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Original Research

Callus induction and somatic embryogenesis in *Stevia rebaudiana* Bertoni as a medicinal plant

Tahereh Keshvari, Abdollah Najaphy*, Danial Kahrizi, Alireza Zebarjadi

Department of Agronomy and Plant Breeding, Razi University, Kermanshah, Iran

Correspondence to: nadjaphy@yahoo.com, anajaphy@razi.ac.ir

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Abstract: *Stevia rebaudiana* (Bert.) from Asteraceae family is a useful medicinal plant that prevents and cures diabetes, blood pressure, weight gain and tooth decay. Due to self-incompatibility in stevia, somatic embryo investigation for artificial seed production is valuable in this plant. In order to evaluate the callus induction characteristics in stevia, a factorial experiment was laid out based on a completely randomized design with three replications. The factors included ten hormone combinations and control, two kinds of media (MS and B5) and two types of explants (leaf and internode). Callus induction characters including the percentage of callus formation, days to callus induction, fresh and dry callus weight were recorded. Analysis of variance showed significant differences ($p<0.01$) among hormone combinations, media and explant types as well as their interactions. The best treatment for callus induction with minimum time to callus formation was 1 mg/l NAA+1 mg/l BAP. The highest fresh and dry callus weight were obtained on B5 medium supplemented by 1 mg/l 2,4-D+1 mg/l BAP (in leaf explant) and 0.25 mg/l 2,4-D+ 0.1 mg/l BAP (in internode explant). These results can be used in suspension culture. To induce somatic embryogenesis in suspension culture, six hormone treatments were investigated. The highest somatic embryogenesis percentage was obtained in MS medium supplemented by 2 mg/l 2,4-D+ 0.5 mg/l NAA+0.5 mg/l BAP.

Key words: Artificial Seed; Callus; Somatic Embryo; Stevia; Tissue Culture.

Introduction

Stevia rebaudiana Bertoni is a small, herbaceous, semi-bushy, tropical perennial shrub belongs to Asteraceae family. It is native to Paraguay and Brazil. The leaves of stevia are the source of diterpenoid glycosides (such as stevioside and rebaudioside A). These compounds are 300 times sweeter than sugar (sucrose) obtained from sugar beet, sugarcane and etc. (1-5). Stevioside is regenerated as a valuable natural sweetener agent because of its relatively good taste, non-caloric and chemical stability. Consequently, stevia does not have any effects on blood sugar and therefore it is friendly to human health. Moreover, it has therapeutic values such as obesity, hypertension, heartburn, hypoglycemia, anticancer and to lower the uric acid levels (6-7).

Stevia is known as a sweet herb, sweet leaf, candy leaf, and honey leaf. Although more than 150 stevia species have been known, *S. rebaudiana* has been become popular because of the significant sweetening properties (8).

Stevia can regenerate by seeds, but due to very low germination percentage, large-scale mechanized production of stevia through seeds is uneconomical. Therefore, the production of homogeneous populations for sweetening levels and composition is difficult. Biotechnological approaches, specifically plant tissue culture can be used for rapid propagation and conservation of such valuable and endangered plant species which are difficult to propagate by conventional methods. Recently, this technique also used to produce active biochemical constituents in very short span (9-10).

A mass of unorganized parenchyma cells which is called callus can derive from plant tissues after plating onto *in vitro* culture medium that is supplemented by plant growth regulators, such as auxins. Many callus cells are totipotent for converting to different plant body (11).

Somatic embryogenesis is the most useful process in plants and can be utilized for biotechnological application such as clonal propagation, production of synthetic seeds and genetic transformation. The direct somatic embryo is produced from organized cells whereas indirect somatic embryo appears after an intervening callus phase. Somatic embryos look like zygotic embryos in the aspect of the anatomical and physiological features (12). Somatic embryogenesis has been reported for more than hundred plant species, but there are a few reports from Asteraceae (13).

This study was aimed to optimize callus induction and somatic embryogenesis in *Stevia rebaudiana*.

Materials and Methods

Plant material and callus induction

Stevia rebaudiana material was provided by Zagros Bioidea Co., Razi University Incubator. The plants were thoroughly washed for 15 minutes under running tap water and cleaned with a solution of Tween 20 (two drops in 100 ml of water) for 5 min, and again washed with sterile distilled water, then were surface sterilized in 70 % (v/v) ethanol for 30 seconds. Further steriliza-

Table 1. PGRs treatments used for callus induction in *S. rebaudiana.*

Code	Treatment
T1	1mg/l BAP+1mg/l NAA
T2	2mg/l 2,4-D+0.5mg/lBAP+1mg/l NAA
T3	4mg/l 2,4-D
T4	1mg/l 2,4-D+1mg/l NAA
T5	2mg/l 2,4-D+2mg/l BAP+2mg/l NAA
T6	0.2mg/l2,4-D+0.1mg/l BAP
T7	3mg/l 2,4-D+2mg/l BAP+3mg/l NAA
T8	1mg/l 2,4-D+1mg/l BAP
T9	2mg/l 2,4-D+1mg/l BAP
T10	0.4mg/l 2,4-D
Control	-

tion was done under aseptic conditions under a laminar air flow cabinet by hypochlorite (2% w/v) for 8 min followed by washings with sterile distilled water for 3 to 4 times. The explants were inoculated onto the medium. Two types of explants (leaf & internode) and two media (MS & B5) in addition to different concentrations and combinations of 2, 4-D, NAA and BAP and the control medium without plant growth regulator (PGRs) (Table 1) were used to determine the better treatment for callus induction. Media were solidified using agar and pH value was adjusted to 5.7± 0.1. Callus induction characters including percentage of callus formation, fresh and dry callus weight were recorded after a month.

Somatic embryogenesis

For somatic embryogenesis, two different types of media (MS and B5) with six different PGR combinations were prepared (Table 2). Subculture was done into the similar media that auxin had been decreased to half or zero. The calli which produced somatic embryos were characterized.

Table 2. PGRs treatments used for somatic embryogenesis in *S. rebaudiana.*

Code	Treatment
1	0.5mg/lNAA+ 0.5mg/lBAP+0.5mg/l 2,4-D
2	0.5mg/lNAA+ 0.5mg/lBAP+2mg/l 2,4-D
3	0.4mg/l2,4-D
4	0.5mg/l BAP+2mg/l 2,4-D+ 50mg/l glutamine
5	0.2mg/lBAP+2mg/l2,4-D+0.2mg/Ltdz
6	4mg/l2,4-D+0.4mg/lKin

Growth conditions

The explants in all experiments were maintained at 25±2°C in a constant temperature growth room, under cool white fluorescent light using a 16-hour photoperiod provided by a cool fluorescent light intensity of 2500 lux.

Statistical analysis

To evaluate the characteristics of callus induction in stevia, a factorial experiment was laid out based on a completely randomized design with three replications. The factors included ten hormone combinations and control, two kinds of media (MS and B5) and two explants (leaf and internode). Data analysis was done using SAS and SPSS softwares. LSD test was used for mean comparisons. Diagrams were drawn by Excel.

Results

Callus characteristics analysis

Analysis of variance showed significant differences ($p<0.01$) among hormone treatments, media and explant types and their interactions (Table 3). The callus characteristics including the percentage of callus induction, callus fresh weight and callus dry weight in medium B5 were higher than MS. As well as, more calli was produced by internode as compared with leaf explants (data not shown). The details of mean separations for hormone × media × explant interaction have been shown in Figure 1.

Out of different treatments evaluated, the shortest time for callus induction was recorded six days using 1mg/l BAP+1mg/l NAA for both media and explant types. Little callus was observed in control treatment after 18 days. The highest callus induction percent (100%) for both of media and explants were obtained using 1mg/l BAP+1mg/l NAA (Figure 1). Some other treatment also led to full callus production.

The highest callus fresh weight was recorded for leaf explant in B5 medium using 2mg/l 2,4-D+2mg/l BAP+2mg/l NAA, while more callus dry weight belonged to 0.2mg/l 2,4-D+0.1mg/l BAP for the same explant and media. B5 medium containing 1 mg/l 2,4-D + 1mg/l BAP showed to be the most effective on the fresh and dry weight of callus in internode explants. Callus induction in stevia from internode and leaf explants has been shown in Figure 2. More amount of 2,4-D (4mg/l 2,4-D) decreased fresh and dry callus weight (Figure 1).

Table 3. Analysis of variance for callus characteristics in *S. rebaudiana*.

Source of Variation	Df	Mean Squares		
		Callus induction (%)	Callus fresh weight	callus dry weight
Hormone	10	88.44**	0.7008**	0.0048**
Media	1	5.12**	0.3399**	0.0017**
Explant	1	1.93*	1.1557**	0.0092**
Hormone × media	10	5.17**	0.0720**	0.0005**
Hormone × explant	10	2.82**	0.1795**	0.0017**
Media × explant	1	0.48$^{n.s}$	0.0570^{ns}	0.0004^{ns}
Hormone × media × explant	10	2.73**	0.0508**	0.0002**
Error	88	0.41	0.0171	0.00005
CV (%)	-	7.73	26.91	18.96

^{ns}, * and ** are non significant, significant at 5% and 1%, respectively.

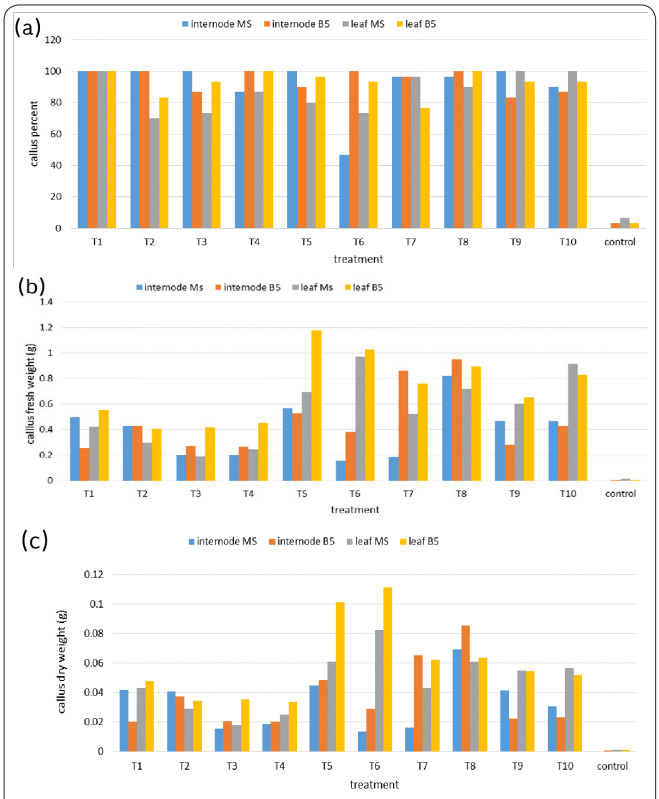

Figure 1. Effect of growth regulator, explant and media on a) callus induction (**LSD $_{0.01}$=1.36**); b) callus fresh weight (**LSD $_{0.01}$=0.28**); c) callus dry weight (**LSD $_{0.01}$=0.015**) in *S. rebaudiana.*

This experiment showed that B5 medium has the suitable competency for callus production.

Somatic embryogenesis

Subculture was done at the interval of two weeks in two steps (half PGR and free). All studying treatments produced light green and yellow calli (100%) at the first step but, after transfer to secondary medium, the tissue of the calli that has been inoculated by 0.5 mg/l BAP + 2mg/l 2,4-D + 50mg/l glutamine; 0.2mg/l BAP+2mg/l 2,4-D + 0.2mg/l TDZ and 4mg/l2,4-D+0.4mg/l Kin turned brown and later became extinct. Rest of the treatments survived and produced sparkling embryonic calli and after 35 days, the embryos were formed gradually on their surface. Finally, globular embryos and early heart embryo appeared after 45 days (Figure 3). Figure 4 shows the evaluation of the potential of the different combination of BAP, NAA and 2, 4-D in formation of somatic embryos of *S. rebaudiana* using the leaf as explant in two media (MS and B5).The highest efficiency of somatic embryogenesis (63%) was observed in MS medium supplemented with 0.5mg/l NAA+ 0.5mg/l BAP+2mg/l 2, 4-D (Figure 4).

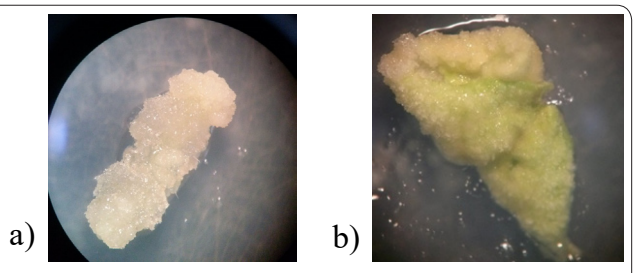

Figure 2. Callus induction in *S. rebaudiana* from a) internode, b) leaf.

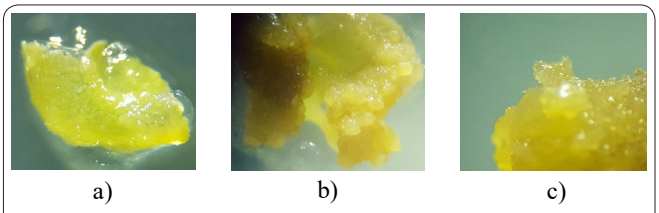

Figure 3. Somatic embryogenesis process in *S. rebaudiana* stages: a) somatic embryogenic calli; b) globular embryos formation; c) heart shape embryo.

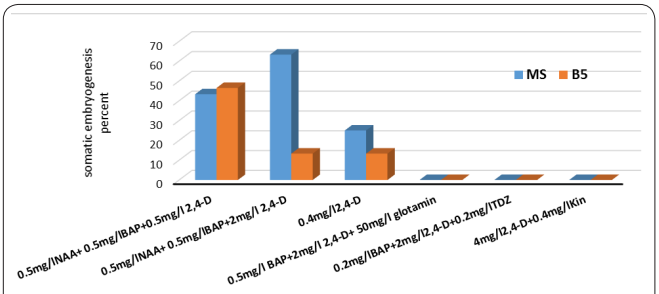

Figure 4. Comparison of different growth regulators and media for somatic embryogenesis in *S. rebaudiana.*

Discussion

The type of medium and explant are important factors in quantity and quality of calli obtained from explants. In our experiment, B5 was more appropriate medium than MS for stevia callus production. This is probably due to more potassium nitrate or less ammonium nitrate in the B5 medium. Generally, leaf explants led to more callus fresh and dry weight. Guruchandran and Sasikumar (2013) reported that among the three stevia explants (leaf, node and shoot tip), highest callus induction was observed in leaf explants (14). Singh *et al*. (2017) also emphasized on using of leaf for callus production and *in vitro* propagation of stevia (15). Interestingly, very little calli were produced under control treatment, free PGRs (Figure 1a), indicating the high response of stevia explants for callus production under *in vitro* conditions.

A higher amount of 2,4-D (4mg/ l 2,4-D) in T3 decreased fresh and dry callus weight (Figure 1). Another study by Abdelmaksood *et al*. (2017) showed that a combination of auxin and cytokinin was a better treatment for the high frequency of callus (16). The role of 2,4-D is undeniable for producing callus but another investigation showed that 2,4-D in combination with kinetin is the best for callus induction. Meanwhile, NAA and BAP were superior for callus permanence in stevia (17).

Callus induction using different concentrations of hormones in MS medium has been reported while so far, no reports described the callus production in the B5 medium in stevia. Our experiment showed that B5 medium has the appropriate competency for callogenesis (Figure 1).

Percentage of somatic embryogenesis in MS medium was higher than B5. The embryos appeared mostly in slicing point of explants. It seems that scarification of tissue can stimulate embryogenesis (18). According to Bi *et al* (2007) and Amali *et al*. (2014), increasing 2,4-D can decrease the formation of embryogenic calli (19-20).

In this study, we optimized callus induction and somatic embryogenesis in *stevia.* The embryos can be used for producing artificial seeds to overcome self-incompatibility and facilitate propagation of the valuable plant.

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