

The Effect of Some Plant Growth Regulators (PGRs), Coconut milk and Casein Hydrolysate on Somatic Embryogenesis of Stevia (*Stevia rebaudiana*) as an Anti-Diabetic Medicinal Plant

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Research Article

Abstract

Background: Stevia (*Stevia rebaudiana*), a sweet herb and anti-diabetic medicinal plant, could be replaced by artificial sweeteners and even table sugar. It grows in South America.

Methods and findings: Somatic embryogenesis of leaf and bud explants was studied in two experiments in a completely randomized design with 5 replications. At first experiment, different concentrations of 6-benzyladenine (BA), α -naphthaleneacetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) on Murashige and Skoog (MS) culture medium were tested. According to the results the highest percentage of embryogenesis callus obtained in T2 (MS medium supplemented with 1 mg/l BA+2 mg/l NAA) and T6 (0.01 mg/l BA+1 mg/l 2,4-D) (10.87%) and the lowest one (1.25%) observed in T11 (0.5 mg/l NAA+0.5 mg/l 2,4-D). As well as the highest embryogenesis percentage was obtained in MS medium supplemented with 0.01 mg/l BA and 1 mg/l 2,4-D. In second experiment, the effect of casein hydrolyzate in 5 concentrations (0, 50, 100, 150 and 200 mg/l) and coconut milk in two levels (0 and 55 mL) on *stevia* embryogenesis was investigated. According to the result 78.82% of bud and 25.09% of leaf explants were induced embryo in MS medium. Coconut milk and casein hydrolysate did not show significant difference on callus induction. Resulted embryos were transferred to MS medium containing 0.1 mg/l GA₃ for organogenesis, and some of the embryos were grown to complete plant.

Conclusion: In this study the most embryo induction was obtained by using MS medium without casein hydrolysate and the lowest one obtained by 50 mg/l casein hydrolysate. It proposed that type and concentration of different PGRs for embryogenic callus induction and somatic embryogenesis of Stevia investigated.

Keywords: Casein hydrolysate; Coconut milk; Somatic embryogenesis; *Stevia rebaudiana*.

Abbreviations: ANOVA: Analysis of Variance; MS: Murashige and Skoog; BAP: Benzylaminopurine; TDZ: Thidiazuron; IBA: Indole-3-butyric acid; 2, 4-D: 2,4-Dichlorophenoxyacetic acid; GA₃: Gibberellic Acid

BA: 6-Benzyladenine; NAA: Naphtalin Acetic Acid; PGR: Plant Growth Regulators

1. Introduction

Stevia rebaudiana Bertoni, is a small, herbaceous, semi-bushy, perennial shrub of Asteraceae family. This plant is native to certain regions of South America-Brazil and Paraguay [1]. It is one of the important antidiabetic medicinal plants. Stevioside and rebaudioside of its leaves taste about 300 times sweeter than sugar [2]. It is a major source of sweetener and has enormous commercial importance. Stevia has various properties such as regulating blood sugar, preventing hypertension and tooth decay, anti-bacterial, anti-candidal, anti-fungal, anti-viral, diuretic, hypoglycemic, vasodilator and treatment of skin disorders [3,4].

Heterozygosity, self-incompatibility and low germination percentage are the most important problems in *stevia* seed propagation [5]. Because of lower number of individuals that can be obtained simultaneously from a single plant, classical vegetative propagation is not suitable [6]. To overcome all, multiplication and improvement of this medicinal plant, biotechnologically approaches specially tissue culture is the desirable alternative for rapid mass propagation of Stevia. Several studies have been reported clonal propagation of this plant [7-10].

Somatic embryogenesis and organogenesis have been the common pathways for clonal propagation of superior medicinal plant species. Somatic embryogenesis enables large numbers of plantlets to be produced within a short span of time. In Stevia, somatic embryogenesis has been reported from leaves [11-13]. Somatic embryogenesis in *S. rebaudiana* reported by Filho et al. [14] for the

first time. Their results showed that combination of 10 or 25 mM 2,4-D and 1 mM BA in MS medium were effective for somatic embryogenesis. The embryos directly induced, without intermediate callus development. Filho et al. [15] cultured floret explants on MS medium supplemented with 2,4-D (9.05 and 18.10 mM) and kinetin (0 to 9.29 mM) and after 10 days, observed embryogenic callus. They reported that embryogenic callus was light green or light yellow color, it had compact structure and globular somatic embryos observed on its surface. Khan et al. [16] were found the highest direct regeneration frequency from leaves of stevia plant on MS medium supplemented with 1 mg/l BAP+1 mg/l NAA, while indirect regeneration from callus was obtained on MS medium fortified with 1 mg/l BAP+2 mg/l NAA. Ahmad et al. [17] after 30 days of stevia culture on MS medium+2.0 mg/l BA+2.0 mg/l 2,4-D was observed the best callogenic response.

Somatic embryogenesis of *Stevia rebaudiana* was successfully achieved from axillary buds using nodal and leaf as explants in basal culture medium (MS) with vitamins, sucrose (30 g/l), agar (0.9% w/v) and supplemented with 2, 4-D (2.0 mg/l)+BAP (0.2 mg/l)+TDZ (0.2 mg/l).

Naranjo et al. [18] obtained the highest number of somatic embryos in MS medium fortified with glutamine, adenine and coconut milk and supplemented by the combination of 2,4-D (18.09 μ M) and 2iP (7.38 μ M) as a plant growth regulator. Somatic embryos in the regeneration medium containing gibberellic acid (GA_3) (0.29 μ M) and activated charcoal regenerated to the seedlings. In a small aromatic tree, *Murraya koenigii*, embryogenic callus was obtained from 90% zygotic embryonic axis and 70% cotyledon explants in MS basal medium supplemented with 8.88 μ M BA and 2.675 μ M NAA [19]. BA could increase multiplication rate, vitrification and somaclonal variation in Stevia plant [20].

Because of low energetic nature of stevia, many people especially diet conscious consumers are interested to use it as an alternative to chemical

sweeteners. Regeneration of *S. rebaudiana* by somatic embryogenesis is very important, since this technique can be used for clonal propagation of this plant within a short span of time. The goal of this study was investigation the effect of some PGRs and other additives on callus and embryo induction.

2. Materials and Methods

2.1 First experiment

S. rebaudiana explants were collected from tissue cultured plantlets. Leaf and bud explants were obtained from matured plants were grown in a primary MS medium supplemented with different concentrations of BA. The explants (leaf and bud) were transferred on MS medium basal medium. This medium fortified with different PGRs at various concentrations consisted of BA (0, 0.01, 0.5, 1 and 2 mg/l), NAA (0, 0.5, 1 and 2 mg/l), 2, 4 dichlorophenoxy acetic acid (2, 4-D) (0, 0.5, 1, 1.5, 2 and 2.5 mg/l) and supplemented with various sucrose concentrations (30, 40, 100 g/l) (Table 1). The pH of the media was adjusted at 5.8 before gelling the medium with (7.5 or 8 g/l) agar. The media were sterilized by autoclaving at a pressure of 1.2 atm. (121°C) for 15 min. Solid medium cultures were incubated either in 16/8 h photo period (25 μ mol m⁻² s⁻¹) after stored in darkness for 4, 6 or 16 days (Table 1). According to Table 1, treatments designed in 13 groups.

The cultures were incubated at 25 \pm 1°C with a photoperiod of 16 h at 3000 lux light intensity of cool white fluorescent light. All the experiments were performed twice with 5 cultures per treatment. Observations were made every day and data were recorded after 4 weeks of inoculation.

The secondary medium was consisted of MS medium without growth regulators with 30 g/l sucrose and 7 g/l agar for gelling. The explants were cultured for 5 weeks in primary medium and then embryogenic callus were transferred to the secondary medium to progress embryos. The color of callus was initially white in turning yellow and then after about 30 days showed green color.

Table 1. Different treatments for *S. rebaudiana* callus and embryo induction in first experiment.

Treatments	BA (mg/l)	NAA (mg/l)	2,4-D (mg/l)	Sucrose (g/l)	Agar (g/l)	Ammonium nitrate (mg/l)	Darkness (days)	Callus induction	Embryo induction
T1	1	1	0	40	8	-	4	5.3 ^{cd}	2.27 ^c
T2	1	2	0	40	8	-	4	10.87 ^a	10.88 ^{abc}
T3	2	1	0	40	8	-	4	10.46 ^{ab}	19.04 ^a
T4	0	0	2	40	7.5	-	6	9.2 ^{abc}	8.84 ^{bc}
T5	0.01	0	0.5	40	7.5	-	6	4.6 ^{cd}	14.28 ^{ab}
T6	0.01	0	1	40	7.5	-	6	10.87 ^a	12.24 ^{abc}
T7	0.01	0	1.5	40	7.5	-	6	8.36 ^{abc}	8.16 ^{bc}
T8	0.01	0	2	40	7.5	-	6	6.27 ^{bc}	8.84 ^{bc}
T9	0.01	0	2.5	40	7.5	-	6	8.36 ^{abc}	9.52 ^{abc}
T10	0.5	0.5	1	30	7.5	-	4	5.85 ^{cd}	8.16 ^{bc}
T11	0	0.5	0.5	30	7.5	-	4	1.25 ^d	5.42 ^c
T12	1	0	2.5	100	7.5	-	16	5.85 ^{cd}	3.40 ^c
T13	1	0	2.5	100	7.5	1650	16	7.53 ^{abc}	4.00 ^c

2.2 Second experiment

Effect of casein hydrolysate and coconut milk on embryogenic callus of stevia was investigated. In the next step, the effect of different concentrations of casein hydrolysate (0, 100, 150 and 200 mg/l) and coconut milk (0 and 55 ml/l) their combination on the best somatic embryogenesis medium of previous experiment (MS supplemented with 0.01 mg/l BA+1 mg/l 2, 4-D) tested. These media solidified with 7.5 g/l agar; pH was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. In this section, leaf and axillary buds used as explant.

The cultured were incubated 6 days in darkness then transferred to 3000 lux light intensity in 16/8 h photo period ($25 \mu\text{mol m}^{-2}\text{s}^{-1}$) and temperature of $25 \pm 1^\circ\text{C}$. All the experiments were performed twice with 5 cultures per treatment. After 5 weeks, embryogenic callus observed and after 8 weeks embryos formed. Embryogenic callus (in globular phase) transferred in two different media:

- 1) MS medium supplemented with 0.05 mg/l BA
- 2) MS medium contained B5 vitamins (2 mg/l glycine+1 mg/l nicotinic acid+10 mg/l thiamin+1 mg/l pyridoxine)+100 mg/l myoinositol+0.05 mg/l BA+1 g/l active charcoal

For organogenesis, embryos transferred to MS medium supplemented with 0.1 mg/l GA and darkness treatment for 10 days. Then all embryos transferred to 3000 lux light intensity in 16/8 h photo period ($25 \mu\text{mol m}^{-2}\text{s}^{-1}$).

2.3 Hardening

Rooted explants were exited from the glass jars, washed carefully with sterile water to remove agar media, placed in the plastic cups filled with sterilized peat moss and perlite by the 1: 3 ratio (Figure 3e). Plastic bags on the pots, used for maintaining humidity in pots. These plants incubated at $25 \pm 1^\circ\text{C}$ with a photoperiod of 16 h at 3000 lux light intensity of cool white fluorescent light. After hardening established plantlets, were re-potted in small pots containing sterilized garden soil and sand (1:1) (Figure 3f).

2.4 Observation recorded and statistical analysis

Data with regarded to frequency of multiple callus and embryo induction (mean \pm SD) was recorded after 30 days of culture. Each treatment had five replicates and each single explant was considered as an experimental unit. All data are statistically analyzed by analysis of variance (ANOVA). Significant differences among the treatments were tested by Duncan's multiple range test at 5% level using SAS 9.1 software.

3. Results

3.1 First experiment

According to the results the most percentage of

embryogenic callus obtained in T2 (1 mg/l BA+2 mg/l NAA) and T6 (0.01 mg/l BA+1 mg/l 2,4-D) (10.87%) and the lowest one (1.25%) observed in T11 (0.5 mg/l NAA+0.5 mg/l 2,4-D) (Table 1). These treatments showed friable proliferated callus with greenish yellow color, smooth and compact which produced somatic embryos (Figure 3). Between two kinds of explants, the highest callus induction (61.5%) observed in leaf explants (Figure 1).

The highest percentage (19.04%) of somatic embryogenesis was induced in T3 (2 mg/l BA+1 mg/l NAA), But T1 explants induced the lowest (2.27%) embryos (Table 1).

3.2 Second experiment

In the second experiment coconut milk, combination of coconut milk and casein hydrolysate, kind of explant and their interaction didn't show significant difference on callus induction.

Using 100 mg/l casein hydrolysate could induce more callus than other treatments (data not shown). But the most embryos induced in MS medium without casein hydrolysate and the lowest one obtained by 50 mg/l casein hydrolysate (Figure 2). Higher concentrations of casein hydrolysate (150 and 200 mg/l) resulted with no further increase in the callus induction (Figure 2). Bud explants in MS medium showed the best result in embryo induction (Table 2).

While transferring the explants in MS medium without coconut water resulted in the highest embryos

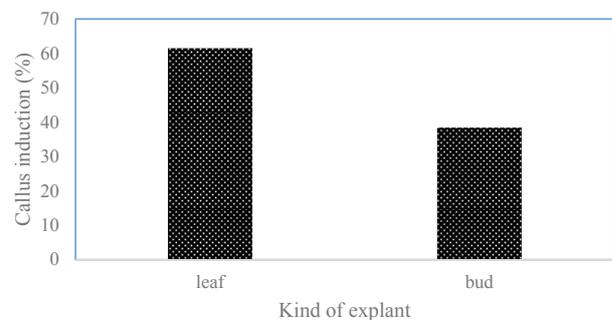


Figure 1. The effect of kind of explant on Stevia callus induction in first experiment.

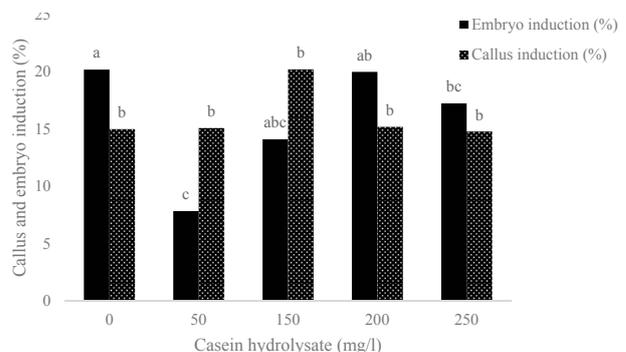


Figure 2. The effect of different concentrations of casein hydrolysate on embryo and callus induction in Stevia in second experiment.

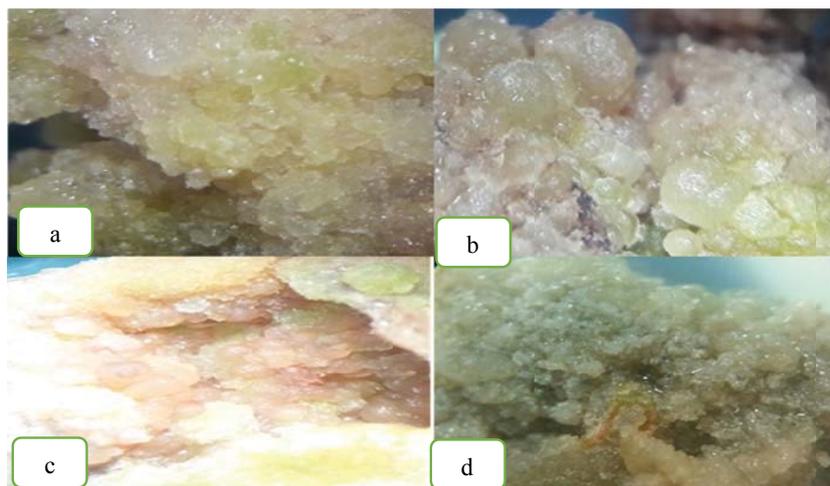


Figure 3. a) Leaf derived yellowish green and compact embryogenic calli b) Globular stage embryo and c) callus that are in globular phase. d-f) A developed progress of somatic embryogenesis isolated from a clump of callus after the torpedo-stage embryo during the cotyledonary phase of *Stevia rebusiana* Bertoni.

Explant		Coconut water (ml/l)	Culture media
Leaf	Bud		
13.2 ^{cd}	42.35 ^a	0	MS
5.49 ^{cd}	5.88 ^{cd}		MS+B5
5.69 ^{cd}	16.86 ^b	55	MS
0.39 ^d	13.3 ^{bc}		MS+B5

Table 2. The interaction between culture media, coconut milk and kind of explant on embryo induction of *Stevia* in second experiment.

(42.35%) (Table 2) and the lowest one obtained in MS medium supplemented with B5 vitamins and coconut water (Table 2).

4. Discussion

In this study the most percentage of embryogenic callus observed in MS medium supplemented with BA+NAA or 2,4-D. By callus culture in various conditions, differentiation of adventitious shoots, roots or even embryos can take place.

Das et al. [16] investigated the callus culture of *stevia* on different strength of MS medium supplemented with various plant growth regulators. Their results, showed using the half strength medium supplemented with 2,4-D (1.0 mg l⁻¹) and kin (0.2 mg l⁻¹) was the best culture medium for callus induction and NAA (0.1 mg l⁻¹) and BAP (2.0 mg l⁻¹) combination was the best for maintenance.

Combination of BA+NAA resulted the maximum percentage of somatic embryogenesis. Swanson et al. [21] also yielded friable callus by culture of leaf explants of *stevia* in MS medium with vitamins, sucrose (30 g l⁻¹) and agar (0.9 % W V⁻¹) and supplemented with NAA(0.5 mg l⁻¹) and BA(0.5 mg l⁻¹). By eliminating the agar and modulating the medium PGRs concentration, facilitated differentiation of the callus tissue.

According to the results, embryogenesis in *Stevia*

was affected by the concentration of casein hydrolysate and kind of explant and culture media. Coconut milk and casein hydrolysate did not show significant difference on callus induction.

Das et al. [22] found that glutamine (50 mg/l) and casein hydrolysate (100 mg/l) produced greenish, healthy, nodular calli with more embryogenic potential and less necrotic lesion in comparison with only PGR supplemented basal medium which further performed better in regeneration medium for producing large number of regenerated shoots. It was similar to our results. Casein hydrolysate contains several substances such as carbohydrates, protein, fat, several vitamins, phenolic compounds, fatty acids and mixture of up to 18 amino acids. It is found that any of these substances (single or in combination) cannot improve callus growth [23].

Coconut milk contains some growth hormones and is commonly use in tissue culture [24]. When containing auxin added to the culture medium, the coconut milk can induce plant cells to divide and grow rapidly. In the present study addition of coconut milk into the media culture didn't show positive effect on callus induction. These results were not in agreement with [25].

According to the results, culture media contain casein hydrolysate induced 60.01% embryos, while coconut milk media produced only 30.99%. So, casein hydrolysate showed effective role in *Stevia* embryogenesis.

5. Conclusion

In conclusion, the present study demonstrates type and concentration of PGRs was effective in embryogenic callus induction and somatic embryogenesis of *Stevia*. Also kind of explant was important. Somatic embryogenesis was affected by casein hydrolysate concentration, but coconut milk didn't show significant effect.

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