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ORIGINAL ARTICLE

Adaptation to *ex vitro* conditions of *Stevia rebaudiana* (Bertoni) Hemsl. regenerants

L.P. Khlebova[1], A. Orazov[2], A.M. Titova[1], A.V. Pirogova[1]

[1]Altai State University, 61 Lenina prospect, Barnaul, 656049, Russia.
[2]Al-Farabi Kazakh National University, Almaty, Kazakhstan
E-mail: hlebova61@mail.ru

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Stevia rebaudiana (Bertoni) Hemsl. belongs to Asteraceae family and is of great importance for pharmaceutical and food industries. Stevioside obtained from the leaves of this plant is regarded as a valuable natural sweetener. Low seed fertility is one of the most important problems in stevia production. It multiplies almost exclusively in a vegetative way. Plant tissue culture is an efficient method for mass propagation of *S. rebaudiana*. We studied the effect of various concentrations of auxins on rooting stevia shoot cuttings under *in vitro* conditions. We found that adding 0.6-1.0 mg l^{-1} IBA or 0.2 mg l^{-1} IAA to the B_5 medium is effective for rooting the shoot fragments of this species. The regenerants were adapted to *ex vitro* conditions for 3 weeks on a hydroponic setup filled with a solution of mineral salts according to the quarter-strength Murashige and Skoog (MS) basal medium modified by the content of KH_2PO_4 and NH_4NO_3. Using a triple concentration of KH_2PO_4 (510 mg l^{-1}) during the first week of adaptation and a full concentration of NH_4NO_3 (1650 mg l^{-1}) over the next 2 weeks ensures 100% acclimatization of stevia regenerants to *ex vitro* conditions. The replacement of agar in the nutrient medium with a perlite-vermiculite mixture in the ratio of 1 : 3 stimulated the transition of regenerants to the photomyxotrophic type of nutrition. The use of a porous substrate provided a decrease in humidity inside the culture vessels, which led to forming both leaves with well-functioning stomata and a branched root system with root hairs. The stevia regenerants propagated *in vitro* on a porous substrate did not require special conditions for the *ex vitro* acclimatization. The yield of surviving plants in the greenhouse was 100%.

Key words: *Stevia rebaudiana* Bertoni; Natural sweetener; *In vitro* propagation; Auxin; Rooting; Acclimatization; Porous substrate; Photomyxotrophic micropropagation

Introduction

The introduction of medicinal plants, sweeteners, among which the most practically valuable is *Stevia rebaudiana* (Bertoni) Hemsl. (Asteraceae), is of great importance for pharmaceutical and food industries. Stevia is a South American species, which is indigenous to Paraguay. Among 154 representatives of the genus *Stevia*, *S. rebaudiana* is one of two species which produce sweet steviol glycosides. Stevia extract have been used as a natural sweetener and customary medicine by the indigenous inhabitants of South America for several hundred years. *S. rebaudiana* began to be cultivated in the countries of Latin America and Southeast Asia in the late 50s of the last century. Extensive attempts have been undertaken to introduce it, as a crop, in a number of countries, including the United States, Canada, Russia, Ukraine, Korea, Japan, India. Stevia is currently grown mainly in China, and the main market is in Japan (Verzilina, 2005; Kustova, 2013; Gantait et al., 2014). The introduction of *S. rebaudiana* in a temperate climate of Russia showed a high ability of plants to adapt to the soil and climatic conditions in the Central Black Earth Region, Stavropol Territory, Primorsky Territory (Sikorskaya, 2004; Kononova et al., 2012; Galdina, 2015).

The species is diploid and has 22 chromosomes, which are characteristics for most of the American member of the genus *Stevia* (Frederico et al., 1996). The plant is a herbaceous perennial shrub with annually dying and newly growing brittle squarish stem reaching 80 cm in length. The leaves are located opposite, sessile. The inflorescence is a centripetal head. The ray florets are small arranged in an irregular fashion, with white long petals (Skaria et al., 2004). This rare plant contains a whole complex of sweet substances, diterpene glycosides, which are contained in all organs of the plant. Stevioside, rebaudioside A, rebaudioside C and dulcoside A are the main components of stevia (Chatsudthipong and Muanprasat, 2009). The amount of steviol glycosides has been found to decline in the following order: leaves, flower, stems, seeds, roots. The greatest interest in practical terms is stevioside, whose content in leaves reaches 6-10% of total dry weight. The maximal content of stevioside in leaves is achieved during the formation of flower buds and then it gradually declines. The compound is 50-400 times sweeter than sucrose, very low in calories and is used in the prevention of diabetes and obesity (Madan et al., 2010). It has been established that the presence of steviosides normalizes metabolism, restores the antitoxic function of the liver, improves the process of lipid peroxidation and does not increase the blood sugar content (Gantait et al., 2014).

The peculiarity of stevia is that it multiplies almost exclusively in a vegetative way. Seed germination is very poor because of infertility (Kumar, 2013). In addition, plant grown from seed does not allow the production of homogenous plant population resulting in great variability in chemical composition (Brandle and Telmer, 2007; Kovylyaeva et al., 2007). It is usually propagated by cuttings, sprouts in the lower parts of the stem or by dividing the bush (Smitha and Umesha, 2011). Depending on the density of planting, it is necessary to plant from 50,000 to 100,000 plants on 1 hectare. Obtaining this quantity of planting material using conventional breeding methods takes quite a long time. Therefore, tissue culture is a way to create a large number of high-quality stevia planting material (Ahmed et al., 2007; Pande and Gupta, 2013; Islam and Tareq, 2015; Bhingradiya et al., 2016; Ghaheri et al., 2017; Ghorbani et al., 2017; Singh et al., 2017). It is known that the stage of adaptation of regenerants to *ex vitro* conditions is one of the responsible and time-consuming in the process of *in vitro* plant propagation. The death of regenerants significantly

reduces the efficiency of micropropagation. The present study was aimed at the developing an efficient protocol for successful *in vitro* rooting of stevia shoot fragments and subsequent acclimatization of regenerants to *ex vitro* conditions.

Materials and Methods

Materials for the study were sterile stevia plants grown *in vitro*. To obtain them, small segments (about 1.5 cm in length) of stevia shoots were introduced into *in vitro* culture after washing in running tap water for 30 minutes and sterilizing in a 2% solution of lysoformin for 15 minutes. Then, the explants were washed 4 times with sterile water and placed on B_5 medium containing 4.5% sucrose, 0.8% agar and 0.6 mg l^{-1} IBA. The pH of the nutrient medium was adjusted to 5.7-5.8 prior to autoclaving at 121°C for 20 min. The cultures were incubated at a temperature of 25 ± 2°C with 55-60% relative humidity, under conditions of a photoperiod (16/8 hours light/dark cycles) provided by cool white fluorescent tubes with 3000 lux intensity. After 18-20 days of cultivation, sterile shoots developed. For further clonal micropropagation, plant stems were divided into fragments with two oppositely located axillary buds and placed on B_5 medium containing various concentrations of IBA (0.05-3 mg l^{-1}) and IAA (0.05-3 mg l^{-1}). The duration of the passage was 21 days. The growth and development of regenerants were evaluated by the frequency of rhizogenesis (%), the number of roots (pcs./explant), the total length of the roots (cm), the height of the shoot (cm). In addition, the presence or absence of callus on the basal part of the shoot was marked. After 21 days of cultivation, rooted plants were removed from the flasks, carefully washed with sterile water to remove the agar medium. The regenerants were adapted to *ex vitro* conditions for 3 weeks on a hydroponic setup filled with a solution of mineral salts according to the quarter-strength Murashige and Skoog (MS) basal medium modified by the content of KH_2PO_4 and NH_4NO_3. The experiment was performed in 5 replicates. Five explants per each replicate were used. Mean values were compared according to least significant differences test (LSD) at $P<0.05$.

The photomixotrophic technique included cultivating segments of stevia stems in 250 ml flasks on a 1 : 3 mixture of perlite and vermiculite with the addition of a solution of mineral salts according to the basal medium B_5. The perlite-vermiculite mixture (2 g), a solution of mineral salts (25 ml), 0.2 mg l^{-1} IAA were placed in a flask and autoclaved at 121 °C for 30 minutes. Regenerants were cultured on the substrate for 3 weeks. Cultivation conditions were as described above. Rooted stevia shoots were planted in a greenhouse in pots in a substrate consisting of peat moss, vermicompost and sand in equal proportions. Peat moss has a high acidity, therefore, to normalize the acid-alkaline balance, dolomite flour is added to the substrate. The experiment was performed in 3 replicates. Acclimatization success was estimated by the number of surviving plants (%).

Results

The plants of *S. rebaudiana* raised from elite germplasm through tissue culture are genetically pure plants, free from all pathogens. They have excellent vigor and can be planted throughout the year (Jain et al., 2014). Plant regeneration of stevia from *in vitro* culture can be achieved by either organogenesis or embryogenesis. At present, direct regeneration of plantlets via adventitious shoot bud induction from nodal explants is considered to be the preferred method (Singh et al., 2017). Some researchers use MS medium without growth regulators during the reproduction of stevia *in vitro*. However, not all shoot fragments are able to regenerate roots or their number is insufficient for subsequent growth under *ex vitro* conditions. In addition, root hairs often do not develop on the roots. It also complicates the acclimatization of regenerants in the soil under non-sterile conditions. Therefore, we investigated the effect of various growth regulators on the induction of root regeneration in shoot cuttings and the subsequent growth and development of regenerants. In the nutrient medium B_5, various concentrations of IBA and IAA were introduced as the most frequently used rhizogenesis inducers. The results of the experiment are presented in Figure 1.

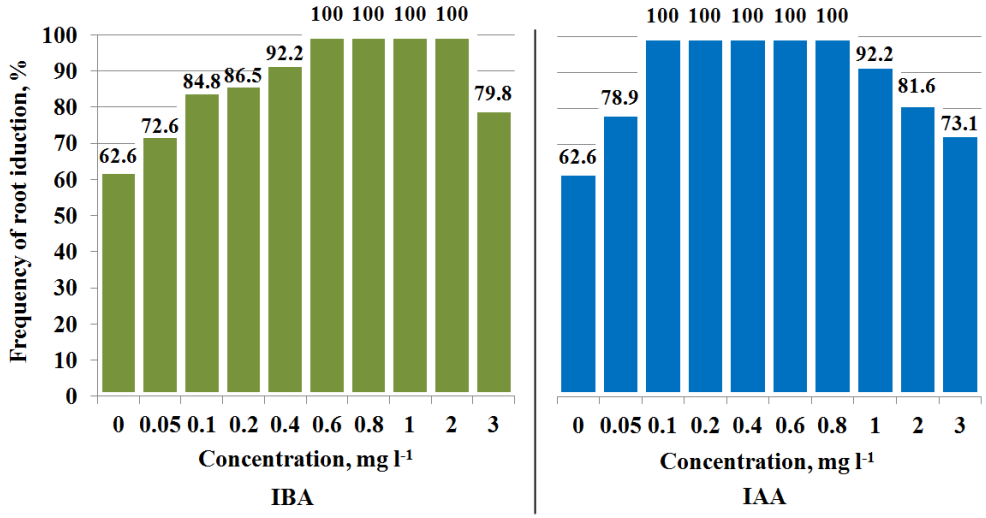

Figure 1. Effect of auxins on *in vitro* root induction in *Stevia rebaudiana* (Bertoni) Hemsl. after cultivation for 21 days.

The total length of the roots regenerated in one shoot varied depending on the IBA concentration. It increased in comparison with the control, reaching a maximum value with the addition of 1 mg l^{-1}. Then the parameter was significantly decreased when using 2-3 mg l^{-1} of the auxin. The length of shoots was maximum at a growth regulator concentration of 1 mg l^{-1} (Table 1).

Table 1. The influence of auxins on the development of *Stevia rebaudiana* (Bertoni) Hemsl regenerants in the culture of axillary buds.

Auxin	Concentration, mg l^{-1}	Number of roots, pcs./shoot	Total root length, cm	Shoot length, cm	Callus	The yield of adapted plants,%
Control	0.0	4.2 ± 0.6[a]	4.7 ± 0.9[a]	3.1 ± 0.5[a]	-	78.6 ± 3.5[a]
IBA	0.05	5.4 ± 0.5[a]	5.7 ± 0.8[b]	2.9 ± 0.3[a]	-	77.4 ± 4.1[a]
	0.1	6.8 ± 0.4[b]	6.5 ± 0.4[b]	3.2 ± 0.4[a]	-	85.2 ± 4.3[b]

	0.2	7.4 ± 0.3[b]	7.7 ± 0.6[c]	2.9 ± 0.4[a]	-	83.2 ± 3.9[b]
	0.4	8.7 ± 0.6[c]	8.1 ± 0.8[c]	3.0 ± 0.6[a]	-	84.7 ± 5.0[b]
	0.6	9.9 ± 1.1[d]	10.2 ± 1.0[d]	2.8 ± 0.2[a]	-	100.0 ± 0.0[c]
	0.8	11.6 ± 1.2[e]	9.0 ± 1.1[e]	2.9 ± 0.3[a]	-	100.0 ± 0.0[c]
	1.0	10.9 ± 0.9[d]	10.6 ± 1.2[d]	3.7 ± 0.4[b]	-	100.0 ± 0.0[c]
	2.0	10.6 ± 1.2[d]	3.6 ± 0.3[f]	1.9 ± 0.1[c]	+	50.2 ± 4.1[d]
	3.0	10.1 ± 0.9[d]	3.4 ± 0.2[f]	1.9 ± 0.2[c]	+	47.1 ± 3.5[d]
IAA	0.05	3.9 ± 0.4[a]	4.5 ± 0.4[a]	2.9 ± 0.3[a]	-	76.7 ± 4.7[a]
	0.1	7.3 ± 0.8[b]	6.5 ± 0.6[b]	3.8 ± 0.4[d]	-	77.9 ± 3.9[a]
	0.2	10.1 ± 1.2[d]	11.0 ± 1.3[d]	3.8 ± 0.3[d]	-	100.0 ± 0.0[c]
	0.4	4.2 ± 0.5[a]	4.9 ± 0.7[a]	3.0 ± 0.2[a]	-	78.4 ± 4.5[a]
	0.6	4.2 ± 0.5[a]	5.1 ± 0.5[a]	2.8 ± 0.4[a]	-	77.9 ± 4.6[a]
	0.8	2.6 ± 0.4[f]	3.5 ± 0.3[f]	2.1 ± 0.3[c]	+	60.1 ± 3.8[e]
	1.0	2.6 ± 0.5[f]	3.5 ± 0.3[f]	1.8 ± 0.3[c]	+	57.3 ± 2.7[e]
	2.0	2.4 ± 0.3[f]	2.7 ± 0.4[f]	1.7 ± 0.2[c]	+	46.4 ± 4.6[d]
	3.0	1.2 ± 0.1[g]	1.6 ± 0.2[g]	1.7 ± 0.1[c]	+	48.6 ± 4.2[d]

Note: Parameters have been recorded after 3 weeks of transfer in rooting media. Data are in the form of mean ± SEM, and means followed by the same letter within the columns are not significantly different at $P<0.05$.

The stimulating effect of IAA on the development of the root system in stevia shoots was observed at concentrations of 0.1-0.2 mg l^{-1}. When the hormone was added at a concentration of 0.4-0.6 mg l^{-1}, the number and length of the roots did not differ from the control. Increasing the dose of IAA in the nutrient medium caused a decrease in the parameters of the root system. In addition, the use of higher concentrations both of IBA (2-3 mg l^{-1}) and IAA (0.8-3 mg l^{-1}) stimulated the development of callus on the basal shoots and inhibited their rooting. According to Debnath (2008) data, the best result of stevia rooting was found when 2 mg l^{-1} IBA was added to the MS nutrient medium. Ahmed et al. (2007) discovered that micro cuttings taken from the *in vitro* proliferated shoots initiated the maximum frequency of rooting (97.66%) in MS medium fortified with 0.1 mg l^{-1} IAA. Thus, according to our research, the adding 0.6-1.0 mg l^{-1} IBA or 0.2 mg l^{-1} IAA to the B_5 medium is effective for the *in vitro* rooting of the shoot fragments of *S. rebaudiana*.

When plants are propagated in tissue culture, the stage of adaptation of regenerants to *ex vitro* cultivation is one of the most important (Pospisilova et al., 1999; Pérez et al., 2015; Vahdati et al., 2017). This is the final stage of micropropagation and the death of most of the planting material significantly reduces its effectiveness. For the successful adaptation of regenerants, it is necessary to create optimal conditions for the further growth and development of both the aerial part of plants and their root system. The proposed methods for acclimatizing various plants to *ex vitro* conditions are very diverse (Pospisilova et al., 2007, 2009; Pérez et al., 2015; Huh et al., 2017; Emara, 2018; Hoang et al., 2019). To adapt the stevia regenerants, we used a hydroponic setup. Plants were fixed in cassettes and placed in a vegetative cuvette filled with a nutrient solution (30 l) containing the concentration of mineral salts according to the quarter-strength MS basal medium prescription. During the first week of adaptation, we used the tripled concentration of KH_2PO_4 (510 mg l^{-1}) in comparison with the content of this substance in MS basal medium (170 mg l^{-1}). Over the next 2 weeks, regenerants were adapted in a solution containing mineral salts of the quarter-strength MS basal medium, with the exception of the concentration of NH_4NO_3, which was left unreduced (1650 mg l^{-1}). Thus, the adaptation period was 3 weeks.

The yield of adapted plants on average for all options was 75.2%. Significant differences in comparison with the control were observed in the cultivation of stevia explants on the medium with the addition of 0.1-3.0 mg l^{-1} IBA, as well as 0.2 or 0.8-3 mg l^{-1} IAA. The maximum yield of surviving plants (100%) was found to occur when both 0.6-1 mg l^{-1} IBA and 0.2 mg l^{-1} IAA were added to the culture medium. In cases where callus develops on the basal part of the stevia shoot fragments, short thick roots without root hairs are usually formed. As a result, despite the high proportion of *in vitro* rooted plants, the yield of regenerants adapted to *ex vitro* conditions is low. This can probably be explained by a violation of the vascular connection between the roots and stems. In addition, in poorly developed lateral roots, changes in the vascular system and hypertrophy of the cortical layer are also noted.

One of the possible approaches to solving the problem of adaptation is to create conditions for regenerants during *in vitro* cultivation, under which they do not form the so-called "cultural phenotype". This specific phenotype includes a number of physiological, anatomical and metabolic features of plants induced by culture conditions (Isah, 2015; Martins et al., 2019). These changes relate primarily to leaves. When cultivated in a nutrient medium, plants are provided with photomixotrophic nutrition. Heterotrophic micropropagation is provided by sucrose, and autotrophic one is due to the process of photosynthesis. The carbon dioxide in the vessel is quickly exhausted, therefore, inhibition of autotrophic growth occurs. A deficit of carbon dioxide causes a corresponding adaptation of the assimilation tissue of the leaves, a change in the functional activity of the photosynthetic apparatus and the state of the closing stomata cells. In addition, air humidity close to saturating, the absence of a gradient of water potential between the evaporating surface of the leaves and the atmosphere lead to inhibition of transpiration. As a result, the water-holding capacity in leaves of regenerants is reduced because of the weak activity of the stomatal apparatus, poorly developed cuticle, and reduced osmotic potential (Pospisilova et al., 1999; Xiao et al., 2011; Barupa et al., 2018; Krisantini and Wiendi, 2018). Thus, a specific complex of *in vitro* culture factors causes adequate adaptive responses of plants. But *in vivo*, after transferring regenerants to *ex vitro* conditions, such adaptations are impracticable, since irreversible dehydration of regenerated plants occurs. "Cultural phenotype" is also determined by the characteristics of the root system of regenerants. It is often characterized by the absence of root hairs and second-order roots. For this reason, regenerants have a small feeding area and low absorption capacity, which also negatively affects plants at the stage of their adaptation to *ex vitro* conditions.

Therefore, at the last stage of micropropagation, it is necessary to develop these *in vitro* conditions under which regenerants will be able to switch from heterotrophic nutrition to mixotrophic or autotrophic ones. This way of plant nutrition will create the prerequisites for the successful development of plants during acclimatization under new *ex vitro* conditions. Photoautotrophic tissue culture is *in vitro* propagation without or with a reduced sugar levels in the culture media. As a result, the growth or accumulation of carbohydrates by explants depends on photosynthesis and inorganic nutrient uptake (Nguyen and Kozai, 2005; Kozai, 2010). The photomixotrophic technique involves various approaches.

They are an increase in light intensity, a decrease in sugar concentration, an addition of porous materials such as vermiculite, gum and paper pulp to liquid or agar media, CO_2 enrichment either by using a permeable gas filters for vessel caps or by supplying CO_2 directly into the vessels which ensures gas exchange. These manipulations during the micropropagation lead to changes in cuticular

wax deposit, increased stomatal density, ion exchange in guard cells of stomata during opening and closure, increased mesophyll layers (Mitra et al., 1998; Xiao et al., 2011; Isah, 2015; Pérez et al., 2015; Emara, 2018; Krisantini and Wiendi, 2018; Hoang et al., 2019; Martins et al., 2019). In addition, an important advantage of the technique is drastic reduction in contamination due to non-inclusion of sugar in the medium and use of non-agar supporting matrix (Kaur, 2015). The photomixotrophic technique affects growth parameters of regenerants during multiplication and rooting and can help plantlets cope with the challenging *ex vitro* conditions during acclimatization stage.

In our study, to increase the adaptive ability of stevia regenerants during acclimatization, we replaced agar with a mixture of perlite and vermiculite in the last subculture medium. A mixture (2 g) in a ratio of 1 : 3 (perlite : vermiculite) was placed in a 250 ml flask into which B_5 liquid nutrient medium was added for rooting. Regenerants cultured on the substrate for 3 weeks. The use of a porous substrate provided a decrease in humidity inside the vessels. As a result, leaves with well-functioning stomata formed on growing shoots. These stevia regenerants well tolerated a change in growing conditions. They were planted in the ground and did not require shelter with plastic wrap to create a special chamber for high humidity. The moist substrate remaining on the roots after removing the plants from the flasks contained residues of the nutrient medium and thereby provided regenerants with mineral substances in the first days of their acclimatization to *ex vitro* conditions. The survival rate of stevia regenerants in the greenhouse was 100%. In addition, the use of such a composition for rooting stevia regenerants is more economical, as it reduces the cost of the substrate.

Conclusion

We concluded that the adding 0.6-1.0 mg l^{-1} IBA or 0.2 mg l^{-1} IAA to the B_5 medium is effective for the *in vitro* reproduction and rooting the shoot fragments of *Stevia rebaudiana* (Bertoni) Hemsl. A two-stage technique for adapting regenerated plants to *ex vitro* conditions using hydroponic setup filled with a solution of mineral salts according to the quarter-strength MS basal medium modified at each stage of adaptation by the content of KH_2PO_4 and NH_4NO_3 characterized by high efficiency and allows you to get plants with a well-developed root system and shoot. Using a triple concentration of KH_2PO_4 (510 mg l^{-1}) during the first week of adaptation and a full concentration of NH_4NO_3 (1650 mg l^{-1}) over the next 2 weeks ensures 100% acclimatization of stevia regenerants to *ex vitro* conditions. The replacement of agar in the nutrient medium with a perlite-vermiculite mixture in the ratio of 1 : 3 stimulated the transition of regenerants under *in vitro* conditions to the photomyxotrophic type of nutrition. The use of a porous substrate provided a decrease in humidity inside the culture vessels, which led to forming both leaves with well-functioning stomata and a branched root system with root hairs. The stevia regenerants propagated *in vitro* on a porous substrate did not require special conditions for the *ex vitro* acclimatization (for example, shelters with plastic bags to provide increased humidity) and can be transferred from culture flasks to a greenhouse or field. The yield of surviving plants in the greenhouse was 100%.

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