Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 6 Special issue [3] 2017: 442-446

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804
NAAS Rating 4.95

FULL LENGTH ARTICLE **OPEN ACCESS**

Micropropagation of Sweet Herb *Stevia rebaudiana* through Leaf Explant

N. S. Chavan*, S. D. Mardane, V. J. Pachpend
Department of Plant Biotechnology, K. K. Wagh College of Agril. Biotechnology, Nashik, Maharashtra.
Email. nschavan@kkwagh.edu.in , vpachpend123@gmail.com

ABSTRACT
The present investigation entitled micropropagation of Stevia rebaudiana was conducted to study micropropagation stages of Stevia rebaudiana, rapid multiplication of Stevia rebaudiana within short span time, to produce large number of callus and to produce genetically identical plant i.e. homozygous plant of Stevia rebaudiana. Micropropagation was done by using leaf segment as explants which were initiated on Muashige and Skoog (1962) media supplemented with four different concentration of BAP (1.0, 1.5, 2.0, 2.5 mg/l) combination with NAA (1.0, 1.5, 2.0, 2.5 mg/l) after the three weeks of initiation maximum number of calls was obtained in MS medium supplemented with 2.0 mg/l BAP and 2.0 mg/l NAA. (Average number of callus 10 and survival percentage was 80%). Callus was regenerated from leaf explants of Stevia rebaudiana. Best shoot induction was observed medium containing 5.0 mg/l BAP were highest callus per explants. The subculture of shoot was done on Muashige and Skoog media supplemented with different concentration of IAA (2.0 mg/l) and in combination with kinetin (5.0 mg/l) for the root formation.
Key words: *Stevia rebaudiana, micropropagation, callus segment, MS media, BAP, kinetin and NAA.*

Received 28.07.2017 Revised 07.08.2017 Accepted 27.08. 2017

INTRODUCTION

Stevia rebaudiana (2n=22) is a small, herbaceous, semi bushy, perennial shrub of belongs to family "*Asteraceae*", native to the Amambay region in the North East of Paraguay. It occurs in the neighbouring parts of Brazil and Argentina (Soejarto, 2002) and it became known by the European due to its discovery by Moises Bertonii in 1899. It is a natural sweetener plant commonly known as "Sweet Weed", "Sweet Leaf", "Sweet Herbs" or "Honey Leaf" (Chalapathi and Thimmegowda, 1997; Liu and Li, 1995; Inamake *et al*., 2010; Mandaca *et al*., 2012). Total number of species in this genus estimated to 150-300 (Ibrahim *et al*., 2008) which are distributed in the new world, from the South-Western United States to the Northern Argentina. Stevia is a diploid plant, having 11 pairs of chromosomes (Frederico *et al*., 1996) with critical day length of 13 hours (Zaidan *et al*., 1980). It is self-incompatible plant and the pollination behaviour is entomophilous (Yadav and Singh, 2010). The plant growth requires mild temperature between 15^0C & 38^0C and relative humidity of about 80 per-cents. *Stevia rebaudiana* plant has antibacterial effect and impedes the growth of bacteria. *Stevia* is anti-inflammatory (Dr. David Jockers, 2015), antimicrobial (Enketeswar Subudhi, 2008), antiviral (Yasukawa *et al*., 2002), anti-hypertension (LA Ferri, 2006) and anti-rotavirus (Boonkaewwan *et al*., 2008). It is also cardiotonic, hypoglycemic, hypotensive, vasodialator and diuretic (Ferri *et al*., 2006). Steviol and isosteviol may also offer therapeutic benefits, as they have anti-hyperglycemic, antihypertensive, anti-fungal, anti-tumour, antidiarrhoeal, diuretic, and immunomodulatory effects (Chatsudthipong & Muanprasat, 2009). Stevia has many diversified uses in confectionary, bakery, beverages and medicines. It also contains natural trace elements like calcium, magnesium and iron. *Stevia rebaudiana* is available as a sweetener in Japan, South Korea, Malaysia, Taiwan, Russia, Israel, Mexico, Paraguay, Uruguay, Venezuela, Columbia, Brazil, and Argentina.

Micropropagation helps to accelerate large scale multiplication, improvement and conservation of the plant. The main advantage of micropropagation is the production of many plants that are clones of each other. It is the only viable method of regenerating genetically modified cells or cells after protoplast fusion. It is useful in multiplying plant which produces seeds in uneconomical amounts, or when plants are sterile and do not produce viable seeds or when seed can't be stored. Limited work has been done on

micropropagation of *Stevia rebaudiana* species. However, the multiplication rate achieved by this investigation was very low. The objective of current study was to develop a large scale production and aseptic growth of *Stevia rebaudiana*. Tissue culture of such important stevia species is recommended for faster growth and large scale production in minimum time period.

MATERIALS AND METHODS

Materials

Mother plant was taken from Department of Olericulture, Kerala Agriculture University (KAU). Different explants that are newly formed shoots of stevia plant and leaves were used for study. The chemicals used for present study were of good quality from various agencies including Hi media, Flesher and Research laboratory. For *in vitro* culture experiments Murashige and Skoog (1962) basal salts were used. For the standardization of *in vitro* plant regeneration, different auxins like 1-naptheleneacetic acid (NAA), and cytokinins like 6-benzyl amino purine (BAP) at different concentrations were used.

Methodology

Collection of explants and surface sterilization

Initially the explants of Stevia plant were excised from plants maintained insect and disease free at field with blade and nodal segments were cleaned under running tap water for about 15 to 20 min. each under laminar air flow hood and followed by three times rinsing in sterile distilled water. Axillary bud and stem segments were further soaked in fungicide (Bavestin) solution (0.1 %) for 1to 2min. and then again washed with sterile distilled water. Subsequectly they were dipped in 70% Ethanol for 2min and mercuric chloride with 1to 2 ml Tween 20 for 20 min thereafter they wash with four times of sterilized distilled water complete sterilization of Axillary bud and stem explants.

Inoculation of nodal and shoot tip explants

MS medium fortified with different growth hormones were tested for micropropagation. For callus induction MS media fortified with BAP, was tested separately. The explants were stem segment (with axillary bud) and leaves segment were excised with sterilized forceps and scalpels under laminar air flow and 3-4 explants were inoculated on a single bottle. Bottles were incubated under 16 h photo periods at 25 ± 2^0C. Explants were sub cultured on same medium at 30 days interval.

Callus multiplication

The multiplication of callus within 10-20 days of subculturing in the same media and hormone concentration required to be multiplied rapidly for that it was cultured on MS media with same or different hormone (BAP) concentration which shows development of new shoot and multiplication of culture in some hormone treatment. That used to find out in which concentration the multiplication rate was higher. MS with BAP mg/l shows significant rate of multiplication among all treatment within 40-50 days.

Rooting

Multiplied shoots were transferred on MS (Half) with different growth hormone auxin (IAA + kinetin) treatment was tried out to find out which one shows higher rooting. After inoculation of shoot rooting was observed in days which is kept for about 15 days and then prepared for hardening for farm of greenhouse. Transfer the plantlet explants in potting mix comprising of coco peat and garden soil pH 5.4 in hardening glasses.

Cultural conditions

After inoculation of the nodal and shoot tip explants in vitro its observed that the regeneration of shoot requires typical photoperiod (16 hrs) with 25°C temperature and in the case of multiplication favorable effect of controlled culture condition during *Stevia rebaudiana* multiplication (pH 5.8), with a temperature range of 25±2°C, 16 hrs photoperiod, 60% RH and 1000-2000 lux light intensity. The *Stevia rebaudiana* explants were observed thoroughly for contamination.

RESULTS AND DISCUSSION

The explants were sterilized by using different chemical and a standard protocol was developed for sterilization of *Stevia rebaudiana* explants. Observations of inoculated explants after 3, 5 and 7 days showed contamination free culture. For this treatment of $HgCl_2$ (1 mg/l) used for 10 min was given to avoid fungal and bacterial contamination respectively. Under aseptic conditions, leaves and nodal segment were removed from the mother plants without any damage. The explants were inoculated on MS media containing different concentration of growth hormone (BAP, NAA and kinetin). The cultures were then incubated at 25 ± 2^oC with a photoperiod regime of 16 hr at 1000 lux light intensity and at 50 to 60% humidity. Results present in table 4.1 and 4.2 clearly indicate the significant effect of growth regulator concentration on establishment of explants for callus induction from leaf segment (Fig. 1, C). MS medium subjected with 2.0 mg/l concentration of BAP was better suited for callus induction as compared to the

nodal segment. When the leaf explants were inoculated on MS medium fortified with 2.0 mg/l BAP and 2.0 mg/l NAA showed best result for callus induction (Table 2 ; Fig. 1, C). Nodal segment explants (Fig. 1, AB) showed lowest initiation of shoot as compared to leaf explants. MS medium subjected with BAP in concentration of 1.5 mg/l and 2.0 mg/l have shown shoot induction after 28 days (Fig. 1, D; Table 3). BAP was found to be best growth regulator when used in combination with NAA. The callus initiations were subjected to the regeneration on the different media and the growth was observed after 4 weeks of sub culturing. The media with all concentration of BAP and NAA showed multiple shoot regeneration and proliferation. The average no. of shoots formed on all concentration of BAP and NAA was ranging from 1 to 2 shoots per callus. Best growth was observed on the 5 mg/l BAP concentration where the average no. of shoots generated per callus was 2. The result showed that the ratio of no. of shoot formed per explants was increased with the increasing concentration of BAP and NAA. The BAP promotes cell division and shoot formation. (Fig 1, D; Table 3). For root regeneration studies, shoots were transferred in MS media with IAA and kinetin. In present investigation, rooting was observed from cultures on MS medium supplemented with 2.5 mg/l IAA in combination with 0.5 mg/l kinetin (Fig 1, E; Table 4).

Table No. 1: Effect of different concentration BAP on callus induction from leaf segment of *Stevia rebaudiana*. Data was recorded after four weeks of culture.

Supplement (BAP mg/l)	Type of explants inoculated	No. of explants inoculated	No. of explants regenerated	% of explants induced callus	Weight of callus (mg)	Days of shoot initiation
0.5	Leaf segment	10	-	0	-	-
1.0	Leaf segment	10	-	0	-	-
1.5	Leaf segment	10	3	30	42.2	20
2.0	**Leaf segment**	**10**	**5**	**50**	**61.0**	**20**
2.5	Leaf segment	10	-	0	-	-

Table No. 2: Effect of BAP combine with NAA on callus induction from leaf segment of *Stevia rebaudiana*. Data was recorded after four weeks of culture.

Supplement (BAP + NAA mg/l)		Type of explants inoculated	No. of explants inoculated	No. of explants regenerated	% of explants callus induction	Weight of callus (mg)	Days of shoot initiation
BAP	NAA						
2.0	1.0	Leaf segment	10	4	40	68.0	21
2.0	1.5	Leaf segment	10	6	60	72.0	21
2.0	**2.0**	**Leaf segment**	**10**	**8**	**80**	**134.5**	**21**
2.0	2.5	Leaf segment	10	7	70	83.3	21

Table No. 3: Observation of various combination of hormone BAP on shoot formation by using callus culture of *Stevia rebaudiana* after 3 weeks.

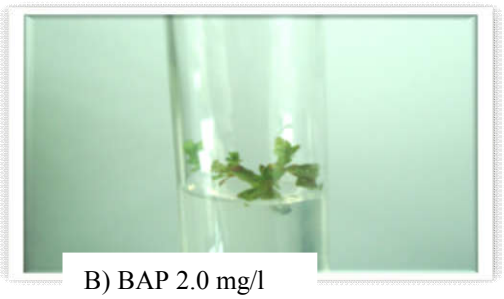
Sr. No.	Source of culture	MS + concentration of growth hormones (BAP mg/l)	No. of shoots	Length of shoot (cm)	Performance
1	Callus of *Stevia rebaudiana*	1.0	-	-	-
2		2.0	1	2.1	+(Fair)
3		3.0	1	2.6	+ (Fair)
4		4.0	2	4.2	++(Good)
5		**5.0**	**3**	**6.4**	**+++(Very good)**

Table No. 4 : Observation of various combination of hormone IAA combine with kinetin on root formation of *Stevia rebaudiana* after 3 weeks.

Sr. No.	Source of culture	MS + concentration of growth hormones (mg/l)		No. of root	Length of root (cm)	Performance
		IAA	Kinetin	-	-	-
1	Callus of *Stevia rebaudiana*	1.0	0.5	1	2.0	+(Fair)
2		1.5	0.5	1	2.3	+(Fair)
3		2.0	0.5	1	3.2	+(Fair)
4		**2.5**	**0.5**	**8**	**4.5**	**+++++(Excellent)**
5		3.0	0.5	-	-	-

Fig. 1: Regeneration of *Stevia rebaudiana* A, B) Shoot induction from nodal explants C, D, E) Callus, shoot and root regeneration

A) BAP 1.5 mg/l



B) BAP 2.0 mg/l

C) Callus Induction

D) Shooting regeneration

E) Root regeneration

CONCLUSION

Stevia Rebaudiana is an important medicinal plant. It is becoming a vulnerable species due to its low seed germination percentage and poor seed viability. The methods of vegetative propagation are not efficient to save this rare species. The productions of large numbers of stevia plant were possible through micropropagation technique. In *Stevia rebaudiana* MS medium containing 2.0mg/l BAP + 2.0 mg/l NAA was most suitable for callus multiplication. I also observed improvement in callus multiplication by different concentration of BAP (1.0, 1.5, 2.0, 2.5, 3.0 mg/l) with IAA (1.0, 1.5, 2.0, 2.5, 3.0 mg/l). Best shooting response was observed on medium containing 5.0 mg/l BAP. Best root formation was observed on MS media containing IAA (2.5 mg/l) in combination with kinetin (0.5 mg/l).

REFERENCES

1. Boonkaewwan C., Ao M., Toskulkao C. and Rao M. C. **(2008)**. Specific immunomodulatory and secretory activities of stevioside and steviol in intestinal cells. *J. Agric. Food Chem*. **56(10):** 3777-3784.
2. Chalapathi M. V. and Thimmegowda S. **(1997)**. Natural non-calorie sweetener in stevia (*Stevia rebaudiana* Bertoni)**:** A future crop of India. *Crop Res. Hisar.* **14(2):** 347-350.
3. Chatsudthipong V. and Muanprasat C**. (2009)**. Stevioside and related compounds**:** Therapeutic benefits beyond sweetness. *Pharma. Therap*. **121:** 41-54.
4. Ferri L. A., Alves D. P. W., Yamada S. S., Gazola S., Batista M. R. and Bazotte R. .B. **(2006)**. Investigation of the antihypertensive effect of oral crude stevioside in patients with mild essential hypertension. *Phytother. Res*. **20:** 732-736.
5. Frederico A. P., Ruas P. M., Marinmoralaes M. A., Ruas C. F. and Nakajima J. N. **(1996)**. Chromosome studies in some *Stevia (Compositae)* species from southern Brazil. *Braz. J. Genet*. **19:** 605-609.
6. Ibrahim A. I., Nasr I. M., Mohammed R. B and El-Zefzafi M. M. **(2008)**. Plant growth regulators affecting *in vitro* cultivation of *Stevia rebaudiana. Sugar tech.* **10:** 254-259.
7. Ibrahim I. A., Nasr M. I., Mohammad B. R. and El-Zafzafi M. M. **(2008)**. Nutrient factors affecting *in vitro* cultivation of *Stevia rebaudiana*. *Sugar Tech*. **10(3):** 248-253.
8. Inamake M. R., Shelar P. D., Kulkarni M. S., Katekar S. M. and Tambe R. **(2010)**. Isolation and analytical characterization of stevioside from leaves of stevia rebaudiana Bert: (Asteraceae). *Int. J. Res. Ayu. Pharm*. **2:** 572-581.
9. Liu J. and Li S. F. Y. **(1995)**. Separation and determination of stevia sweeteners by capillary electrophoresis and high performance liquid chromatography. *J. Liq. Chromato.* **18 (9):** 1703- 1719.

10. Soejarto D. D., Kinghorn A. D. and Farnsworth N. R. **(2002)**. Potential sweetening agents of plant origin.II. Field search for Sweet tasting Stevia species. *Econ. Bot.* **37:** 71-79.
11. Yadav K., Singh N. and Aggarwal A. **(2011)**. Influence of arbuscular mycorrhizal (AM) fungi on survival and development of micropropagated *Acorus calamus* L. during acclimatization. *Int. J. Agric. Technol*. **7(3):** 775-781.
12. Yasukawa K., Kitanaka S. and Seo S. **(2002)**. Inhibitory effect of stevioside on tumor promotion 12-Otetradecanoylphorbol- 13-acetate in two-stage carcinogenesis in mouse skin. *Biol. Pharm. Bull*. **25:** 1488-1490.
13. Zaidan L. B. P., Dietrich S. M. C. and Felippe G. M. **(1980)**. Effect of photoperiod on flowering and stevioside content in plants of *Stevia rebaudiana Bertoni*. *Jap. J. Crop Sci*. **49:** 569-574.

CITATION OF THIS ARTICLE

N. S. Chavan, S. D. Mardane, V. J. Pachpend. Micropropagation of Sweet Herb *Stevia rebaudiana* through Leaf Explant. Bull. Env. Pharmacol. Life Sci., Vol 6 Special issue [3] 2017: 442-446