Journal of Agricultural Technology 2013 Vol. 9(7):2005-2011
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Direct regeneration from axillary bud explants of *Stevia rebaudiana* Bertoni- A medicinal plant

Shende, C.B[*]. and Manik, S.R.

Mohasinbhai Zaveri Mahavidhyalaya Desaiganj (Wadsa) Dis Gadchiroli, P.G. Department of Botany SGB Amravati University Amravati- 444602 India

Shende, C.B. and Manik, S.R. (2013) Direct regeneration from axillary bud explants of *Stevia rebaudiana* Bertoni- A medicinal plant. Journal of Agricultural Technology 9(7):2005-2011.

Young axillary buds were used as explants for rapid multiplication of *Stevia rebaudiana*. The explants were cultured on medium containing basal salts of Murashige and Skoogs (MS) and various concentrations of BAP for shoot induction. Treatment containing 1.50 mg/*l* BAP produced higher mean number of shoots per explants. The regenerated shoots were successfully rooted on MS medium supplemented with 1.00 mg/*l* NAA. The rooted plantlets were acclimatized on sterilized Soil, Sand and Manure at 1:1:1 ratio. A total of 80% plants survived through this process.

Keywords:*Stevia rebaudiana*, multiplication, growth regulator.
Abbreviations: BAP – (Benzyl amino purine), 2,4-D – (2,4 Dichlorophynoxy acetic acid), MS – (Murashige and Skoog (1962) basal medium), NAA-(α-napthalene acetic acid).

Introduction

The word wide demand for high potency sweeters are considerably increasing in recent years with the introduction of stevia as modern crop. *S. rebaudiana* is a perennial herb of family Asteraceae commonly known as candy leaves or natural non-caloric sweet plant which was officially discovered by Dr. M.S. Bertoni during early 20th century. It could be found growing in semi-arid habitat ranging from grassland to scrub forest to mountain terrain. The plant has gained wide access to pacific Rim countries, where in recent decades it is being cultivated domestically (Lewis, 1992). Stevia plants grow better having temperature range of 0-40°C. So these conditions allow stevia growing annually in areas having diverse environment. However, its cultivation on commercial basis is by seeds (Carneiret *et al.*, 1997). The beneficial effects focused the importance of stevia and its availability throughout the year, but the main problem lies with the stevioside content of stevia plants raised through

[*]Corresponding author: Shende, C.B.; e-mail: shendecb@gmail.com

seeds which varied widely (Das *et al.*, 2006). Now a days, Stevia plants and stevioside are being used as sweeteners in South America, Asia, Japan, China and some countries of Europe. The Stevia is more frequently used in the food industries of Japan, Korea and Brazil (Gupta *et al.*, 2010) To avoid such segregation and also to improve the yield of stevioside it is necessary to propagate a genetically homogeneous population from selected elite plants of desirable characters. *In vitro* techniques can serve as an alternative method to conventional techniques which give disease free and resistant plants (Nepovim and Vanek, 1998). The micro propagation of plants through shoot tip or axillary bud culture allows recovery of genetically stable and true to true progeny (George and Sherrington, 1984).

There are few reports of *In vitro* clonal propagation stevia using leaf, nodal, intermodal segment and shoot tips (Yukiyoshi *et al.*, 1984, Salim *et al.*, 2006). A stevia leaf produces a variety of high potency natural source, low calorie (non sucrose) sweeteners with 300 times sweeter than sucrose (Soeiarto *et al.*, 1983). These sweet compounds passes through the digestive process without chemically breaking down, making safe to control sugar level (Strauss, 1995).

Materials and methods

The *in vitro* clonal propagation of *S. reabaudiana* was carried out at Department of Botany J.B. College of Science, Wardha. The axillary buds were surface sterilized with 0.2% $HgCl_2$ solution for 5-6 minutes, washed with sterile distilled water for three times and inoculated on simple MS medium with various concentration of BAP. All the cultures were placed under light in growth room at 25± 2°c. For *in vitro* shoot multiplication the initiated shoots were sub cultured on MS medium containing 3% sucrose and 0.5, 1.0, 1.5, 2.0 Mg/L BAP. While for root induction excised micro shoots (3.0-3.5 cm) were cultured on MS medium supplemented with 2.0% sucrose and 0.25, 0.50, 1.0, 1.5 & 2.00 Mg/L NAA. All the cultures were incubated in growth room at 25± 2°c. with photoperiod of 16h and 2500 lux white fluorescent light. The rooted plantlets were first transferred to plastic pots containing garden soil, sand and manuare in (1:1:1) and placed in humidity chamber at 2.8± 2°c temperature and 70-90% humidity. After two weeks, the plants were then shifted in shade house under low light intensity.

Results and discussions

In vitro proliferation and multiplication

The axillary buds of *S. rebaudiana* proliferated within 5-6 days of culture on BAP supplemented MS medium.In *Stevia rebaudiana* the explants were properly sterilized and established when treated with 0.5% $HgCl_2$ at 3-5 minutes. Krishnan *et al.* (1995) reported the exposure to 0.05-.1% $HgCl_2$. The exposure to 0.05-0.1 % HgCl2 for more than 7 minutes was lethal to the tissue although it reduced the percentage of contamination significantly in *Trichopus zeylanicus.* When the explants were culture on 0.25 to 1.50 mg/L BAP containing media 70-80% of them produced shoots. The axillary shoots proliferated and elongated 2.5-3.5cmwith in 3 weeks. In these experiment MS medium containing 1.0 mg/L BAP showed the response of multiple shoot formation with 3.40 0.24 microshoots per explants (Table1).The BAP at1.0 mg/l improved the response of shoot multiplication and higher concentration of BAP above 1.0 mg/l decreased the number of shoots (Anand and Hariharan, 1997). When explants were cultured on BAP 1.50 mg/l only 35-50% was proliferated to shoots. Of all the concentrations, the best concentration was 1.0 mg/l BAP. Other researcher also reported the influence of BAP on shoot multiplication in different plant species.The shoot tips proliferated and elongated with in three weeks of culture on MS medium fortified with with 2.0 mg/l (Francis *et al.*, 2007). Bondarev (2001) reported that micro cuttings with apical or axillary were most effective explants for large scale multiplication. When cultured on the hormone free MS medium and this allowed producing the plants by 3-4 months. The addition of little concentrations of BAP to the MS medium was established to induce adventitious shoot formation by increasing a propagation coefficient. Sivaram and Mukundan (2003) reported the efficient regeneration of shoot apex, nodal and leaf explants of *s. rebandiana* when cultured on MS medium supplemented with 8.87 µm BAP. Effectiveness of BAP on the Clonal multiplication reported by Balachandran*et al* (1990) in Curcuma and Zingiber (MS + 3.0 mg/L BAP), Hosoki and sagawa (1977) in Ginger (MS+1ppm BA) and Change and Criley (1993) in *Alpinia purpurata* and A. *calcarata* also shows a specific effect of BAP on its Clonal multiplication.

Rood Induction from microshoots

In vitro microshoots (2-3cm) were excised from multiple culture and grown on MS medium without or with growth regulators. Initiation of roots from microshoots was very slow in medium without NAA. The percentage of

shoots that formed roots and number of roots / shoot significantly varied at different concentrations of NAA within two weeks of microshoots transfer. The optimum rooting (65%) in microshoots was observed on MS medium containing 1.0 mg/l NAA (Table2). Ninety four percent root formation was recorded in the *in vitro* proliferated shoots on MS with 0.50 mg/l NAA (Rahman *et al*., 2004). The 1 or 2 mg/l NAA for rooting observed rendered rooted shoots with fasciculated roots formed on each shoot (Sharma and Mohan, 2006). Effect of NAA on root induction was positive, however auxin concentration had a positive effect on the number of roots/ shoot and it was higher in the case of NAA. Sivanesan *et al.* (2007) reported that 1.0 mg/L NAA in medium showed maximum rooting in regenerated shoots of *S. rebaudina*. In root induction experiment 1.0 mg/L NAA was found better as maximum roots proliferated, that results one better than reported by Sivanesan *et al* (2008).The MS medium containing NAA 1.5mg/l produced 2-4 roots/shoot, rhizogenesis was inhibited in the presence of high concentration of NAA (Harikrishnan *et al*,1997). NAA and I BA were commonly used for root induction. Sharma and Mohan (2006) reported MS medium with 2.o Mg/L NAA without charcoal produced more number of roots in *C. borivilianum.*

Table 1. Effect of different concentrations of BAP on multiple shoot formation and shoot length in *S. rebaudiana*

Ms+ media +BAP (mg/l)	% of explants with multiple shoots	No. of shoots per explant (Mean ± SE)	Average shoot length (Mean ± SE)
0.25	12	1.27± 0.16	2.44± 0.13
0.50	46	1.67±0.21	2.51± 0.16
0.75	43	2.40 ± 0.21	2.56 ± 0.21
1.00	50	3.40 ± 0.24	3.04 ± 0.10
1.50	52	3.21 ± 0.21	2.62 ± 0.19

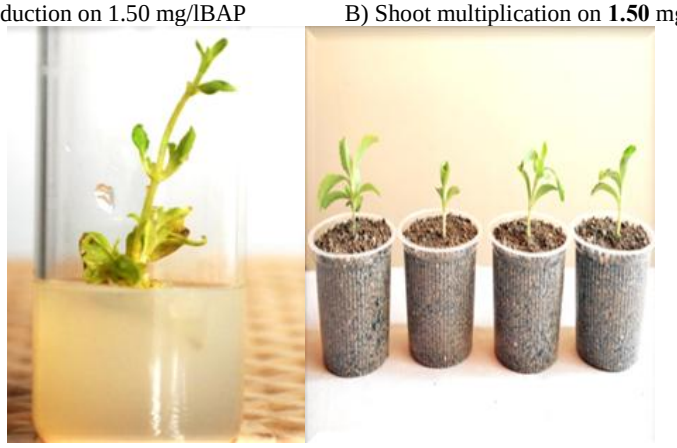
Table 2. Effect of different concentrations of NAA on root formation and average root length in*S. rebaudiana*

Ms+ media +NAA (mg/l)	% of microshoot rooted	No. of roots per explant (Mean ± SE)	Average root length (Mean ± SE)
0.25	14	3.83 ± 0.17	3.23 ± 0.27
0.50	30	4.00 ± 0.14	2.89 ± 0.37
0.75	18	2.86 ± 0.14	1.96±0.21
1.00	65	6.20 ± 020	2.90 ± 0.38
1.50	08	3.83 ± 0.12	1.88 ± 0.26

Acclimatization and field establishment

Rooted plantlets were shifted to plastic pots containing sterilized soil, sand and manuare at 1:1:1 ratio covered with transparent polythene bags and placed in acclimatization room at 28°C with 70-90 % humidity. The temperature was gradually increased to 30,32 and 36°C after every week. A total number of 90% plants survived through this process. The acclimatization of plantlets by gradual increase in temperature and use of polythene bags to control humidity improved the survival of plants during hardening process.The net pots were placed inside the green house for acclimatization at 70% relative humidity and 25-30% temperature. The total 80% plants survived with normal growth and morphology in *Curcuma angustifolia* (Shukla *et al.*, 2007). The application of BAP (0.25-0.50mg/l) was effective for acclimatization of plantlet in *Curculigoorchioides* (Shende *et al.*, 2012). Many researchers used different parameters to increase the survival rates of plants (Kramarenko, 1999).

A) Shoot Induction on 1.50 mg/lBAP B) Shoot multiplication on **1.50** mg/l BAP



C) Root Induction on **1.00** mg/l NAA D) Hardened plant on 1:1:1 soil : sand : manure

Conclusion

A protocol for *in vitro c*lonal propagation of *S. rebaudiana* has been optimized and the *in vitro* raised plantlets have been established in local environment. This study will be helpful to establish and cultivate *S. rabaudiana* for commercial scale production in different local environments.

References

Anand, P.H.M. and Hariharan, M. (1997). *In vitro* multiplication or greater galangal(*Alpiniagalanga* Linn willd) – A medicinal plant. Phytomorphology 47(1):45-50.

Balachantran, S.M.Bhat, S.R. and Chandel, K.P.S. (1990). *In vitro*Clonalmultiplication of turmeric (*Curcuma* spp.) and ginger (*Zingiberofficinale* Rose",) Plant Cell Report 8, pp.521-524.

Bondarev, N. (2001). Peculiarities of propagation and development of *S. rebaudiana*Bertoni Plants.*In vitro* procceding of International conference of Horiculture September 3-6, LedniceCzoch Republic 2:431-434.

Chang, B.K.W. andCrilly, A. (1993) Clonal propagation of pink Ginger *in vitro*.HortSci 28:1203

Das, K., Dong, R. and Rajasekharan, P.E. (2006). Establishment and maintenance of Callus of *Stevia rebaudiana*Bertoni under aseptic environment. Natural product Radiance 5(5):373-376

Francis, S.V., Senapati, S.K. and Rout, G.R. (2007). Rapid Clonal propagation of *Curculigoorchioides*Gaertn an endangered medicinal plant.*In vitro* Cell Dev Biol-Plant 43:140-143

Gupta, P., Sharma, S and Saxsena, S. (2010). Callusing in *Stevia rebaudiana* (Natural sweetener) for Steviol Glycoside production. International Journal of Agricultural and a Biological Sciences 1(1):30-34

Harikrishnan, K.N., Martin, K.P., Anand, P.H.M and Hariharan, M. (1997). Micropropagation of Sweetflag(*Acorus calamus*)- A medicinal plant. Journal of Medicinal and Aromatic Plant Sciences 19:427-429

Krishnan, P.N.Sudha,C.G. and Seeni, S. (1995). Rapid propagation through shoot tip culture of *Trichopus zeylanicus* Gaertn. a rare ethnomedicinal plant. Plant Cell Reports14:708-711

Lewis, W.H. (1992). Early uses of *stevia rabaudiana* leaves as sweetner in Paraguay.Econ. Bot 46:336-337.

Nepovim, A., and Vanek, T. (1998). *In vitro* propagation of S. *rebaudiana* plants using multiple shoot culture.Plantamedica 64(8):775-776.

Rahman, M.M. Amin, M.N. Jahan, H.S. andAhmed, R. (2004). *In vitro* regeneration of plantlets of *Curcuma longa* Linn. a valuable Spice plant in Bangladesh. Asian Journal of plant Sciences 3(3):306-309

Salim, M.U. Shaheed, M.H.C.Monoztaba, M.M.K.Belal, M.U.Ahmad, R., and Baten, M.A. (2006). *In vitro* propagation of *S. rebaudiana* Bert in Bangladesh.Atr. J. Biotechnol 5:1238-1240.

Sharma, V., &Mohan, J.S.S. (2006). *In vitro* clonal propagation of *Chlorophytumborivilianum* Sant et Fernand a rare medicinal herb from immature floral buds along with inflorescence axis. Indian Journal of Experimental Biology 41:77-82

Shende, C.B. Undal, V.S. and Chaudhari, U.S. (2011). *In vitro* propagation of *Curculigo orchioides* from rhizome bud. Journal of Agricultural Technology 8(1):353-362

Shukla, S.K., Shukla, S., Koche, V., & Mishra, S.K. (2007). *In vitro* propagation of Tikhur (*Curcuma angustifolia* Roxb) a Starch yielding plant. Indian Journal of Biotechnology 6:74-76

Sivanesan, I. (2007). Direct regeneration from apical bud explant of *Withania somnifera* Dunal 6:125-127

(Received 29 January 2013; accepted 22 December 2013)