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Cell Dedifferentiation in *Stevia Rebauiana* as a Pharmaceutical and Medicinal Plant

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Stevia is a natural and non-caloric sweetener which most used in food and drug industries. In the present study, we focused on optimization of cell dedifferentiation and callus induction in stevia. In order to evaluation of growth regulators and explant types effects on callus induction, a factorial experiment was carried out in two factors and based on completely randomized design in four replications. The factor A included different levels of benzene adenin in three levels (0.0, 0.5 and 1.0 mg/l), Naphthalene acetic acid in four levels (0.0, 0.5, 1 and 2 mg/l), 2-4-D in three levels (0, 1 and 2 mg/l). The factor B comprised two levels of explant (leaf and stem) that were evaluated. The experiment was performed on Tissue Culture Lab in Kermanshah Industrial University. Analysis of variance for data results showed that there were significant differences among levels of plant explants for callus induction ($P<0.05$). However differences among above levels were not significant for callus growth rate. There were significant differences among plant growth regulators levels for callus induction and callus growth rate ($P<0.01$). Interaction effects were not significant for above two traits. Means comparison for plant growth regulators via Duncan's test ($p<0.5$) showed that medium plants supplemented with N0.5B0.5 was the best medium for callus induction (97.5%). The medium included N1B1 showed the highest callus growth rate (0.1 mm/day). In conclusion, we described a role of plant growth regulators and explant types on callus induction in stevia.

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Introduction

Stevia (*Stevia rebauiana* bertoni) belongs to Asteraceae family is commonly known as sweet weed, sweet leaf, sweet herb and honey leaf in perennial herb [1-3] (Figure 1). The leaves of stevia that contain glycoside diterpenes with chemical and pharmacological characteristics are appropriate for use in human diet as a natural sweeter and calorie-free drug. The glycosides have several types such as rebaudioside A, B, C, D, E and F, steviolbioside A and C are identified [5]. The previous results showed that glocosides are key factor in sweetening at stevia [6]. Since rebaudiside A has shown the best quality for food industries [7]. These materials had head stability, not yeasting and discoloring in pH 3-9 and 100 ˚C. Therefore, stevia was used as source of sweet at products such as baking, confectionery, juice, jam, biscuit and others [8]. The propagation through seed was not adequate. Stevia seeds show poor germination percentage so the propagation through seeds is not effective and propagated through cuttings cannot produce many plants. So, tissue culture technique is the only method for rapid propagation of stevia plants [9]. The different methods for propagations of stevia have been done by addition of different growth regulators in tissue culture such as NAA and IBA [10]. Due to many uses of stevia in drug industries, propagation of it was done by tissue culture. Also, growth regulators such as NAA and BA and 2-4-D were more prevalent hormones in this industry. So, this experiment was designed based on it. In this study, a factorial experiment was carried out in two factors and based on completely randomized design.

This plant is commonly known as sweet weed, sweet leaf, sweet herb and honey leaf in perennial herb belonging to the family Asteraceae [4].

Fig. 1. A sample of stevia medicinal and pharmaceutical plant.

Materials and methods

Materials

All chemicals and reagents were of analytical The all reagents for tissue culture were purchased from the Merk Company (Germany) and Zagros Bioidea Co. (Razi University Incubator). The other chemicals with analytical grade were from Merck. In current study, the impacts of different factors such as explant type and different quantities of growth regulators on callus induction in stevia were studied. This research was performed in 2013 in Tissue Culture Laboratory in Kermanshah Industrial University and Zagros Bioidea Co., Razi University Incubator. Samples of leaf and stem of stevia were collected from Department of Agronomy and Plant Breeding, Razi University. In this study, In order to optimize the conditions of growth regulators and explant types for the high level growth of stevia with tissue culture, two factors based on completely randomized designs were applied. Factor A: different levels of benzil adenin (BA) in three levels (0, 0.5, 1 mg/L),

Naphthalene Acetic Acid (NAA) in three levels (0.0, 0.5, 1, 2 mg/ L) and 2,4-dichlorophenoxyacetic acid (2,4-D) in three levels (0, 1 and 2 mg/l). Then 15 plant growth regulator compositions had been evaluated (Table 1). Factor B comprised two levels of explant (leaf and stem) that were evaluated in four replications.

Table 1. The 15 plant growth regulator compositions that have been evaluated in callus induction experiment in stevia.

No.	NAA	2,4-D	BA	Abbreviation
1. 1	0.5	0.0	0.0	N0.5B0
2. 2	1.0	0.0	0.0	N1B0
3. 3	2.0	0.0	0.0	N2B0
4. 4	0.0	1.0	0.0	D1B0
5. 5	0.0	2.0	0.0	D2B0
6. 6	0.5	0.0	0.5	N0.5B0.5
7. 7	1.0	0.0	0.5	N1B0.5
8. 8	2.0	0.0	0.5	N2B0.5
9. 9	0.0	1.0	0.5	D1B0.5
10. 10	0.0	2.0	0.5	D2B0.5
11. 11	0.5	0.0	1.0	N0.5B1
12. 12	1.0	0.0	1.0	N1B1
13. 13	2.0	0.0	1.0	N2B1
14. 14	0.0	1.0	1.0	D1B1
15. 15	0.0	2.0	1.0	D2B1

Four weak after incubation of explants in 25°C and dark conditions, the callus induction was measured and recorded. For callus growth rate evaluation, the callus diameters were recorded in 20, 27, 34, 41 and 48 days after incubation. The measurement was done according to growth rate as mm/day.

Statistical analysis

Statistical significance was assumed at the $p<0.05$ level. All of the statistical analyses were performed using MSTATC software.

Results and discussion

After incubation of explants on media, the calli were induced (Figure 2). Analysis of variance results demonstrated that there was a significant and non-significant difference among explant types for callus induction and callus growth rate respectively. Also, there were significant differences ($p< 0.01$) among levels of growth regulators for callus induction and callus growth rate.

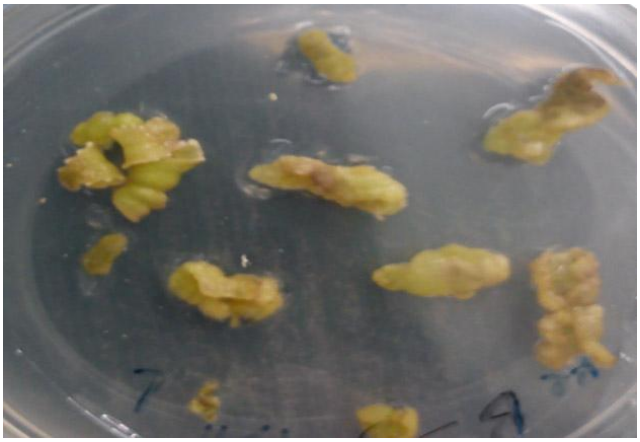

Fig. 2. Callus induction in *S. rebaudiana.*

Interaction effects of different levels of growth regulators and explant types didn't show significant effects on callus induction and growth rate in stevia (Table 2).

Table 2. Analysis of variance effects of plant growth regulators (PGRs) and explant type on callus induction and callus growth rate in stevia

Source of variations	df	Mean squares	
		Callus induction	Callus growth rate
Explant	1	569.494*	0.017ns
PGRs	14	386.236**	1.000**
Explant × PGRs	14	108.606ns	0.024ns
Error	90	91.265	0.021
CV(%)		4.88	8.63

Where ns (non-significant), *(significant at P<0.05) and ** (significant at 0.01)

Mean comparison for effect of explant on callus induction showed that stem (72.33%) produced more callus than leaf (59.00%) explant.

Many authors reported that the analysis of variance showed that there are significant differences among plant growth regulators (PGRs) levels and explant types and their interactions for callus induction percentage and callus growth rate [11-14].

Duncan's mean comparison showed that there was no significant different between stem (0.065 mm/day) and leaf (0.060 mm/day) explants for callus growth rate. This Duncan's results confirmed the ANOVA results.

Means comparison for plant growth regulators via Duncan's test (p<0.5) showed that medium plants supplemented with N0.5B0.5 showed the highest callus induction (97.5%). This composition statistically hadn't any difference with D1B0.5 (87.5%), D2B0.5 (87.5%), D2B1 (85.0), N2B0.5 (82.5%), N2B0 (72.5%), N1B1 (72.5%), N0.5B1 (67.5%) and N2B1 (65.0%). The D1B0 composition demonstrated the lowest callus induction (22.5%) (Table 3).

Table 3. Mean comparisons for effects of plant growth regulators (PGRs) on callus induction and callus growth rate in stevia. Where N (NAA), B (BA) and D (2,4-D).

PGRs	Callus induction (%)	Callus induction rate (mm/d)
N0.5B0	60.0 bcd	0.0495 bcde
N1B0	37.5 de	0.0417 def
N2B0	72.5 abc	0.0812 abc
D1B0	22.5 e	0.0230 ef
D2B0	45.0 cde	0.0294 ef
N0.5B0.5	97.5 a	0.04637 cdef
N1B0.5	60.0 bcd	0.0855 ab
N2B0.5	82.5 ab	0.0980 a
D1B0.5	87.5 ab	0.0665 abcd
D2B0.5	87.5 ab	0.0802 abc
N0.5B1	67.5 abcd	0.0961 a
N1B1	72.5 abc	0.1007 a
N2B1	65.0 abcd	0.5375 bcde
D1B1	42.5 cde	0.0124 f
D2B1	85.0 ab	0.0732 abcd

The same letter in each column show the non-significant difference at P<0.05

The medium included N1B1 showed the highest callus growth rate (0.1 mm/day). However this composition has not significant different with N2B0.5 (0.0980 mm/day), N0.5B1 (0.0961mm/day), N1B0.5 (0.0855 mm/day), D2B0.5 (0.0802 mm/day), D2B1 (0.0732 mm/day) and D1B0.5 (0.0665 mm/day). The D1B1 composition showed the lowest callus growth rate (0.0124 mm/day) (Table 3).

Taware *et al.,* (2010) established an efficient plant regeneration via shoot and callus organogenesis in *Stevia rebaudiana* (Bertoni). Explants were cultured on MS medium containing different concentrations of cytokinins and auxins. Callus optimization was observed on MS medium supplemented with 0.1mg/l 2,4-D [15].

Conclusion

In this experiment it was found that there were significant differences among stem and leaf stevia explants for callus induction. However above differences were not significant for callus growth rate. There were significant differences among plant growth regulators levels for callus induction and callus growth rate. Results showed that medium plants supplemented with N0.5B0.5 and N1B1 showed the highest callus induction and callus growth rate respectively. In this paper it was demonstrated the role of plant growth regulators and explant types on callus induction in stevia as a medicinal plants.

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Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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