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Efficient Protocol for Indirect Shoot Regeneration from Leaf Explants of *Stevia rebaudiana* (Bert.) – An Important Calorie Free Biosweetner

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Summary

Callusing response of various field grown and *in vitro* grown explants such as leaf was studied using various auxins (NAA/IAA/2,4–D) alone or in combination with cytokinins namely, BA/Kn. Either NAA/IAA in combination with BA or Kn induced profuse light green to dark green coloured, fragile to nodular callus formation from leaf explants. At higher concentration of auxins occasional root formation directly from the leaf explants was also observed. Indirect shoot organogenesis was achieved from the callus using BA (2.0 mg/L) where highest number of shoots (8.5 ± 0.1) with maximum frequency (86%) was regenerated. All the *in vitro* raised shoots with a length of 3-5 cm were transferred to rooting medium supplemented with different concentrations of auxins such as IBA and NAA (0.1–1.0 mg/L). The best rooting response was observed on 0.5mg/l IBA. The well rooted plantlets were transferred to polybags containing soil + vermiculite in 1: 1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions for maximum survivability.

Key Words: *Stevia rebaudiana*, Leaf explants, 2, 4-D, NAA, IAA, BA, Kn, Callus induction, Plant regeneration

Introduction

Micropropagation is an alternative to the conventional methods of vegetative propagation with the objective of enhancing the rate of multiplication [1]. Plant tissue culture has been viewed as an important technology for enhancing the capability of selected elite high yielding varieties, so as to boost production and productivity.

Stevia rebaudiana (also called as sweet leaf or sugar leaf) belongs to the family of Asteracae. *Stevia* is an outstanding herb bearing leaves of very refreshing sweet taste and remarkable health promoting qualities. It is native to Northeastern Paraguay and has been used for centuries as a natural sweetener [2]. It is a perennial herbaceous plant being about 60-80 cm tall. *Stevia* leaf contains secondary metabolites such as stevioside, rebaudioside A, rebaudioside C and dulcoside A [3]. Experiments proved that stevioside which is responsible for sweetness is 300 times sweeter than sucrose apart from being a calorie free sugar [4].

Stevia is non-toxic, non-calorie, non-plaque, non-fermentative, flavour enhancing, non-carcinogenic, non-addictive sweetness for children and an intense sweetener compared to sucrose. Apart from this due to calorie free property it is absolutely safe for diabetics, phenyl ketonuria patients and slimming people [5]. Economically the plant has much in store for bakery, confectionary and beverage sectors. *Stevia* leaf tea offers excellent relief for an upset stomach. Like cucumber, a wet *Stevia* leaf bag provides a cooling effect to eyes and helps to reduce weight and blood sugar management. The addition of *Stevia* powder also helps in rejuvenating the pancreatic gland [6]. Stevioside and rebaudioside induce insulin secretion [7] and the former acts as anti-tumour agent [8].

The present study was aimed to understand the effect of different plant growth regulators at various concentrations on *in vitro* callus induction and indirect plant regeneration of *Stevia rebaudiana*.

Materials and Methods

Collection of Plant material

The plants are collected from Suveda Ayurvedic Pharmacy, P.V.Puram, Tirupati, Andhra Pradesh, India and grown in the nursery of Department of Biotechnology, S.V. University, and Tirupathi, India.

Surface sterilization

Explants were washed thoroughly under running tap water to remove the traces of dust etc. followed by treatment with 10% teepol/tween-20 for 5 minutes. Then the explants were sterilized in 70% ethanol for a minute, and finally with 0.01% $Hgcl_2$ for 1-2 minutes and washed 3-4 times with sterile double distilled water.

Culture medium

Young leaf and explants (1-2 cms) were inoculated on MS medium [9] containing 3% sucrose and gelled with 0.8% agar supplemented with various concentration of auxins such as 2,4–D, NAA and IAA in combination with cytokinin BAP. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20 minutes at 121^0C for 15 lbs pressure.

Sub culturing

Sub culturing was carried out at regular intervals of thirty days. Visual observations of the cultures were taken for every transfer and the effects of different treatments were quantified on the basis of percentage of cultures showing response.

Culture conditions

The growth room conditions maintained for *in vitro* cultures were 26 ± 2^0C and 60-70% relative humidity, light intensity was 3000 lux with a photoperiod of 18 hrs day light and 6 hrs dark. Each experiment was conducted at least thrice with 20 replicates per treatment

Results

Induction and proliferation of callus from leaf explants

Callus is dedifferentiated and unorganized mass of parenchyma cells formed by the proliferation of parent tissue. Callus tissue is a good source of genetic variability and adventitious shoot formation.

From the two month old field grown plants the leaves are excised and are used as explants for the callus induction. Leaf segments (0.5 – 1.0 cm^2) were inoculated on MS medium fortified with different concentrations of auxins (NAA, IAA and 2, 4-D) singly and in combination with cytokinins (BA and Kinetin) gave varied callusing response (Table-1; Figure-1). The explant failed to produce callus on MS medium lacking growth regulators but the swelling of the explant is observed.

Callus produced from leaf segments were dark green, light green, creamish green and brown and segments were dark green, light green, creamish green and brown and nodular to fragile in nature. NAA, 2, 4-D along with BA were observed to be potent hormonal combinations for profuse callus production from leaf explants. In the combination of NAA (1.5 mg/L and 2.0 mg/L) and BA (0.1 mg/L) a profuse yellowish green coloured callus was formed. In the lower concentrations of NAA (0.5 mg/L and 1.0 mg/L) with BA (0.1 mg/L) less amount of callus was formed in comparison to higher concentrations. In the combination of NAA (2.0 mg/L) with kinetin (0.2 mg/L) formed profuse green coloured fragile to nodular callus. 2, 4-D, along with BA was noted to be a potent hormonal combination for stimulating callus induction from leaf explants. Light green fragile callus was formed at the lower concentration of 2, 4-D (0.5 mg/L) along with BA (0.5 mg/L). As the concentration of 2, 4-D increased light brown to dark brown fragile callus was formed even in the presence of BA (0.5 mg/L). IAA at the concentration of 2.0 mg/L along with BA (0.1 mg/L) showed profuse light green coloured nodular callus formation.

During the present investigation, young leaf explants of *in vitro* raised plants were culture on MS medium supplemented with different hormonal combinations of 2, 4-D, NAA, BA and Kinetin exhibited varied response after 3-4 weeks of culture. NAA (2.0 mg/L) alone is able to produce abundant light green callus. The combination of NAA (1.0 mg/L) along with BA (0.1 mg/L) and NAA (1.0 mg/L) with BA (0.5 mg/L) showed profuse green organogenic callus formation (Table-2; Figure-1). In 2, 4-D alone supplemented medium explants showed poor brown coloured fragile callus formation. Whereas, 2, 4-D in combination with BA (0.5 mg/L) showed profuse creamish brown coloured callus. In the present study, it was found that NAA and 2, 4-D in combination with BA are superior for the production of callus from leaf explants.

Table 1: Effect of different concentrations of auxins such as NAA, IAA and 2, 4-D singly and in combination with BA and Kn on induction of callus from leaf explants of field grown *S. rebaudiana* plants

Plant growth regulator (mg/L)					Intensity of callus formation	Nature of callus
NAA	2,4-D	IAA	BA	Kinetin		
-	-	-	-	-	-	Swelling of explant
0.5	-	-	0.1	-	C^{++}	Light green, nodular
1.0	-	-	0.1	-	C^{++}	Light green, fragile
1.5	-	-	0.1	-	C^{+++}	Yellowish green, compact
2.0	-	-	0.1	-	C^{+++}	Yellowish green, compact
0.5	-	-	-	0.2	C^{+}	Creamish green, fragile
1.0	-	-	-	0.2	C^{++}	Light green, nodular
1.5	-	-	-	0.2	C^{++}	Light green, fragile
2.0	-	-	-	0.2	C^{+++}	Dark green, nodular
-	0.5	-	0.5	-	C^{++}	Light green, fragile
-	1.0	-	0.5	-	C_{+++}	Light brown, fragile
-	2.0	-	0.5	-	C^{++}	Dark brown, fragile
-	1.0	-	-	0.5	C^{++}	Light brown, fragile
-	2.0	-	-	0.5	C^{++}	Light brown, fragile
-	-	1.0	0.1	-	C^{++}	Light green, fragile
-	-	2.0	0.1	-	C^{+++}	Light green, nodular
-	-	1.0	-	0.1	C^{+}	Light green, nodular
-	-	2.0	-	0.1	C^{++}	Dark green, compact

Observation: After 4 weeks; C^{+}-Poor callus; C^{++} - Moderate callus C^{+++} - Profuse callus

Table 2: Effect of different concentrations of plant growth regulators added to MS medium on induction of callus from leaf of *in vitro* grown *S. rebaudiana*

Plant growth regulator (mg/L)				Degree of callus formation	Nature of callus
NAA	2,4-D	BA	Kn		
1.0	-	-	-	C^{++}	Light green, fragile
2.0	-	-	-	C^{+++}	Light green, nodular
-	1.0	-	-	C^{++}	Brown, fragile
-	2.0	-	-	C^{++}	Brown, fragile
0.1	-	0.1	-	C^{+}	Light green, compact
0.5	-	0.1	-	C^{++}	Light green, compact
1.0	-	0.1	-	C^{+++}	Green, compact
1.0	-	0.5	-	C^{+++}	Green, nodular, organogenic
-	0.5	0.5	-	C^{++}	Creamish brown, fragile
-	1.0	0.5	-	C^{+++}	Creamish brown, fragile
-	2.0	0.5	-	C^{+++}	Brown, nodular
1.0	-	-	0.5	C^{++}	Green, organogenic
-	1.0	-	0.5	C^{+}	Light brown, organogenic

Observation: After 4 weeks; C^{+}- Poor callus; C^{++} - Moderate callus C^{+++} - Profuse callus

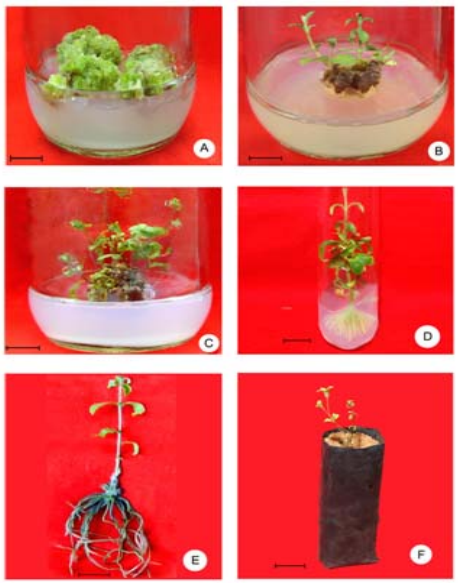

Figure-1: Callus formation from the leaf explants of *in vitro* grown plants
A) MS + NAA (1.0 mg/L) + BA (0.1 mg/L) (bar 1cm = 0.9 cm)
Indirect shoot regeneration from the leaf derived callus
B) MS + BA (2.0 mg/L) (bar 1cm = 0.9 cm)
C) MS + BA (0.5 mg/L) + IAA (1.0 mg/L) (bar 1cm = 1.0 cm)
In vitro root organogenesis from regenerated shoots
D) MS + IBA (0.5 mg/L) (bar 1cm = 1.6 cm)
In vitro root formation from regenerated shoots
E) MS + IBA (0.5 mg/L) (bar 1cm = 1.0 cm)
Hardening and acclimatization of *in vitro* regenerated plants
F) A tissue cultured plant in polybag after hardening (bar 1cm = 2 cm)

Indirect shoot organogenesis from leaf derived callus

Vegetative plant parts especially leaves are desirable explants for *in vitro* improvement because of regeneration from these explants would preserve the genetic homozygosity of the parent genotype. The presence of cytokinin along with auxin is necessary for indirect adventitious shoot induction. The induction of callus and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators such as BA, Kn, NAA and IAA in the culture medium.

Among the growth regulators tested, BA (2.0 mg/L) induced maximum frequency of shoot regeneration. The maximum shoot number (8.2±0.1) was observed when it was grown on the MS medium with BA (2.0 mg/L). But the maximum shoot length (5.4±0.4) was observed in BA (0.5 mg/L) along with kinetin (0.5 mg/L) and NAA (0.1 mg/L) (Table-3; Figure-1). The minimum regeneration frequency and shoot length (3.0±0.1 cm) was noted

at BA (0.5 mg/L) in combination with IAA (0.5 mg/L). Minimum number of shoots (2.4±0.1) were also observed in BA (0.5 mg/L) along with IAA (1.0 mg/L). Kinetin when used alone did not show any shoots. In the present study BA alone and along with NAA exhibited better morphogenesis. *In vitro* derived shoots with a length of (3-5cm) were excised and transferred to MS medium supplemented with different concentrations of auxins such as IBA and NAA (0.1–2.0 mg/l). In all the concentrations tried, exogenous supply of auxins favoured the root formation and root primordial appeared between 7-10 days of inoculation. High rooting frequency (98%) with highest number of roots (30.3±1.85) were obtained in IBA (0.5 mg/L). The maximum root length (3.8±0.10) was observed at NAA (0.1 mg/l) (Table-4; Figure-1). Higher concentrations of IBA and NAA showed the organogenic callus formation.

Table 3: Indirect shoot organogenesis from callus obtained through leaf explants of *S.rebaudiana*

Plant growth regulator (mg/L)				Regeneration frequency (%)	Mean number of shoots/ callus	Mean shoot length (cm)	Nature of callus
BA	KN	NAA	IAA				
0.5	-	0.1	-	64	5.0 ± 0.2	3.4 ± 0.05	Yellowish green, fragile
1.0	-	0.1	-	79	6.2 ± 0.5	4.6 ± 0.1	Green, nodular
0.5	0.5	0.1	-	81	5.9 ± 0.1	5.4 ± 0.4	Yellowish green, fragile
1.0	0.5	0.1	-	85	7.1 ± 0.1	4.1 ± 0.2	Yellowish green, fragile
0.5	-	-	0.5	60	3.0 ± 0.2	3.0 ± 0.1	Green, nodular
0.5	-	-	1.0	54	2.4 ± 0.1	3.2 ± 0.2	Light green, nodular
-	1.0	-	0.1	-	-	-	Green, compact
-	1.0	-	0.5	-	-	-	Light green, compact
1.0	-	-	-	80	6.4 ± 0.4	4.2 ± 0.1	Green, nodular
2.0	-	-	-	86	8.5 ± 0.1	3.1 ± 0.3	Green, nodular
-	1.0	-	-	-	-	-	Whitish green, nodular
-	2.0	-	-	-	-	-	Light green, nodular

Observation: After 4 weeks; Values are mean ± SE of 20 independent determinations.

Table 4: Root organogenesis of *in vitro* derived shoot lets in MS half strength medium supplemented with various concentrations of auxins such as IBA and NAA.

Plant growth regulator (mg/L)		Frequency of root formation (%)	Mean number of roots / shoot	Mean length of root (cm)	Callus
IBA	NAA				
-	-	-	-	-	-
0.1	-	86	10.3 ± 0.66	3.06 ± 1.77	-
0.5	-	88	21.7 ± 0.85	3.17 ± 0.08	-
1.0	-	96	27.0 ± 0.57	3.76 ± 0.14	-
2.0	-	98	30.3 ± 1.85	2.73 ± 0.49	-
3.0	-	80	22.5 ± 0.75	2.12 ± 0.80	C^{++}
-	0.1	82	8.3 ± 0.88	4.9 ± 0.62	-
-	0.5	85	12.2 ± 0.94	3.4 ± 0.12	-
-	1.0	91	16.6 ± 1.20	2.53 ± 0.08	C^{+}
-	2.0	94	15.6 ± 0.88	1.53 ± 0.26	C^{+}
-	3.0	81	7.5 ± 0.62	1.20 ± 0.15	C^{++}

Observation: After 4 weeks; values are mean ± SE of 20 independent determinations; C^{+}- Poor callus; C^{++} - Moderate callus, C^{+++} - high callus

Acclimatization and hardening

Well rooted plantlets were separated from the culture tubes, washed and transferred to polybags containing soil + vermiculite in 1:1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions. Rooted shoots showed the maximum percentage of survival.

Discussion

Callus is differentiated and unorganized mass of parenchyma cells formed by the proliferation of parent tissue. Callus tissue is a good source of genetic variability and adventitious shoot formation [10]. NAA, 2, 4-D along with BA were observed to be potent hormonal combination for profuse callus production from leaf explants. In which light green to dark green colour, nodular to fragile callus was formed. Similar callusing response was noted in *Justicia genderussa* [11] *Diathus caryophyllus* [12]. The effectiveness of 2, 4-D and NAA in combination with cytokinins in inducing callus might be due to their role in DNA synthesis and mitosis [13]. In 2, 4-D supplemented medium light brown to dark brown colour fragile callus formation takes place. Brown colour of the callus showed sensitivity of plant tissues to 2, 4-D. This is in agreement with earlier reports in *Ipomea aqualtica* [14].

Callus induction is a prerequisite for adventitious shoot formation and also for the other *in vitro* genetic improvement including induction of somaclonal variations and embryoids. Among the growth regulators tested BA induced maximum frequency of shoot regeneration. Similar *in vitro* response was

reported in *Piper longum* [15], *Pithecallobium saman* [16]. In the present study BA along with NAA exhibited better morphogenesis. These findings were in line with previous reports in *Asteracantha longifolia* [17]. Indirect shoot regeneration through callus phase obtained from leaf explants was earlier reported in many plants like *Spillanthus acmella* [18] and *Justicia gendarussa* [11].

Conclusion

A callus culture system offer many advantages as a model system for several biological investigations. Hence, the present study of indirect plant regeneration via callus phase is most effective in micropropagation and conservation of this economically important medicinal herb *Stevia rebaudiana.*

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