Abstract

Stevia rebaudiana Bertoni is important medicinal plant which is native between southern Brazil and northern Paraguay. A reliable protocol for shoot organogenesis and regeneration was developed for an antidiabetic medicinal plant species. Regeneration via indirect shoot organogenesis was established from two types of explant (leaf and stem) on MS medium [1] with different concentrations and combinations of plant growth regulators (PGR) such as 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and gibberellic acid (GA$_3$) for inducing organogenic callus of S. rebaudiana. In the present investigation the highest percentage of response was shown by 90% and 80% callogenesis from leaf and stem explants on MS medium supplemented with 2.0 mg/l 2,4-D + 0.5 mg/l BAP + 0.5 mg/l GA$_3$ (MSC$_3$) was observed. The high frequency of regenerants was observed from S. rebaudiana leaves at 0.5 mg/l 2,4-D + 2.0 mg/l BAP + 0.5 mg/l GA$_3$ (MSR$_3$). Optimal shoot multiplication and rooting were obtained on 2.0 mg/l BAP and 0.1 mg/l indole-3-butyric acid (IBA), respectively, followed by successful acclimatization of regenerants (90% survival) to greenhouse condition. The purpose of this study was to establish a reliable S. rebaudiana regeneration protocol that can be used to facilitate the cultivation, propagation and planting of this plant species.

Key words: stevia, callus from leaf and stem, indirect organogenesis

Introduction. Stevia rebaudiana Bertoni, belonging to the family Asteraceae, is a perennial sweet herb. It is a native medicinal plant of Paraguay [2]
and is a new alternative source of calorie-free sweetener having no carbohydrates. The leaves of this plant produce diterpene glycosides (stevioside and rebaudiosides). Pure stevioside is 30 times sweeter than sugar $[^3,4]$. The dry extract from the leaves also contains flavonoids, alkaloids, water-soluble chlorophylls and xanthophylls, hydroxycinnamic acids (caffeic, chlorogenic, etc.), neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils, and trace elements. The additional benefits are: zero calories, zero carbohydrates, not causing spikes in blood sugar levels, having non-fermenting ability and maintaining thermal stability at 100°C. The leaf extract of the stevia plant has been used in the treatment of diabetes $[^5]$. It also enhances weight reduction, prevents dental caries, and has antimicrobial properties. It is non-carcinogenic, a quality distinguishing it from artificial sweeteners $[^6]$. The favourable soil and climatic conditions in Bulgaria also allow the successful growth of $S. rebaudiana$, but only as an annual plant because rhizomes cannot survive in low soil temperatures in winter. There are different factors that influence the process of callus and organogenesis, such as initial explants and concentration and combination of growth regulators. Plant organogenesis was the common pathway for in vitro clonal propagation of superior medicinal plant species. Callus along with multiple shoots were obtained from nodal segments of $S. rebaudiana$ on half strength MS media supplemented with various concentrations of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) in concentrations 0.2, 0.5, 1.0, 1.5 mg/l $[^7]$. The analysis of stevioside and rebaudioside A in callus cultures by LC-MS method has shown that MS medium with 2,4-D and BAP and glucose or dextrose (3%), exposure of callus cultures to light for 12 h photoperiod has promoted accumulation of both stevioside and rebaudioside A in one-month-old leaf derived callus cultures $[^8]$. Stem and leaf segments were inoculated on MS medium supplemented with different concentrations of BAP and NAA for callogenesis $[^9]$. Apical meristem explants showed better advantages for in vitro cultivation of $S. rebaudiana$ since they present less contamination and higher survival at the induction stage, even when exhibiting the highest oxidation among explants, which did not influence the decrease in their survival $[^10]$. Overall, this system results in mass multiplication of $S. rebaudiana$ with simultaneous production of callus within short period $[^7]$. The objective of this study was to develop an efficient callogenesis from leaf and stem explants and indirect shoot organogenesis for $S. rebaudiana$.

**Materials and methods.** This investigation was conducted in Plant Tissue Culture Laboratory at the Department of Plant-Soil Interactions, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences. $S. rebaudiana$ plants of the following origins was studied: St USA (Stevia – United States of America). Various plant explants (leaf and stem) taken from in vitro healthy and clean $S. rebaudiana$ plants in active growth used to induce fast-growing callus. To obtain the primary callus, the sterilized explants cultured on MS nutrient medium with different ratios of growth regulators of auxin and cytokinin nature. Callus did
not form at MSC0 and MSR0-hormone-free medium as a control. Callus induction occurred under controlled conditions (22 ± 1°C, 70% humidity, 16/8 h photoperiod, 40 µmol/m²s⁻¹ illumination by Philips 36 W cool white fluorescent tubes) and scored after four weeks intervals. The calluses were further subcultivated on fresh MS containing 2,4-D, BAP and GA3 (Table 1). After three subcultures, the callus characteristics were taken into account. Fresh weight of callus was recorded after the first and third cultivation. The percentage of callus forming shoot was reported after the third subculturing. For indirect organogenesis, the leaf and stem derived calluses on MSC1–MSC3 media were transferred on PRG-free medium as a control (MSR0) and on media with 2,4-D/BAP/GA3 (MSR1–MSR3, respectively) (Table 1). For shoot multiplication, the regenerated plants were cultivated on MS with two different BAP concentrations for four weeks of culture (Table 2). The explants were placed in the culture vessels (tubes 140 × 20 mm), with 2–3 explants per 10 ml of medium. For rooting, S. rebaudiana regenerants were transferred to half strength MS (½ MS) medium with 2.0% sucrose (control variant) and to IBA containing medium for rooting for three weeks (Table 2). S. rebaudiana regenerants were moved to a growth chamber (24 ± 1°C, 16/8 h photoperiod, 50 µmol/m²s⁻¹ illumination by fluorescent lamps) for ex vitro acclimatization. They were planted individually to small plastic pots (8 cm diameter) containing mixture of soil:sand:perlite (2:1:1, v/v/v) covered with a transparent polythene membrane to ensure high humidity (90%) and then opened after three weeks. The survival rate of the acclimatized plants was determined after six weeks. After two months, the plants were transferred to greenhouse.

**Statistical analysis.** Each treatment involved at least 20 samples and the data are presented as mean ± SE. The data were statistically analyzed using analysis of variance (ANOVA) for comparison of means, and significant differences were calculated according to Fisher’s least significance difference test at the 5% significance level using a statistical software package (Statgraphics Plus, version 5.1 for Windows).

**Results and discussion.** Callus induction. Callus induction was obtained on MS media supplemented with various concentrations of 0.5, 1.0 and 2.0 mg/l of auxin 2,4-D and cytokinins 0.5 mg/l BAP and 0.5 mg/l GA3. Callus did not show regeneration when cultured on PGR free medium. Callus was initiated from leaf and stem explants of S. rebaudiana after the four weeks of culture. The impact of PGRs was to investigate on stevia callus induction and growth from in vitro tissue. In the present study, the effects of 2,4-D, BAP and GA3 on callogenesis from leaf and stem explants of S. rebaudiana plants were investigated (Table 1, Fig. 1). The highest frequency of callus formation (95% for leaf and 80% for stem explants) was observed when high auxin 2,4-D (2.0 mg/l) and low BAP and GA3 (0.5 mg/l) concentrations were applied (MSC3). Good response for percentage of callus induction (70% and 80%, respectively) was obtained from the leaf explants on MS medium, when the medium was supplemented with the two
Table 1
Effect of PGRs on callus induction and shoot regeneration from leaf and stem explants of *S. rebaudiana*

<table>
<thead>
<tr>
<th>Type of culture</th>
<th>PGRs in MS medium, mg/l</th>
<th>Leaf explants</th>
<th>Stem explants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,4-D</td>
<td>BAP</td>
<td>GA$_3$</td>
</tr>
<tr>
<td>MSC$_0$</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>MSC$_1$</td>
<td>0.5</td>
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<tr>
<td>MSC$_2$</td>
<td>1.0</td>
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<tr>
<td>MSC$_3$</td>
<td>2.0</td>
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<td>MSC$_0$</td>
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<td>MSC$_2$</td>
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<tr>
<td>MSC$_3$</td>
<td>2.0</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>Regeneration</td>
<td>Shoot regeneration, %</td>
<td>No shoots/callus</td>
<td>Shoot regeneration, %</td>
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<tr>
<td>MSR$_0$</td>
<td>0</td>
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<tr>
<td>MSR$_1$</td>
<td>0.5</td>
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<td>MSR$_2$</td>
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<td>1.0</td>
<td>0.5</td>
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<tr>
<td>MSR$_3$</td>
<td>0.5</td>
<td>2.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The data are presented as means of an average sample of 20 plants per treatment ± SE. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing ANOVA multifactor analysis.
combinations of growth regulators (MSC\textsubscript{1} and MSC\textsubscript{2}). The colour and texture of the callus varied depending on the type of explants as well as the concentrations of growth regulators used in the media. The calluses were compact green and whitish with friable appearance (Fig. 1a, b, e, f). The frequency of callus induction on all the medium was high but leaf explants were more responsive than the stem ones. The callus cultures are the preferred type of plant in vitro cultures, and they are used also in \textit{S. rebaudiana} to produce healthy biomass under controlled growth conditions from which medicinally potent natural products could be extracted. Previous reports on \textit{S. rebaudiana} showed that the type of explants used as callus source significantly affect callus- and organogenesis \cite{10}. The results in Table 1 show the mean of fresh weight of callus induced by different concentrations of 2,4-D/BAP/GA\textsubscript{3}. The cytokinins BAP and GA\textsubscript{3} and auxin 2,4-D combination had a positive effect on the growth of callus mass compared with that of the control. The highest mean of fresh weight of callus from leaf and stem explants was induced by MSC\textsubscript{3} (0.550 g and 0.450 g, respectively), followed by MSC\textsubscript{2} (0.380 g and 0.340 g, respectively) induced lowest amount of fresh weight of callus MSC\textsubscript{1} (0.320 g and 0.305 g, respectively). Fresh weights show the same trend in callus subculturing in \textit{S. rebaudiana}. BLINSTUBIEN\accentuml{E} et al. \cite{9} showed that leaf and stem segments were inoculated on MS medium supplemented with different concentrations of NAA and BAP for callus genesis and callus growth by \textit{S. rebaudiana}. In many cases, callus mass from the leaf explants was higher in comparison to that from the stem segments, so the leaf explant is more suitable for obtaining a higher callus mass of \textit{S. rebaudiana}. Culture medium supplemented with 2.0 µM NAA

\begin{table}
\centering
\caption{Effect of PGRs on \textit{S. rebaudiana} shoots and roots induction of regeneration from callus derived from leaf explants}
\begin{tabular}{lll}
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& Shoot multiplication, MS & \\
\hline
Medium variants & Shoot formation, % & Number of shoots per explant & Shoot height, cm \\
\hline
1.0 mg/l BAP & 80 & 5.8 ± 0.29\textsuperscript{a} & 1.5 ± 0.08\textsuperscript{a} \\
2.0 mg/l BAP & 95 & 8.2 ± 0.41\textsuperscript{b} & 1.8 ± 0.09\textsuperscript{b} \\
\hline
Rooting, 1/2 MS & & & \\
\hline
Root formation, % & Number of roots per plant & Root length, cm \\
\hline
1/2 MS (control) & 40 & 1.3 ± 0.07\textsuperscript{c} & 0.8 ± 0.04\textsuperscript{c} \\
0.1 mg/l IBA & 95 & 4.5 ± 0.23\textsuperscript{c} & 1.6 ± 0.08\textsuperscript{b} \\
0.5 mg/l IBA & 85 & 2.2 ± 0.11\textsuperscript{b} & 0.9 ± 0.05\textsuperscript{a} \\
\hline
\end{tabular}
\end{table}

The data are presented as means of an average sample of 20 plants per treatment ± SE. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing ANOVA multifactor analysis

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Fig. 1. Callus induction and indirect shoot organogenesis of *S. rebaudiana*: Green callus from leaf explants on MSC$_3$ (a, b); Green friable callus with shoot organogenesis from leaf-derived callus on MSR$_3$ (c); Micropropagation of regenerants from leaf-derived callus (d); Green callus from stem explants on MSC$_3$ (e); Callus with indirect shoot organogenesis from stem-derived callus on MSR$_3$ (f); Regenerants from stem-derived callus (g); Micropropagation of regenerants from stem-derived callus (h); In vitro rooting plant regenerants on $1/2$ MS medium with 0.1 mg/l IBA (i); Ex vitro acclimatized plants in soil:sand:perlite (2:1:1 v/v/v) (j); Plant regenerants in greenhouse condition (k)

was the most appropriate for mass increasing of the callus developed from the leaf explant $^{[9]}$. Auxin plays vital role in callus induction especially 2,4-D. The potential of 2,4-D as the most efficient growth regulator was well documented in

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several plant species \[8,13\]. These results are consistent with previous reports that callus derived from explants of varying potentials results in different morphogenic responses.

**Callus growth and maintenance.** Subcultures of callus was grown and maintained on medium of 2,4-D/BAP/GA\(_3\) for three times. These totipotent cells gave rise to shoots when transferred to the same plant growth regulator. Indirect shoot induction was raised from callus of leaf and stem explants.

**Indirect shoot organogenesis.** Shoot regeneration was achieved from the callus of the two types of explants when transferred to shoot regeneration medium supplemented with 2,4-D, BAP and GA\(_3\) at different concentrations and combinations. The maximum percentage of shoot regeneration from the callus of two explant types was obtained with the combination of 2,4-D, BAP and GA\(_3\) on MSR\(_3\) medium. However, the best response in terms of minimum number of days (18 days) taken to regenerate shoots and maximum number of shoots was obtained using the leaf explants at 0.5 mg/l 2,4-D + 2.0 mg/l BAP + 0.5 mg/l GA\(_3\) (Table 1). The medium containing auxin and cytokinin yielded shoot or root cultures, respectively. Since the leaf-derived callus was more responsive to subsequent shoot induction than the stem one, we focused on shoot regeneration from leaf-derived callus of *S. rebaudiana*. Callus proliferation significantly increased after subculturing of the regenerated callus. The highest frequency of indirect shoot organogenesis and the number of shoots per callus occurred on MSR\(_3\) medium. However, the best response in terms of minimum number of days (18 days) taken to regenerate shoots and maximum number of shoots was obtained using the leaf explants at 0.5 mg/l 2,4-D + 2.0 mg/l BAP + 0.5 mg/l GA\(_3\) (Table 1). The medium containing auxin and cytokinin yielded shoot or root cultures, respectively. Since the leaf-derived callus was more responsive to subsequent shoot induction than the stem one, we focused on shoot regeneration from leaf-derived callus of *S. rebaudiana*. Callus proliferation significantly increased after subculturing of the regenerated callus. The highest frequency of indirect shoot organogenesis and the number of shoots per callus occurred on MSR\(_3\) (60% and 2.3 shoots), followed by MSR\(_2\) (50% and 1.8 shoots), which suggested 2,4-D and BAP and GA\(_3\) as more appropriate to use for shoot organogenesis (Fig. 1c, g).

**Shoots and roots induction, and ex vitro acclimatization.** The effect of cytokinins on *S. rebaudiana* shoot induction from callus, especially BAP, was recognized in previous studies, as well \[11,12\]. To multiply the number of shoots, the regenerated *S. rebaudiana* shoots were further cultivated on MS containing cytokinin at different concentrations (1.0 and 2.0 mg/l BAP) for the stage of micropropagation (Table 2). The maximum frequency of shoot formation (95%) was observed at 2.0 mg/l BAP, which induced the maximum number of shoots (8.2) with longest shoot (1.8 cm) induction of regeneration from callus derived from leaf explants (Fig. 1d) and is lower than that of regeneration from callus derived from stem explants after four weeks of culture (Fig. 1h). For root induction, the regenerated shoots were rooted after transferring to \(\frac{1}{2}\) MS supplemented with 2.0% sucrose and 0.1 mg/l and 0.5 mg/l IBA (Table 2, Fig. 1i). The best parameters were detected in the medium containing 0.1 mg/l IBA – up to 95% rooting and increased number of roots (4.5), with average root length of 1.6 cm per regenerated plant. IBA induced shoot cultures to grow roots; it possesses both shooting and rooting property. The effectiveness of IBA in rooting has been reported for medium plants like *S. rebaudiana* \[12\].

The regenerated in vitro plants revealed a good adaptability ex vitro on potting mixture consisting of soil, sand and perlite (2:1:1 v/v/v), which was found to
be the most appropriate for *S. rebaudiana* ex vitro adaptation (Fig. 1j). During the adaptation, the plants continued to grow and develop, which is considered an indication of successful ex vitro acclimatization. The plants were then transferred to greenhouse conditions. About 90% of the plant regenerants survived during acclimatization and were successfully transferred to greenhouse (Fig. 1k). The present protocol describes the shoot regeneration of *S. rebaudiana* through callusing which facilitates the rapid propagation of this valuable, medicinal plant.

**Conclusion.** A method for efficient callusogenesis and indirect shoot organogenesis of *S. rebaudiana* was studied. From the study, we can conclude that calluses were induced from leaf and stem explants. The highest significant value of callus fresh weight was induced from leaf explants by MSC$_3$. The highest potential for organogenic differentiation from callus could be observed from leaf explants on MSR$_3$ medium. An efficient protocol for in vitro propagation of *S. rebaudiana* from leaf and stem explants has been developed through indirect organogenesis. The best shoots and roots from indirect organogenesis were induced by cytokinin of BAP (2.0 mg/l) on MS medium and auxin of IBA (0.1 mg/l) on $\frac{1}{2}$ MS medium. The protocol established will facilitate mass propagation and conservation of this medicinally important plant. In vitro technology is important to keep plants from extinction and increase the production of natural stevia products for pharmaceutical uses.

**REFERENCES**


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