Full Length Research Paper

The use of long-term *Scutellaria altissima* callus cultures for shoot regeneration, production of bioactive metabolites and micropropagation

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Different types of seedling explants were used to induce *Scutellaria altissima* callus formation. The explants were cultured on Murashige and Skoog (MS) agar medium supplemented with 0.1 mg l\(^{-1}\) indole-3-acetic acid (IAA), 0.2 mg l\(^{-1}\) thidiazuron (TDZ) and Schenk and Hildebrandt (SH) agar medium containing 0.1 mg l\(^{-1}\) naphthalene acetic acid (NAA), 0.2 mg l\(^{-1}\) 6-benzylaminopurine (BAP), 0.5 mg l\(^{-1}\) 2,4-dichlorophenoxyacetic acid (2,4-D). Hypocotyl explants had the highest frequency of callus formation and the calli had the best shoot regeneration potential. Two types of hypocotyl-derived calli named SH-type and MS-type were maintained for two years under *in vitro* conditions and characterized in terms of shoot proliferation and ability to produce bioactive metabolites (baicalin, wogonoside, verbascoside). The results indicate that the SH-type callus grown for two years under dark conditions was characterized by high regeneration efficiency: 13 shoots per culture with a length of about 4 cm. The callus also accumulated a high concentration of flavone glycosides: baicalin (32.4 mg g\(^{-1}\) dry weight) and wogonoside (7.5 mg g\(^{-1}\) dry weight). On the other hand, MS-type calli of the same age cultured under photoperiod light conditions produced the highest amount of verbascoside (10.6 mg g\(^{-1}\) dry weight). Inter simple sequence repeat (ISSR) analysis of *S. altissima* shoots developed via callus organogenesis showed their genetic similarity to shoots originated from the seeds. The regenerated plantlets from calli *S. altissima* shoots were direct rooted *ex vitro* in pots and acclimatized in the greenhouse with a survival rate of 90 to 95% after 12 weeks. No morphological abnormalities were observed in the micropropagated plants.

Key words: Callus culture, inter simple sequence repeat (ISSR) analysis, flavonoids, *Scutellaria altissima*, shoot regeneration, verbascoside.

INTRODUCTION

*Scutellaria altissima* L. (Lamiaceae) commonly known as tall skullcap, is a perennial herb described in mountain regions of South Europe, East Asia and North America. The plant is one of about 300 species of the *Scutellaria* genus. Among them, *Scutellaria baicalensis* is the most widely-used species in Chinese folk medicine (Shang et al., 2010). The extract of *S. baicalensis* roots is used in the treatment of cancer, hepatitis, leukemia, arteriosclerosis, bronchitis and other diseases (Zobayed et al., 2004). It has antibacterial and antiviral activity (Shang et al., 2010). The activities have been mainly linked to the presence of flavonoids (Chang et al., 2002; Gao et al., 1999; Lim, 2003). Of 50 various flavonoids isolated from *S. baicalensis*, flavone glycosides, such as...
baicalin and wogonoside are major components, as well as the aglycones wogonin, baicalein and chrysin. The compounds were isolated from the roots of *S. altissima* (Beshko et al., 1975).

Plant *in vitro* techniques, because of their independence from seasonal and geographical conditions, are recognized as alternative methods for rapid propagation and for studies of secondary metabolite production and accumulation. So far, only a few species of the *Scutellaria* genus, that is, *S. baicalensis*, *Scutellaria lateriflora*, *Scutellaria racemosa* and *Scutellaria costaricana*, have been studied in *in vitro* culture (Cole et al., 2008; Tascan et al., 2007; Wilczyńska-Barska et al., 2012).

In this report, a procedure for the production of *S. altissima* organogenic callus cultures from hypocotyl seedlings is described. The calli were maintained for two years, during which, their potential shoot regeneration ability and secondary metabolite production were determined. Three compounds, that is, baicalin, wogonoside and verbascoside, were quantified by ultra high performance liquid chromatography (UHPLC). In order to determine whether shoots regenerated from *S. altissima* callus were genetically stable, inter simple sequence repeat (ISSR) analysis was performed. The procedure for *ex vitro* rooting of *S. altissima* shoots and plant acclimatization was also described.

**MATERIALS AND METHODS**

**Plant**

Seeds of *S. altissima* provided by the Garden of Medicinal Plants of Wrocław were sterilized in 1% sodium hypochlorite for 15 min, then rinsed three times in sterile distilled water. Sterilized seeds were placed on Murashige and Skoog (MS, 1962) agar (0.7%) medium containing kinetin (0.02 mg l\(^{-1}\)) and gibberellic acid (1 mg l\(^{-1}\)) and germinated in the growth room at 26 ± 2°C in the dark.

**Callus induction**

The hypocotyls, cotyledons and roots of 3-week-old seedlings of *S. altissima* were excised and used as explants for callus induction. The explants were placed horizontally on MS agar medium supplemented with 0.1 mg l\(^{-1}\) indole-3-acetic acid (IAA) and 0.2 mg l\(^{-1}\) thidiazuron (TDZ). Others were placed on Schenk and Hildebrandt (SH, 1972) agar medium containing 0.1 mg l\(^{-1}\) naphthalene acetic acid (NAA), 0.2 mg l\(^{-1}\) 6-benzylaminopurine (BAP) and 0.5 mg l\(^{-1}\) 2,4-dichlorophenoxyacetic acid (2,4-D). The cultures were incubated at 26 ± 2°C under a 16 h light and 8 h dark cycle with a light intensity of 40 µM m\(^{-2}\) s\(^{-1}\) provided by cool white fluorescent lamps. After 3 weeks, the frequency of callus formation (calculated as the percentage of explants forming a callus) and its growth were recorded. The experiments were repeated three times, using 6 to 10 of each type of the explant for each experiment.

**Shoot organogenesis from calli**

Calli generated from different seedling explants (hypocotyl, cotyledon and root) on callus induction medium were separated, cut into small pieces of about 450 mg and transferred to fresh medium with the same composition. The cultures were maintained under the conditions described earlier. The percentage of callus cultures inducing shoot bud/shoot and the average number of adventitious shoots per callus were recorded after 4 weeks. The experiment was repeated three times (in 1 to 4 passages) and each attempt consisted of at least 7 cultures.

**Shoot organogenesis during long-term callus culture**

Hypocotyl-derived calli after five subcultures were selected and designated as SH-type callus (cultures growing on SH medium supplemented with 0.1 mg l\(^{-1}\) NAA, 0.2 mg l\(^{-1}\) BAP and 0.5 mg l\(^{-1}\) 2,4-D) or MS-type callus (cultures growing on MS medium supplemented with 0.1 mg l\(^{-1}\) IAA and 0.2 mg l\(^{-1}\) TDZ). For long-term maintenance, the SH-type callus was cultivated in an 8-week subculture in the dark, whereas the MS-type callus was subcultured every 6 weeks and grown under photoperiods consisting of 16 h light and 8 h dark cycles.

The rate of shoot regeneration, that is, the average number of adventitious shoots per culture, and the average length of the regenerated shoots were determined in both callus types at the end of the growth period. The two parameters were recorded in cultures maintained for 6, 12, 18 and 24 months. Three replicates of 10 callus cultures were used for each age and for each callus type.

**Acclimatization *ex vitro***

Shoots over 1 cm long were taken from the 18-month-old SH-type and MS-type calli and were directly transplanted to pots with a mixture of sand, peat and soil (3:3:4 v/v/v). They were kept in the greenhouse under controlled temperature and natural light. The number of shoots transferred into pots was 26 from the MS-type callus and 30 from the SH-type callus. To maintain high humidity, the potted shoots were covered with glass caps. The covers were gradually opened after 3 days and completely removed after 2 weeks. The survival rate, defined as the percentage of shoots that survived the transfer from *in vitro* to *in vivo* growth conditions was determined 12 weeks after the shoots were introduced into the greenhouse.

**DNA isolation**

Genomic DNA was extracted from the fresh leaves of 6 shoots regenerated from the MS-type calli (growing on MS medium supplemented with 0.1 mg l\(^{-1}\) IAA and 0.2 mg l\(^{-1}\) TDZ) and 6- from the SH-type calli (growing on SH medium with 0.1 mg l\(^{-1}\) NAA, 0.2 mg l\(^{-1}\) BAP and 0.5 mg l\(^{-1}\) 2,4-D) according to the modified CTAB method of Khan et al. (2007). The extracted DNA was tested for purity and concentrations using a Nanospectrophotometer (Implen).

**ISSR analysis**

ISSR-PCR amplification was performed in a 25 µl volume containing DNA solution (100 ng), 3 µl ISSR oligodeoxynucleotide primer (Birt Gdansk), 2.5 µl 10x reaction buffer (Birt Gdansk), 2.5 µM MgCl\(_2\) (Birt Gdansk), 200 µM dNTP mix (Birt Gdansk) and 2U of Taq DNA polymerase (Birt Gdansk). A total of 8 ISSR primers (Birt Gdansk) were tested using plant DNA (Table 1). The choice of primer was based on the probability of its relative abundance in the *Scutellaria* genome (Guo et al., 2009).

The PCR program comprised 43 cycles, in which denaturation was carried out at 95°C for 5 min, followed by segment denaturation at 95°C for 1 min, annealing from 48 to 51°C
Table 1. List of 8 primers used in ISSR analysis in *Scutellaria altissima* shoots.

<table>
<thead>
<tr>
<th>Primer number</th>
<th>DNA sequence</th>
<th>Number of amplified bands</th>
<th>Number of polymorphic bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>807</td>
<td>5'-(AG)_8T-3'</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>811</td>
<td>5'-(GA)_8C-3'</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>812</td>
<td>5'-(GA)_8A-3'</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>813</td>
<td>5'-(CT)_8T-3'</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>830</td>
<td>5'-(TG)_8A-3'</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>834</td>
<td>5'-(AG)_8G-3'</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>835</td>
<td>5'-(AG)_8C-3'</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>836</td>
<td>5'-(AG)_8A-3'</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

(depending on primer type) for 1 min, extension at 72°C for 2 min and final extension for 10 min at 72°C. Amplification was carried out in a MJ Mini programmable peltier thermocycler (Bio Rad).

Amplifications were checked by separation on 1.5% agarose gel electrophoresis for 2 h at a constant 80 V with 1x TBE (running buffer). Finally, the gel was stained with ethidium bromide, visualized under ultraviolet rays and documented using a compact documentation system for superior results (Minibis Pro, DNR). The sizes of the amplification products were estimated according to a 100 to 1000 bp ladder (A&A Biotechnology).

For comparison, the DNA of 3-week-old plantlets grown in the greenhouse initiated from the same seeds as the *in vitro* culture was also analyzed. Amplified DNA markers were scored as band present or not, both in the regenerated shoot and the mother plants. The analysis was performed for all the samples two times with each selected primer.

**UHPLC analysis**

The lyophilized callus tissues (250 mg) were extracted three times with 30 ml methanol:water 7:3 (v/v) at 15 min in an ultrasonic bath. The extracts were combined and evaporated under reduced pressure. The dry residue was dissolved in 2 ml of methanol and subjected to UHPLC analysis. For the analysis, a modified method described by Wilczyńska et al. (2012) was used. Chromatographic analysis was performed using an Agilent Technologies 1290 Infinity UHPLC apparatus equipped with a diode array detector (DAD), a binary solvent delivery pump, vacuum degasser, an autosampler (5 µl injection volume) and thermostatted column compartment. The analysis was performed on a Zorbax Eclipse Plus C18 column (100×3 mm; 1.8 µm Agilent Technologies) at 27°C. The mobile phase consisted of 0.1% formic acid in acetonitrile (v/v) (solvent A) and 1.0% formic acid in water (v/v) (solvent B). A gradient program was applied as follows: 0 to 15 min (20 to 30% solvent A), 15.1 to 17 min (99% solvent A) at flow 0.3 ml min⁻¹. The column was equilibrated with 20% solvent A (0.1% formic acid in acetonitrile) for 2 min between injections. The detection wavelength was set at 320 nm. Baicalin, wogonoside and verbascoside were identified by comparison of their retention times (Rt for baicalin = 8.74, Rt for wogonoside = 13.9 and Rt for verbascoside = 3.49), UV spectra and mass spectra with those of the standard compounds and published data (Funes et al., 2009; Islam et al., 2011).

The quantification of these compounds was achieved using calibration curves. Baicalin was purchased from Sigma-Aldrich, wogonoside from ChemFace (China), while verbascoside was provided by phytoplan. The linear standard curves were obtained by plotting standard concentration (baicalin: 220 to 1100 µg ml⁻¹, wogonoside: 320 to 1600 µg ml⁻¹ and verbascoside: 200 to 1000 µg ml⁻¹) as a function of peak area. The calibration curves obtained for baicalin, wogonoside and verbascoside gave good correlation values (0.976, 0.999 and 0.998, respectively). The compound contents were expressed as mg g⁻¹ of dry weight.

**LC-MS/MS analysis**

Samples were analyzed using an API LC/MS/MS system (Applera, USA) with an electrospray ionization (ESI) source equipped with a Dionex (Germany) HPLC system. Detection was performed in both positive and negative ion modes with the conditions set as follows: drying gas (N₂) 11.0 L/min, temperature 350°C, nitrogen nebulizer pressure 40 psi, capillary voltage 4.0 kV, a detector gain of 1600 V, fragmentation voltage 100 V and full scan range from 100 to 900 m/z. Separation of the compounds was performed on a Hypersil Gold RP column (C18, 2.1 mm × 150 mm, 5 µm) protected by a Hypersil Gold C18 guard column (2.1 mm × 10 mm, 5 µm). The column was maintained at 27°C and the solvent system was as described earlier.

**Statistical analysis**

The results were expressed as the means ± standard error (SE) of repeated independent experiments. The significance of differences between the means of various parameters was determined using the Kruskal-Wallis test at p = 0.05.

**RESULTS**

**Callus induction and shoot organogenesis**

For callus induction, explants (hypocotyls, cotyledons and roots) from aseptic 3-week-old seedlings of *S. altissima* were incubated on two agar solidified media: SH supplemented with 0.1 mg l⁻¹ NAA, 0.2 mg l⁻¹ BAP, 0.5 mg l⁻¹ 2,4-D or MS containing 0.1 mg l⁻¹ IAA, and 0.2 mg l⁻¹ TDZ. These media were selected for callus formation according to earlier published data for *S. baicalensis* (Li et al., 2000) and the results of our own preliminary experiments (data not shown).

The response of the *in vitro* derived explants was evaluated on day 21 of their primary culture (Table 2). After this time, callus induction frequency varied between 50 and 100% depending mainly on explant type. The explants started to form a callus after 1 week of culture.

Hypocotyls had the highest callus induction ability.
Regardless of the medium used, all hypocotyls formed large calli within 3 weeks of culture. The calli were compact, light green or yellow in colour and developed a dark green nodular structure on their surface. The nodular structures started differentiating meristematic centers from which shoot buds and shoots developed in subsequent callus subcultures.

As shown in Table 3, hypocotyl-derived calli were the most competent for shoot organogenesis. After the 4th subculture, almost 90% of the calli formed shoots with an average shoot regeneration rate of 4.1 to 4.4 shoots/callus within 4 weeks. The hypocotyl-derived calli of S. altissima were further subcultured for about 2 years to investigate their capacity for shoot regeneration during long-term culture. The calli were maintained on the media of the same composition as the media for callus induction. Two callus types were established according to the culture medium used: the SH-type callus and MS-type callus (Figure 1). The calli of SH type were yellowish and compact with a high tendency to produce shoots and roots. The roots, however, had no direct vascular connection with the shoots. Furthermore, with each subculture, a strong inhibition of callus growth was observed and shoot development was observed to predominate. In response to this, and to allow the callus to be further used as material for shoot regeneration, it was grown under dark conditions starting from the 5th passage. However, the shoots developed slowly in the dark and a prolonged period of subculture up to 8 weeks was needed. Under these conditions, an average of about 13 shoots per callus was formed. These results show that the number of formed shoots did not decrease during continuous subculture (Figure 2). The average length of the shoots was almost 4 cm (Figure 3). After 8 weeks of culture in the dark, the shoots were thin, etiolated with a reduced number of leaves (Figure 1A). However, they became green when they were transplanted to light conditions in the greenhouse.

The use of MS medium with 0.1 mg l\(^{-1}\) IAA and 0.2 mg l\(^{-1}\) TDZ led to better callus growth, but significantly fewer shoots were regenerated from the MS-type callus compared to the SH-type callus. A mean number of 5 shoots/callus with a length of about 2 cm was produced on MS medium after 6 weeks of culture (Figures 2 and 3). The shoots regenerated from the MS-type callus had thicker stems and contained a greater number of larger leaves (Figure 1B) in comparison with shoots developed from the SH-type callus.

### Molecular analysis

In this study, to evaluate the genetic stability of shoots regenerated from the SH-type and MS-type calli of S. altissima, an ISSR analysis was carried out. Good quality bands were taken into consideration in this study. The total number of bands for each primer varied from 1 to 10, with an average of 4 bands per primer (Table 1). The amplification products ranged from 300 to 1100 bp in size. Each primer generated a unique set of amplification products which were monomorphic for all analyzed shoots, which suggests that all shoots, irrespective of their source, showed a genetic similarity with green house-grown plants initiated from the same seeds as in vitro culture. Figure 4 shows examples of amplified banding patterns produced using ISSR primer number 836.

The results of our study show that S. altissima is a species with a high morphogenetic potential, and the genetic stability of the obtained plants is one of the most important factors determining an effective in vitro regeneration procedure, whereby a large number of shoots can be obtained in a short time.

### Production of compounds

The baicalin, wogonoside and verbascoside content were measured by UHPLC in S. altissima organogenic calli cultured for one and two years on SH medium (SH-type callus) or MS medium (MS-type callus) (Figure 5). The morphogenetic ability of the calli is described in the

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**Table 2. Effect of explant type on S. altissima callus induction after 3 weeks of culture.**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Explant type</th>
<th>Callus formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Frequency (%)</td>
</tr>
<tr>
<td>MS (0.1 mg l(^{-1}) IAA and 0.2 mg l(^{-1}) TDZ)</td>
<td>Cotyledon</td>
<td>58.6</td>
</tr>
<tr>
<td></td>
<td>Hypocotyl</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>92.3</td>
</tr>
<tr>
<td>SH (0.1 mg l(^{-1}) NAA, 0.2 mg l(^{-1}) BAP and 0.5 mg l(^{-1}) 2,4-D)</td>
<td>Cotyledon</td>
<td>70.0</td>
</tr>
<tr>
<td></td>
<td>Hypocotyl</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>50.0</td>
</tr>
</tbody>
</table>

Intensity of callus growth: + callus tissue covering less than 50% of the explant surface, ++ callus tissue covering 50 to 90% of the explant surface, +++ callus tissue covering more than 90% of the explant surface. Each experiment consisted of 6 to 10 explants and each was repeated three times.
Table 3. Evaluation of morphogenetic potential of *S. altissima* calli initiated from different seedling explants for four passages (passage 1 to 4).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Explant type</th>
<th>Shoot regeneration* during passage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>MS (0.1 mg l(^{-1}) IAA and 0.2 mg l(^{-1}) TDZ)</td>
<td>Cotyledon</td>
<td>33.3 (2.2±0.3)</td>
</tr>
<tr>
<td></td>
<td>Hypocotyl</td>
<td>50 (2.6±0.7)</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>22.2 (3.5±0.2)</td>
</tr>
<tr>
<td>SH (0.1 mg l(^{-1}) NAA, 0.2 mg l(^{-1}) BAP and 0.5 mg l(^{-1}) 2,4-D)</td>
<td>Cotyledon</td>
<td>25.0 (1.0±0.0)</td>
</tr>
<tr>
<td></td>
<td>Hypocotyl</td>
<td>44.4 (2.0±0.4)</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>28.6 (2.0±0.6)</td>
</tr>
</tbody>
</table>

*Results include only buds/shoots with normal morphology. Observation was taken on 4 weeks. In parenthesis, number shoots per callus was presented. Values represent means ± SE.

Figure 1. Shoot regeneration from hypocotyl-derived callus of *S. altissima* on SH medium containing NAA (0.1 mg l\(^{-1}\)), BAP (0.2 mg l\(^{-1}\)) and 2,4-D (0.5 mg l\(^{-1}\)) (SH type callus) (A) or MS medium supplemented with IAA (0.1 mg l\(^{-1}\)) and TDZ (0.2 mg l\(^{-1}\)) (MS type callus) (B) (bar = 1 cm)
According to the data presented in Figure 6, the SH-type callus produced higher levels of both flavones, that is, baicalin and wogonoside, than the MS-type, but the verbascoside content in the former was lower. The highest amount of baicalin (32 mg g\(^{-1}\) dry weight) was detected in a 2-year old SH-type callus. The level was almost twice that found in the one-year old SH-type callus and 3 to 4 times higher than that seen in the MS-type callus. The callus tissues of \textit{S. altissima} synthesized also wogonoside, the concentration of which ranged from 1.0 to 7.5 mg g\(^{-1}\) dry weight, depending on the analyzed callus type and age of culture (Figure 6). The highest amount of wogonoside was achieved in the SH-type callus maintained over two years under in vitro conditions: the value was about 7 times higher than in the MS-type callus.
Figure 4. ISSR profiles of regenerated shoots of *S. altissima* using primer 836. L1 - DNA marker 100-1000-bp DNA ladder, lane M – DNA of mother plant (seedlings from the same seeds as *in vitro* culture), lanes 1-6 – DNA of regenerated shoots from MS-type callus after 6 weeks, lane 7-12 – DNA of regenerated shoots from SH-type callus after 8 weeks.

Figure 5. UHPLC chromatogram of the SH-type callus extract of *S. altissima*. 1-vervascoside, 2- baicalin, 3- wogonoside.

The results of this study indicate that SH-type and MS-type of *S. altissima* callus cultures also have the ability to synthesize verbascoside (Figure 6). In this case, the MS-type callus was more productive. The amount of the phenylethanoid in the callus (about 10 mg g⁻¹ dry weight) was about twice that detected in the SH-type callus.
by the same letter do not differ statistically at p ≥ 0.05. The values are the means ± SE. The means for the same metabolites followed by the same letter do not differ statistically at p ≥ 0.05. The results were recorded 6 weeks (MS - 1) or 2 years (SH - 2) in vitro culture. The results were recorded 6 weeks (MS-type callus) or 8 weeks (SH-type callus) after transferal into the fresh medium. The values are the means ± SE. The means for the same metabolites followed by the same letter do not differ statistically at p ≥ 0.05.

### Table 4. MS fragmentation of the investigated compounds by HPLC-ESI-MS/MS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Positive ion mode</th>
<th>Negative ion mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verbascoside</td>
<td>625.2 [M+H]+</td>
<td>623.2 [M-H]; 669.6 [M+HCOO]; 461.2 [M-H-CaA]</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The experiments were conducted with shoots excised from the MS- and SH-type calli of *S. altissima*. They rooted directly during the transfer to ex vitro conditions. Twelve weeks after transfer from culture medium into pots with mixture of sand, peat and soil, the survival rate of the acclimatized plants was evaluated: 90% for plants originated from the SH-type callus and 95% from the MS-type callus. All regenerated plants of *S. altissima* were grown in the green house.

**Plant regeneration**

The experiments were conducted with shoots excised from the MS- and SH-type calli of *S. altissima*. They rooted directly during the transfer to ex vitro conditions. Twelve weeks after transfer from culture medium into pots with mixture of sand, peat and soil, the survival rate of the acclimatized plants was evaluated: 90% for plants originated from the SH-type callus and 95% from the MS-type callus. All regenerated plants of *S. altissima* were grown in the green house. Nevertheless, in both types of callus, the organogenic capacity was high and was not decreased during two years of cultivation. The loss or decrease of regeneration potential in the callus tissue is one of serious limitations for the use of an indirect organogenic system in plant micropropagation. Such a problem was noted for example in calli of *Centaurium erythraea* (Piątczak and Wysokińska, 2003) and *Sorghastrum nutans* (Yao et al., 1987). Shoots of *S. altissima* regenerated from calli were rooting ex vitro after being transferred to soil and the survival rate of plantlets obtained in this way was 90 to 95%. This rate was higher than that previously observed regenerative than other seedling explants in *Fraxinus americana* (Palla and Pijut, 2011) or *Arctium lappa* (He et al., 2006).

In further experiments, the capacity for shoot regeneration and production of secondary metabolites using two morphologically different calli derived from the hypocotyls of *S. altissima* were evaluated. The results of the study demonstrated that significantly more adventitious shoots were regenerated from an SH-type callus (>10) compared to an MS-type callus (<10). Nevertheless, in both types of callus, the organogenic capacity was high and was not decreased during two years of cultivation. The loss or decrease of regeneration potential in the callus tissue is one of serious limitations for the use of an indirect organogenic system in plant micropropagation. Such a problem was noted for example in calli of *Centaurium erythraea* (Piątczak and Wysokińska, 2003) and *Sorghastrum nutans* (Yao et al., 1987).

Shoots of *S. altissima* regenerated from calli were rooting ex vitro after being transferred to soil and the survival rate of plantlets obtained in this way was 90 to 95%. This rate was higher than that previously observed...
in *S. altissima* plantlets derived from axillary shoots after *in vitro* rooting on MS medium supplemented with IAA (40%) (data not shown). *Ex vitro* rooting is a promoting method in that the micropropagation cycle is shortened by avoiding the rooting stage, and cost and labor are reduced. Chen and Yeh (2007) have reported that *ex vitro* rooting reduced production costs in tissue culture by 35 to 75%. *Ex vitro* rooting has been successfully applied to several plant species such as *Siratia grosvenori* (Yan et al., 2010) and *Ceroxega bulbosa* (Phulwaria et al., 2013). For these plants, root induction was achieved by the basal dipping of *in vitro*-developed shoots in auxin solvent for a short period. In the present study, regenerated *in vitro* *S. altissima* shoots can be directly transferred to the green house without dipping in auxin solution. Both of the methods of *ex vitro* rooting mentioned earlier were used by Martin (2003) for *Rotula aquatica*, however, a lower percentage of survival was achieved using direct *ex vitro* rooting than a combination of *in vitro* rooting and auxin dipping. On the other hand, Brondani et al. (2012) showed that acclimatization was more successful when rooting *Eucalyptus benthamii* plantlets by using an *ex vitro* (without auxin treatment) than *in vitro* method, that is 65 to 87% of acclimatization for *ex vitro* rooting versus 32 to 40% of acclimatization for *in vitro*.

Genetic mutations are frequently observed in shoots/plants regenerated from callus, particularly when the subculturing period is prolonged, because of the non-uniform nature of callus tissue or stress conditions during *in vitro* culture (Nehra et al., 1992; Mo et al., 2009). However, ISSR analysis indicates that this was not found in cases of shoots regenerated from the *SH*-type and MS-type *S. altissima* calli. The ISSR method is an effective and simple tool to analyze variations among *in vitro* regenerated plants (Shen et al., 2006). In plant genome analyses, microsatellites are frequently used to fingerprint genotypes (Rongwen et al., 1995). Uniformity among regenerated plants was observed for *Solanum melongena* (Xing et al., 2010) and *Zingiber rubens* (Montanty et al., 2011). Bennici et al. (2004) suggest that a lack of somaclonal variation in the regenerants of some plants could be connected with genetic factors responsible for the maintenance of their genetic stability or culture conditions.

A literature search reveals only one study on the baicalin level in field-grown plants of *S. altissima*. Zagórka (2006) notes that the aerial parts of the plants accumulate 0.3 mg baicalin/g of dry weight. A comparison of the data with ours indicates that both organogenic callus types of *S. altissima* synthesized far more baicalin than the shoots of intact plants. It is apparent that *in vitro* conditions support the production of the flavone. The baicalin content in the *SH*-type callus of *S. altissima* was higher than in most *S. baicalensis* *in vitro* regenerated plantlets cultured in various types of bioreactor (Zobayed et al., 2004). In five bioreactor sys-

tems used by the authors for shoot growth, the baicalin level ranged between 10 to 30 mg g\(^{-1}\) dry weight. Only plantlets grown in an Low Volume Terminal (LVT) system, that is, a large vessel with a gelled medium under a forced ventilation system, produced significantly higher amounts of the compound: about 50 mg g\(^{-1}\) dry weight.

The second flavone glycoside, wogonoside, was earlier detected in the roots of *S. altissima* (Beshko et al., 1975), but so far, quantification of the compound has not been determined. The present study shows that the quantity of wogonoside in the SH-type callus (7.5 mg g\(^{-1}\)) was similar to that found in leaves (7.9 mg g\(^{-1}\) dry weight) and stems (5.6 mg g\(^{-1}\) dry weight) of *S. baicalensis*, but lower compared to the roots of the plant (12.63 mg g\(^{-1}\) dry weight) (Islam et al., 2011).

This is the first report describing the presence of verbascoside in *S. altissima*. Verbascoside was previously found in *in vitro* hairy roots cultures of *S. lateriflora* and *S. baicalensis* in quantities of 18.5 and 17.5 to 29.5 mg g\(^{-1}\) dry weight, respectively (Nishikawa et al., 1999; Wilczyńska-Barska et al., 2012). The compound has been found in naturally growing *S. lateriflora*, *S. racemosa*, *S. tomentosa*, *S. wrightii* or *S. baicalensis* (Islam et al., 2011), its contents ranging from 0.5 to 2.3 mg g\(^{-1}\) dry weight, depending on plant species and organ. Literature data, together with our results, suggest *in vitro* culture conditions have a beneficial effect on the production of the compounds.

Our results indicate that organogenic callus cultures of *S. altissima* are good sources of some flavones characteristic of *Scutellaria* species. They also reveal that the flavonoid level was higher in the *SH*-type callus, whereas the MS-type callus was better for verbascoside biosynthesis. Given the different media used, including the difference in growth regulators and varying time intervals at which measurements were taken, it is difficult to explain the variation in metabolite production between the analyzed calli of *S. altissima*. One possible reason for the differences observed in flavonoid biosynthesis may be related with the different levels of organogenesis of both calli. Beside shoots, a lot of roots from the *SH*-type callus are differentiated. The roots are the main site of flavonoid accumulation in plants belonging to *Scutellaria* spp. (Islam et al., 2011).

It is also important to note that both examined callus types described in the work did not lose their ability to accumulate flavonoids and verbascoside over a period of 24 months in *in vitro* culture under standard growth conditions. It is possible that long-term stability in secondary metabolite production can be attributed to organ regeneration from the calli. This confirm the results of numerous research that organized tissue culture seem to be more appropriate for the production of secondary metabolites than cultures of undifferentiated tissues (Jain et al., 2011; Palacio et al., 2012). The positive correlation between metabolite synthesis and morphological
differentiation suggest that organogenesis is the key regulatory factor which stimulates production of metabolites in vitro.

Conclusion
This study reports the establishment of two types of hypocotyl-derived S. altissima callus, which maintained high shoot proliferation capacity during a long-term subculture of 2 years. These shoots could be used in a propagation program. Rooting took place by a direct ex vitro method and the survival of obtained plantlets in green house was high (90 to 95%). The ISSR analysis revealed that the regenerated in vitro S. altissima shoots showed similarity with seed-derived plants. The study also demonstrated the suitability of organogenic callus types of S. altissima for the production of the flavones baicalin and wogonoside, as well as the phenylethanoid verbascoside. Among the two analyzed callus types, the SH-type callus was more suitable for flavonoid production, especially baicalin. It will be interesting to determine the accumulation of baicalin and other secondary metabolites in the regenerated plants of S. altissima in future studies.

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