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In vitro plant regeneration of *Stevia rebaudiana* through indirect organogenesis

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Abstract
Stevia rebaudiana plants produce non caloric sweeteners that can be used as an alternative to sugar. In the present study reliable protocol was developed to obtain healthy plants of *S. rebaudiana* through callus cultures. Leaf discs and nodal explants were cultured on MS medium supplemented with different concentrations and combinations of PGRs. MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA was found to be the best medium for callus induction. Leaf disc was found to be the best explant type for callus initiation. Leaf derived calli were transferred into shoot induction media and MS medium supplemented with 0.5 mg/L NAA and 2.0 mg/L BAP was the best medium. Shoots with more than 5.0 cm were transferred into root induction media and half strength MS medium supplemented with 0.5 mg/L IBA was the best medium for root induction.

Keywords: *Stevia rebaudiana*, callus induction, shoot induction, root induction, explants, organogenesis

1. Introduction
Stevia rebaudiana (Bert.) Bertoni (family Asteraceae) is a herbaceous plant and commonly known as honey leaf, sweet leaf or candy leaf herb. It is one of the most valuable medicinal plant and widely grown for its sweet taste. Glycosides produced by *Stevia* leaves are about 300 times sweeter than sucrose, due to the presence of Steviol Glycosides [1]. *S. rebaudiana* can be utilized as a substitute to sucrose. They are natural sources of non-caloric sweetener and used as an alternative to the synthetic sweetening agents. Its extracts are used today as a food additive as a non caloric sweetener in many countries.
Stevia is a semi humid subtropical plant and propagation is usually done by stem cuttings, but requires high labour inputs. Although rooted plants were established, survival percentage is very low. Propagation through seeds is not a common method owing to the problem of low seed production, low viability and poor germination capacity. Propagation using the seed also causes great variability on stevioside level and composition [2]. Therefore micropropagation, or *In vitro* culture appears to be the best method to overcome those problems and has the potential to produce genetically uniform, large quantity of stevia plantlets within a short period of time.
The demand for sugar in Sri Lanka is likely to go up steadily in coming years. Therefore to meet this demand for sugar, *S. rebaudiana* will elegantly meet the requirement of sugar in Sri Lanka including the growing demand in pharmaceuticals, confectionary and soft drink industries. Therefore this research was conducted to develop a tissue culture protocol for mass propagation of *S. rebaudiana* through indirect organogenesis.

2. Materials and Methods
Mother plants were collected and maintained in a shade house. Seeds were collected from those and they were surface sterilized. Initially, seeds were washed with few drops of liquid detergent, Teepol (0.01% w/v) for 5 min. Thereafter, the detergent was completely drained out by washing it vigorously under running tap water for 30 min. Then the seeds were transferred into the laminar air flow cabinet. After that they were treated with 0.2% Carbendazim for 5 min and 10% Clorox for 10 min. Each step was followed by two successive washings in sterile distilled water. Then seeds were treated with 70% ethanol for 30 sec followed by washing with sterile distilled water twice. Finally surface sterilized seeds were cultured on MS medium supplemented with 3.0 mgL^{-1} GA_3 for *In vitro* germination.
MS medium [3] supplemented with 30.0 g/L sucrose and 8.0 g/L agar was used as the basal medium. The pH of the all media was adjusted to 5.8 ± 0.5. Temperature of the culture room was maintained at 25 ± 1 C^0 and PAR (Photosynthetically Active Radiation) was provided for 18 hours per day. There were at least 20 replicates in each treatment and growth regulators free MS medium was used as the control. Completely Randomized Design (CRD) was used in all experiments.

2.1 Determination of the effect of plant growth regulators on callus induction
Effect of different concentrations and combinations of PGRs on callus induction from leaf disc (1.0 cm^2) and internodal explants (1.0 cm) were determined. Leaf discs and nodal segments were taken from two weeks old *In vitro* germinated seedlings and they were inoculated into MS medium supplemented with different concentrations and combinations of BAP, NAA, 2.4-D and Kin. The swelling of the cut edges and number of days taken to initiate calli was observed daily and mean dry weight of callus was determined after six weeks of incubation.

2.2 Determination of the best explant source for callus induction
Leaf discs (1.0 cm^2), internodes (1.0 cm) and nodal segments (1.0 cm) from *In vitro* raised seedlings were used

as explants. MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA combination was used for this experiment. Swelling of the cut edges and initiation of callus was observed regularly. Color, nature of calli and number of days taken to initiate calli from different explant types was observed over a period of six weeks. Dry weight of calli obtain from each explant type was recorded after six weeks.

2.3 *In vitro* shoot induction from callus

Three weeks old leaf disc derived calli were cut into 1.0 cm^2 pieces and transferred into MS medium supplemented with different concentrations and combinations of BAP and NAA for shoot initiation. Number of days taken to initiate shoots, number of shoots per callus, mean shoot lengths were observed over a period of six weeks.

2.4 *In vitro* rooting of tissue cultured shoots

Six to eight weeks old *S. rebaudiana* multiple shoots (> 5.0 cm) were used for *In vitro* root induction. Healthily grown multiple shoots were carefully separated into single shoots. Then the shoots were transferred into half strength MS medium supplemented with different concentrations of IBA and NAA. Number of roots per shoot and root lengths (roots lesser than 1.0 cm were neglected) were measured after six weeks of incubation by taking rooted plantlets out of the vessel.

3. Results & Discussion

3.1 Determination of the effect of plant growth regulators on callus induction

Initiation of callus was observed after 6 days. At the beginning a swollen appearance was observed at the cut ends of the explants. The whole surface of the explants was covered with the callus within 5-6 weeks of incubation. Among all the treatments, MS medium supplemented with 2.0 mgL^{-1} BAP and 1.0 mgL^{-1} NAA (C2) found to be the best treatment for callus induction. It took 6.5 ± 0.85 mean number of days for calli initiation and highest mean dry weight of callus (2.97 ± 0.50 g). Other than C2 treatment, C5,C1 and C3 also took considerably lesser number of days for callus initiation and show high callus weight than all the other treatments.

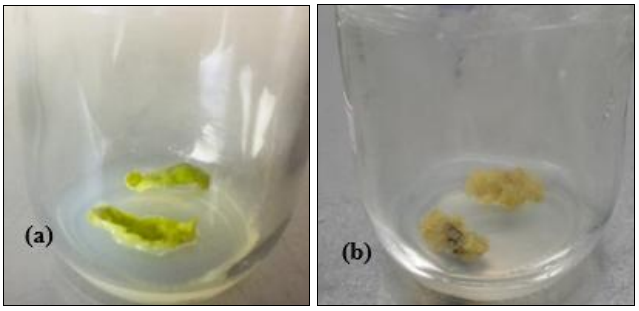

Fig 1: (a) Swelling of cut edges of leaf disc explants, (b) callus formation

Table 1: Effect of different plant growth regulator combinations used for callus induction

Treatment code	BA P conc. (mg/L)	NAA conc. (mg/L)	Kin conc. (mg/L)	2,4-D conc. (mg/L)	Mean no. of days to initiate calli± SD	Mean mass of calli after 6 weeks (g) ± SD
C0(Control)	0	0	0	0	0.0 ± 0.00	0.00 ± 0.00
C1	1	1	0	0	7.9 ± 0.88	2.76 ± 0.19
C2	2	1	0	0	6.5 ± 0.85	2.97 ± 0.50
C3	3	1	0	0	8.1 ± 0.99	2.47 ± 0.34
C4	1	2	0	0	8.5 ± 0.85	2.05 ± 0.24
C5	2	2	0	0	7.6 ± 0.84	2.72 ± 0.21
C6	3	2	0	0	9.6 ± 0.97	1.82 ± 0.33
C7	1	3	0	0	9.1 ± 1.19	1.95 ± 0.28
C8	2	3	0	0	8.1 ± 0.99	2.36 ± 0.29
C9	3	3	0	0	10.3 ± 1.06	1.57 ± 0.34
C10	0	0	0.5	0.5	12.2 ± 1.22	1.99 ± 0.27
C11	0	0	1	0.5	10.5 ± 1.08	2.25 ± 0.30
C12	0	0	2	0.5	11.8 ± 1.75	2.13 ± 0.21
C13	0	0	0.5	1	11.6 ± 1.08	2.02 ± 0.39
C14	0	0	1	1	12.6 ± 1.26	1.93 ± 0.35
C15	0	0	2	1	12.1 ± 1.10	1.96 ± 0.19
C16	0	0	0.5	2	14.5 ± 1.08	1.79 ± 0.20
C17	0	0	1	2	13.6 ± 1.35	1.86 ± 0.33
C18	0	0	2	2	13.2 ± 1.14	1.93 ± 0.32

Results showed that, MS medium supplemented with 1.0 mg/L Kin and 0.5 mg/L 2,4-D found to be the best treatment in Kin and 2,4-D combination indicating high Kin and low 2,4-D concentrations are favorable for callus initiation. According to the results all the treatment combinations of Kin and 2,4-D took longer period of time to initiate calli than BAP and NAA combination. Therefore BAP and NAA are more effective than Kin and 2,4-D for callus induction.

Previous studies also highlighted the importance of BAP, NAA, Kin and 2,4-D in callus induction and growth of Stevia. Sikdar *et al* [4] reported that, highest callus induction was obtained from Stevia explants cultured on MS medium supplemented with 2.0 mgL^{-1} BAP and 2.0 mgL^{-1} NAA. Another study reported that, Stevia explants showed 60% response when they were cultured on MS medium supplemented with 2.0 mg/L BAP and 0.8 mg/L NAA [5]. In both of these studies BAP and NAA were used as the PGR for callus induction and got better results which confirmed the results of the current study. Also BAP found to be the best cytokinin and 2.0 mg/L concentaration was found to be the best concentration of it.

3.2 Determination of the best explants source for callus induction

According to the observations, callus obtained from leaf disc explants was green in color and hard in nature. However the callus obtained from other types of explants (Intermodes and nodal segments) were greenish yellow in

color and friable in nature. According to the results, days taken to initiate calli from nodal segments (7.8 ± 1.03) were significantly lower than days taken to initiate calli from leaf (12.5 ± 1.58) and intermodal (13.5 ± 1.35) explants. Although leaf explants took more days to initiate calli than nodal segments, they have significantly higher dry calli weight (3.37 ± 0.14 g) after 6 weeks than other explants derived calli (nodal segments- 2.75 ± 0.16 g and internodes- 2.59 ± 0.09 g).

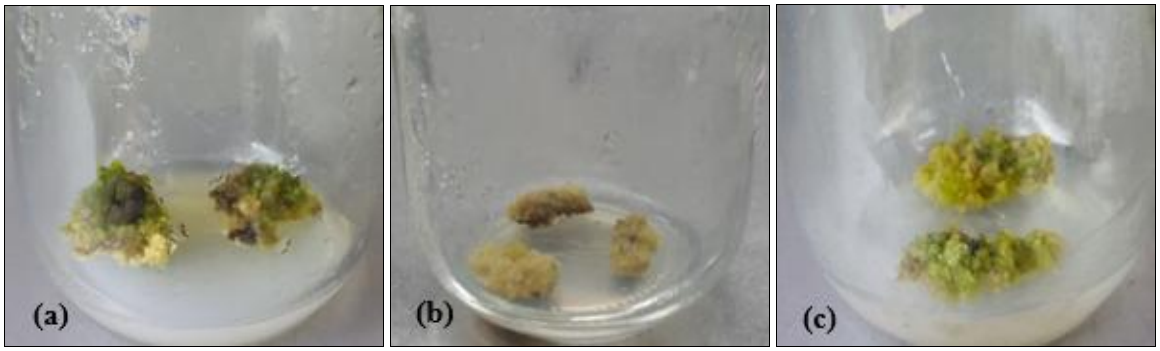

Fig 2: (a) Leaf callus, (b) intermodal callus, (c) nodal callus

Table 2: Effect of different explant types used for callus induction

Type of explant	Mean no. of days to initiate calli± SD	Mean dry mass of calli (g) ± SD
Leaves	12.5 ± 1.58	3.37 ± 0.14
Internodes	13.5 ± 1.35	2.59 ± 0.09
Nodal segments	7.8 ± 1.03	2.75 ± 0.16

Singh *et al* [1] reported that, calli obtained from leaf explants were hard and served as the best explants for callus production. Guruchandran and Sasikumar [6] reported that among the three explants (leaf, internode and nodes) best callus induction was occurred in leaf explant. In a similar study 100% callusing was obtain from leaf explants cultured on combination of NAA and 2,4-D [5]. Studies suggested that leaf explants could serve as a best explant for callus production and present study confirmed it. They also reported that, calli obtained from leaf explants were shiny green while with nodal explants it was yellow. Results of the present study confirmed it.

3.3 *In vitro* shoot induction from callus

Shoot regeneration in calli pieces were observed after 2 weeks of incubation. Among treatments, the best medium for the shoot regeneration from leaf disc explants was MS medium supplemented with 2.0 mgL^{-1} BAP and 0.5 mgL^{-1} NAA (S6). It took lowest number of days to initiate shoots (15.2 ± 1.13) and have highest mean number of shoots per explants (7.9 ± 0.56) and highest mean shoot length after 6 weeks (5.75 ± 0.48 cm). Other than S6 treatment, S5 (1.0 mg/L BAP and 0.5 mg/L NAA) and S9 (2.0 mg/L BAP and 1.0 mg/L NAA) also have significant effect on shoot regeneration from calli obtained from leaf discs.

Table 3: Effect of different plant growth regulator combinations used for *In vitro* shoot induction

Treatment code	NAA conc. (mg/L)	BAP conc. (mg/L)	Mean no. of days to initiate shoots ± SD	Mean no. of shoots ± SD	Mean shoot length (cm) ± SD
S0(Control)	0.0	0.0	0.0 ± 0.00	0.0 ± 0.00	0.00 ± 0.00
S1	0.5	0.0	29.8 ± 1.22	4.8 ± 0.91	3.65 ± 0.41
S2	1.0	0.0	29.1 ± 1.19	5.0 ± 0.66	3.80 ± 0.42
S3	2.0	0.0	28.7 ± 1.33	5.1 ± 0.99	4.35 ± 0.85
S4	0.5	0.5	31.3 ± 1.49	3.8 ± 0.78	3.45 ± 0.43
S5	0.5	1.0	16.8 ± 0.78	7.2 ± 0.91	4.85 ± 0.47
S6	0.5	2.0	15.2 ± 1.13	7.9 ± 0.56	5.75 ± 0.48
S7	1.0	0.5	32.7 ± 1.41	3.8 ± 0.78	3.45 ± 0.55
S8	1.0	1.0	19.8 ± 0.91	6.5 ± 0.85	4.65 ± 0.47
S9	1.0	2.0	17.5 ± 1.26	6.7 ± 1.16	4.85 ± 0.74
S10	2.0	0.5	33.7 ± 1.41	3.7 ± 0.94	3.35 ± 0.53
S11	2.0	1.0	21.6 ± 1.26	6.2 ± 1.31	4.35 ± 0.66
S12	2.0	2.0	22.1 ± 1.66	6.2 ± 0.78	4.45 ± 0.59
S13	0.0	0.5	35.3 ± 1.33	2.4 ± 0.84	2.45 ± 0.59
S14	0.0	1.0	36.7 ± 1.49	2.4 ± 0.84	2.15 ± 0.33
S15	0.0	2.0	36.9 ± 1.10	2.1 ± 0.87	2.15 ± 0.41

When consider the overall *In vitro* shoot induction it was observed that, as BA concentration increases and simultaneously decrease NAA concentration, the mean number of days to initiate shoots decreases while increase the mean number of shoots per cluster, mean shoot length and mean number of leaves per shoot. Specifically, when BA concentration is higher than NAA it increases the rate of shoot initiation.

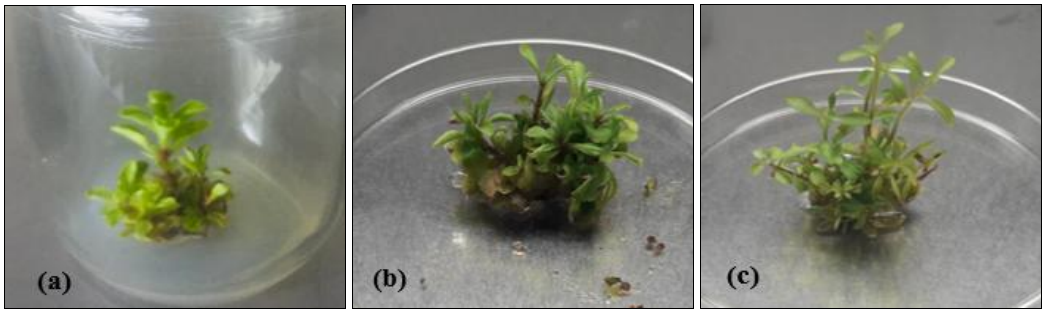

Fig 3: (a), (b), (c) stages of *In vitro* shoot initiation from callus

Organogenesis is an outcome of the process of dedifferentiation followed by redifferentiation of cells. The balance between exogenous auxin and cytokinin in the medium is essential for organogenesis. Thiyagarajan and Venkatachalam [2] studied the effect of different concentrations of BAP in combinations with various auxins on multiple shoot bud regeneration. Of the three auxins combinations tested (IAA/IBA/NAA), BAP with IAA combination was found to be best for induction of highest percent (92%) of multiple shoot bud development, followed by BAP and NAA (83%) and BAP along with IBA (75%) combinations.

According to Razak *et al* [7] BAP was proved to be the most efficient cytokinin for multiple shoot bud regeneration compared to KIN. Both the plant regeneration frequency and the number of shoot buds per culture increased with increasing the BAP concentration up to the 1.0 mg/l. However, shoot bud regeneration frequency as well as the number of shoot buds were declined when the BAP concentration was increased beyond 1.0 mg/l in the medium.

3.4 *In vitro* rooting of tissue cultured shoots

According to the results IBA showed significantly higher root formation than NAA. Half strength MS medium without any PGR (control) also showed root formation, but it is significantly lower than the root formation in media supplemented with PGRs. Half Strength MS medium supplemented with 0.5 mg/L IBA (R1) was found to be the best medium for *In vitro* root induction. It has the highest mean number of roots per explants (15.7 ± 1.49) and highest mean root length (4.64 ± 0.76 cm) which were significantly higher than the other treatments, after 6 weeks of incubation.

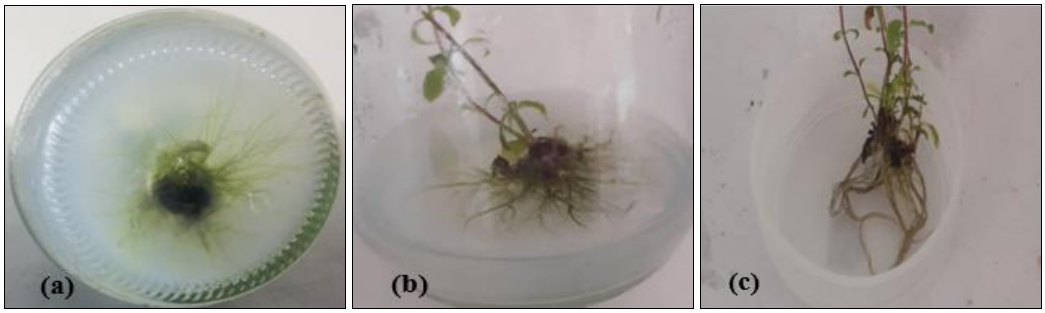

Fig 4: (a)(b)(c) *In vitro* root induction

Table 4: Effect of different plant growth regulator concentrations used for *In vitro* root induction

Treatment code	IBA conc. (mg/L)	NAA conc. (mg/L)	Mean no. of roots ± SD	Mean root length (cm) ± SD
R0 (Control)	0.0	0.0	5.8 ± 1.03	1.50 ± 0.52
R1	0.5	-	15.7 ± 1.49	4.64 ± 0.76
R2	1.0	-	12.9 ± 0.87	3.75 ± ± 1.16
R3	1.5	-	11.3 ± 0.82	2.75 ± 0.48
R4	-	0.5	10.7 ± 1.16	2.35 ± 0.62
R5	-	1.0	9.5 ± 1.26	2.45 ± 0.49
R6	-	1.5	10.5 ± 1.71	1.95 ± 0.59

Results indicate that lower concentrations of IBA and NAA induces root proliferation and root elongation. However, high concentrations of auxins inhibit root elongation and instead enhance adventitious root formation [8]. Newly developing parts of the roots were thin and white in colour and later became thick and brownish.

It is reported in literature that use of lower concentrations of MS salts and different types of auxins for the *In vitro* root induction. Sikdar *et al* [4] reported that, half strength of MS medium was more promising than full strength in root formation of *S. rebaudiana*. In every hormonal treatment, half strength of MS basal medium showed better root formation response than that of full strength. The highest response of 86.67 ± 6.67% rooting was found in half strength of MS basal medium supplemented with NAA 1.0 mg/L. According to Razak *et al* [7] the maximum number of roots (30.12 ± 2.1) induced was observed in the MS medium supplemented with 1.0 mg L-1 of IBA. The roots in the IBA-containing mediumwere thick and strong but short. Hwang [9] reported the maximum numbers of roots using a treatment of 1.0 mg L-1 IBA in the MS medium with up to 100% rooting. Tadhani *et al* [10] also obtained the highest rate of root induction in 1.0 mg/L IBA medium.

4. Conclusions

The best medium for callus induction is MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA and leaf disc is the best explants type for callus initiation. MS medium supplemented with 0.5 mg/L NAA and 2.0 mg/L BAP is the best medium for shoot induction and the best medium for root induction is half strength MS medium supplemented with 0.5 mg/L IBA.

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