Micropropagation of *Stevia rebaudiana* plants

Marta Teresa Rokosa¹* Danuta Kulpa²

¹Laboratory of Plant Physiology and Entomology, Department of Bioengineering, Faculty of Environmental Management and Agriculture, West Pomeranian University of Technology in Szczecin, ul. Julussa Słowackiego 1770-953 Szczecin, Poland. E-mail: marta.rokosa@zut.edu.pl.
²Department of Genetics, Plant Breeding and Biotechnology, Faculty of Environmental Management and Agriculture West Pomeranian University of Technology in Szczecin, Szczecin, Poland.

ABSTRACT: The aim of the study was to develop optimum composition of plant growth regulators in media for the propagation and rooting of shoots of stevia (*Stevia rebaudiana* Bertoni) in in vitro cultures. Single-node shoot fragments obtained from plants propagated on MS medium were placed onto media supplemented with: BAP, 2iP and KIN at concentrations: 0.5, 1, 2 and 5 mg dm⁻³, whereas at the rooting stage with addition of: IAA, IBA and NAA at concentrations 1, 2, 4 and 8 mg dm⁻³. The highest number of shoots and leaves was reported for plants propagated on MS medium enriched with 0.5 mg dm⁻³ BAP. The greatest number of the longest roots was developed by stevia on the MS medium enriched with 1 mg dm⁻³ IAA.

Key words: micropropagation, plant growth regulators, cytokinins, auxins, steviosides.

INTRODUCTION

*Stevia rebaudiana* Bertoni is a plant belongs to the *Asteraceae* family. It is a shrub growing to 80 cm height, having leaves with a lanceolate shape. The stem is woody, the flowers are abundant and possess white-violet color (LEMUS-MONDACA et al., 2012). Its leaves contain diterpene glycosides, such as steviosides, rebudiosides, rubosides steviolbiosides and dulcosides, the sweetness of which is up to 300-fold higher than traditional sugar and thus it is known as sweet leaf (BONDAREV et al., 2001, DASI et al., 2011, GANTAIT et al., 2015, GEUNS, 2003, LEMUS-MONDACA et al., 2012, SIVARAM & MUKUNDAN, 2003). In many countries, stevia is used as a healthier substitute for cane or beet sugar, commonly used to sweeten many dishes. The plant has been used in the area of its origin (South America) to sweeten various food products, including seafood, marinated vegetables, desserts, beverages and confectionery for centuries (KHAN et al., 2013). The sweetener produced from stevia leaves, contrary to typical sucrose, is low in calories and low-glycemic, thus it is recommended for persons with diabetes (BESPALHOK-FILHO & HATTORI, 1997, GOETTEMOELLER - LUCKE, 2010). Sweetening substances present in stevia did not cause dental caries (MATHUR - SHEKHAWAT, 2013).

Moreover, stevia leaves contain alkaloids, flavonoids, water-soluble chlorophylls and xanthophylls, hydroxyccinnamic acids (caffeic, chlorogenic etc.), water-soluble inert oligosaccharides, free sugars, amino acids, lipids, essential oils, trace elements, vitamins, polyphenols and other antioxidants (CARBONELL-CAPELLA et al., 2013, TAVARINI & ANGELINI, 2013).

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RESUMO: O objetivo do estudo foi desenvolver uma composição ótima de reguladores de crescimento em meios para a propagação e enraizamento de brotos de estévia (*Stevia rebaudiana* Bertoni) em culturas in vitro. Fragmentos de parte aérea obtidos de plantas propagadas em meio MS foram colocados em média suplementada com: BAP, 2iP e KIN nas concentrações: 0.5, 1, 2 e 5 mg dm⁻³, enquanto no estádio de enraizamento com adição de: IAA, IBA e ANA nas concentrações 1, 2, 4 e 8 mg dm⁻³. O maior número de brotações e folhas foi encontrado para plantas propagadas em meio MS enriquecido com 0.5 mg dm⁻³ de BAP. O maior número de raízes mais longas foi desenvolvido por estévia no meio MS enriquecido com 1 mg dm⁻³ de IAA.

Palavras-chave: micropropagação, reguladores de crescimento de plantas, citocininas, auxinas, esteviosídeos.
In addition, *S. rebaudiana* leaf extract has antibacterial and fungicidal properties, with an inhibitory effect on the growth of such bacteria as *Staphylococcus aureus*, *Streptococcus mutans*, *Bacillus subtilis* and *Escherichia coli* (RAZAK et al., 2014). Moreover, its antihypertensive, anti-inflammatory, anti-cancer, anti-diarrheal, diuretic and immunomodulatory properties have been demonstrated (CARBONELL-CAPELLA et al., 2013). *In vitro* and *in vivo* studies carried out on humans and animals under the WHO (World Health Organization) supervision, demonstrated that consumption of steviosides and rebaudiosides did not induce mutagenicity, teratogenicity, and carcinogenicity (LEMUS-MONDACA et al., 2012, KHALIL et al., 2014).

Considering its valuable properties, as well as highly impeded traditional stevia propagation methods (seeds demonstrate very low germination percentage, and the progeny plants exhibit high variability in terms of chemical composition and sweetness level), the need for development of an efficient method for mass clonal propagation of stevia in *in vitro* conditions appears to be valid (SOLIMAN, 2014, NOWER, 2014, KUMAR, 2013, DESHMUKH & ADE, 2012, ANBAZHAGAN et al. 2010, PANICKER et al. 2007, HWANG, 2006, SIVARAM & MUKUNDAN, 2003). However, in order for this to be possible, the parameters of the conducted culture must be precisely determined, and in particular the content of plant growth regulators in the medium. Research should be conducted on the effects of different concentrations of plant growth regulators on the growth and development of stevia. Currently, there are no studies using the factors and conditions used in this study. Thus, the objective of this study was to determine the impact of selected growth regulators on the propagation and rooting of stevia plants in *in vitro* cultures.

**MATERIALS AND METHODS**

*Initiation stage.* Stevia seeds (Vilmorin Clause & Cie, Paris, France) constituted the study material. For the purpose of disinfection, the seeds were soaked in water with the addition of detergent-Ludwik for 20 minutes. Subsequently, they were rinsed several times under running water and immersed for 30 seconds in 70% ethanol solution. Initially disinfected seeds were shaken in 10% sodium hypochlorite solution for 15 minutes and were rinsed three times with sterile water. Then were placed individually to 15 ml test tubes containing 3 ml MS medium (Murashige and Skoog, 1962). Thus obtained sterile shoots, propagated several times on MS medium constituted the population for the establishment of the subsequent experiment.

**Propagation stage**

In the subsequent stage, 1 cm long single-node shoots were placed on MS media with 0.5, 1, 2 or 5 mg dm$^{-3}$ addition of cytokinins: BAP (6-benzyl aminopurine), 2iP (2-isopentyladenine) or KIN (kinetin). Twenty passages were carried out to multiply plant material.

**Rooting stage**

The 1 cm long nodal fragments was taken from plant originated from propagation phase, and were placed onto MS media supplemented with auxins: IAA (indole-3-acetic acid), IBA (indole-3-butyric acid) and NAA (1-naphthaleneacetic acid) at concentrations 1, 2, 4 and 8 mg dm$^{-3}$. Explants placed onto MS medium without addition of plant growth regulators constituted control in both experiments.

At all study stages, the pH of media was set at 5.7 using 0.1 M solutions of hydrochloric acid (HCl) sodium hydroxide (NaOH). Media were supplemented with 8 g dm$^{-3}$ agar (media were gelled), 30 g dm$^{-3}$ sucrose and 100 mg dm$^{-3}$ inositol. The cultures were carried out in 300 ml jars, each containing 30 ml, which were sterilized at 121 °C for 20 min. To each jar, 6 explants were introduced and each combination comprised of 7 jars. The established cultures were placed in a growth chamber, on shelves illuminated with white light for 16 hours, at 40 µmol·m$^{-2}$·s$^{-1}$ PAR intensity, temperature 25±1 °C. After 5 weeks the number and length [cm] of shoots, number, and length [cm] of roots, as well as plant weight [g], were determined.

**Statistical analysis**

Results of biometric measurements were analyzed using ANOVA and the significance of differences at $\alpha=0.05$ between the tested media was assessed by means of Tukey test.

**RESULTS AND DISCUSSION**

**Propagation stage**

The study allowed to demonstrate the addition of BAP and 2iP cytokinins influenced the increase in the number of side shoots. Their highest number was reported for media supplemented with 0.5 and 1 mg dm$^{-3}$ BAP (Figure 1)-close to two-fold
higher compared to plants propagated on control medium. With the increase of BAP concentration, the number of produced shoots decreased. A reverse tendency was observed for KIN, where its increased concentration resulted in the formation of a higher number of shoots. Plants propagated on media with the addition of cytokinins produced a higher number of leaves (from 12.06 to 21.17) relative to control plants (9.60). The exception consisted of plants propagated on media with the addition of the lowest KIN concentrations (0.5 and 1.0 mg·dm⁻³). The highest number of leaves was determined in the case of the lowest BAP concentration (0.5 and 1 mg·dm⁻³) (Table 1).

The major properties of cytokinins are: stimulation of cell division and release of lateral bud dormancy (GASPAR et al., 1996). Based on the completed study, it can be determined that addition of cytokinins to media has a stimulatory effect on the formation of the above-ground parts of stevia (Stevia rebaudiana Bertoni) plants—under their influence, the number of shoots and leaves has typically increased. An identical result was obtained by Nower (2014), where plants growing on media enriched with BAP at 0.5 and 1 mg·dm⁻³ concentration produced the highest number of shoots. Moreover, the study of ZAYOVA et al. (2013) demonstrated that a medium with the

![Figure 1](image-url) - Stevia growing on MS with 1 mg · dm⁻³ BAP (A), without growth regulators (B) and with 5 mg · dm⁻³ KIN.

### Table 1 - Mean values of morphological characters of plants propagated on media with variable cytokinin content.

| Cytokinin concentration [mg·dm⁻³] | Length of shoots [cm] | No. of shoots | No. of leaves | Fresh weight [g] | No. of roots | Length of roots [cm] |
|----------------------------------|-----------------------|---------------|--------------|------------------|-------------|---------------------|
| **MS**                           | **BAP**               |               |              |                  |             |                     |
| 0.5                              | 2.80 b⁷               | 1.42 e        | 9.60 g       | 0.053 f         | 0.64 bc     | 0.29 cd             |
| 1                                | 2.78 b                | 3.04 a        | 19.04 ab     | 0.157 ab        | 0.40 bc     | 0.13 de             |
| 2                                | 2.42 c                | 3.38 a        | 21.17 a      | 0.157 a         | 0.00 e      | 0.00 e              |
| 5                                | 1.90 f                | 2.63 b        | 17.08 bc     | 0.124 cd        | 0.00 e      | 0.00 e              |
| **2iP**                          | **KIN**               |               |              |                  |             |                     |
| 0.5                              | 2.96 b                | 2.17 c        | 14.88 c      | 0.167 a         | 0.00 e      | 0.00 e              |
| 1                                | 2.20 cde              | 2.69 b        | 15.39 cd     | 0.123 cd        | 0.00 e      | 0.00 e              |
| 2                                | 1.80 f                | 2.05 cd       | 12.06 ef     | 0.100 de        | 0.00 e      | 0.00 e              |
| 5                                | 1.96 ef               | 2.33 bc       | 18.64 b      | 0.173 a         | 0.00 e      | 0.00 e              |
| 0.5                              | 3.65 a                | 1.79 de       | 11.46 fg     | 0.132 bc        | 1.21 a      | 0.81 a              |
| 1                                | 2.35 cd               | 1.75 de       | 10.79 fg     | 0.076 ef        | 0.29 cde    | 0.41 bc             |
| 2                                | 2.81 b                | 2.05 cd       | 14.09 cde    | 0.156 ab        | 0.74 b      | 0.63 ab             |
| 5                                | 2.45 c                | 2.50 b        | 15.17 c      | 0.183 a         | 0.21 de     | 0.16 de             |

*means marked with the same letters do not differ significantly at the significance level 0.05.
addition of BAP at 1 mg·dm\(^{-3}\) concentration enables
the production of a higher number of shoots in stevia
than at 0.5 mg·dm\(^{-3}\) concentration. YÜCESAN et al.
(2016) demonstrated that addition of BAP and KIN
at concentrations between 0.1 – 2 mg·dm\(^{-3}\) results in
the development of an equal number of shoots, yet
the longest shoots were obtained when higher KIN
concentrations were used (0.5, 1 and 2 mg·dm\(^{-3}\)).
The study of DAS et al. (2013), kinetin added to the
medium at the concentration of 2 mg·dm\(^{-3}\) was the
most efficient growth regulator stimulating growth and
development of stevia shoots. In the present
study, plants growing on medium with the addition
of kinetin produced a lower number of shoots than
plants growing on media enriched with BAP and
2iP; however, they attained a greater height than the
latter combinations. SOLIMANI et al. (2014) showed that
the highest number of shoots is formed on medium enriched with 2 mg·dm\(^{-3}\) BAP. This has been
confirmed by the results obtained by RAMIREZ-
MOSQUEDA et al. (2016).

The addition of the following cytokinins
to the medium: BAP and 2iP, added at 1–5 mg·dm\(^{-3}\)
concentrations influenced shortening of the shoots relative to control plants. Cytokinins applied in the experiment had a negative impact on the elongation of the developed shoots. Shoots of plants propagated on the test media had lower or equal height compared to control plants. The exception consisted of plants propagated on the medium with the addition of 0.5 mg·dm\(^{-3}\) KIN (3.65 cm), whose shoots were significantly longer than in plants propagated on MS medium (Table 1). The present study results are similar to the results procured by NOWER (2014), who was also able to demonstrate a positive influence of kinetin on the stevia shoot elongation growth; however, the most favorable result was obtained upon using a medium with the addition of 1.5 mg·dm\(^{-3}\) KIN. Also, the study carried out by DAS et al. (2011) showed that kinetin causes stevia shoot elongation growth. However, in that study, the most favorable result was obtained by using kinetin concentration of 2 mg·dm\(^{-3}\). Conversely, a study conducted by CHOTIKADACHANARONG & DHEERANUPATTAN (2013) showed that stevia developed longest shoots when explants were introduced onto medium without kinetin addition. Such differences can be attributed to the physiological condition of the explant, which is determined by genetic factors. Multiple passages foster mutation (ROKOSA & MIKICIUK, 2017) In this study the plants originated from several-year-old culture - in the CHOTIKADACHANARONG & DHEERANUPATTAN’S (2013) study plants could be younger or older (there is no information).

The present experiment also demonstrated that all cytokinin types used in the experiment had a stimulatory effect on the number of leaves developed. The highest number of leaves characterized plants propagated on medium with the addition of BAP at 0.5 and 1 mg·dm\(^{-3}\) concentration (Table 1). Further increase in its concentration reduced a number of leaves. A reverse situation was observed for media enriched with 2iP and KIN, where increasing cytokinin concentration resulted in increased number of stevia leaves (Table 1). The study of SIVARAM & MUKUNDAN (2003) demonstrated a complete inhibition of stevia leaf growth propagated on media enriched with kinetin, and the 4 mg·dm\(^{-3}\) (8.87 μM) addition of BAP stimulated their quantity. A similar result was also obtained by ANBACHIAGANA et al. (2010), where kinetin inhibited stevia leaf development, and BAP at 2.5 mg·dm\(^{-3}\) concentration produced the highest number of leaves.

Inhibition of root system formation is the
typical effect of cytokinin action. Inhibitory effect of all cytokines on root system formation compared to plants growing on control medium was also observed in the present study. Formation of the short and low number of roots was only observed on the control medium, medium enriched with KIN (independently of the concentration) or 0.5 mg·dm\(^{-3}\) in BAP (Table 1). The above observations are in line with the study of TREJGELL et al. (2013), who demonstrated the inhibitory effect of BAP at a concentration above 0.5 mg·dm\(^{-3}\) on the formation of roots of dandelion (Taráhacum piennicum), an Asteraceae representative. Moreover, the study carried out by PANICKER et al. (2007) on a chrysanthemum species, Dendranthema grandiflora cv. Arka Swarna (Asteraceae), demonstrated that the highest number of roots was formed on the medium without addition of cytokinins. Furthermore, rhizogenesis was completely stopped when cytokinin concentrations were elevated in the media. Similar results were also obtained by IBRAHIM et al. (2008).

In the majority of cases, the addition of cytokinins produced an increase of stevia plant mass as compared to control (Table 1). Plants propagated on medium with 1 mg·dm\(^{-3}\) KIN addition constituted an exception, with their mass being similar to control plants. The lowest BAP concentrations (0.5 and 1 mg·dm\(^{-3}\)) and higher KIN concentrations (2 and 5 mg·dm\(^{-3}\)) had the strongest stimulatory effect on plant mass increase. In the study of CHAKRABARTY & DATT (2008) on Gerbera jamesonii, also classified in

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the *Asteraceae* family, a positive impact of cytokinin addition to medium on the formation of plant fresh weight was also observed.

**Rooting stage**

In the second experiment, the influence of auxins on the development of stevia in *in vitro* cultures was examined. It was determined that the addition of auxins at higher concentrations has an inhibitory effect on the development of the above-ground plant part (Figure 2). Stevia rooted in media supplemented with the lowest used IAA concentration (1 mg·dm⁻³) developed shoots that were longer (5.24 cm) than in control plants (4.47 cm). Increased concentration of all auxins used in the experiment resulted in decreasing shoot length (Table 2). Moreover, the study demonstrated that addition of IAA auxin to medium, independently of its concentration and IBA and NAA auxins at lowest concentrations did not influence the number of shoots produced (Table 2; average 1.51). Plants rooted in media supplemented with higher IBA concentrations (4 and 8 mg·dm⁻³) and NAA (2, 4 and 8 mg·dm⁻³) developed a lower number of shoots (average 0.62) than control plants (1.4). The number of stevia leaves on media with IAA addition was similar to control plants (Table 2). However, an increase of the IBA and NAA concentration (to 4 and 8 mg·dm⁻³) resulted in a reduced number of leaves. It was demonstrated that plants propagated on media supplemented with lower IBA concentrations (1 and 2 mg·dm⁻³) are characterized by higher mass relative to control. Increased auxin concentration resulted in reduced plant mass.

The plants produced from 6.08 to 0.16 roots. Similarly to the aforementioned characters, it was also here where the highest number of roots were formed in media with the addition of lower plant growth regulator concentrations – 1 and 2 mg·dm⁻³ (Figure 3). Their highest number was determined in the case of plants propagated on medium supplemented with 1 mg·dm⁻³ IBA (6.08). With the increase of plant growth regulator concentrations, the number of roots produced decreased (Table 2). Similar results were obtained by RAZAK et al. (2014), where the highest number of roots was developed on the medium with the same composition. These authors were able to produce the same result in the case of root length - the longest roots were formed on medium with the addition of low IAA concentrations. Increasing concentration of auxins added to media resulted in a reduction of the number and length of roots produced in both in the present experiment, as well as in the work of RAZAK et al. (2014). HWANG (2006) also confirmed the results of the aforementioned studies. The highest number of roots also developed on a medium enriched with 1 mg·dm⁻³ IBA, and the percentage or plants with developed roots was 100%. Similar results were obtained by ZAYOVA et al. (2013). According to their study, the highest number of roots is produced by stevia on medium with 0.1 mg·dm⁻³ IBA (100% of plants developed roots), whereas the longest roots can be observed in plants growing on medium with 0.5 mg·dm⁻³ IAA addition. These results are also confirmed by ANBAZHAGAN et al. (2010) and IBRAHIM et al. (2008).

Conversely, the study of NOWER (2014) showed that the highest number of roots was developed on medium with 0.1 mg·dm⁻³ IAA, whereas longest roots developed on medium enriched with 1 and 2 mg·dm⁻³ IBA. However, similar to the present study, NOWER (2014) showed that highest plants with the highest number of leaves grew on the medium with 1

![Figure 2 - Stevia growing on MS with 1 mg · dm⁻³ IAA (A), 8 mg · dm⁻³ IBA (B), 2 mg · dm⁻³ IBA (C), without growth regulators (D) and with 8 mg · dm⁻³ NAA (E).](image)
mg dm\(^{-3}\) IAA addition. What is more, DESHMUKH & ADE (2012) stated that the highest number of the longest roots was formed in stevia growing on the medium with 0.1 mg dm\(^{-3}\) IAA. Moreover, such plants exhibited the highest contribution of rooted individuals (95%). As in the present study, those authors observed the inhibitory influence of higher auxin concentrations on root formation. Similar
results were obtained by YÜCESAN et al. (2016), who was able to procure the highest number of roots and 100% rooted plants on MS medium with 0.25 mg∙dm$^{-3}$ IAA addition and SOLIMAN et al. (2014), who obtained the highest number of roots and 98.72% rooted plants on MS medium with 0.5 mg∙dm$^{-3}$ IAA.

The longest roots were developed by stevia shoots rooted on control medium (2.96 cm) and on media with the lowest (1 mg∙dm$^{-3}$) auxin concentration. Increase in the IAA and NAA concentration (4 and 8 mg∙dm$^{-3}$) resulted in a reduction of the number of roots formed, as well as their length relative to plants rooted in control medium. Plants propagated on media supplemented with auxins at those concentrations developed roots measuring from 0.12 cm to 1.24 cm (Table 2).

The NAA turned out to be the least efficient growth regulator for stevia rooting in RAZAK et al. (2014), HWANG (2006), NOWER (2014), DESHMUKH AND ADE (2012) and ANBAZHAGAN et al. (2010), as well as in the present study. A completely different result was obtained by THIYAGARAJAN & VENKATA CHALAM (2012). Those authors demonstrated that NAA is a more efficient growth regulator stimulating root formation in stevia than IAA and IBA. As in length of shoots case described, this difference can be attributed to the physiological condition of the explant, which is determined by genetic factors. In THIYAGARAJAN & VENKATSCHALAM’S (2012) study mother plants were 3 months old and there was small number of passages. Conversely, in the study of THIYAGARAJAN & VENKATA CHALAM (2012) the highest number (8.3) of the longest (6.13 cm) roots were produced on medium enriched with 0.5 mg∙dm$^{-3}$ NAA. Moreover, those plants exhibited the highest contribution of rooted individuals (86%).

CONCLUSION

On the basis of the conducted study it can be determined, that the used cytokinins: KIN, BAP and 2iP, and auxins: IAA, IBA, and NAA added to mineral medium according to Murashige and Skoog (1962) had a very significant impact on the growth and development of stevia (Stevia rebaudiana Bertoni) in in vitro cultures.

Stevia should be propagated on Murashige and Skoog (1962) mineral medium enriched with 0.5 mg∙dm$^{-3}$ BAP. Plants propagated on such substrate are characterized by high mass and produce numerous shoots and leaves.

Stevia shoots should be rooted on medium with Murashige and Skoog (1962) medium enriched with 1 mg dm$^{-3}$ IBA. Plants growing on such medium were characterized by the highest number of long roots.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS’ CONTRIBUTIONS

The authors contributed equally to the manuscript.

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