS as the basic medium. This demonstrates that alterations in hormonal ratios cannot be the sole mechanism controlling in vitro developmental processes [31]. Stimulatory effect of higher levels of copper in basal medium have been reported for many dicotyledonous and monocotyledonous plants including Triticale [32], *Oryza sativa* [23], *Tinospora cordiofoila* [33], *Sorghum bicolor* [24], *Eleusine coracana* [34], *Triticum aestivum* [25], *Capsicum annum* [26].

Figure: Modified micropropagation method of *S. rebaudiana* (a) Shoot regeneration (b) Initiation of multiple shoot formation (c) Multiple shoot formation in $CuSO_4$ containing MS medium (d) Root formation (e)Transplantation of regenerated plantlets in small and big plastic pots (f) Established regenerated plant

Copper is the vital component of electron transfer reactions mediated by proteins, such as Cytochrome-c oxidase, and plastocyanin involved in photosynthetic activity [35]. Therefore, optimum copper level in MS medium positively affects development of membrane system of chloroplast and chlorophyll content. There is significant enhancement in biomass also, as various Cu-containing enzymes involved in electron transport, protein and carbohydrate biosynthesis also play a role in plant regeneration [27]. Copper and cobalt have received attention for their stimulatory effect on secondary metabolites. [36-39]. The naphthoquinone pigment shikonin from Lithospermum erythrorhizon was the first plant secondary metabolite produced in industrial scale from plant cell cultures. Rooted plant required transplantation, so the plantlets were transplanted to small plastic pots (Fig. e). The pots were then transferred to the big plastic pots (Fig. e). Finally established regenerated plants were transferred to the field (Fig. f). About 77% plants were successfully grown.

In conclusion optimization of the amount of micronutrient copper in MS medium has a positive effect on in vitro morphogenesis and total biomass in stevia. These findings can play an important role in field studies as optimum copper levels can be maintained in field conditions to produce high yielding *S .rebaudiana* variety which has high stevioside content.

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