Journal of Phytology 2011, 3(5): 69-73
ISSN: 2075-6240
www.scholarjournals.org

JOURNAL OF PHYTOLOGY

www.journal-phytology.com

JP-Tissue Culture

Direct Shoot Organogenesis from Leaf Explants of *Stevia rebaudiana*

D. Preethi[1], T.M. Sridhar[1] and C.V. Naidu[2*]

[1]*Department of Biotechnology, Sri Venkateswara University, Tirupathi-517502, A.P., India*
[2]*Department of Biotechnology, Dravidian University, Kuppam-517426, A.P., India*

Article Info

Article History

Received : 19-02-2011
Revised : 03-04-2011
Accepted : 07-04-2011

*Corresponding Author

Tel : +91-877 2260386
Fax : +91-8570278209

Email: challagundlav@yahoo.co.in

Summary

In the present investigation, an attempt has been made to standardize a protocol for direct shoot regeneration from leaf explants of *Stevia rebaudiana*. Among the combination of plant growth regulators tested in field grown leaf explants maximum number of shoots (10.4±0.21) was obtained on MS medium supplemented with BA (1.0 mg/L), Kn (0.5 mg/L) and IAA (0.1 mg/L), where as in *in vitro* derived leaf explants maximum number of shoots (28.7±0.84) was obtained on MS medium supplemented with BA (2.0 mg/L), Kn (0.5 mg/L) and NAA (0.1 mg/L). 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 (0.1–2.0 mg/L). The best rooting response was observed on 2.0mg/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, BAP, Kn, NAA, IAA, IBA, Shoot regeneration

Introduction

Micropropagation offers many advantages over conventional methods such as rapid multiplication of valuable genotypes, expeditious release of improved varieties, the independence of seasonal supply, germplasm preservation, and clonal uniformity – all combine to increase the value of this technique [1].

Stevia botanically called as *Stevia rebaudiana* (also called sweet leaf, or sugar lear) is a genus of about 150 species of herbs and shrubs in the asteraceae family [2]. The herb is nutrient rich, containing substantial amounts of protein, calcium, phosphorus, sodium, magnesium, zinc, rutin, vitamin A, vitamin C and other nutrients, yet has no caloric value [3]. *Stevia* has many favourable and exciting health benefits and it is completely non-toxic. In 1931, two French chemists isolated the glycosides that give *Stevia* is taste. These extracts were named as stevioside, rebaudioside A, rebaudioside C and dulcoside. Stevioside and rebaudioside induces insulin secretion [4]. Besides, stevioside acts as anti-tumour agent [5]. *Stevia* possess antifungal and antibacterial property also in addition to its other versatile uses [6]. *Stevia* contain a stevioside, a secondary metabolite responsible for the sweetness and the leaf by itself is about 20 to 30 times sweeter than sugar. Experiments proved that stevioside is 300 times sweeter than sucrose, apart from being a calorie free sugar [7]. Hence, *Stevia* has been named as calorie free "Biosweetener of high quality". It is estimated that about 30 million Indians are presently suffering from diabetes and it is estimated that by 2025 India's contribution to the diabetic global population would be a whooping 80 million. Therefore the wave of 'sugar free' has become a welcome trend. Stevia shows calorie free high potency sweetener, does not contain calcium cyclamate, saccharin and aspartame and causes no side effects [8]. As a response, many no-calorie synthetic alternatives of sugar popularly known as artificial sweeteners have been discovered and replacing sugar in food and beverage industry.

In the present investigation experiments were performed to determine the role of different plant growth regulators on direct shoot regeneration from field grown, *in vitro* derived leaf explants 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, Tirupathi, A.P., 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

Leaf explants (0.5-1.0 cms) were inoculated on MS medium [9] containing 3% sucrose and gelled with 0.8% agar supplemented with different plant growth regulators such as cytokinins (BA or BA-Kn) along with auxins (NAA or IAA or IBA). 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

Subculturing 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^{0}C 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

Direct shoot organogenesis from leaf explants of field grown plants of Stevia rebaudiana

Effect of cytokinins alone on direct shoot regeneration

After 10 days of incubation, enlargement of most of the leaf explants was observed. Direct formation of shoots was observed after three weeks from mid rib region or basal petiolar region or apical region of the leaf. Plant growth regulator BA alone without any combination of hormones also induced shoots ranging from 1.4 – 3.1 with a shoot length ranging from 1.25 – 2.30 cm. When kinetin alone was tested for shoot initiation and elongation there was no formation of shoots observed at the concentration of 0.5 mg/L and 1.0 mg/L and very less number of shoots (1.9 ± 0.40) was recorded at 2.0 mg/L with a mean shoot length of 2.85 ± 0.10 cm (Table-1).

Effect of auxins in combination with cytokinins on direct shoot regeneration

BA along with kinetin and other auxins such as NAA, IAA and IBA showed increase in number of shoots. Kinetin when used in the combination of BA and NAA showed good response. The combinations of kinetin (0.5 – 2.0 mg/L), BA (1.0 mg/L) and NAA (0.05 mg/L) were tested for shoot initiation and elongation. The number of shoots formed was (5.2 – 8.9) and shoot length ranges in between 1.25 – 2.55 cm. A maximum number of (10.4 ± 0.21) shoots were observed in BA (1.0 mg/L), Kn (0.5 mg/L) and IAA (0.1 mg/L) (Table-1; Figure-1). The maximum shoot length (4.23 ± 0.10) was obtained in BA (2.0 mg/L), Kn (0.5 mg/L) and IAA (0.1 mg/L). The combination of BA, Kn and NAA was also studied. This combination also induced *de novo* shoot formation from leaf explants of field grown plants. The second best concentration observed was MS medium supplemented with BA (2.0 mg/L), kinetin (0.5 mg/L) and NAA (0.1 mg/L) in which the second highest number of shoots (8.1 ± 0.35) was observed. BA (0.5 – 3.0 mg/L) in combination with kinetin (0.5 mg/L) and NAA (0.1 mg/L) induced mean number of shoots (4.0 – 8.1) with a mean shoot length ranged from 1.74 – 2.25 cm. BA (0.5 – 2.0 mg/L) in combination with kinetin (1.0 mg/L) and IBA (0.1 mg/L) induced multiple shoots from leaf explants, where the mean number of shoots ranged from 2.5 – 5.5 and mean shoot length ranged from 1.4 – 2.30 cm . The combinations of BA (0.5 – 2.0 mg/L), Kinetin (0.5 mg/L) and IAA (0.1 mg/L) were also tested for their effect on direct organogenesis. The number of shoots formed (5.5 – 10.4) and the shoot length (3.2 – 4.23 cm) was recorded.

Among all the concentrations tested, combination of BA, Kn and IAA induced maximum number of shoots from field grown leaf explants.

Table-1: *In vitro* direct multiple shoot organogenesis from leaf explants of field grown *S. rebaudiana* plants; Observation: after 4 weeks; values are mean ± SE of 20 independent determinations.

Plant growth regulator (mg/L)					Number of shoots / explant	Shoot length (cm)
BA	Kn	NAA	IAA	IBA		
0.5	-	-	-	-	-	-
1.0	-	-	-	-	1.4 ± 0.62	1.25 ± 0.62
2.0	-	-	-	-	2.3 ± 0.33	2.30 ± 0.65
3.0	-	-	-	-	3.1 ± 0.54	1.63 ± 0.08
-	0.5	-	-	-	-	-
-	1.0	-	-	-	-	-
-	2.0	-	-	-	1.9 ± 0.40	2.85 ± 0.10
0.5	0.5	0.1	-	-	4.0 ± 0.55	1.95 ± 0.38
1.0	0.5	0.1	-	-	6.3 ± 0.28	1.74 ± 0.50
2.0	0.5	0.1	-	-	8.1 ± 0.35	2.25 ± 0.62
3.0	0.5	0.1	-	-	7.6 ± 0.07	2.05 ± 0.19
0.5	0.5	-	0.1	-	5.5 ± 0.65	3.90 ± 0.24
1.0	0.5	-	0.1	-	10.4 ± 0.21	3.2 ± 0.62
2.0	0.5	-	0.1	-	6.2 ± 0.72	4.23 ± 0.10
1.0	0.5	0.05	-	-	6.0 ± 0.89	1.92 ± 0.33
1.0	1.0	0.05	-	-	8.9 ± 0.42	2.55 ± 0.90
1.0	2.0	0.05	-	-	5.2 ± 0.71	1.25 ± 0.38
0.5	1.0	-	-	0.1	2.5 ± 0.64	1.4 ± 0.10
1.0	1.0	-	-	0.1	5.5 ± 0.33	2.30 ± 0.65
2.0	1.0	-	-	0.1	3.8 ± 0.92	1.95 ± 0.24

Table-2: Direct shoot oreganogenesis from leaf of *in vitro* regenerated *S.rebaudiana* plants; Observation: after 4 weeks; values are mean ± SE of 20 independent determinations

Plant growth regulator (mg/L)				Number of shoots/ explant	Length of shoot (cm)
BA	Kn	NAA	IAA		
0.5	0.5	0.1	-	14.0 ± 0.66	1.90 ± 0.55
1.0	0.5	0.1	-	19.5 ± 0.20	2.63 ± 0.32
2.0	0.5	0.1	-	28.7 ± 0.84	1.65 ± 0.40
1.0	-	0.05	-	12.1 ± 0.92	2.05 ± 0.63
2.0	-	0.05	-	15.4 ± 0.35	1.95 ± 0.22
0.5	1.0	-	0.05	14.8 ± 0.15	3.30 ± 0.55
1.0	1.0	-	0.05	10.6 ± 0.26	3.95 ± 0.19

Table 3: 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	22.7 ± 0.85	3.17 ± 0.08	-
1.0	-	96	28.0 ± 0.50	3.76 ± 0.14	-
2.0	-	98	32.4 ± 0.34	2.70 ± 0.50	-
-	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^{+}

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

Figure-1

Direct regeneration of shoots from leaf explants
A. Shoot bud induction from leaf explant of field grown plants MS + BA (2.0 mg/L) (bar 1cm = 1.1 cm); B. MS + BA (0.5 mg/L) + Kinetin (0.5 mg/L) + NAA (0.1 mg/L) (bar 1cm = 1.2 cm); C. MS + BA (1.0 mg/L) + Kinetin (1.0 mg/L) + IBA (0.1 mg/L) (bar 1cm = 0.9 cm);
D. Shoot multiplication from explant of *in vitro* grown plants MS + BA (1.0 mg/L) + Kinetin (0.5 mg/L) + IAA (0.1 mg/L) (bar 1cm = 1.0 cm);
***In vitro* root organogenesis from regenerated shoots**
E. MS + IBA (0.1 mg/L) (bar 1cm = 1.2 cm)
Hardening and acclimatization of *in vitro* regenerated plants
F. Regenerated plant in root trainer (bar 1cm = 2 cm)

In vitro direct shoot regeneration from leaf explants of in vitro regenerated S. rebaudiana shoots

Effect of auxins in combination with cytokinins on direct shoot regeneration

The leaves which are excised from the *in vitro* regenerated shoots of *S. rebaudiana* were successfully used for the regeneration of plants and *in vitro* micropropagation of *S. rebaudiana*.

BA (0.5 – 2.0 mg/L) in combination with lower concentrations of NAA (0.05 – 0.1 mg/L) or BA (0.5 – 2.0 mg/L) in combination with kinetin (1.0 mg/L) and IAA (0.05 mg/L) was used to produce the direct shoot regeneration from leaf of *in vitro* regenerated *S. rebaudiana* plants and the results were tabulated. BA (2.0 mg/L) with kinetin (0.5 mg/L) and NAA (0.1 mg/L) resulted in maximum number of shoots (28.7 ± 0.84) (Table-2; Figure-1). The maximum shoot length (3.95 ± 0.19) was observed in the combination of BA (1.0 mg/L), kinetin (1.0 mg/L) and IAA (0.05 mg/L). At lower concentrations BA favoured the shoot length and as concentration of BA increased the shoot length was decreased. It is evident from the combinations of BA 2.0 mg/L with NAA 0.05 mg/L and BA 2.0 mg/L, Kn 0.5 mg/L and NAA 0.1 mg/L. The combination of BA (0.5 – 1.0 mg/L) with kinetin (1.0 mg/L) when used with IAA (0.05 mg/L) showed the reduced shoot number (10.6 – 14.8) over the NAA (14 – 28.7).

Well developed 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 (32.4±0.34) were obtained in IBA (2.0 mg/L) (Table-3 and Figure-1).

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

Growth and organogenesis in *in vitro* depends on the interaction between endogenous growth substances and the synthetic growth regulators which may be added to the medium. The induction of direct shoot regeneration depends on the nature of the plant organ from which the explant was derived and is highly dependent on plant [10]. Leaf bits excised from the leaf explants resulted in callus formation at cut ends. Most of the media tested were induced shoot regeneration via direct organogenesis and the primordia always regenerated directly from the leaf without forming callus. After some days of incubation, meristemoids differentiated from the abaxial epidermal cells, which by further cell division gave rise to small protrusions of tissue which gradually became green and organized into a growing plant as described in *Pathomorphe umbellata* [11].

The combination of cytokinin and auxin seems to have a synergistic effect as cytokinins enhance cell division, stimulate axillary bud and adventitious shoot proliferation and auxins regulate cell elongation, tissue swelling and shoot expansion. The effect of auxins and cytokinins on optimizing shoot regeneration has been reported in several spices, such as *Catalpa ovata* [12] *Echinacea purpurea* [13] and *Kigelia pinnata* [14]. The effect of BA and IBA was studied earlier in *Platanus acerifolia* wild [15] and *Daphne georum* [16]. The combined effect of BA and Kn on the direct adventitious shoot development is well documented in *Piper longum* [17]. Direct multiple shoot regeneration was obtained in *Murraya koenigii* using the combinations of BA and IAA and kinetin with IAA [18]. Among all the concentration tested combination of BA, Kn and IAA induced maximum number of shoots from leaf. These results are in line with the observations reported in *Lillium* [19] and *Cajanus cajan* [20]. Age of the excised leaves was the major factor controlling morphogenetic potential as is also true in *Cleisostoma racimeferum* [21]. According to the earlier reports [22] the adventitious shoot regeneration from carnation leaves obtained from green house grown plants is more efficient than from leaves of *in vitro* grown plants. But in *S. rebaudiana* more number of adventitious shoot formation were observed in the *in vitro* grown plants when compared with the field grown.

The differential response of field grown leaves and *in vitro* grown leaves could be due to the varying concentrations of the growth regulators used in the medium, endogenous levels of growth regulators and explant type [23]. The morphogenic potential of younger tissues is attributed due to more physiological and biochemical activity [24].

Conclusion

Direct regeneration from leaf is another alternative step for clonal propagation and germplasm conservation. Hence in the present study an approach has been made to standardize an efficient protocol for *in vitro* propagation of highly commercial *Stevia rebaudiana* using leaf explants.

References

[1] Govil S. and Guptha S.C. 1997. Commertialization of plant tissue culture in India. Plant Cell Tiss. Org. Cult., 51 : 65- 73.

[2] Soejarto D.D., Compadre C.M., Medon P.J., Kamath S.K. and Kinghorn A.D. 1983. Potential sweetening agents of plant origin, 11. Field search for sweet tasting *Stevia* receipes. Econ. Bot., 37: 71-79.

[3] Miyzaki Y and Watanable H. 1974. Studies on the cultivation of *Stevia rebaudiana* Bertoni; on the propagation of the plant. Jap. J. Trop. Agric., 17: 154-157.

[4] Matsukubo T. 2006. "Sucrose substitutes and their role in caries prevention", Int. Dent. J. SCN; 56(3): 119-130.

[5] Jeppesen P.B., Gregersen S., and Rolfsen S.E. 2003. Antihyperglycemic and blood pressure-reducing effects of stevioside in the diabetic Goto-Kakizaki rat. Metabolism. 52: 372-378.

[6] Reziwanggu Audula, Per Bendix Jeppesen, Stig Eric Rolfsen D., Jianzhong Xiao and Kjed Hermansen. 2004. Rebaudioside A potently stimulates insulin secretion from isolated mouse islates: Studies on the dose-glucose and calcium-dependency. Metabolism. 53 (10): 1378-1381.

[7] Bridel M. and Lavielle R. 1931. The sweet principle of Kaa-he-e (*Stevia rebaudiana*). J. Pharm. Chem., 14: 99-154.

[8] Tofazzal Islam M.D. 2006. *Stevia rebaudiana* News: Is there any safe and natural alternative to sugar? From this Financial Express. 8-26.

[9] Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

[10] George E.F. 1993. Plant propagation by tissue culture. Part - I. The Technology Exgetics Ltd., U.K.

[11] Pereira A.S., Bertoni B.W., Beatriz Appezz-da-Gloria, Alba R.B., Araujo, Ana Helena Januario, Miriam V. Lourenco and Suzelei C. Franca. 2000. Micropropagation of *Pothomorphe umbellate* via direct shoot organogenesis from leaf explants. Plant Cell Tiss. Org. Cult., 60: 47 - 53.

[12] Lisowska K and Wysokinska H. 2000. *In vitro* propagation of *Catalpa ovata* G. Don . Plant Cell Tiss. Org. Cult., 60 : 171 - 176.

[13] Koroch A., Juliani H.R., Kapteyn J. and Simon J. 2002. *In vitro* regeneration of *Echinacea purpurea* from leaf explants. Plant Cell Tiss. Org. Cult., 69: 171-176.

[14] Thomas T.D. and Puthur J.S. 2004. Thidiazuron inuced high frequency shoot organogenesis in callus from *Kigelia pinnata* L. Bot. Bull. Acad. Sin., 45:307-313.

[15] Liu G. and Bao M. 2003. Adventitious shoot regeneration from *in vitro* cultured leaves of London plane tree (*Platanus acerifolia* Willd.). Plant Cell Rep., 21: 640 - 644.

[16] Mala J. and Bylinsky V. 2004. Micropropagation of endangered species *Daphne cneorum*. Biologia Plantarum. 48(4): 633 - 636.

[17] Sarasan V., Thomas E., Lawrence B. and Nair G.M. 1993. Plant regeneration in *Piper longum* L. (Piperaceae) through direct and indirect shoot development. J Spice Arom. Crops. 2: 34 - 40.

[18] Deepu Mathew and Prasad M.C. 2007. Multiple shoot and plant regeneration from immature leaflets of *in vitro* origin in curryleaf (*Murraya koenigii* SPERG). Indian J. Plant Physiol., 12(1):18 - 22.

[19] Loretta Bacchetta, Patrizio C., Remotti, Claudia Bernardini and Francesco Saccardo 2003. Adventitious shoot regeneration from leaf explants and stem nodes of *Lilium.* Plant Cell Tiss. Org. Cult., 74:37 - 44.

[20] Misra P. 2002. Direct differentiation of shoot buds from leaf explants of *Cajanus cajan* L. Biological Plantarum. 45(3):347 - 351.

[21] Temjensangba and Chitta Ranjan Deb 2005. Regeneration of plantlets from *in vitro* raised leaf explants of *Cleisostoma racimeferum* Lindl. Indian J. of Expt. Biol., 43:377 - 381.

[22] Van Altvorst A.C., Koehorst H.J.J., Bruinsma l. and Dons J.J.M. 1994. Improvement of adventitious shoot formation from carnation leaf explants. Plant Cell Tiss. Org. Cult., 37: 87 - 90.

[23] Das G. and Rout G.R. 2002. Direct plant regeneration from leaf explants of *Plumbago* species. Plant Cell Tiss. Org. Cult., 68: 311 - 314.

[24] Misra A.K. and- Bhatnagar S.P. 1995. Direct shoot regeneration from the leaf explants of cucumber (*Cucumis sativa* L.). Phytomorphology. 45:47.