View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Directory of Open Access Journals

JOURNAL OF HORTICULTURE AND POSTHARVEST RESEARCH
2018, VOL. 1(2), 97-104

Journal of Horticulture and Postharvest Research

Journal homepage: www.jhpr.birjand.ac.ir

University of Birjand

A new protocol for direct regeneration of stevia plant (*Stevia rebaudiana* Bertoni.) by tissue culture techniques

Soghra Rezaie[1], Maryam Dehestani-Ardakani [2*] and Kazem Kamali[3]

1, 2 Department of Horticultural Science, Faculty of Agriculture & Natural Resources, Ardakan University, Ardakan, Iran

3, Department of Soil Science, Faculty of Natural Resources, Yazd University, Yazd, Iran

ARTICLE INFO

Article history:

Received 7 January 2018

Revised 27 February 2018

Accepted 7 March 2018

Available online 15 June 2018

Keywords:

BA

IBA

in vitro culture

proliferation

stevia

DOI: 10.22077/jhpr.2018.1273.1010

P-ISSN: 2588-4883

E-ISSN: 2588-6169

***Corresponding author:**

Department of Horticultural Science, Faculty of Agriculture & Natural Resources, Ardakan University, Ardakan, Iran.

E-mail: mdehestani@ardakan.ac.ir

© This article is open access and licensed under the terms of the Creative Commons Attribution License http://creativecommons.org/licenses/by/4.0/) which permits unrestricted, use, distribution and reproduction in any medium, or format for any purpose, even commercially provided the work is properly cited.

ABSTRACT

Purpose: Due to pharmaceutical values of Stevia plant (*Stevia rebudiana* Bertoni.), this study was done to introduce a new protocol for rapid mass propagation of it through tissue culture. **Research Method:** In MS medium shoot proliferation of stevia by six concentrations of BA (0, 0.1, 0.2, 0.3, 0.4, 0.5 mg l^{-1}) and root induction by four concentrations of IBA (0, 0.025, 0.05, 0.1 mg l^{-1}) was investigated. Rooting of cuttings was done both *in vitro* and *ex vivo* conditions. **Findings:** According to the results, the most number of stems obtained in MS medium containing 0.3 mg l^{-1} BA. The highest length of stems obtained in MS medium without BA and the most number of leaves observed in MS medium supplemented with 0.4 mg l^{-1} BA. In *in vitro* situation, the most number and length of roots obtained in MS medium without IBA. The most number of rooted cuttings was obtained in IBA solution after 72 hours and about 70% of rooted cuttings were healthy. **Research limitations:** It had no limitation to report. **Originality/value:** In conclusion, it seems that the potential of producing root and shoots in stevia plant is extremely high, so its proliferation is possible using low concentrations of plant growth regulators in *in vitro* culture.

INTRODUCTION

Diabetes is one of the most common diseases in human societies. Therefore, the use of natural sweeteners is especially important if people with diabetes can also consume it. *Stevia rebaudiana* Bertoni is a Sweet herb and anti-diabetic medicinal plant, from Asteraceae family (Aggarwal et al., 2011; Altaf et al., 2013). This plant is native to certain regions of South America-Brazil and Paraguay (Abd Alhady, 2011). It is a major source of sweetener and has enormous commercial importance. It could be replaced by artificial sweetener and even table sugar in the future. *Stevia* has various properties such as regulating blood sugar, preventing hypertension and tooth decay, antibacterial, anticandidal, antifungal, antiviral, diuretic, hypoglycemic, vasodilator and treatment of skin disorders (Mathur & Begum, 2015; Singh & Rao, 2005). Stevioside and rebaudioside are two important glycosides in leaves that taste about 300 times sweeter than sugar (Geuns, 2003).

Stevia plant propagates by both sexual and asexual methods. Heterozygoty, self-incompatibility and low germination percentage are the most important problems in *stevia* seed propagation (Toffler & Orio, 1981). Because of the lower number of individuals that can be obtained simultaneously from a single plant, vegetative propagation is not suitable (Sakaguchi & Kan, 1982). To overcome all, multiplication and improvement of this medicinal plant, tissue culture is the best alternative for the rapid mass propagation of *Stevia* (Borroto et al., 2008). Several factors such as the type of explant, kind and concentration of plant growth regulators (PGRs) play important role in successfulness culture establishment of *Stevia* (Babber et al., 2001). Kind of explants considerably influences on multiplication and proliferation in tissue culture. Successful initiation of *in vitro* direct organogenesis in stevia has been attained using different explants, for instance, shoot tips (Ibrahim et al. 2008), leaves (Jain et al., 2009; Kalpana et al., 2010), nodal segments (Ahmed et al., 2007; Modi et al., 2012), and axillary buds (Das et al., 2011). However, among different explants in stevia tissue culture, nodal segments have been extremely applied. Between two methods of direct and indirect regeneration for clonal propagation of stevia, the development of adventitious shoots directly from the explants is more suitable compared to the callus mediated regeneration.

Majumder and Mahbubur Rahman (2016) investigated the effect of different explants (shoot apex, leaf, and nodal explants) in organogenesis of stevia. They found indirect organogenesis in leaf segment and direct organogenesis in shoot apex. But in case of the nodal segment, both direct and indirect organogenesis was observed.

Tomaszewska-Sowa et al. (2015) obtained enormous shoots using sterile nodal segments that were cultured on Murashige and Skoog (MS) (1962) media and supplemented with phytohormones. They observed the highest efficiency of shoots proliferation at the presence of 0.5 mg dm^{-3} Benzylaminopurine (BAP). The longest stems and the largest number of leaves and the greatest width of leaf were observed when 0.5 mg dm^{-3} GA_3 was applied to the medium. The process of rhizogenesis was intensified by the 0.5 mg dm^{-3} Indole-3-butyric acid (IBA) contained in the nutrient medium.

Ojha and Chadhary (2013) obtained the maximum number of the shoot on the MS medium supplemented with BAP (2.0 mg l^{-1}). The well Developed shoots used for rooting *in vitro* and 100% rooting found in 0.5 mg l^{-1} IBA. The *in vitro* developed rooted plantlets were transferred to medium size pots and they were grown in the greenhouse for hardening and finally, they were planted in the open field. Around 92% of plants were successfully established in natural field condition.

The present study was carried out to examine a new protocol for the rapid mass propagation of *S. rebaudiana* using different PGRs.

MATERIALS AND METHODS

Plant materials

S. rebaudiana explants were collected from tissue cultured plants that were established in the greenhouse. Axillary bud explants (1-1.5 cm long) and 0.1–0.3 cm thick (Fig. 1a) were washed thoroughly for 5 min under running tap water, treated with 0.05% sterile acetic acid (w/v) for 2-3 min followed by treatment with 0.05% sterile acetic acid (w/v) + 0.1% (w/v) $HgCl_2$ for 2–3 min and finally treated with 0.05% sterile acetic acid (w/v) for 2-3 min and washed 3 times with sterile double distilled water. Sterilization was carried out in the laminar airflow.

Shoot induction media

Murashige and Skoog medium (1962) was used supplemented with 3% (w/v) sucrose, solidified with 0.7% (w/v) agar; pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. for investigation the effect of BA on shoot development and multiplication, MS medium fortified with different concentrations of BA (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg l^{-1}). Cultures without BA served as control. In each 30 ml glass jar, 10 ml of media were dispensed. A total number of shoots, length of shoots and number of leaves were measured after 4 weeks of culture. The cultures were incubated at 25 ± 1°C with a photoperiod of 16 h at 3000 lux light intensity of cool white fluorescent light. Data according to the frequency of multiple shoots were recorded after 4 weeks of inoculation.

In vitro and *ex vitro* root induction

After 30 days, the elongated shoots with length more than 3.0 cm were carefully removed from the glass jars and transferred to the rooting media. To determine the effect of BA on root induction, MS medium supplemented with 0, 0.025, 0.05 and 0.1 mg/l IBA under aseptic conditions. The growth regulator was added separately to MS medium containing 3% sucrose and 0.7% (w/v) agar. A total number of roots per shoot and the length of the roots were recorded after 4 weeks of culture.

In another experiment for accelerating and reducing the cost of tissue culture, root formation in shoots examined in *ex vitro* condition. So, micro-cuttings with 6 cm length taken from the *in vitro* proliferated shoots that were transferred to small clear plastic cups (by the volume 50 cc) containing 10 mg l^{-1} IBA (20 ml of solution dispensed in each cup) for four different times (0, 24, 48, 72 hours) (Fig. 1d) and each containing six replications. After that cutting was transferred to pots that were contained sterilized peat moss and perlite (1:3 ratio). These pots kept four days in dark and then incubated at 25 ± 1 °C with a photoperiod of 16 h at 3000 lux light intensity of cool white fluorescent light. A total number of shoots and leaves and the length of shoots were recorded after 4 weeks of culture.

Hardening

Rooted cuttings were exited from the glass jars, washed carefully with sterile water to remove agar media, placed in the plastic cups filled with sterilized peat moss and perlite by the 1: 3 ratio (Fig. 1e). Plastic bags on the pots, used for maintaining humidity in pots. These plants incubated at 25 ± 1 °C with a photoperiod of 16 h at 3000 lux light intensity of cool white fluorescent light. After hardening established plantlets were re-potted in small pots containing sterilized garden soil and sand (1:1) (Fig. 1f).

Statistical analysis

Data with regard to different parameters (number of shoots, length of shoots, number of leaves, number and length of roots) was measured after 30 days of culture. Each treatment had 10 replicates and every single explant was considered as an experimental unit. All data were statistically analyzed by ANOVA using SAS program (ver 9.1). Significant differences among the treatments were tested by Duncan‘s multiple range test at 5% level.

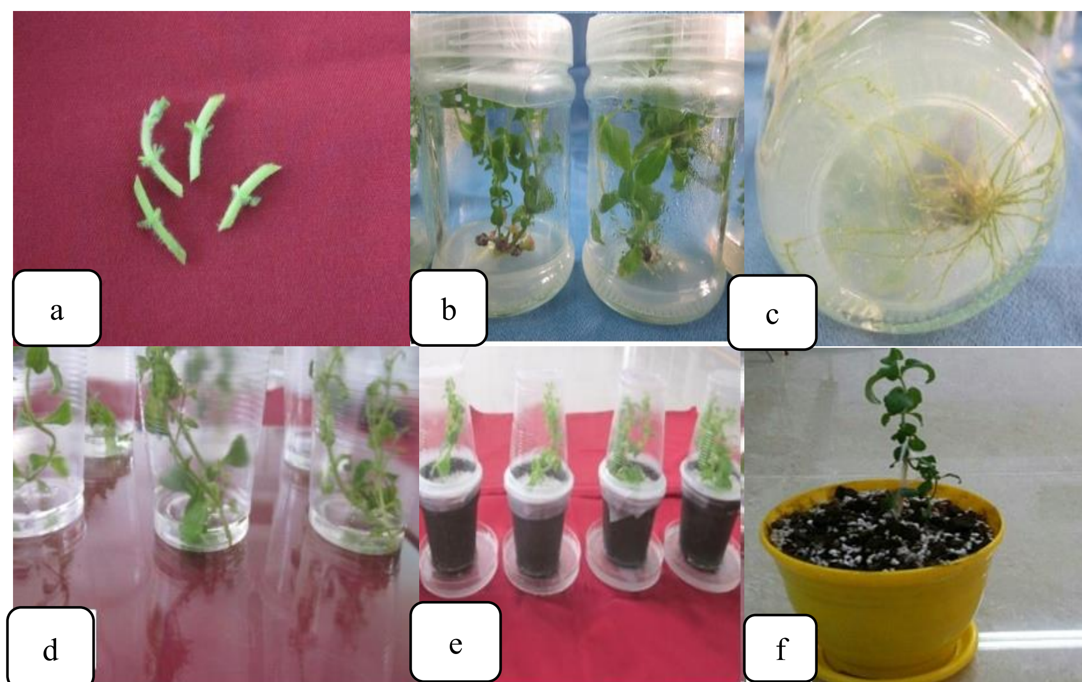

Fig. 1. *In vitro* propagation of *Stevia rebaudiana*. (a) Axillary bud explants used for micro-propagation (b) Establishment of *Stevia rebaudiana* in MS medium and multiplication of shoots (c) *In vitro* rooted plantlet after 6 weeks of culture (d) *Ex vitro* cuttings in IBA solution. (e) Cuttings in plastic cups filled with sterilized peat moss and perlite by the 1:3 ratio in hardening phase (f) A plantlet acclimatized in greenhouse.

Table 1. Analysis of variance of various concentrations of BA in MS media on some growth characteristics of stevia

S.O.V	df	Number of shoots	Length of shoot	Number of leaves
Concentration of IBA	5	8.16**	9.38**	27.12**
Error	54	1.76	1.38	0.84

** Significant at $P < 0.01$.

Table 2. The effect of various concentrations of BA on the number of shoots, length of shoots and number of leaves of stevia

Parameters	BA (mg l^{-1})					
	0	0.1	0.2	0.3	0.4	0.5
Number of shoots	$2.6b^{c}$ †	4.0^{a}	3.7^{ab}	4.2^{a}	2.4^{c}	2.1^{c}
Length of shoots	4.01^{a}	1.66^{b}	1.67^{b}	1.5^{b}	1.79^{b}	1.61^{b}
Number of leaves	10.3^{bc}	7.84^{c}	7.53^{c}	6.99^{c}	19.09^{a}	16.71^{ab}

† Values with the same letter in the each row were not significantly different ($P > 0.01$)

Table 3. Analysis of variance of various concentrations of IBA on the length and number of roots of stevia

S.O.V	df	Length of roots	Number of roots
Concentration of IBA	3	5.48**	58.49**
Error	36	0.90	9.59

** Significant at $P < 0.01$.

Table 4. Analysis of variance of keeping various times in IBA solution on some growth characteristics of stevia

S.O.V	df	Number of leaves	Number of shoots	Length of shoots
Time of IBA	3	14.37**	0.27^{ns}	36.33^{ns}
Error	20	4.16	0.35	17.65

ns, non-significant; **, significant at P < 0.01.

RESULTS AND DISCUSSION

The axillary bud explants were cultured in MS media with different concentration of BA (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg l^{-1}) (Fig. 1b). All measured parameters (number of shoots, length of shoot and number of leaves) have shown significant differences at various concentrations of BA in MS media (Table 1). Data recorded after 30 days of culturing showed that axillary bud explants of *S. rebaudiana* could be established in all tested media including the control medium (without any growth regulators) (Table 2). Our result was in agreement with the findings of Sivaram and Mukundan (2003) reported that BA could induce shoot regeneration in the shoot apex, nodal and leaf explants. They found that higher concentrations of BA resulted in decreasing multiple shoot formation in all the explants of Stevia. The maximum number of leaves obtained in MS medium supplemented with 0.1 and 0.3 mg l^{-1} BA (Table 2). The maximum length of shoots observed in MS medium free from BA (Table 2). The BA didn't show any effect on the length of shoot. The highest number of leaves recorded in MS medium fortified with 0.4 mg l^{-1} BA. Sairkar et al. (2009) obtained the best results (1.55 shoots/explant) on MS medium supplemented with 0.1 mg $^{-1}$ IBA.

Different concentrations of IBA showed a significant difference in the number and the length of roots of stevia (*Stevia rebaudiana* Bertoni.) in *in vitro* condition (Table 3). MS medium without IBA (control) showed better growth in terms of a number of roots (9) and root length (3.48 cm) (Table 3). Also, 0.05 mg l^{-1} IBA, as well as MS free PGR, could form the highest length of the root (3.12 cm). Actually, by increasing the concentration of IBA the number and length of roots didn't increase (Fig. 2). According to the results, the rooting percentage was observed as 100% that could be obtained on MS without IBA and with IBA alone (Fig. 1c). It seems that because of the internal level of phytohormones in stevia was high, so, external IBA had low effect on root induction.

In the *ex vitro* rooting experiment, shoot length was significantly affected by the keeping time in IBA solution, but it didn't show a significant difference in the number of shoots and leaves (Table 4). Keeping cutting for 72 hours in IBA solution was optimum for shoot length (13 cm) (Fig. 3). Other times (0, 12 and 24 hours) didn't show a significant difference in length of shoots (Fig. 3). It should be noted that the rooting medium also acted as shoot elongation medium, (Sivaram & Mukundan, 2003) so we recorded length of shoot.
According to the results, it seems that producing root and shoots in stevia plant is extremely high, so its proliferation is possible by low concentrations of plant growth regulators in *in vitro* culture.

CONCLUSION

In conclusion, this study introduced a new protocol for *in vitro* propagation of *Stevia rebudiana* to optimize the culture media for multiple proliferation of shoot and root. The present study advises the use of MS medium fortified with BA for shoot induction and MS medium with IBA for rooting of Stevia shoots. In order to reduce the cost and accelerating the work, rooting the cutting in *ex vitro* condition is proposed.

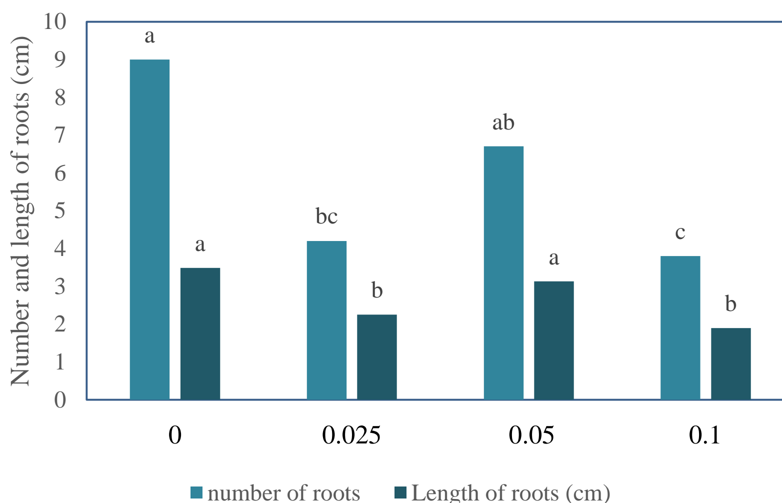

Fig. 2. The effect of various concentrations of IBA on number and length of roots of stevia

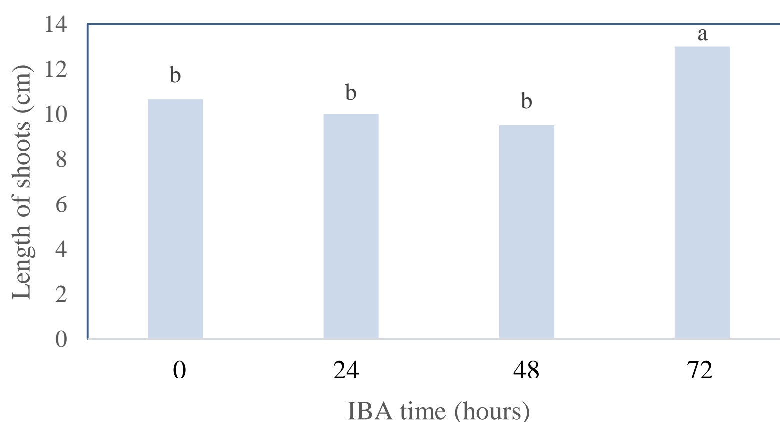

Fig. 3. The effect of different incubation times in IBA solution on shoot length of stevia

ACKNOWLEDGEMENT

We are grateful to Dr. Majid Sadeghinia for valuable help in statistical analysis.

REFERENCES

Abd Alhady, M.R.A. (2011). Micropropagation of *Stevia rebaudiana* Bertoni. A new sweetening crop in Egypt. *Global Journal of Biotechnology & Biochemistry, 6*(4), 178-182.

Aggarwal, A., Singh, N., & Yadav, K. (2011). Influence of arbuscular mycorrhizal (AM) fungi on survival and development of micropropagated Acorus calamus L. during acclimatization. *International Journal of Agricultural Technology*, *7*(3), 775-781.

Ahmed, M.B., Salahin M., Karim, R., Razvy, M.A, Hannan, M.M., Sultana, R., Hossain, M., & Islam, R. (2007). An efficient method for *in vitro* clonal propagation of a newly introduced sweetener Plant (*Stevia rebaudiana* Bertoni.) in Bangladesh. *American-Eurasian Journal of Science and Research, 2*, 121–125.

Altaf, T., Amin, S., Singh, S., & Kaloo, Z.A. (2013). Micropropagation of medicinally important plant species of family Asteraceae– a review. *International Journal of Recent Scientific Research*, *4*(8), 1296- 1303.

Babber, S., Mittal, K., Ahlawat, R., & Varghese, T. (2001). Micropropagation of Cardiospermum halicacabum. *Biologia Plantarum*, *44*, 603-606.

Borroto, J., Coll, J., Rivas, M., Blanco, M., Concepcion, O., Tandron, Y.A., Hernandez, M., & Trujillo, R. (2008) Anthraquinones from *in vitro* root culture of *Morinda royoc* L. *Plant Cell Tissue and Organ Culture, 94*,181–187.

Das, A., Gantait, S., & Mandal, N. (2011). Micropropagation of an Elite Medicinal Plant: *Stevia rebaudiana* Bert. *International Journal of Agricultural Research, 6*, 40-48.

Geuns, J.M.C. (2003). Molecules of interest stevioside. *Phytochemistry*, *64*, 913–921.

Ibrahim, I.A., Nasr, M.I., Mohammed, B.R., & El-Zefzafi, M.M. (2008). Plant growth regulators affecting *in vitro* cultivation of *Stevia rebaudiana. Sugar Technology*, *10*, 254–259.

Jain, P., Kachhwaha, S., & Kothari, S.L. (2009). Improved micropropagation protocol and enhancement in biomass and chlorophyll content in *Stevia rebaudiana* (Bert.) Bertoni by using high copper levels in the culture medium. *Scientia Horticulturae, 119*, 315–319.

Kalpana, M., Anbazhagan, M., Natarajan, V., & Dhanavel, D. (2010). Improved micropropagation method for the enhancement of biomass in Stevia rebaudiana Bertoni. *Recent Research on Science and Technology*, 2, 8–13.

Majumder, S., & Mahbubur Rahman, M.D. (2016). Micropropagation of *Stevia rebaudiana* Bertoni. through direct and indirect organogenesis. *Journal of Innovations in Pharmaceuticals and Biological Sciences*, *3*(3), 47-56.

Mathur, M., & Begum, T. (2015). Shootlets Regeneration and Tissue Culture Studies on *Stevia rebaudiane* Bertoni and *Terminalia bellerica* Roxb. *International Journal of Research of Biotechnology, 3* (1), 25-35.

Modi, A.R., Patil, G., Kumar, N., Singh, A.S., & Subhash, N. (2012). A simple and efficient *in vitro* mass multiplication procedure for *stevia rebaudiana* Bertoni and analysis of genetic fidelity of *in vitro* raised plants through RAPD. *Sugar Technology*, *14*, 391–397.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, *15*(3), 473-497.

Ojha, A., & Chadhary, R. (2013). Callus development and indirect shoot regeneration from leaves and stem explants of *Stevia rebaudiana* Bertoni. *Journal of Cell and Tissue Research*, *13* (2), 3755-3759.

Sairkar, P., Chandravanshi, M.K., Shukla, N.P., & Mehrotra, N.N. (2009). Mass production of an economically important medicinal plant *Stevia rebaudiana* using *in vitro* propagation techniques. *Journal of Medicinal Plants Research*, *3*(4), 266-270.

Sakaguchi, M., & Kan, T. (1982). Japanese researches on *Stevia rebaudiana* (Bert.) Bertoni and stevioside. *CiCult, 34*, 235-248.

Singh, S., Garg, V., Yadav, D., & Beg, M.N. (2012). *In vitro* antioxidative and antibacterial activities of various parts of *Stevia rebaudiana* Bertoni. *International Journal of Pharmacy and Pharmaceutical Science*s. ISSN- 0975-149.

Singh, S.D., & Rao, G.P. (2005). Stevia: the herbal sugar of 21st century. *Sugar Technology*, *7*, 17–24.

Sivaram, L., & Mukundan, U. (2003). *In vitro* culture studies on (*stevia rebaudiana*). *In vitro cellular Developmental and Biology plant, 39*, 520- 523.

Toffler, F., & Orio, O.A. (1981). Acceni sulla pin ata tropicale 'Kaa-he-e' ou 'erba dolce'. *Revista. Society and Science Aliment, 4*, 225-230 [English summary].

Tomaszewska-Sowa, M., Figas, A., & Katarzyna Sawilska, A. (2015). A non-caloric sweetener *Stevia rebaudiana* Bertoni- tissue culture plantlets for organic farming and home gardening. *Infrastruktura i Ekologia Terenów Wiejskich*, (IV/3).

یک پروتکل جدید برای باززایی مستقیم گیاه استویا (*Stevia rebaudiana* Bertoni) با استفاده از تکنیک کشت بافت

صغری رضایی، مریم دهستانی اردکانی و کاظم کمالی

چکیده

به‌علت ارزش دارویی گیاه استویا (*Stevia rebaudiana* Bertoni.)، پژوهش حاضر انجام شد تا پروتکل جدیدی برای تکثیر انبوه و سریع آن از طریق کشت بافت ارائه کند. پرآوری شاخساره گیاه استویا در محیط کشت پایه MS با استفاده از شش سطح BA (صفر، ۰/۱، ۰/۲، ۰/۳، ۰/۴ و ۰/۵ mg l^{-1}) و القای ریشه با چهار سطح IBA (صفر، ۰/۰۲۵، ۰/۰۵ و ۰/۱ mg l^{-1}) مورد بررسی قرار گرفت. این آزمایش در قالب طرح کاملاً تصادفی با ۱۰ تکرار اجرا شد. ریشه‌دار شدن قلمه‌ها در این آزمایش هم در شرایط *in vitro* و هم در *ex vitro* انجام شد. جهت ریشه دار شدن قلمه‌ها در شرایط *ex vitro*، قلمه‌ها در لیوان‌های کوچک شفاف پلاستیکی حاوی IBA ۱۰ mg l^{-1} در چهار زمان متفاوت (۰، ۲۴، ۴۸ و ۷۲ ساعت) و هر کدام در شش تکرار قرار گرفتند و پس از آن به گلدان‌های حاوی پیت ماس و پرلایت به نسبت ۳:۱ منتقل شدند. بر اساس نتایج، بیشترین تعداد ساقه در محیط کشت حاوی mg l^{-1} BA ۰/۳ حاصل شد. بیشترین طول ساقه در محیط کشت فاقد BA و بیشترین تعداد برگ در محیط کشت حاوی BA ۰/۴ mg l^{-1} مشاهده شد. در شرایط درون شیشه، بیشترین تعداد و طول ریشه در محیط کشت فاقد IBA به دست آمد. بیشترین تعداد قلمه‌های ریشه دار شده در محلول IBA پس از ۷۲ ساعت حاصل شد و حدود ۷۰٪ گیاهان ریشه دار شده سالم ماندند. به‌طور کلی، به نظر می‌رسد که شاخه‌زایی و ریشه‌زایی گیاه استویای مورد مطالعه بسیار بالاست به طوری‌که با کاربرد غلظت‌های پایین تنظیم کننده‌های رشدی امکان تکثیر آن در محیط درون شیشه‌ای وجود دارد.

کلمات کلیدی: BA، IBA، کشت درون شیشه‌ای، پرآوری، استویا