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Some biomorphological features of the *Stevia rebaudiana* Bertoni and its *in vitro* cultivation

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Stevia rebaudiana **Bertoni was reproduced vegetatively under greenhouse conditions and its different developmental stages were studied. Seeds were obtained from Absheron cultivated plants. In order to obtain cell culture under *in vitro* conditions, various plant organs were isolated and transferred to nutrient environments that differed in mineral and phytohormonal composition. The reaction of plant tissue culture to the *in vitro* conditions was studied.**

Keywords: *Stevia rebaudiana* Bertoni*, in vitro, morphogenesis, developmental stage, explant, sterilization mode, kallusogenesis*

INTRODUCTION

Stevia Rebaudiana Bertoni (*Asteraceae*) is considered as one of the most promising drugs and nutritional plants. This subtropical perennial herb lasts 8 to 11 years while its aerial part dries every year. The natural habitat of this plant is located in South America (Paraguay, Brazil) and it grows in bush, riverbeds and swamps. It produces kurtin via both seeds and vegetative propagation (Sumida, 1980; Verzilina, 2005). At the same time, the *Stevia* is also known as honey plant, sweet leaf, honey leaf, sweet grass, etc (Lyakhovkin et al, 1990; Pototsky, Pokrovsky, 2004; Ozerova, 2005; Carakostas et al, 2008). The main feature that distinguishes *Stevia* from other crops is that its leaves contain a complex of 8 diterpene glycosides (Tanaka, 1980; Semenova, 2004; Podporinova, 2005, 2007; Amin et al, 2017), which sweetness is 200-350 times higher than sugar's: stevioside, rebaudioside A, B, C, D, E, steviolbioside, duclucid (Zubenko, 1990; Sokolov, 2004; Abou-Arab et al, 2010). Nowadays, stevioside and rebaudioside A are used as natural sweeteners in many countries. European countries, Canada, Australia, New Zealand, Japan, Korea, Great Britain, Russia and China have already started to use *Stevia* plants for nutritional purposes.

Stevioglycosides were first isolated from the leaves of *Stevia rebaudiana* in 1931. Glycosides in 1 kg of dry leaves of the plant completely substitute 30 kg of sucrose and unlike sucrose do not increase the nutritional value, because there are only 18 kcal per 100 gram (Gregersen, 2004; Massoud, 2005; Brahmachari, 2011). Substitution of sucrose with *stevia* products in food and sweet drinks reduces the amount of glucose and insulin in the blood without increasing the energy intake of the diet, thus having a positive effect on the human body (Tsapko et al, 1990; Smolyar, 1990; Gorbatenko, 2003; Kuznetsova, 2014). It should be noted that the daily maximum dosage of steviosides is 4 mg/kg (depending on body weight) (AO, WHO, 1985), which is much less than of sucrose's. In addition, the sweetener extract from *Stevia* is completely natural and its glycemic index is zero (Youssef, 2007).

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In economic terms, E 960 sweetener obtained from *Stevia rebaudiana* costs 3 times cheaper. Addition of dried and cut plant leaves to livestock feed increases meat and wool production (Surkova, 2007; Rastovarov, 2009; Lavrenova, 2018). Furthermore, as recent research shows *stevia* glycosides and the extract obtained from the plant have a therapeutic effect and can be used in the treatment of various tumors (Limarenko, 1995; Massoud et al, 2005; Ghoshet al, 2008).

Extensive use of *Stevia rebaudiana* for various needs (preparation of medicines, food, as well as use as feed additives) attracted attention of scientists to this plant and made it a research target. Unfortunately, this culture isn't still widely known in Azerbaijan, even though the country has favorable soil and climatic conditions for its cultivation. In order to put in place cultivation of *Stevia* in the country first of all it is necessary to study its bioecological and biomorphological features, develop appropriate agricultural methods, conduct breeding work using biotechnological methods, which lets possible the study of the plant response *in vivo* and *in vitro* conditions. Considering the above, the study of *Stevia rebaudiana* in closed ground and *in vitro* conditions has begun for the first time in Azerbaijan.

MATERIALS AND METHODS

Planting material of Stevia rebaudiana brought from Turkey was planted in flower pots with a substrate consisting of soil, sand, humus and rotted manure (2:2:1:0.5).

The plants were cultivated in the greenhouse of the Central Botanical Garden of the Azerbaijan National Academy of Sciences. Stevia *rebaudiana* cuttings were used as the study object, and their various parts were isolated for cultivation under *in vitro* conditions. Leaf blades of large, medium, small leaves, primordial leaves, petioles, parts of the stem and inflorescences were used as explants (Figure 1). The following sterilization options were used to introduce the explants *in vitro*:

1) 1 min in 70% ethanol + 10 min in 5% sodium hypochlorite solution;
2) 1 min in 70% ethanol + 12 min in 5% sodium hypochlorite solution;
3) 1 min in 70% ethanol + 15 min in 5% sodium hypochlorite solution;

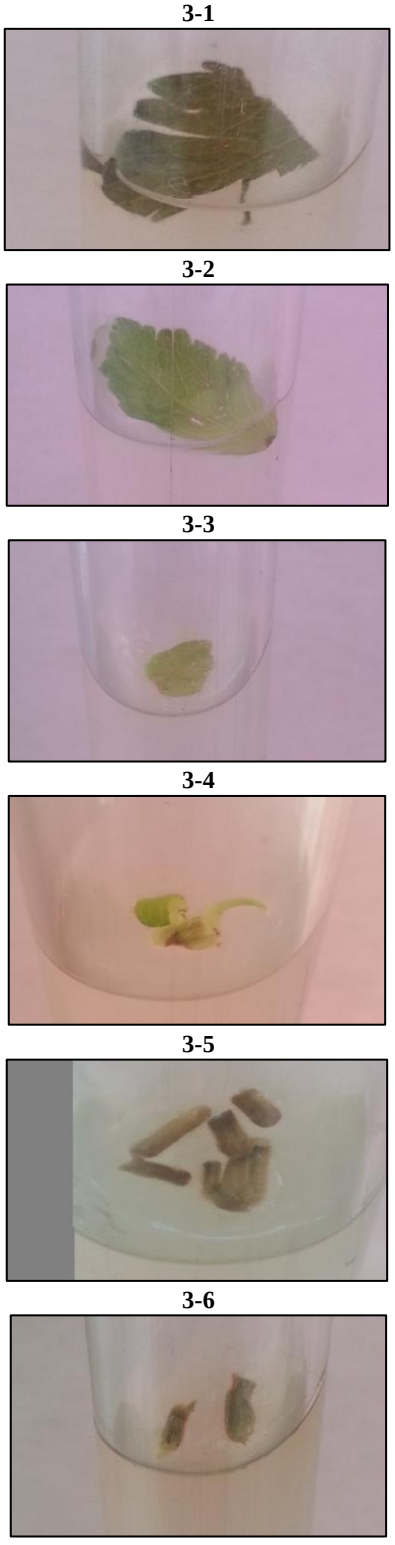

Fig. 1. Explants introduced into in vitro culture: Large (3-1), medium (3-2) and small (3-3) leaves; growth points with primordial leaves (3-4); parts of the stem (3-5); inflorescences (3-6).

4) 1 min in 70% ethanol + 18 min in 5% sodium hypochlorite solution;
5) 1 min in 70% ethanol + 20 min in 5% sodium hypochlorite solution;
6) 3 min in 70% ethanol + 15 min in 5% sodium hypochlorite solution;
7) 3 min in 70% ethanol + 18 min in 5% sodium hypochlorite solution;
8) 5 min in 70% ethanol + 15 min in 5% sodium hypochlorite solution;
9) 5 min in 70% ethanol + 18 min in 5% sodium hypochlorite solution.

After sterilization the seedlings were planted in two nutrient media with different mineral composition: Gamborg (B_5) and Murashige-Skoog (M-S). In order to obtain callus tissue, phytohormones of different concentrations were added to medium where the transplants were planted: 2,4-dichlorophenoxyacetic acid (2,4-D) - 5 mg/l and 8 mg/l; kinetin (KIN) - 5 mg/l and 8 mg/l; α-naphthylic acid (NST) - 0.5 mg/l.

RESULTS AND DISCUSSION

Observations showed that since March plants began to actively vegetate (Fig. 2). In August and early September, buds started to form and by the end of September, flowering was observed. Mass flowering (Fig. 3) continued until the end of October. Fruit ripening and seed formation began in late October.

Based on experimental data obtained in *in vitro* conditions, it can be stated that during the induction of callusogenesis no significant differences were observed on nutrient media with the same phytohormonal but different mineral composition (M-S and B_5). The lack of differences can be explained by a very high level of explant infection and the associated reduction in the number of repeatability. In general, callus formation is not induced when transferring differentiated plant tissues to hormone-free media regardless of the mineral composition of the nutrient medium. Callus and morphogenesis are obtained only from the vegetative organs of plants with active meristematic cells (Sidorov, 1990).

Despite this, each plant is characterized by its endogenous phytohormonal balance and the need for mineral elements. Therefore, for each type of plant and even for each genotype customised regeneration protocol is developed (Kolesnikova, Zhuzhzhalova, 2012). Thus, during induction of callusogenesis for flax both B_5 and M-S media were equally favorable, whereas for alfalfa and cockle the favorable ones were B_5 and M-S respectively. Whereby, various combinations and concentrations of phytohormones were required in order to obtain a cell culture of each of these plant species. It should be noted that in the cockle, a poisonous weed, morphogenic calli formed more intensively on hormone-free M-S medium (Litvinova and Gladkov, 2012), which let us classify this plant as a high auxin type.

Fig. 2. General view of *Stevia rebaudiana.*

Fig. 3. Inflorescences of *Stevia rebaudiana.*

In literary sources, information on the introduction of *Stevia rebaudiana* into an *in vitro* culture is scarce. But the results of some experiments shows that without the use of phytohormones, a *Stevia* cell culture cannot be obtained, despite the fact that this culture belongs to the high auxin type of plants.

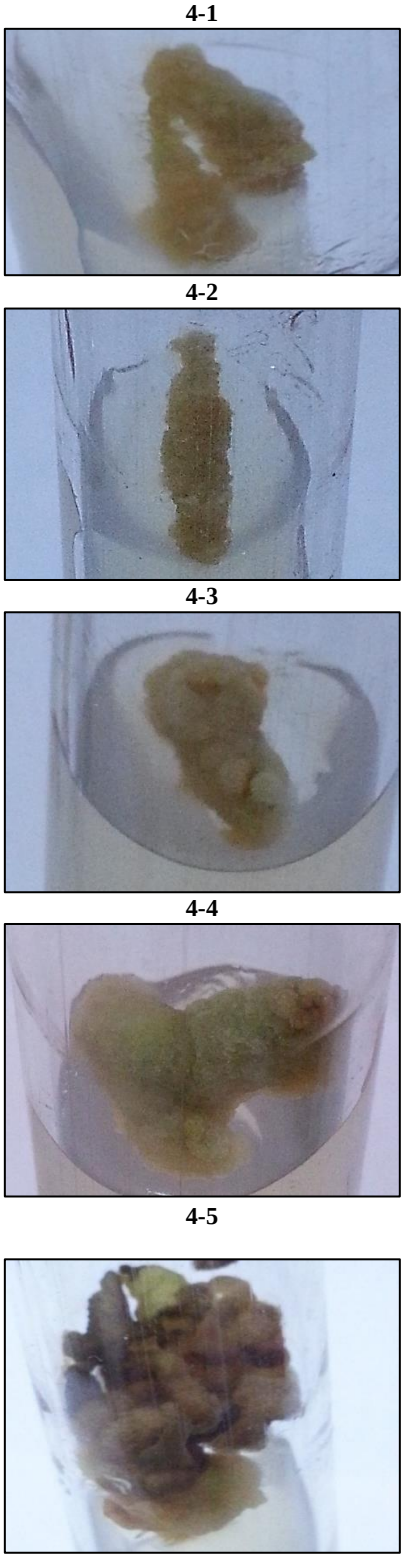

Fig. 4. Callusogenesis on *Stevia rebaudiana* explants: primordial leaves (4-1), stem parts (4-2), small leaf (4-3), middle leaf (4-4), large old leaf (4-5).

Callus and morphogenesis could be obtained from leaf and stem explants only by using auxins (Kolesnikova, Zhuzhzhalova, 2012; Kononova, 2015). Both 2,4-D and cytokinin were used in our experiment, and callus cultures were obtained in each experiment (Figure 4). It can be seen from the pictures that calli are composed of dense greenish, somewhat loose yellowish and hydrated white cells. Due to the fact that numerous explants were infected, many of them were withdrawn from the experiment, so the number of repeatability decreased. Therefore, it was not possible to carry out statistical processing confirming the advantage of one explant over another. However, we can state that in the medium with 5 mg/l 2,4-D and 5 mg/l KIN, the process of callus proliferation was more intensive and there were significantly less white hydrated cells. In experiments of other authors, morphogenic calli were obtained when 0.5 mq/l 2,4-D was introduced into the nutrient medium. In this case, the calli were of medium density and had a light yellow color, while separate meristematic foci were observed (Kolesnikova, Zhuzhzhalova 2012). Undoubtedly, an optimal balance must be maintained between endogenous phytohormones of the explant and exogenous hormones added to the nutrient medium in order to obtain a cell culture *in vitro* and induce morphogenesis. It depends on both the type of implant and the characteristics of the genotype. In our experiment, depending on the type of explant, the structure and shape of the calli was different (Fig. 4). For example, dense, knotty dark-colored calli formed on large old leaves (Fig. 4-5).

This may be due to the accumulation of phenols during explant aging. Despite this, the mass of calli increased significantly after 4 weeks, and calli for the induction of morphogenesis were transferred to a nutrient medium containing 0.2 mg/l BAP.

Stevia seeds obtained from plants grown in greenhouses were also planted on nutrient media. However, during sterilization the seeds were very wet and within 1.5 months cultivation did not germinated (Fig. 5). This may be due to defects in the seeds themselves or destruction processes that occur in the shell of the seeds during sterilization.

It is known that *Stevia rebaudiana* is a self-incompatible plant and is characterized by a very weak germination of seeds and its germination ability is quickly lost (Bodrug, 1995; Verzilina,

2005; Bozhimirov, 2011). However, the lack of germination and wettability of seeds in our experiment is probably associated with adverse greenhouse conditions — whiteflies, which are carriers of the sooty fungus, were seen on plants (Fig. 6).

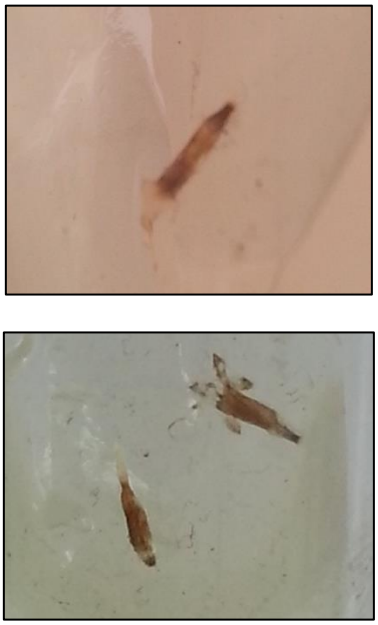

Fig. 5. Seeds of *Stevia rebaudiana* obtained in a greenhouse and planted on an artificial nutrient medium.

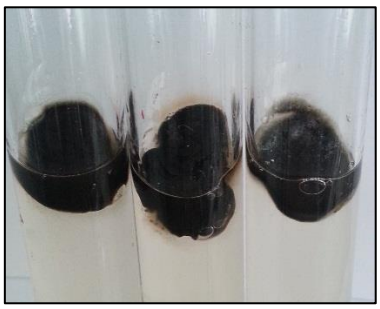

Fig. 6. *Stevia rebaudiana* explants infected with a black fungus.

A similar type of infection was observed in explants isolated, sterilized and transferred to artificial nutrient media. And this indicates the presence of an internal infection in the plants from which the seeds were obtained. Probably, formation of defective seeds is the consequence of internal infection which weaken the plants.

Despite this, as a result of research it can be stated that *Stevia rebaudiana* propagated under the greenhouse conditions exhibited competence for *in vitro* cultivation. The calli obtained as a result of the experiment had a dense structure and numerous meristematic foci. Exactly such calli demonstrated successful morphogenesis (Kolesnikova, Zhuzhzhalova, 2012). This fact indicates the possibility of selection in *in vitro* conditions.

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Stevia rebaudiana Bertoni bitkisinin bəzi biomorfoloji xüsusiyyətləri və onun *in vitro* kulturaya daxil edilməsi

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Stevia rebaudiana Bertoni vegetativ yolla (qələmlə ilə) çoxaldılmış və müxtəlif inkişaf fazaları öyrənilmişdir. Abşeronda örtülü şəraitdə becərilən bitkilərdən toxumlar alınmışdır. *İn vitro* şəraitində hüceyrə kulturasının alınması məqsədi ilə bitkinin müxtəlif orqanları təcrid edilmiş, mineral və fitohormonal tərkibinə görə fərqlənən süni qida mühitlərinə köçürülmüşdür. Bitkinin toxuma kulturasının *in vitro* şəraitinə cavab reaksiyası öyrənilmişdir.

Açar sözlər: *Stevia rebaudiana* Bertoni, *in vitro, morfogenez, inkişaf mərhələləri, eksplant, sterilizasiya rejimi, kallusogenez*

Некоторые биоморфологические особенности растения *Stevia rebaudiana* Bertoni и введение ее в культуру *in vitro*

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Изучались фазы развития растения *Stevia rebaudiana* Bertoni, размноженного вегетативным путем (черенкованием). У растений, выращенных на Апшероне в условиях закрытого грунта, получены семена. С целью получения клеточной культуры изолированные органы растений культивировались на искусственных питательных средах с различным минеральным и фитогормональным составами. Изучена ответная реакция растений на культивирование в условиях *in vitro.*

Ключевые слова: *Stevia rebaudiana* Bertoni, *in vitro, морфогенез, стадии развития, эксплант, режим стерилизации, каллусогенез*