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DIRECT ORGANOGENESIS OF *STEVIA REBAUDIANA IN VITRO* USING NODAL EXPLANTS

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ABSTRACT

Stevia rebaudiana Bertoni is a medicinal herb belonging to the family of Asteraceae. It is a natural sweetener plant, which is estimated to be 300 times sweeter than cane sugar. In this study, reliable protocol was developed for direct organogenesis of *S. rebaudiana* using *in vitro* derived nodal explants. Seeds were collected from mother plants and they were surface sterilized. To optimize the surface sterilization procedure, dark color (fertile seeds) seeds were surface sterilized using different concentrations and in different exposure time of carbendazim and sodium hypochlorite (Clorox). Out of different combinations 0.2% carbendazim for 5 minutes, 10% sodium hypochlorite for 10 minutes and 70% ethanol each followed by two successive washings in sterile distilled water was found to be the best for surface sterilization. Two sets of seeds (fresh, stored) were cultured on MS basal medium supplemented with different concentrations of GA3 for seed germination. According to the results seed viability was lost with time and it affected seed germination. Seed germination was not affected by GA3, but seedling height was affected by it. Seeds germinated on MS medium supplemented with 3.0 mg/L GA3 showed the highest seedling height after 10 days. MS basal medium supplemented with different concentrations of BAP and Kin were tested for shoot bud and multiple shoot induction. Out of different media Ms basal medium supplemented with 2.0 mg/L BAP was found to be the best medium for shoot bud and multiple shoot induction within 60 days.

Keywords: *Stevia rebaudiana, surface sterilization, seed germination, shoot induction, direct organogenesis.*

INTRODUCTION

Stevia rebaudiana is a herbaceous plant belongs to Family Asteraceae. It's also known as sweet leaf herb or honey plant due to its sweetness. It's about 300 times sweeter than cane sugar. The chemical compounds that produce its sweetness are steviol glycosides and there are seven major sweetener compounds and among them Rebaudioside A and Stevioside are the most important two (Singh *et al*., 2017). They are non caloric sweeteners and can be used as an alternative to sugar

and synthetic sweetening agents. Therefore this could be considered as the best alternative natural sweetener for diabetic patients. In addition to its non caloric sweetening property it has many therapeutic values such as anticancer, antimicrobial and anti-inflammatory activity. Therefore Stevia is used as food supplement and sweetener in countries like USA, Japan, Brazil and China (Yadav *et al*., 2011). The demand for sugar in Sri Lanka is likely to go up in coming years. Therefore this will elegantly meet the requirement of sugar in Sri Lanka including demand in pharmaceuticals and soft drink industries.In natural conditions, percentage seed germination is poor and unsuccessful due to small endosperm and infertile seeds. There are reports about propagation of Stevia through stem cuttings, but direct planting of them in field has limitations due to poor rooting (Yadav *et al.,* 2011). Plant regeneration from *in vitro* culture can be achieved by either organogenesis or embryogenesis. Supplement of different plant growth regulators enhances and accelerates the production of *in vitro* plants with good agronomical traits and steviosides content in leaves (Singh *et al*., 2017).

MATERIALS AND METHODS

Mother plants were collected and were maintained in a shade house.

MS medium 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° 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.

Optimizing surface sterilization protocol for seeds

Seeds were collected from mother stock maintained in department shade house and were initially washed with few drops of liquid detergent for 5 minutes followed by running tap water for 30 minutes. After that they were treated with a fungicide (Carbendazim) and Clorox. Each step was followed by two successive washings with sterile distilled water. Then seeds were treated with 70% ethanol for 30 seconds followed by washing with sterile distilled water twice. Finally surface sterilized seeds were cultured on growth regulators free MS medium. To determine the suitable surface sterilization method, effect of 2.0% carbendazim with two different exposure time and Clorox at two different concentrations with different exposure time were tested. Contamination percentage, survival percentage and germination percentage were recorded and best method was used for further experiments.

In vitro seed germination

Seeds were collected from mother plants and two sets of seeds (fresh and ten days stored) were used for the experiment. They were surface sterilized and cultured on MS medium supplemented with different concentrations of (1.00 mg/L- 3.00 mg/L) GA3. Germination percentage and mean shoot length after 10 days were recorded.

Multiple shoot induction *in vitro*

Nodal segments were used as the explants source and taken from two weeks old *in vitro* germinated seedlings. Seedlings were carefully taken out from the culture bottles and approximately 1.0 cm length nodal segments were prepared. They were cultured on MS medium supplemented with different concentrations of (0.5 mg/L - 2.5 mg/L) BAP and (0.5 mg/l-2.5 mg/L) Kin. Number of shoots per explants and mean shoot length after 30 days were recorded.

RESULTS AND DISCUSSION

Optimizing surface sterilization protocol for seeds

After seven days of incubation, seeds in growth regulator free MS medium, it was observed that 0.2% carbendazim with 5 minutes exposure time and 10% Clorox with 10 minutes exposure time (T3) was the most suitable surface sterilization protocol based on the visual observations of the contamination, germination and survival percentage of seeds. It showed low percentage contamination (16.0%) together with highest percentage germination of seeds (61.9%). Though percentage contamination of the seeds treated with 0.2% carbendazim for 10 minutes and 15% Clorox for 10 minutes was zero, none of the seeds were germinated. It was observed that when the Clorox concentration and exposure time increase, percentage contamination decreases, but showed adverse effects on seed viability (Table 1). In addition, treatment T4, T5 and T6 also showed considerably higher germination, which is higher than 45.0% compared to the other treatments.

Table 1: Percentage contamination, percentage survival and percentage germination of seeds with different treatments used for surface sterilization

Treatment code	Conc. Of Clorox (v/v)	Exposure time of Clorox	Exposure time of 0.2% carbendazim (min)	% Contamination	% Survival	% Germination
T1	10 %	5	5	76.0	24.0	16.67
T2	10 %	5	10	68.0	32.0	12.50
T3	**10 %**	**10**	**5**	**16.0**	**84.0**	**61.90**
T4	10 %	10	10	36.0	64.0	50.00
T5	15 %	5	5	28.0	72.0	55.56
T6	15 %	5	10	32.0	68.0	47.05
T7	15 %	10	5	4.0	96.0	0
T8	15 %	10	10	0	100.0	0

Contamination is one of the major problems in plant tissue culture. Therefore determination of suitable sterilization method is necessary for successful establishment of protocol. Urbi and Zainuddin (2015) reported that, effect of Clorox in disinfecting of Stevia is time and concentration dependent and results of the present study confirmed the same fact.

In vitro seed germination

After 10 days of incubation, it was observed that fresh seeds shown higher percentage germination in all media than 10 day stored seeds (Table 2). Seedling height was increased with GA_3 in both types of seeds. Therefore the results indicate that seed storage affect their viability and GA_3 only affect in seedling height. The highest mean seedling height after 10 days was observed in seeds cultured on MS medium supplemented with 3.0 mg/L GA_3 (T4) in both types of seeds (4.38 cm for fresh seeds, 4.10 cm for 10 days stored seeds). Therefore it was the best medium for *in vitro* seed germination (Figure 1).

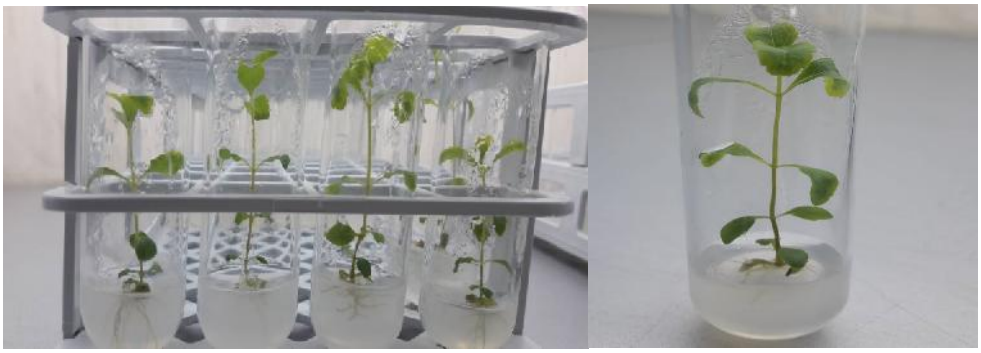

Figure 1*: In vitro* germinated seedlings on MS medium supplemented with 3.0 mg/L GA_3

Gunasena and Senarath, (2017) reported that the height of the *in vitro* germinated *Punica granatum* seedlings exponentially increase with the increase in GA_3 concentration in MS medium. Present study also showed an increase in seedling height with increase of GA_3 concentration.

Table 2. Percentage seed germination and mean seedling height of the seedlings after 10 days in the presence of GA_3

Treatment code	Seed type	GA_3 (mg/L)	% Germination ± SD	Mean height after 10 days (cm) ± SD
T1	Fresh seeds	0.0	52.0 ± 0.51	1.49 ± 0.01
T2	Fresh seeds	1.0	56.0 ± 0.50	3.41 ± 0.14
T3	Fresh seeds	2.0	64.0 ± 0.49	3.94 ±0.09
T4	**Fresh seeds**	**3.0**	**72.0 ± 0.46**	**4.38 ± 0.14**
T5	10 days stored seeds	0.0	20.0 ± 0.41	1.38 ± 0.08
T6	10 days stored seeds	1.0	20.0 ± 0.41	3.76 ± 0.09
T7	10 days stored seeds	2.0	20.0 ± 0.41	3.90 ± 0.1
T8	10 days stored seeds	3.0	24.0 ± 0.44	4.10 ± 0.17

Multiple shoot induction *in vitro*

After 4 weeks of incubation, all the nodal segments cultured on MS medium supplemented with BAP showed higher number of shoots per explants and higher mean shoot length than nodal segments cultured on MS medium supplemented with Kin (Table 3). According to the results, number of shoots per explants and mean shoot length increase with BAP concentration up to 2.0 mg/L. Results indicated that shoot induction was also affected by Kin, but not vary with its concentration. Therefore the best medium for multiple shoot induction was MS medium supplemented with 2.0 mg/L BAP (T4) and there were 8.20 shoots per explants and mean shoot length was 3.94 cm (Figure 2).

Figure 2: Multiple shoots obtained from nodal segments cultured on MS medium supplemented with 2.0 mg/L BAP

The presence of cytokinins in the medium was essential to induce bud break and shoot proliferation from nodal explants. Out of the two cytokinins used, BAP was found to be more effective than KIN for shoot bud development from nodal explants (Thiyagarajan and Venkatachalam., 2012). Debnath (2008) reported that the maximum shoot proliferation was in 2 mg/L BAP and it confirms the observations in the present study as well.

Table 3. Number of shoots per explants, mean shoot length after 30 days in media with different concentrations of BAP and Kin

Treatment code	BAP (mg/L)	Kin (mg/L)	Number of shoots per explants ± SD	Mean shoot length after 30 days (cm) ± SD
T1	0.5	-	5.78 ± 0.09	2.53 ± 0.12
T2	1.0	-	6.05 ± 0.13	2.67 ± 0.13
T3	1.5	-	6.91 ± 0.12	3.38 ± 0.13
T4	**2.0**	**-**	**8.20 ± 0.12**	**3.94 ± 0.14**
T5	2.5		5.19 ± 0.14	2.54 ± 0.13
T6	-	0.5	4.81 ± 0.12	2.43 ± 0.13
T7	-	1.0	4.52 ± 0.13	2.37 ± 0.12
T8	-	1.5	4.32 ± 0.10	2.28 ± 0.12
T9	-	2.0	4.36 ± 0.14	1.97 ± 0.09
T10	-	2.0	4.25 ± 0.18	1.57 ± 0.11

CONCLUSION

The best surface sterilization protocol for *S. rebaudiana* seeds is 0.2% carbendazim with 5 minutes exposure time and 10% Clorox with 10 minutes exposure time. Fresh seeds have higher seed germination percentage than 10 days stored seeds and it indicates seed storage affect their viability. Tissue culture practices revealed that GA_3 only affect in seedling height and highest mean seedling height can be obtain *in vitro* using MS medium supplemented with 3.0 mg/L GA3. Best medium to obtain highest number of shoots per explant and highest mean shoot length is MS medium supplemented with 2.0 mg/L BAP.

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