***In vitro* cultures of *Blackstonia perfoliata* (L.) Hudson and an assay of the secoiridoid content**

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Abstract

In vitro cultures of *Blackstonia perfoliata* (L.) Hudson, a popular medicinal plant, were initiated. Regenerated plants and callus tissue were obtained and then analysed for secoiridoids.

Key words: *Blackstonia*, *Gentianaceae*, *in vitro* cultures, secoiridoid analysis

INTRODUCTION

The species *Blackstonia perfoliata* (L.) Hudson (*Chlora perfoliata* L.) from the family *Gentianaceae* is not a member of Poland's flora. Its natural habitats are in the European and African regions of the Mediterranean (T u t i n 1972, V a n d e r S l u i s 1985). Four subspecies are distinguished in European flora. However Z e l t n e r (1970) considers them to be separate species. A secoiridoid compound, gentiopicroside, has been isolated from entire plants (H e g n a u e r 1966). It occurs in *B. perfoliata* (about 3%), alongside small amounts of sweroside and swertiamarin (V a n d e r S l u i s et al. 1983). Hence this plant is often used in medicine in place of *Radix Gentianae*. Gentiogenal found in *B.*

Abbreviations: GA₃ — gibberelic acid; kin — kinetin; BAP — benzyl-aminopurine; IAA — β -indolyl-3-acetic acid; NAA — α -naphthalene-acetic acid; 2,4-D — dichlorophenoxyacetic acid; AS — adenine sulphate.

perfoliata (Van der Sluis et al. 1983), also termed gentiopical (Ishiguro et al. 1983), is an artefact created by the decomposition of gentiopicroside and swertiamarin. Xanthoncs (Van der Sluis 1985) and flavonoids (Kouadji et al. 1990, Kouadji 1990) have also been found in *B. perfoliata*. Both groups of compounds serve as taxonomical markers of the family *Gentianaceae* and are characterised by biological activity (Sullivan et al. 1977, Hevsteen 1983, Wagner 1985). Terephthalic acids have been found in *B. perfoliata* and species from the genera *Gentiana* and *Swertia*, which is interesting for researchers studying the occurrence of these compounds in the family *Gentianaceae* (Hattimanoli et al. 1988). Figure 1 shows the structures of compounds in the species under study.

In a series of *in vitro* studies of the species of the family *Gentianaceae* (Wesołowska et al. 1985, Skrzypczak et al. 1988a, b, 1991) we attempted tissue culture of *Blackstonia perfoliata*. At the same time we tested the cultures for pharmacologically active compounds. The species mentioned has not been subject to *in vitro* studies to date (Bajaj et al. 1988).

MATERIAL AND METHODS

***In vitro* cultures.** Tissue cultures were initiated using seeds of the species *B. perfoliata* obtained from sites in Greece. The preparation and sterilisation of seeds as well as the culture conditions were as described earlier (Skrzypczak et al. 1988b). For germination, seeds were placed on a Murashige and Skoog medium (1962, MS) supplemented with GA₃ (0.5 or 1.0 mg dm⁻³) and kin (0.25 or 0.5 mg dm⁻³), as well as a half-strength MS medium without plant growth substances. The primary callus culture was obtained on an MS medium with BAP (1.0 mg dm⁻³) and IAA (0.1 mg dm⁻³) or kin (0.5 mg dm⁻³) and NAA (0.25 mg dm⁻³). For the passage of the callus tissue an MS medium was used supplemented with kin (1.0 mg dm⁻³) and 2,4-D (0.5 mg dm⁻³) or AS (40 mg dm⁻³) and NAA (0.5 mg dm⁻³). The callus average growth index was calculated from three determinations. Micropropagation of *B. perfoliata* was done by transferring the seedlings to a modified MS medium based on a composition of macro- and microelements (Zenktele 1984). The medium was enriched with growth substances (mg dm⁻³): zeatin (0.5), IAA (2.0) and kin (2.0), and BAP (1.0).

Chemical analyses. The presence of compounds was checked using thin layer chromatography (TLC) in extracts prepared from aerial parts of plants (herbarial specimens from Greece) and tissues and plantlets obtained *in vitro* (Table 1). Crushed material (0.5 g) was extracted three times under a reflux condenser with 30 cm³ methanol. The extracts were combined and condensed at 40°C under reduced pressure. The residue was dissolved in methanol and transferred to 10 cm³ flasks. The extracts were used for qualitative and quantitative determinations. TLC was carried out on glass plates covered with

Secoiridoids

Gentiopicroside

Swertiamarin

Sweroside

Gentiogenal
(Gentiopical)Xanthones Terephthalic acids Flavonol glycosides

1 kaempferol R=H

2 quercetin R=OH

3 isorhamnetin R=OCH₃

Diglycosides of 1, 2, 3: 3-O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-galactopyranosides

Triglycosides of 1, 2, 3: 3-O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-galactopyranosides-7-O- β -D-glucopyranosides

Fig. 1. The compounds in *Blackstonia perfoliata* (L.) Hudson

silica gel HPTLC 60 F₂₅₄ (Merck). Chromatograms were developed in a 100:17:10 solvent mixture of ethyl acetate-ethanol-water and in a 20:0.1 mixture of chloroform-methanol (acetyl derivatives).

Standard compounds such as gentiopicroside and acetyl derivatives of gentiopicroside, sweroside and swertiamarin were chromatographed simultaneously. To establish the presence of the last three compounds, some extracts obtained from the *in vitro* material (Table 1 —B,D,F) were reduced to dryness and dissolved in acetic anhydride and pyridine. The solution was kept at room temperature for 24 h, then diluted with water and shaken with chloroform. The condensed solutions dissolved in methanol were chromatographed.

Table 1
Gentiopicroside in *Blackstonia perfoliata* (L.) Hudson

Material	Murashige-Skoog medium with mg·dm ⁻³	Gentiopicroside content (%)
A Ground plant	—	1.63
B Rooted shoots	IAA 0.5	5.79
C Shoots	Kin 2.0; IAA 2.0	5.06
D Buds	Kin 2.0; IAA 2.0	2.59
E Differentiated callus	NAA 0.5; SA 40.0	4.24
F Non-differentiated callus	Kin 1.0; 2,4-D 0.5	0.42

Gentiopicroside was quantitatively analysed using a densitometric method following chromatographic resolution. The plant extracts were spotted on the plates 12 mm from each other (as in qualitative analysis) in volumes ranging from 4–15 µl. The standard (8 µl) was spotted at the beginning of each plate. The sample volumes were adjusted experimentally so that the gentiopicroside content in the sample spot was close to that in the standard. The chromatograms were resolved as in qualitative analysis. The equipment and methods used to plot the calibration curve and determine gentiopicroside were those described by Krupinski et al. (1991). The results given in Table 1 are averages of at least 6 determinations.

RESULTS AND DISCUSSION

The *in vitro* cultures of *Blackstonia perfoliata* (L.) Hudson were started from seedlings that grew from seeds placed on an MS medium supplemented with GA₃ and kinetin, as well as on a half-strength MS medium without plant growth substances. The primary callus culture was obtained from the hypocotyl on an MS medium supplemented with BAP and IAA (Fig. 2). Green callus undergoing differentiation was first observed on an MS medium with kinetin and NAA. For the further passage of this tissue an MS medium supplemented with NAA and AS proved to be the best (Fig. 3). The primary callus culture placed on a medium with kinetin and 2,4-D did not undergo differentiation and assumed a yellowish-green colour (Fig. 4). The callus average growth index $[(\text{final weight} - \text{initial weight}) / \text{initial weight}] \times 100$ was 491 after eight passage.

In the primary culture of seedlings on three variants of a modified MS medium (with kinetin and IAA, with BAP, or with zeatin), shoots were obtained from the hypocotyl (Fig. 5). BAP induced the most numerous adventitious buds which grew into a mass of shoots difficult to disentangle. In the presence of zeatin a few shoots developed from each seedling. On the medium supplemented with kinetin and IAA, adventitious shoots were obtained growing in a mass easy to disentangle (Fig. 6).

The above variant of a modified MS medium was found to facilitate the micropropagation of *Blackstonia perfoliata* (L.) Hudson from leaves. In the presence of IAA and kinetin, whole leaves or their segments formed adventitious shoot tips (Fig. 7) which developed into shoots. The shoots obtained *in vitro* rooted on an MS medium with IAA within 4 days and then were transferred to one without growth regulators. Within 3 weeks the shoots rooted at a 90% rate (Fig. 8). Transferred to pots, the plantlets grew to flowering (Fig. 9).

The presence of secoiridoids was tested using the TLC method and various developing phases. As the results show, gentiopicroside is already synthesised in the callus tissue (Fig. 10). On the basis of the comparison of standard acetyl derivatives of gentiopicroside, sweroside and swertiamarin with acetylated extracts, we can conclude that these compounds, too, are present in the tissues obtained *in vitro* (Fig. 11), even though they occur in very small amounts in the mother plant (Van der Sluis et al. 1983).

Quantitative determinations of gentiopicroside were carried out on the obtained material using the densitometric TLC method (Krupińska et al. 1991).

In these determinations it was found that 4-16 µg concentrations of gentiopicroside cause a linear increase in background fluorescence decay under the cited conditions. The concentrations of the compound and the media used are shown in Table 1.

In vitro cultures of *Blackstonia perfoliata* (L.) Hudson

Fig. 2. Seedlings callusing from hypocotyl on MS medium with BAP (1.0 mg dm^{-3}) and IAA (0.1 mg dm^{-3}); $\times 1.9$. Fig. 3. A differentiating callus tissue on MS medium with NAA (0.5 mg dm^{-3}) and AS (40 mg dm^{-3}); $\times 1.6$. Fig. 4. An undifferentiated callus on MS medium with kin (1.0 mg dm^{-3}) and 2,4-D (0.5 mg dm^{-3}) after several transfers; $\times 1.3$. Fig. 5. Adventitious buds developing from the hypocotyl on modified MS medium with zeatin (0.5 mg dm^{-3}); $\times 1.1$.

PLATE II

Micropropagation of *Blackstonia perfoliata* (L.) Hudson

Fig. 6. Developing adventitious shoots on modified MS medium with kin (2.0 mg dm^{-3}) and IAA (2.0 mg dm^{-3}); $\times 1.5$. Fig. 7. Adventitious buds regenerating from leaves on modified MS medium with kin (2.0 mg dm^{-3}) and IAA (2.0 mg dm^{-3}); $\times 1.2$. Fig. 8. A rooted shoot regenerated *in vitro* on modified MS medium with IAA (0.5 mg dm^{-3}); $\times 2.1$. Fig. 9. A flowering plant from an *in vitro* culture; $\times 0.8$.

• PLATE III

Chromatograms in UV₂₅₄ of extracts from *Blackstonia perfoliata* (L.) Hudson HPTLC plates with silica gel 60 F₂₅₄

Fig. 10. G—gentiopicroside, A-F — extracts like in Table 1, developing phase: ethyl acetate-ethanol-water 100:17:10. Fig. 11. Acetylated compounds: G—gentiopicroside; Sm—swertiamarin; Sw—sweroside; B, D, F—acetylated extracts; developing phase: chloroform-methanol 20:0.1 (run twice)

Blackstonia perfoliata from natural localities can contain up to 3% of gentiopicroside (Vander Sluis et al. 1983). The plants which we had at our disposal came from Greece and were herbarial specimens. Their gentiopicroside content amounted to 1.63%. Notable were the results of its determination in the material from *in vitro* cultures. The synthesis of this compound already started in the callus tissues placed on suitable media. In differentiating callus its content was 4.24%, increasing to as much as 5.79% in rooted plantlets. On comparison of these results with the gentiopicroside content in the mother plant we found that in the *in vitro* tissues, its amount had increased fourfold. It is difficult to explain the low content of the compound in shoot tips (2.59%) which appeared on an MS medium supplemented with kinetin and IAA.

Medicinal plants do not frequently synthesise more active compounds in tissue culture than in soil (Phillipson 1990). Our results encourage attempts at suspension cultures, which may be the source of a natural therapeutic agent in the case of a similar content of secoiridoids.

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Hodowla in vitro Blackstonia perfoliata (L.) Hudson i oznaczanie zawartości sekoirydoidów

Streszczenie

W kulturach *in vitro* otrzymano kalus i zregenerowane rośliny *Blackstonia perfoliata* (L.) Hudson, gatunku stosowanego w lecznictwie, lecz obcego florze rodzimej. Najlepszy wzrost kalusa obserwowano na pożywce MS wzbogaconej kin i 2,4-D, natomiast mikrorozmnażanie *Blackstonia perfoliata* prowadzono na pożywce MS z kin i IAA. Ukorzenione roślinki wykazywały pełną zdolność do dalszego rozwoju w glebie.

W zregenerowanych roślinach i tkance kalusowej badano zawartość gencjopikrozydu metodą densytometryczną. Stwierdzono czterokrotny wzrost zawartości tego związku w niektórych tkankach otrzymanych *in vitro* w porównaniu z rośliną gruntową.