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A NON CALORIC SWEETENER *STEVIA REBAUDIANA* BERTONI – TISSUE CULTURED PLANTLETS FOR ORGANIC FARMING AND HOME GARDENING

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Summary

The paper presents the optimization process of obtaining plantlets of Stevia rebaudiana under in vitro culture conditions and describes the method for adjusting the rooted plants to ex vitro conditions. The low viability of seeds and poor germination are the reasons of difficulties in commercial cultivation of this species in Poland. Plant tissue and cell cultures are the techniques helping to overcome these limitations. Numerous shoots were obtained using sterile nodal segments that were cultured on solidified MS media and supplemented with phytohormones. Elongated shoots were transferred onto the rooting medium. Thus in vitro regenerated plantlets were acclimated to greenhouse conditions. The highest efficiency of shoots proliferation was observed at presence of 0.5 mg dm^{-3} 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 into medium. The process of rhizogenesis was intensified by the 0.5 mg dm^{-3} IBA contained in the nutrient medium. Under the influence of this phytohormone roots were the most numerous and the longest. At the stage of acclimatization 1/4 MS salt solution was used for irrigation of plantlets what supports increased of the process from 46% to 70% in comparison to use of water.

Key words: Stevia rebaudiana, micropropagation, plant growth regulators, acclimatization.

List of abbreviations: BAP – 6-benzylaminopurine, GA_3 – gibberellic acid, IBA – indole – 3 – butyric acid, KIN – 6 – furfurylaminopurine, MS – Murashige and Skoog medium, PGR – plant growth regulation.

INTRODUCTION

In connection with the greater attention to human health noted the intensive development of organic farming and the growing importance of healthy food. Dynamic development of civilization and easy, and quick access to synthetically sourced medicinal and health-promoting substances have outclassed the benefits deriving from the nature. The active compounds in plants are often equal to their chemical equivalents in terms of potency. Furthermore, their proper application ensures the effectiveness of the reaction in the absence of negative side effects. Stevia is one of such naturally acting plants. *Stevia rebaudiana* comes from subtropical areas of South America, it is an inconspicuous shrub, a short-day plant. In our country it can be grown in the ground as an annual plant or it can act as a perennial plant if kept indoor during winter. Stevia is called „a sweet plant" (Jitendra *et al*. 2012; Lemus-Mondaca *et al*. 2012). It is up to 300 times sweeter than sucrose. Stevia extract has hypoglycemic properties and does not cause sudden changes in blood glucose level. This extract shows antibacterial, antifungal and anti-inflammatory activities, not to mention that they dilate blood vessels (Goettemoeller and Lucke 2010). Thanks to these properties, stevia may help to reduce the consumption of white sugar and replace it with other natural substances low in calories that might be referred to as high-intensity sweeteners. Steviosides are an alternative to sugar derived from sugar beet or cane. Nine steviol glycosides have been isolated and chemically described. Their content in the fresh leaves depends on the genotype, method of propagation or cultivation and growth conditions and ranges from 4 to 20%. Stevioside has the largest volume share of glycosides identified in the tissues of stevia (5.8 – 9.1% DM of leaves). This compound shows anti-caries properties, reinforces the smell, but at the same time it is responsible for the bitter taste of the leaves. Rebaudioside A is characterised by a slightly better flavour and greater stability but its content in the dry extract of the leaves of *S. rebaudiana* is lower than stevioside because it is 1.8 – 3.8% DM (Puri *et al*. 2011; Lemus-Mondaca *et al*. 2012; Bugaj *et al*. 2013; Kolanowski 2013, Luwańska *et al*. 2015). Furthermore, stevia is a good source of proteins, carbohydrates and fiber, which improves the function of the digestive system and reduces the risk of certain diseases. The leaves contain almost all of the necessary amino acids including tyrosine, and cysteine. Six fatty acids were also identified including palmitic acid, linolenic acid, folic acid and vitamin C. A natural probiotic – inulin was isolated from the roots. The plant ash contains the following minerals: potassium, calcium, magnesium, sodium, sulfur

and important microelements such as zinc, iron, cobalt, copper (Lemus – Mondaca *et al*. 2012; Bugaj *et al*. 2013). Other phytochemical compounds that were extracted from the leaves of *S. rebaudiana* are triterpenes, sterols, essential oils, tannins, alkaloids, cardiac glycosides and saponins, and moreover hydroxycinnamic acids, chlorophylls and xanthophylls. The leaves of stevia are also a rich source of flavonoids, so they show a strong antioxidant capacity (Lemus-Mondaca *et al*. 2012; Bugaj *et al*. 2013; Zayova *et al*. 2013).

Because of the low seed viability and poor germination traditional cultivation of this species is difficult. This limits the commercial cultivation of stevia and reduces the chances of obtaining a homogeneous population, which is associated with instability of the basic features, such as the sweetening power and the composition of glycosides. Propagation without losing valuable traits can be done by seedlings which are easily rooted, but this possibility requires a high volume of work and it becomes rather expensive, and the number of progeny derived from one parent plant is relatively small (Alhady 2011; Jitendra *et al*. 2012). In addition, the problem cultivation of this species in Poland is that it does not tolerate low temperatures and requires covering or storage in the room. Despite these limitations, stevia should be grown in Poland but now this is the most popular plant in India and so is much research on its use and proliferation (Kumar 2014).

Due to these difficulties, *in vitro* tissue cultures is an alternative to rapid propagation of *S. rebaudiana* in a short time. Techniques using the shoot tips and axillary buds enable us to produce genetically uniform individuals regardless of the season. The development of *in vitro* regeneration methods and synthetic seeds production of stevia (Nower 2014) is required for cultivation on a large scale has become easier and to improve the species by genetic engineering (Jagatheeswari and Ranganathan 2012; Modi *et al*. 2012, Jain 2014).

MATERIAL AND METHODS

The initiation of culture and disinfection of plant material

Explants of *Stevia rebaudiana* Bertoni (*Sweety* variety) were collected from *ex vitro* plants growing under greenhouse conditions. Nodal fragments devoid of leaves, length 1.5 cm, were chemically disinfected. In the first stage, the vegetable pieces, after being rinsed in running tap water, were immersed in 70% EtOH for one minute for degrease, venting and initial surface disinfection. Then the explants were treated with 30% ACE with a few drops of Tween 20 for 10 min. Later parts of the shoots were washed three times with sterile distilled water. Thus sterilized nodal explants were inoculated onto phytohormone-free Murashige and Skoog medium (MS) (Murashige and Skoog 1962), supplement-

ed with 3% sucrose and solidified with 0.8% agar at pH of 5.7. Medium were autoclaved at a pressure of 0.5 MPa at 121°C for 25 minutes. The cultures were carried out in a plant growth chamber under a 16-hour photoperiod with light intensity of 40 mM m^2 and temperature of 25 ± 2°C.

Shoot proliferation process

After 6 weeks of shoots culture, individual plants were selected and cut into nodal pieces with one bud of max. 1cm in length (Fig. 1). These explants were inoculated onto MS medium with different concentrations and combinations of 6-benzylaminopurine (BAP) 0.5; 1.0; 2.0 mg dm^{-3} (Sigma – Aldrich, Germany) and kinetin (KIN): 1.0; 2.0 mg dm^{-3} (Sigma – Aldrich, Germany). In each repeat of the experiment 50 explants were used. For the propagation of plants, the nodal fragments were subcultured three times, each time isolating the nodal segments and inoculating them into fresh medium on the same composition. After each stage, the newly formed shoots were counted. The results were subjected to statistical analysis and the proliferation rate was determined.

Elongation of the shoots

Obtained shoots required internod elongation and because of it were culture in the presence of 0.5 mg dm^{-3} gibberellic acid (GA_3) (Sigma – Aldrich, Germany).

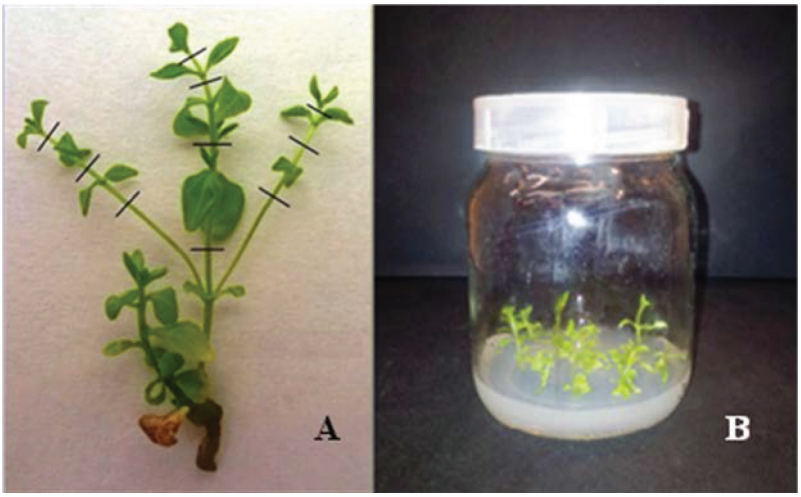

Figure 1. Stevia shoot culture: isolated shoots with marked cutting positions (A), shoot multiplication on MS medium supplemented with 0.5 mg dm^{-3} BAP (B).

Rooting of elongated shoots

Shoots of stevia were transferred onto the rooting MS medium supplemented with 0.5 mg dm^{-3} of indole-3-butyric acid (IBA) (Sigma – Aldrich, Germany).

Acclimatization of seedlings

Properly rooted cuttings were adapted to *ex vitro* conditions. Regenerated plants after removing from the jars and rinsing the agar remains were transferred to plastic pots. Pots were filled with sterilized substrate consisted of a mixture of sand, soil and vermiculite (2:1:1) and its pH ranged from 5.5-6.5. They were hydrated with water and a 1/4 MS salt solution (Sigma – Aldrich, Germany) and in order to reduce excessive transpiration they were protected by the foil tent. The first stage of acclimatization took place in a plant growth chamber under a 16-hour photoperiod with light intensity of 40 mM m^2 and temperature of 25 ± 2°C. After 28 days of adaptation to *ex vitro* conditions, the plants were transferred to greenhouse conditions.

After each stage of the experiment the number and length of shoots and roots were estimated. The number of forming internodes was counted and their length was measured. The width of the leaf blade was also evaluated.

RESULTS

Based on observations of the degree of contamination of the explants disinfected by the chemical method and inoculated onto MS medium, the yield of the decontamination process was estimated at 65%. 200 sterilized nodal segments gave 130 sterile shoots. Stems emerging from lateral buds after the disinfection process, were characterized by a normal plant habit, green color and they did not exhibit the characteristics of the destructive impact of the active substance used in the sterilization process. After 6 weeks of culture of nodal fragments, together with at least one lateral pitch, derived from *in vitro*, cultures were used as secondary explants for shoot proliferation. The explants were inoculated onto appropriate medium with the purpose of the proliferation and growth of shoots. Over the next six weeks the number and length of shoots were being estimated, the number of leaves was determined and the width of the leaf blade was measured. Among the analyzed plants originating from different variants of the experiment, on a medium supplemented with 0.5 mg dm^{-3} BAP gave the highest number of shoots from a single explant (approximately 6.9 individuals). This concentration of BAP preferably also influenced the length of shoots obtained after 6 weeks of culture, and the number of leaves on the shoot. The average length of the shoots obtained on this medium was 4.6 cm, whereas on KIN supplemented media it was almost twice lower. The number of formed leaves was also

dependent on the phytohormonal composition of medium. At low concentrations of BAP in the medium the highest number of leaves on the shoot was observed. Higher concentrations of BAP resulted in a larger leaf blade. In the case of shoots obtained on the medium at concentration of BAP mg dm^{-3} the average leaf width was 4.2 mm. However, both BAP and KIN at concentrations of 1.0 mg dm^{-3} in the highest degree stimulated the growth of leaf blade – in these conditions was the width of the leaf blade was 4.5 cm (Table 1).

Table 1. The effect of growth regulators on shoot multiplication processes stevia in *in vitro* cultures

Plant growth regulator (mg dm^{-3})			Average number of shoots/explant (mean ±SD)	Average shoot length (cm) (mean ±SD)	Average no. of leaves per shoot (mean ±SD)	Average width of the leaf blade (mm) (mean ±SD)
BAP	KIN	GA_3				
0.5		-	6.90±2.65	4.60±0.63	9.30±1.03	4.00±0.40
1.0		-	5.64±2.50	3.90±0.52	8.70±0.97	4.20±0.60
	0.5	-	2.24±0.90	2.80±0.43	7.30±0.87	3.40±0.20
	1.0	-	2.67±1.20	2.40±0.39	6.10±0.56	3.90±0.40
0.5	0.5		**7.80± 2.45**	3.80±0.34	8.70±0.78	3.80±0.90
1.0	1.0	-	3.45±1.53	3.20±1.03	8.20±0.67	4.50±0.70
-	-	0.5	6.50± 1.85	**8.29±2.82**	**10.70±1.00**	**6.60± 0.50**

*indicators computed for the explants each; results are mean ± SD (standard deviation) of 40 replicates

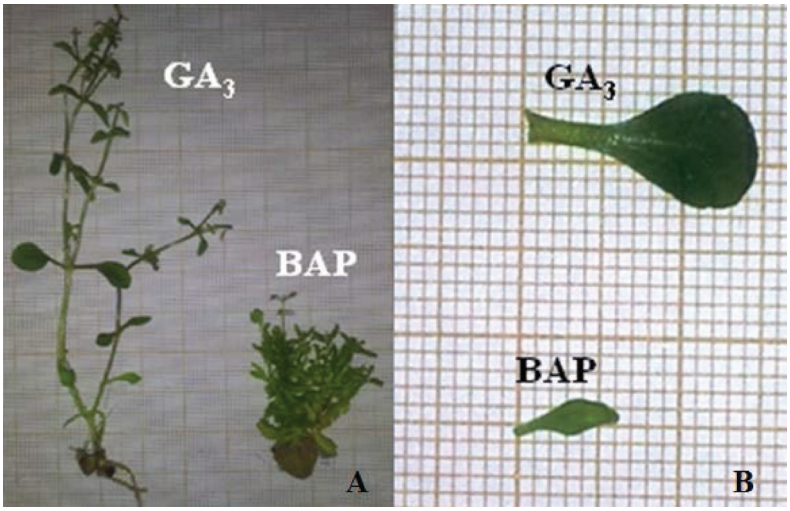

Figure 2. The differences in the length of the shoots (A) and the size of the stevia leaf blade (B) under the influence of applied plant hormones.

The shoots with the highest potential for proliferation obtained on the medium with 0.5 mg dm^{-3} BAP were characterized by relatively short internodes, were thin and small. For the purpose of internodes elongation and obtaining plants with a relatively normal habit, longer with more and bigger size of leaves and suitable for induction of rhizogenesis, the shoots were passaged on MS medium with mg dm^{-3} GA_3. The presence of this phytohormone lead to achieving a significant increase in shoot length (8.29 cm) (Fig. 2A). Plantlets after 6 weeks of growth at the presence of GA_3 were more similar in habit and morphology to the donor plants. The application of gibberellic acid also resulted in an increase in the number of leaves (10.7) and width of leaf blade (6.6 mm) (Fig. 2B).

Plantlets obtained, after several weeks of the culture process, on the medium with different concentrations and combinations of cytokines formed the roots which appeared to be too short and there were only few of them. Intensive root growth was observed in the presence 0.5 mg dm^{-3} IBA and higher number of roots was observed too. It has been proved that the process of rooting proceeded more intensively if it were microseedling after the shoot elongation on the medium containing GA_3. The number of roots obtained in this case was even twice higher than in the other variants. The presence of IBA in the medium had a slightly less influence on length of the roots. The difference in root length ranged 1.0 and 0.3 cm (Table 2).

Table 2. The effect of growth regulators on rooting of shoots stevia process in *in vitro* cultures

Plant growth regulator (mg dm^{-3})			Average number of roots/ shoot (mean ±SD)	Average root length (cm) (mean ±SD)	Average number of roots/ shoot (mean ±SD) at the presence of 0,5 mg dm^{-3} IBA	Average root length (cm) (mean ±SD) at the presence of 0,5 mg dm^{-3} IBA
BAP	KIN	GA_3				
0.5		-	3.40±0.54*	2.30±0.28	5.60±0.52	3.20±0.08
1.0		-	3.60±0.65	3.00±0.34	4.80±0.67	3.60±0.73
	0.5	-	2.30±0.89	2.40±0.45	4.60±0.43	3.10±0.53
	1.0	-	2.80±0.76	2.30±0.47	3.60±0.32	2.90±0.37
0.5	0.5		3.80±1.02	2.10±0.80	5.20±0.77	3.00±0.19
1.0	1.0	-	3.10±0.98	3.10±1,00	5.40±1.00	4.10±0.98
-	-	0.5	**4.20±1.34**	**4.30±1.00**	**7.90±0.50**	**4.60±1.00**

*indicators computed for the explants each; results are mean ± SD (standard deviation) of 40 replicates.

A total of 200 seedlings were placed under a controlled conditions (Fig. 3A). In order to improve the process of acclimatization 100 plants were watered with an aqueous solution of MS salts, and another 100 with tap water. Observations of

the effect of irrigation of the plants, have shown that the survival and acclimatization in the group of plants watered with water was 46%. However, among the plants watered with MS salts 70% of them have demonstrated growth and have successfully adapted to the greenhouse conditions (Fig. 3B, Fig. 4).

Figure 3. Rooted stevia plant during acclimatization (A), advanced stage of acclimatization in greenhouse (B).

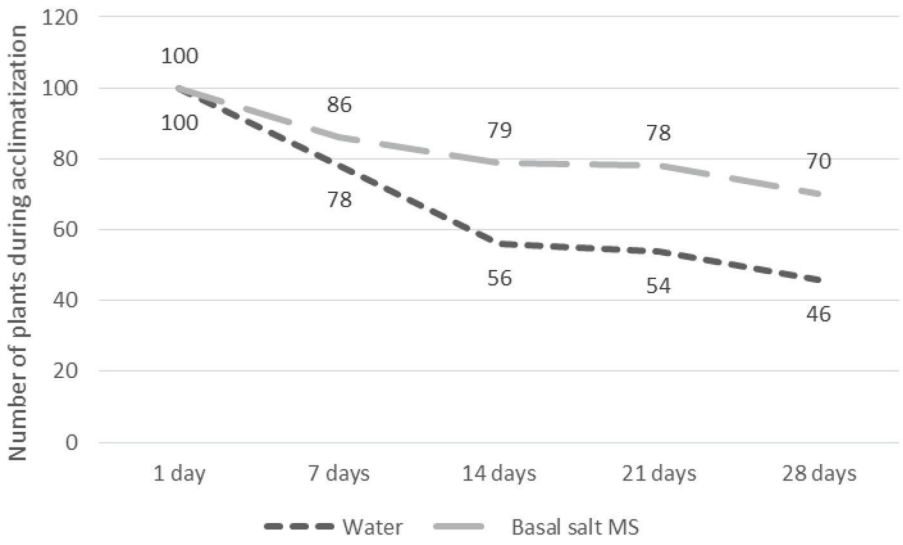

Figure 4. The effect of using an aqueous solution of MS salts for watering the plants at the stage of acclimatization in an *ex vitro* conditions.

DISCUSSION

Due to the large interest in healthy food we describe the optimized micropropagation method for obtaining plantlets of *Stevia rebaudiana* Bertoni, the variety of *Sweety* under *in vitro* culture conditions. This produced seedlings can normally be used for organic field crops and success can be an alternative to sugar beet. The regenerant plant of stevia were obtained by inoculating for the culture sections of the bud side shoots, whose source were the plants collected from *ex vivo* cultivation. This type of explants, also those from greenhouse conditions, were used for proliferation of shoots in a study conducted by Ahmed *et al*. (2007), Jitendra *et al*. (2012) and Thiyagarajan and Venkatachalam (2012). For disinfection of isolated in *ex vitro* conditions shoot apical fragments Jagatheeswari and Ranganathan (2012) used a 0.05% solution of $HgCl_2$, yielding 80% effective decontamination. Due to the high toxicity of this compound, in our own experience explants were used for the decontamination of a 30% solution of ACE with the addition of Tween observing the decontamination of 65%. Sterilized explants quickly started to grow and did not exhibit the destructive action of disinfectants.

In the described experiment, stevia explants were grown in MS medium, but B5 medium were used also (Giridhar *et al*. 2010). In the experiment the effect of cytokines on the process of propagating plants under *in vitro* conditions has been evaluated. The application of growth regulators in diverse concentrations and combinations gave different results. There have been differences in the efficiency of the process of multiplication in terms of number of shoots obtained from the explant, as well as the variability of growth parameters, ie.: shoot length, number of leaves and leaf blade width. The collected results show the intensification of the processes of shoot induction and proliferation under the influence of both KIN and BAP at a concentration of 0.5 mg dm^{-3}, which confirms the theory that the proper relative proportions of growth regulators of auxin and cytokinin group determine the direction of morphogenetic changes (Lal *et al*. 2010; Yadav *et al*. 2011a; Yadav *et al*. 2011b). The efficacy of plant growth regulation (PGR) combinations in the process of obtaining the stevia shoots has been already found because in the presence of the same concentrations of cytokinin in the medium 17.5 shoots were obtained from the explant, whereas in the absence of BAP in the medium 10.2 shoots were observed, 13.7 shoots were obtained from the explants using BAP without KIN (Verma *et al*. 2011). The impact of BAP, on morphogenesis processes in shoot culture of stevia was also evaluated by Ibrahim *et al*. (2008). Described by these authors of numerous analysis of the influence of BAP concentration (from 0.25 to 2.50 mg dm^{-3}) on the biometric features of the explants indicate that the more stimulating effect showed a stronger concentration from those described in our experiment. However, the use of high concentrations of this PGR inhibited the growth of shoots – the length of

their size was significantly shorter from those obtained on the control MS media without growth regulators.

Ahmed *et al*. (2007) and Thiyagarajan and Venkatachalam (2012) also tested the effect of BAP or BAP and KIN combination on the shoot proliferation. Benzylaminopurine at concentration of 0.5 to 2.0 mg dm^{-3} generated from 6 to 15 shoots per explant. However, in the studies conducted by Jitendra *et al* (2012), at comparable concentrations of BAP, from one nodal fragment only three new shoots were developed. The combined effect of BAP and KIN was also weaker, ranging from 4 to 9 new shoots. In this case it was proved that the most efficient medium was that containing 1.5 mg dm^{-3} BAP and 0.5 mg dm^{-3} KIN. The addition of KIN to the medium noticeably stimulated the growth of shoot length. In such culture conditions Ahmed *et al*. (2007) reported that the stems were about 2 cm longer than without this cytokinin. Positive effects were also obtained by BAP supplemented with a small amount of IAA, IBA or NAA (Jitendra *et al*. 2012; Thiyagarajan and Venkatachalam 2012). A beneficial effect of KIN on shoot proliferation was noticed by Das *et al* (2011) getting the highest number of shoots. The study results which are quoted above show that BAP stimulates the development of axillary buds and new shoots formation definitely better than KIN. Whereas, there is no doubt that KIN intensifies the process of shoot elongation. According to our observations KIN also affected the number and size of leaves of stevia in *in vitro* culture (the leaf blades of approx. 5 mm were found). In our experiment short internodes of stevia plants growing on culture media, containing BAP or KIN, was observed. In order to obtain longer internodes, GA_3 was applied in accordance with Giridhar *et al* (2010). The addition of GA_3 to the medium greatly influenced the elongation of shoots. This procedure allowed us to obtain regenerants which were approx. 12 cm long. These shoots were characterized not only by greater length, but also they had more leaves and wider leaf blades. They were more vital and had better shape. Our observations confirm results of Verma *et al* (2011) and Guruchandran and Sasikumar (2013), indicating the inductive effect of GA_3 in *S. rebaudiana* plant regeneration. In our study the plants that were received from media enriched with cytokinin or gibberellic acid were placed on the rooting medium containing 0.5 mg dm^{-3}. IBA. Elongated shoots transferred from the medium with GA_3 underwent the process of rhizogenesis with the highest intensity. Detailed observations show that in such cases there was the formation of root with a length of 4.6 cm. Their average number was 7.9. Similar observation has been reported by Alhady (2011) and Jitendra *et al* (2012), they also found positive role of IBA during *in vitro* rooting of *S. rebaudiana*. In contrast Chotikadachanarong and Dheeranuupattana (2013) and Guruchandran and Sasikumar (2013) reported that it is not only IBA but 1.0 mg dm^{-3} NAA had also a positive effect on the processes of roots formation. However, both Ibrahim *et al*. (2008) and Alhady (2011) confirmed the best rooting

response of shoots on medium containing IBA compared to medium supplemented with NAA.

In order to obtain commercial *S. rebaudiana* plants suitable for growing in the field or under cover, pre-rooted shoots should be subjected to a process of acclimatization to *ex vitro* conditions. For this purpose plantlets were transferred to plastic pots filled with organic and mineral substrate. By optimizing this stage propagated plants were placed in controlled conditions and watered with a 1/4 MS salt solution. This treatment improved the efficiency of acclimatization by 24%. Our results are in agreement with those reported by Jitendra *et al*. (2012), which describe the positive effect of applying a MS salt solution on the plants habit and their growth and development. The presence of exogenously applied ions, in the aqueous salt solution which was used for irrigation, weakened the negative stress response of plants to the process of acclimatization and allowed to obtain plants of *S. rebaudiana* species distinguished by a typical habit and growth rates.

CONCLUSIONS

It might be stated, bearing in mind the results of *in vitro* culture, that the MS medium supplemented with BAP is useful for mass propagation of *Stevia rebaudiana* Bertoni.

Growth parameters of plants, such as the length of shoots and the number and size of the leaves are dependent on the composition of the medium. BAP and GA_3 application to the medium during the *in vitro* culture stimulates plant growth and causes symptoms such as increasing the number and size of leaves, which are a source of valuable glycosides. The process of rooting was stimulated in the highest degree by the presence of IBA in the medium. The presence of salt ions in the MS during the acclimatization increases the efficiency of the process. Plants from *in vitro* cultures can be a source of seedlings for planting organic crops and for home gardens.

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