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Effect of L: Glutamine on *in vitro* callus induction and embryogenic potential of *Stevia rebaudiana* Bert

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Abstract
The present investigation was carried out to find out the effect of L-Glutamine on *in vitro* callus induction using different explants from 3-4 months old plantlets of *S. rebaudiana* reported by (Vasudevan *et al*., 2004). Best response was obtained on MS medium supplemented with L- Glutamine at 15 mg/l in combination with 2, 4 –D (1.5mg/L) and BAP (0.5mg/L). Glutamine at this concentration induced 88 % callus with 37.77 and 104.24 mg callus weight on 14th and 30th day, respectively. This combination can only able to produce greenish, healthy nodular calli with more embryogenic potential and less necrotic lesion which further performed better in regeneration medium fortified with 2,4 –D (0.5mg/L), BAP (1.5mg/L) and L Glutamine (15 mg/L) produced large number of shoots.

Keywords: *Stevia rebaudiana*, organogenesis, callus, l-glutamine, growth regulators

Introduction
Stevia rebaudiana Bertoni, belonging to the family Asteraceae. It consists of 154 species of which *S. rebaudiana* produces sweet steviol glycosides (Robinson 1930, Soejarto *et al.*, 1982) [9]. Leaves of this plant produces of zero calorie Stevioside and rebaudioside, a non-nutritive, high potency sweetener and substitute to sucrose being100 times sweeter than sucrose. (Ishima and Katayama 1976; Tanaka, 1982) [5, 13]. It reduces the calories from foods which make it a suitable replacement of sugar for calorie conscious as well diabetic people. Stevia is also useful in lowering blood sugar. It can be used in tooth pastes to avoid tooth decay and reduces the tooth cavity. Stevia leaf powder possesses great anti-wrinkle qualities in it. Stevia leaf powder can be mixed with chicken feed for the treatment of osteoporosis. It is regularly used in preparation of food products. Brandle and Rosa 1992 reported that Japan alone, estimated 50 tons of stevioside is used annually with sales valued in order of $220 million Canadian Dollar. The plant is indigenous to the northern regions of South America and grows wild in the Highlands of Amambay and near the source of the river Monday (a border area between Brazil and Paraguay). The seeds of Stevia show a very low germination percentage (Nakamura and Tamura 1985) [8]. Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition (Nakamura, 1985) [8]. Vegetative propagation too is limited by the lower number of individuals that can be obtained simultaneously from a single plant (Sakaguchi and Kan, 1982) [11]. Due to the above-mentioned difficulties, tissue culture is the only alternative for rapid mass propagation of Stevia plants. The action of amino acids and additives as a supplement of growth regulator will be use full in the establishment of reliable operation to induce somatic embryos; callus induction is the best way to create somaclonal variations in plant.

Materials and Methods
Collection of Plant material
The plants are collected from Horticultural Research Station Yercaud, Tamilnadu, India and maintained in the nursery of Department of Botany, Bharathidasan University, Tiruchirappalli, India.

Fig 1

Surface sterilization
Leaf, node and shoot tip from 3-4 months old plantlets were used as an explant, explants were washed thoroughly under running tap water to remove the traces of dust etc. The explants were surface sterilized by dipping in 70% ethanol for 30 Sec, then dipping in 2.5% (v/v) sodium hypochlorite solution for 4 min with constant shaking, followed by three rinses with sterile distilled water and transferred to culture tubes containing Murashige and Skoog's medium (MS) under aseptic conditions.

Callus culture

Leaf, shoot tip and node explants were cultured in Murashige and Skoog's medium (1962) containing 0.8% agar (w/v), 3% sucrose (w/v), 2, 4 – Dichlorophenoxy acetic acid (2, 4-D, 0.5 – 2.5 mg/L) in combination with BAP (0.5mg/L) were tested for the induction of organogenic callus. The pH of the medium was adjusted to 5.7 before autoclaving at 121°C for 15min. The cultures were maintained at 25°C less than 16 h photo periods with the light intensity of 3000 lux. Explants were subjected to two sub cultures at an interval of ten days each in the MS medium supplemented with the same concentration of growth regulators.

Effect of L-Glutamine on callus induction

MS (Murashige and Skoog's medium 1962) supplemented with 2,4 – Dichlorophenoxy acetic acid (2,4-D, 1.5 mg/L) BAP (0.5mg/L) in combination with L-Glutamine (5 - 25 mg/L) were tested for the induction of organogenic callus from leaf explant. The pH of the medium was adjusted to 5.7 before autoclaving at 121°C for 15min. The cultures were maintained as described above. Explants were subjected to two sub cultures at an interval of ten days each in the MS medium supplemented with the same concentration of growth regulators.

Embryogenic potential of callus

To find out the efficiency of embryogenic potential the callus was cultured in MS media supplemented with growth regulator BAP (1.5 mg/L) in combination with 2,4-D (0.5 mg/L), L-Glutamine (15 mg/L). The pH of the medium was adjusted to 5.8 and 0.8% agar was added prior to autoclaving. The cultures were maintained at 25°C and high light intensity (90 µE-m^{-2}, $\delta^{-}1$) were the optimal conditions for somatic embryo maturation and sub cultured twice at an interval of ten days each in the MS medium supplemented with the same concentration of growth regulators.

Results and Discussion

Callus culture

Callus producing ability of various explants such as shoot tip, leaf, nodal explants of *S. rebaudiana* were tested on Murashige and Skoog's medium medium fortified with different concentrations of 2, 4-D in combination with Benzyl amino purine. After two weeks of incubation Callus proliferated at the cut end of the explants. Among the three explants best response was observed in leaf explant compared to shoot tip and nodal explant. (Figure a & b) Maximum callus induction occurred in the MS medium was fortified with BAP (0.5mg/l) and 2, 4 –D (1.5mg/l). (Table 1) Earlier **(**Sairkar *et al.*, 2009) reported that 2.0 mg/L of 2, 4-D with 1.0mg/L kinetin induced callus after 30 days.

Effect of L-Glutamine on callus induction.

Leaf explants were inoculated on MS medium with 2,4-D (1.5mg /L), BAP (0.5 mg/L), sucrose (3%), agar (0.8%) and L-Glutamine (5-25 mg /L)**.** Callus occurred at the cut end of leaf explants after two weeks of initiation of culture. Best response was obtained on medium supplemented with L-Glutamine at 15 mg/L. Glutamine at this concentration induced 88 % by Duncan's multiple range test (Duncan, 1955), nodular callus with 37.77 and 104.24 mg weight on 14th and 30th day, respectively. Table 2 (figure d)

Callus induction percentage by using L-Glutamine at 15mg as growth adjuvant was 1.45% increased than the medium with only optimum growth regulators combination. Surprisingly utilization of L-Glutamine at 25 mg/L could not reveal any significant result. Glutamine act as a source of nitrogen, increase explants responsiveness to callus induction medium and also indirectly increase endogenous auxin and cytokinin levels during the induction period (Coruzzi and Last 2000) [2]. (Gamborg and Miller 1968) [3] reported that addition of L-Glutamine enables the cells to maintain a high growth rate for a longer period. In the present investigation it was clearly established that L-Glutamine played vital role in the induction and maintenance of lesion and necrosis free embryogenic calli which can ultimately produce maximum number of regenerated shoots. Glutamine was metabolized extensively to glutamate in the supplied explants and recovery of L-Glutamate as the main component suggested transport in this form. L-Glutamine or other amino acids, which can be readily transferred to other amino acids and incorporated into proteins (Li *et al.*,1993, Higashi *et al.*, 1996) [4]. So, from the studies it was revealed that the MS medium supplemented with L-Glutamine produced the best result regarding callus induction and maintenance for regeneration in comparison with only growth regulator supplemented media.

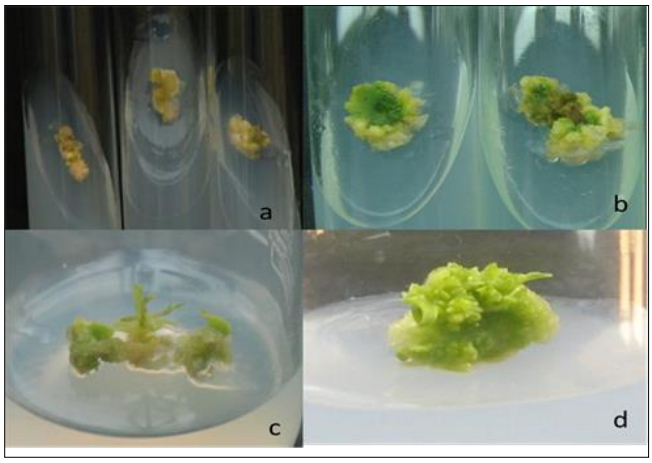
Table 1: Effect of 2,4-D in combination with BAP (0.5 mg/L) on callus induction

S. No	PGR (2,4-D mg/L + BAP 0.5mg/L)	Explants		
		Leaf	Node	Shoot tip
1	2,4-D 0.5mg/L	--	--	--
2	2,4-D 0.1mg/L	+	--	--
3	2,4-D 1.5mg/L	++	+	+
4	2,4-D 2.0mg/L	+	--	--
5	2,4-D 2.5mg/L	--	--	--

(--) No response
(+) Response up to first subculture
(++) Better response up to second subculture

Table 2: Effect of L- Glutamine in combination with 2,4-D (1.5 mg/L) and BAP (0.5 mg/L)

L- Glutamine Mg/L	Induction period 14 days Callus induction %	Callus weight on 14 th day (mg)	Callus weight on 30th day(mg)	Callus Health
5	69	31.04	96.51	Nodular Less Embryogenic
10	76	33.26	98.73	Nodular Less Embryogenic
15	88	37.77	104.24	Nodular More Embryogenic
20	64	26.77	89.48	Nodular Less Embryogenic
25	No response	-	-	No Response



a & b Initiation of callus induction from the cut ends of leaf explant.
c. Nodular callus with less embryogenic potential
d. Nodular callus with more embryogenic potential

Fig 1: Effect of L-Glutamine on callus induction.

Conclusion

Tissue culture techniques offer enormous potential for the selection and improvement of economically important plants. However, *in vitro* culture is a highly complex phenomenon and the success depends on a number of factors such as nature of plant genotype, stage and age of explant, size of explant, method of inoculation, culture media and hormonal influence are the most important areas of interaction which determine the success. Optimal growth and morphogenesis of tissues may vary according to their nutritional requirements. The required amino acids for optimal growth are usually synthesized by most plants, however, the addition of certain amino acids or amino acid mixtures is particularly important for establishing cultures of cells. Amino acids provide plant cells with a source of nitrogen that is easily assimilated by tissues and cells faster than inorganic nitrogen sources. Hence in the present study it was concluded that L glutamine greatly enhanced the mass and shoot regeneration capacity. To enhance the efficiency of somatic embryogenesis manipulated by addition of various organic extracts and amino acids, callus induction is the best way to create somaclonal variations in plant.

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