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Research Paper

Impact of different growth regulators supplemented in MS medium on induction of callus from leaf explants of natural sweetener, *Stevia rebaudiana* (Bertoni)

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Abstract

***Stevia rebaudiana* (Bertoni) is commonly known as natural plant sweetener or sweet herb was taken for tissue culture study. From the healthy plant, cultivated in the local garden fresh leaves were collected and inoculated in MS medium supplemented with 3% sucrose and six different concentration of 2,4-D and three different concentrations of BAP and kinetin, either alone 2,4-D or with BAP or with kinetin. The growth rate, colour and texture and percentage of response of call using were recorded and the mean of the data were tabulated in the table. From the table in may be noted that MS + 2.0 mg/l 2,4-D alone induced callusing in 68.00 %, while MS + 2.0 mg/l 2,4-D + 0.5 mg/l BAP induced callusing in 52 % of the explants. 78.15 % of response with respect to callus induction was observed in MS + 2.0 mg/l 2, 4-D + 1.0 mg/l KN which was followed by 70 % in MS + 2.0 mg/l 2,4-D + 0.5 mg/l KN. Similarly higher growth rate was also noted in the same concentration where higher percentage of response was found. In this culture conditions calli were green and compact in the texture. These calli were sub culture in the same medium aften 21st days.**

Keywords: *Stevia rebaudiana*, callus, natural sugar plant, compact, subculture

Abbreviation: BAP- 6, Benzyl ammino purine, KN- Kinetine, 2,4-D- 2,4- Dichloro phenoxy acetic acid

Introduction

Stevia rebaudiana(Bertoni) of astenaceae commonly known as nature plant sweetener or sweet herb or sugar plant, is a perihal shrub which may grow up to 1 meter. The sugar is a non- caloric and is being used as an alternative to artificially produced sugar substitute[1]. Stevia leaf is 300times sweeter than sugar, obtained from sugar beet & sugar cane with zero caloric value [2]. Stevia was discovered in 1887 by South American natural scientist Antonio bertoni .Tissue culture studies of stevia have been done by different workers. Tamura *et al.*[3] used shoot tips for micro propagation of stevia has also been done by Yukiyoshi *et al.*[4], Ferreira and Handro[5], Miyagawa *et al.*[6] and Lumsden *et al.*[7]. They used leaf, shoot tips and inter nodal segment for the induction of multiple shoots. Tissue culture studies for mass propagation has also been done by Akita *et al.*[8], Sivaram and Mukundan[9], Faisal *et al.*[10], Uddin *et al.*[10], Ahmed *et al.*[1], Debnath[12]. These workers reported that in vitro techniques are alternative or complement to conventional procedures of propagation and genetic improvement. This

could facilitate obtaining disease free homogenes population with high level of sweeting compounds, lage foliar mass, resistant to drought, herbicides, breaking of stems and branches.

Arpita *et al.*[13] also reported techniques of micro propagation of *Stevia*, these workers assessed efficacy of different plant growth regenerates shoot multiplication after inoculating shoot buds in MS medium, supplemented with different concentrations of cytokinins and auxins. As the leaves of the plant contain glycosides, which have chemical and pharmacological characteristic so it is suitable for use in human diet as a natural calorie free agent. Diterene glucosides steviol is 300 times sweeter than glucose. There are plenty of literature with respect to tissue culture of *S. rabaudians* for micro propagation *but* few reports are for callus induction. Because of this ideas, present study were performed to initiate callus on the leaf explants of *Stevia rebaudiana.*

Materials and Methods

Seedling of *Stevia rebaudiana*was obtained from local trader of medicinal plants and was maintained in the garden of the Department.

Culture Medium: Murashige and Skoog's[14] basal medium was prepared from the stock solutions of all the ingredientswhich were prepared earlier. Required amounts from stocks solution were taken and diluted to 500 ml by adding sterilized distilled water. To this the growth regulators viz, 2, 4-D at 0.5-3.0 mg/l alone and with BAP and KN 0.5- 2.0 mg/l were added separately. 3% sucrose was added as concentration with pH of the above medium was adjusted to 5.8 with the help of N KOH or HCl before the addition of 0.8% agar solution as gelling agent. Finally the volume was made 1000 ml by adding extra sterilized distilled water. The medium was dispersed in to 25 X 150 mm culture tubes as 15 ml aliquots and 50 ml in 250 ml culture flasks. The mouths of the culture tubes and flasks were properly plugged with suitable cotton plugs covered with muslin clothes. Further the culture tubes were grouped in 8-10 with the help of rubber bands and were wrapped with aluminum foil. Both the culture tubes and flasks containing culture medium were autoclaved at 15 lb pressure and at 121 c for 20 min. these cultures were taken and allowed to cool at room temperature and finally stored in the freeze for 3 days. All the culture showing contamination were discarded, where as pure one were used for inoculation.

Preparation of explants: Young and healthy leaf from field grown plants of *Stevia rebaudiana* were collected from Botanical Garden, Department of Botany, B.R.A. Bihar University, Bihar, India. These explants were properly surface sterilized by thoroughly washing under running tap water in a conical flask whose mouth was covered with cheese cloth for one hour. Leaves were treated with 70% alcohol for 20-30 seconds followed by 0.1% $HgCl_2$ for one to two minutes. The materials kept in sterilized water in a conical flasks, were shaken manually for 3-4 minutes to remove even the traces of $HgCl_2$ completely. Above materials were preserved in pre sterilized water soaked cheese cloth at low temperature.

Inoculation: Above prepared leaf explants was taken and cut into in pieces (1 cm^2) with the help of sharp and pre sterilized blade. Inoculation was done in aseptic condition of laminar airflow chamber. One pieces of leaf segment was placed in culture medium in such way that the dorsal surface was contact of the medium. These culture were incubated in the culture room at 26 ± 1^0C under 12 hrs light period of 3000 lux, generated with white fluorescent tube light (Philips), and with a relative humidity of 60- 65%. 20 cultures were done on an alternate day. Cultures showing contamination were discarded. Data were collected after 20^{th} days and analyzed.

Experimental design and statistical analysis

The experimental design was completely randomized, corresponding to factorial MS basal medium × Growth regulator (BAP, KN and 2, 4-D), in triplicate with 18 plant of each. For statistical analysis, means ± SD were calculated. Data were analyzed using a one-way analysis of variance (ANOVA). The values were compared using Duncan´s test ($p < 0.05$), using SPSS version 20.0' software.

Results and Discussion

Explants showing response were green while other becomes dark after 5^{th} day of inoculation. The table 1 clearly reveals that leaf explants of *Stevia rebaudiana* responded in different ways in different culture conditions with respect to callus induction. MS + 2.0 mg/l 2, 4-D alone initiated callusing in 68.00±0.34 % of the explants, which was the maximum.

Table 1: Impact of different growth regulators suplimented in MS medium on callusing in leaf explants of *Stevia rebaudiana* Bertoni

Growth regulators mg/l			% of callus induction	Callus conditions		
2,4-D	+ BAP	+ KN		Colour	Texture	Growth
0.5	+ 00	+ 00	--	--	--	--
1.0	+ 00	+ 00	26.07±0.41	White	Loose	++
1.5	+ 00	+ 00	56.03±0.49	White	Compact	+++
2.0	+ 00	+ 00	68.00±0.34	Green	Compact	++++
2.5	+ 00	+ 00	38.24±0.18	White	Loose	++
3.0	+ 00	+ 00	27.51±0.24	White	Loose	+
0.5	+ 0.5	+ 00	--	--	--	--
1.0	+ 0.5	+ 00	31.80±0.28	White	Loose	++
1.5	+ 0.5	+ 00	47.66±0.27	White	Compact	+++
2.0	+ 0.5	+ 00	51.78±0.28	White	Compact	+++
0.5	+ 1.0	+ 00	--	--	--	--
1.0	+ 1.0	+ 00	28.34±0.28	White	Loose	++
1.5	+ 1.0	+ 00	46.04±0.10	White	Loose	++
2.0	+ 1.0	+ 00	51.04±0.47	White	Compact	+++
0.5	+ 2.0	+ 00	--	--	--	--
1.0	+ 2.0	+ 00	27.70±0.29	White	Loose	+
1.5	+ 2.0	+ 00	41.72±0.45	White	Loose	++
2.0	+ 2.0	+ 00	46.05±0.15	White	Loose	++
0.5	+ 00	+ 0.5	--	--	--	--
1.0	+ 00	+ 0.5	35.82±0.30	White	Loose	++
1.5	+ 00	+ 0.5	53.84±0.32	White	Loose	+++
2.0	+ 00	+ 0.5	70.27±0.18	Green	Compact	++++
0.5	+ 00	+ 1.0	--	--	--	--
1.0	+ 00	+ 1.0	42.07±0.12	White	Compact	++
1.5	+ 00	+ 1.0	57.85±0.14	White	Compact	+++
2.0	+ 00	+ 1.0	78.15±0.41	Green	Compact	+++++
0.5	+ 00	+ 2.0	--	--	--	--
1.0	+ 00	+ 2.0	37.90±0.32	White	Loose	++
1.5	+ 00	+ 2.0	53.20±0.25	White	Compact	+++
2.0	+ 00	+ 2.0	66.10±0.45	Green	Compact	+++

The response was zero at 0.5 mg/l concentration. Similarly, 3.0 mg/l the percentage of response was 28 only. MS + 2.0 mg/l 2, 4 –D + 0.5 mg/l BAP induced callusing in 51.78±0.28 % of the explants, while MS + 2.0 mg/l 2, 4-D + 0.5 mg/l KN induced callusing in 70.27±0.18 % of the explants. Highest percentage of response i.e. 78.15±0.41 % was noted in MS + 2.0 mg/l 2, 4-D + 1.0 mg/l KN.

It was further noted that growth rate of calli was excellent in the medium where the percent response was the highest. This was followed by better and good. Similarly, the above concentration the calli were green and compact, while in others they were either white and loose or white and compact.

From the table 1 it may be concluded that both the lowest and highest concentration of growth regulators had less impact on callusing. When six concentrations of 2, 4-D were used alone both 0.5 mg/l and 3.0 mg/l had lower impact than 2.0 mg/l, where the percentage of response was 68.00±0.34 %. Likewise MS + 2.0 m/l 2, 4-D + 0.5 mg/l BAP induced callusing in 51.78±0.28 % of the explants while MS + 2.0 mg/l 2, 4-D + 2.0 mg/l BAP in 46.05±0.15 % only. This was true in case of KN where 0.5 mg/l and 1.0 mg/l with 2.0 mg/l 2, 4-D had better response than that of the 2.0 mg/l (figure 1).

Callus induction in *Stevia rebaudiana* has been done by Kinghorn and Soejarto[15], Sivaram and Mukundan[9], Patel and Sah[16], Gupta *et al.*[17], Sikdar *et al.*[18] and these workers observed that BAP + NAA at different concentration induced calli in the nodal segments, explants, whereas 2, 4-D +KN at various concentration when supplemented in MS basal medium was suitable for induction of callus in leaf explants of *Stevia rebaudiana*. These observations are in agreement with the present findings. Similarly finding in this work that both lower and higher concentration were less effective for callusing also corroborate with the findings of the above workers.

Figure 1: Explants taken from *Stevia rebaudiana* showing different stage of callusing at different concentration of growth regulator: A. Stevia plant in Garden, B. Stevia plant in greenhouse condition, C. Initiation of callus, D. Callus formation after 20th days of culture, E. 30th days of culture after inoculation, F. Subculture and organogenesis in callus after 40th days of culture, G. Subculture and organogenesis in callus after 45th days of culture, H. Subculture and organogenesis in callus after 60th days of culture

Conclusion

Plant biotechnology is considered in a wide source which comprises the various culture methods of the plant agent and parts of facilitate experiments approaches with a large objective of the developmental biology for crop improvement. Calli obtained in the present work can be used for the extraction of different secondary metabolites as well as for sub culture in the presence of elicitors, so that rate of production of secondary metabolites may be enhanced than that found in vivo.

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