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IN VITRO CLONAL PROPAGATION OF *PRIMULA VERIS* L. AND PRELIMINARY PHYTOCHEMICAL ANALYSIS

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A method of in vitro clonal propagation of cowslip *Primula veris* L. (Primulaceae) is reported. This is a species of medicinal importance, protected by law in Poland. MS medium Murashige and Skoog (1962) with 6 benzyladenine BA (4.44 μM) and 2,4-dichlorophenoxyacetic acid 2,4-D (1.13 μM) was found to be optimal for in vitro cowslip propagation from shoot tips under the conditions of culture. Rooting was best in the presence of indole-3-butyric acid IBA (2.45 μM). In vitro seedlings were transferred to pots and then acclimated. After transplanting to a garden they showed further growth and development, including flowering and fruiting. Phytochemical analysis (2D-TLC) revealed that the flavonoid compounds in leafy shoots from in vitro culture were similar to those in leaves from field cultivation.

Key words: *Primula veris* L., cowslip, in vitro clonal propagation, flavonoids.

INTRODUCTION

Primula veris L. (cowslip) of the family Primulaceae occurs throughout Europe except for northern areas, and also in Asia and the Caucasus. The plant grows in warm, sunny, dry habitats, most frequently on meadows and pastures but also in open deciduous forests (Hegi, 1965; Valentine et al., 1972).

It is a medicinal plant rich in triterpene saponins, phenol glucosides and flavonoids. This species is under partial legal protection in Poland, where it is widespread in the lowlands and in lower mountain locations (Zając and Zając, 2001; Mirek et al., 2002). Some of its natural sites are endangered as a result of plowing or intensive grazing of xerothermic grassland. The plant is also picked or dug up for decorative purposes, and under certain conditions permission can be obtained to gather its flowers from natural sites for the pharmaceutical industry (Piękoś-Mirkowa and Mirek, 2003).

Its roots and rhizomes (*Primulae radix*), which are raw material for the pharmaceutical industry, contain up to 10% triterpene saponins, mainly primulasaponin A (primula acid) and primulasaponin B, as well as the phenolic glucosides primulaveroside and primveroside. Also used by the pharmaceutical industry are the flowers (*Primulae flos*), containing ~2% triterpene saponins, phenolic glucosides, and flavonoids: 3',4',5'-trimethoxyflavone (Huck et al., 1999), quercetin and its derivatives, kaempferol, and 3-limocitrin glucoside (Hegnauer, 1990; Harborne and Baxter, 1993). Similar flavonoids, with the exception of methoxyflavones, as well as 3',4'-dihydroxyflavonglucoside were detected in leaves of *P. veris* (Hegnauer, 1969; 1990; Karl et al., 1981; Harborne, 1994). *Primulae radix* is an expectorant, applied in infections of the throat, pharynx, and bronchi. *Primulae flos* is added to expectorant and diuretic mixtures. Extracts from cowslip rhizomes, roots and flowers are components

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of many herbal preparations, such as Bronchicum, Pectosol, Tussipect and Sinupret (Hegnauer, 1969, 1990; Neubauer and März, 1994; Strzelecka and Kowalski, 2000).

The valuable secondary metabolites produced in the underground organs of *P. veris* can be obtained only from cultivated plants, whereas flowers can be harvested from natural habitats (Draxler et al., 2002). In the majority of *Primula* species, including *P. veris*, there are well-known difficulties with seed germination under both greenhouse and field conditions (Coumans et al., 1979; Draxler et al., 2002; Morozowska, 2002). Another way to obtain pharmaceutical raw material is in vitro plant regeneration, producing material from a protected plant species in a relatively short time.

Previously, in vitro micropropagation has been described for *P. obconica* L. by Coumans et al. (1979), who regenerated shoots from inflorescence tips. Bajaj (1981) derived plants of the same species from anthers earlier subjected to freezing in liquid nitrogen. Plants were obtained directly from microspore-derived embryos and indirectly via differentiation of callus tissue. Plant regeneration from cell suspension-derived protoplasts of *P. malacoides* Franch. and *P. obconica* L. was described by Mizuhiro et al. (2001). In vitro propagation of *P. acaulis* (L.) Grufb. (= *P. vulgaris* Huds.) via proliferation of axillary buds was achieved by Merkle and Götz (1990). In 2001, Borodulina et al. worked out a method of clonal propagation for two hybrid species: *Primula* × *polyantha* Mill. and *Primula* × *pruhonicensis* Zeman ex Berg. Recently, Schween and Schwenkel (2002) described a protocol concerning regeneration of *P. vulgaris* Huds. and *P. elatior* (L.) Hill shoots via organogenesis from callus.

The main goal of this study was to establish optimal conditions for in vitro clonal propagation of *P. veris*, not described so far in the available literature. We also make a preliminary comparative phytochemical analysis of material from the plants obtained through in vitro culture and from plants cultivated in the field.

MATERIALS AND METHODS

PLANT MATERIAL

The seeds for initiation of in vitro culture were derived from plants transferred from natural populations to experimental plots of the Department of Botany, Agricultural University, Poznań, Poland. The natural populations were located in the Wielkopolska region, near Kalina (at the northern shore of Wierzbiczańskie Lake), Zagórów (east of Skokum, in the Nadwarciański Landscape Park, on the edge of the Warta Valley), and Lednogóra (in the Lednicki Landscape Park, on Ostrów Lednicki Island in Lednickie Lake). Voucher specimens are deposited in the Herbarium of the Department of Pharmaceutical Botany, Medical University, Poznań (PBMA).

TABLE 1. Media used for *Primula veris* in vitro propagation

MS medium variant (supplements in μM)	Explant type	Micropropagation phase
BA (2.22) + NAA (0.27) KIN (9.30) + IAA (11.42) BA (4.44) + 2,4-D (1.13)	seedling tips	proliferation of lateral buds
BA (4.44) + 2,4-D (1.13)* BA (3.08) + 2,4-D (1.13)* BA (2.22) + 2,4-D (1.13)* BA (0.88) + 2,4-D (1.13)* KIN (9.30) + IAA (11.42) BA (2.22) + NAA (0.27)	shoots from multishoots	multiplication
IBA (2.45) without supplements	shoots	rooting

*media used for morphological measurements of shoots

PRETREATMENT AND IN VITRO CULTURE

Seeds were stratified at 4˚C for 14–30 days, then flooded with water and kept at 25˚C for 48 h until imbibition. Then the seeds were sterilized and placed on medium. Two methods of sterilization were used. In the first method, batches of 60–70 seeds from each population were sterilized with 70% ethanol for 30 sec, then with 100 cm^3 30% solution of Clorox commercial bleach with three drops of Tween 80 for 25–30 min. In the second method, batches of 100 seeds from each population were sterilized with 0.1% solution of $HgCl_2$ instead of Clorox for 7 min. After that, in both methods the seeds were rinsed with sterile distilled water several times and laid out until germination on solid MS medium (Murashige and Skoog, 1962) with gibberellic acid GA_3 (2.90 μM) and kinetin KIN (2.33 μM) or with only GA_3 (2.90 μM).

In our experiments the nutrient media, containing 3% sucrose, were solidified with 0.8% Difco Bacto Agar. The medium pH was adjusted to 5.6 before autoclaving at 121˚C for 20 min. The cultures were maintained in a growth chamber under a 16 h photo-

TABLE 2. In vitro germination of seeds from different populations of *Primula veris*

MS medium variant (supplements in μM)	Disinfectant	% of germinated seeds			
		Seed origin			Mean
		Kalina	Lednogóra	Zagórów	
GA_3 (2.90)	30 % Clorox	28.4	3.5	9.7	13.8
GA_3 (2.90) + KIN (2.33)		50.0	3.5	11.1	21.5
GA_3 (2.90)	0.1 % $HgCl_2$	0.9	5.6	3.7	3.4
GA_3 (2.90) + KIN (2.33)		0.9	8.3	8.3	5.9

period (cool-white fluorescent lamps, 60 μM $m^{-2}s^{-1}$) at 21 ± 2˚C.

In the first phase of micropropagation, tips of 4-week-old sterile seedlings (~3 mm long), were placed as initial explants on three variants of MS medium (Tab. 1) in Erlenmeyer flasks (vol 100 cm^3) containing 20 cm^3 medium. The regenerated multishoots were divided into separate shoots and then subcultured for 4–5 weeks on the same media. Multishoots arising from one explant (seedling tip) were considered the source of one clone culture. After obtaining an adequate number of regenerated multishoots, they were again divided into separate shoots, cultured for 14 days, and then rooted in vitro (Tab. 1). The experiment was carried out in three replicates, each with dozens of shoots.

In the second phase of micropropagation, we tested four variants of media in order to find the most suitable medium for in vitro cowslip propagation. We took morphological measurements of shoots within the two clones that grew the best. The shoots used in this experiment were derived from multishoots developed on one medium variant [MS with 4.44 μM 6-benzyladenine (BA) and 1.13 μM 2,4-dichlorophenoxyacetic acid (2,4-D)]. The separate shoots used for propagation were rosettes of 3–4 leaves. Each shoot was weighed before placing it on a tested medium (Tab. 1) and the longest leaf was measured. After 4 weeks of shoot culture on the four tested media, these measurements were repeated. Similarly, the number of regenerated shoots arisen during 4 weeks of in vitro culture was assessed. All measurements were taken in 3 replicates for 10 shoots of clone A and for 8 shoots of clone B. The obtained results were statistically analyzed. The influence of the tested media on the increase in leaf length and shoot mass was assessed by ANCOVA, and the influence on the number of regenerated shoots by one-way ANOVA. If an influence of the applied media on the examined features was noted, multiple comparisons were performed with Tukey's test.

In the third phase of micropropagation, the rooted shoots of *P. veris* obtained in vitro were transferred to pots with a sterile mixture of soil and sand (3:1). At the initial stage of growth, the plants were covered with glass to increase humidity. The plants were watered with sterilized tap water and acclimated at room temperature for ~3 weeks. Then 25 plants were planted in the ground in the experimental garden of the Department of Botany, Agricultural University, Poznań, and their further development was observed.

THIN-LAYER CHROMATOGRAPHY

Shoots of *P. veris* cultured in vitro and leaves from field cultivation were dried at 40˚C and then analyzed by two-dimensional thin-layer chromatography (2D-TLC fingerprinting) to determine the presence of flavonoid compounds. Methanol extracts were prepared and partitioned to obtain chloroform, butanol and water fractions. Butanol and chloroform fractions were subjected to 2D-TLC on cellulose-covered plates (Merck) developed in phases (v/v): dimension I – 1-butanol, acetic acid, water (4:1:5), upper phase; dimension II – acetic acid, water (15:85) (Budzianowski and Skrzypczak, 1995). The distribution of compound spots on the chromatograms was analyzed under UV light (λ = 365 nm, λ = 254 nm) before and after the plates were sprayed with reagents characteristic of flavonoid compounds: 1% ethanol solution of $AlCl_3$ and a few min exposure to 105˚C heat, or 0.1% ethanol solution of 2-aminoethanol diphenylborate (NA, Naturstoffreagenz A).

RESULTS AND DISCUSSION

Seeds of *P. veris* germinated in vitro 30–60 days after being placed on medium. Seeds from different natural populations differed in germinability. In view of difficulties in seed sterilization, the second method was tried, employing 0.1% $HgCl_2$ instead of

TABLE 3. Average initial value and increase in leaf length and shoot weight, and average number of regenerated shoots of clone A and clone B of *Primula veris*

Character	MS medium variant (supplements in μM)							
	MS BA (4.44) 2,4-D (1.13)		MS BA (3.08) 2,4-D (1.13)		MS BA (2.22) 2,4-D (1.13)		MS BA (0.88) 2,4-D (1.13)	
	Clone							
	A	B	A	B	A	B	A	B
Initial leaf length [mm]	25.1	18.6	24.8	32.1	26.8	27.8	22.5	21.1
Leaf length increase [mm]	8.1	15.0	9.9	10.8	9.7	10.0	13.2	9.2
Initial shoot weight [mg]	84.0	59.6	81.5	69.0	75.6	90.5	107.2	81.1
Shoot weight increase [mg]	861.4	681.4	965.8	765.8	1019.2	888.5	918.5	602.4
Number of regenerated shoots	5.9	2.4	4.2	3.1	3.8	4.0	4.1	2.1

TABLE 4. Empirical values of F statistic and critical F test values in clone A and clone B of *Primula veris*

Evaluated character	Source of variability	Empirical value of F statistic		Critical value of F test ($\alpha = 0.05$)	
		Clone			
		A	B	A	B
Number of regenerated shoots	Medium	9.84*	1.37	2.69	2.92
Leaf length increase	Initial length (concomitant variable)	22.30*	0.03	3.94	4.17
	Medium	1.74	0.20	2.70	2.92
Shoot weight increase	Initial weight (concomitant variable)	33.28*	75.46*	3.97	4.17
	Medium	1.30	2.32	2.72	2.92

*significance of differences for $\alpha = 0.05$

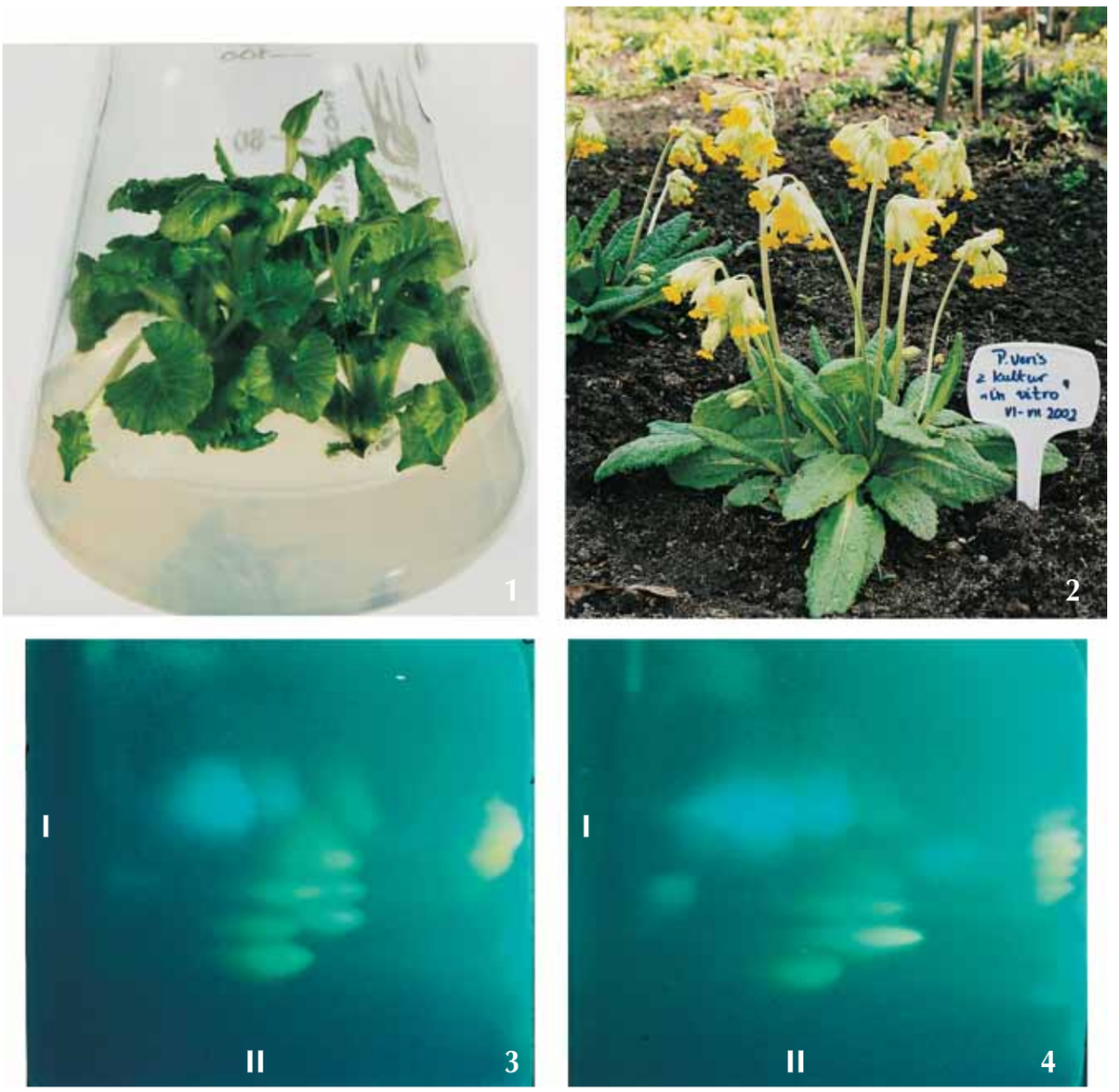
30% Clorox. After 3–10 days, some fungal infections developed with both methods. It is known that endophytes occur in many cowslips, which increases the risk of losing breeding material and seeds as a result of infection (Schween and Schwenkel, 2002). In a study of in vitro culture of *Anagallis arvensis* (Primulaceae), seeds were disinfected with 1% chloramine T for ~15 minutes (Bajaj, 1999).

In our study the largest number of seedlings was obtained on medium supplemented with GA_3 (2.90 μM) and KIN (2.33 μM): an average 21.5% of the seeds sterilized with 30% Clorox germinated, compared to 5.9% of those sterilized with 0.1% $HgCl_2$. On medium supplemented with only GA_3 (2.90 μM) the percentage of sprouted seeds was much lower (Tab. 2). Morozowska (2002) examined the influence of different GA_3 concentrations on germination of *P. veris* seeds using tests carried out according to ISTA (1996) standards, and found that GA_3 (0.9 and 1.8×10^{-3} M) promoted breaking of *P. veris* seed dormancy under laboratory conditions: the percentage of germinated seeds was 64.2–97.1% depending on the applied concentration of GA_3, whereas the time required to germinate the maximum number of seeds varied from 25 to 60 days.

Seedling tips transferred to different MS medium variants proliferated lateral buds. The regenerated shoots, transferred to the tested medium variants (Tab. 1), developed into multishoots.

The most suitable variant of MS medium for *P. veris* propagation in both the initial stage (proliferation of lateral buds) and for further multiplication appeared to be medium supplemented with BA (4.44 μM) and 2,4-D (1.13 μM), on which an average 7 new shoots were obtained from each explant. Medium supplemented with KIN (9.30 μM) and indole-3-acetic acid (IAA; 11.42 μM) favored the increase in multishoots and rooting of *P. veris* at a lower multiplication coefficient (3–4).

Schween and Schwenkel (2002) obtained callus tissue in the presence of 2,4-D (18.08 μM) and thidiazuron (TDZ; 2 mg/l) from flower elements of *P. vulgaris* Huds. and *P. elatior* (L.) Hill, regenerating vegetative buds. They recorded the highest multiplication coefficient for 2,4-D at a concentration of 2 mg/l (= 9.04 μM). A higher concentration of 2,4-D (8



Figs. 1–2. *Primula veris* L. from in vitro culture. **Fig. 1**. Multishoot formation after 4 weeks on MS medium containing BA (4.44 µM) and 2,4-D (1.13 µM). **Fig. 2**. In vitro regenerated plants flowering in experimental garden. **Figs. 3–4.** 2D-TLC of butanolic fractions from intact leaves **(3)** and shoot cultures **(4)** of *Primula veris* L. in UV_{365} after spraying with $AlCl_3$ (I – first direction; II – second direction).

mg/l = 36.16 µM) decreased the multiplication coefficient of the appearing buds. Those authors suggested that further reduction of the 2,4-D concentration below 9.04 µM might increase the coefficient of shoot regeneration. In the process of callus regeneration the authors observed an interaction between BA and 2,4-D. Callus induction of explants on medium containing BA was significantly lower than on media with other cytokinins. In our study the highest multiplication coefficient was obtained in the presence of BA (4.44 µM) and 2,4-D (1.13 µM).

Medium with BA (2.22 μM) and α-naphthaleneacetic acid (NAA; 0.27 M) was also suitable for initiation of lateral buds: 4–5 shoots from one. Coumans et al. (1979) obtained vegetative shoot production on fragments of *P. obconica* L. leaf blades in the presence of BA and NAA at concentrations similar to those used in our study. The same authors found that flower buds were produced at higher BA concentrations (4.44 μM to 44.4 μM). When the BA concentration was low (~4.44 μM), vegetative buds were produced instead of flower buds. On MS medium with BA (4.44 μM) and NAA (5.36 μM), numerous vegetative buds were produced on inflorescence tips. Borodulina et al. (2001) obtained the highest multiplication coefficient (12–32 depending on the hybrid) on medium B_5 (Gamborg, 1968) enriched with BA (10.0 μM), NAA (0.5 μM) and GA_3 (5.0 μM). The concentrations of two of the three growth regulators applied by Borodulina et al. (2001) were twice those in our study, in which the multiplication media had no GA_3.

In the course of our study, the growth and development of in vitro plantlets from the two most numerous clones of cowslip were compared on the basis of morphological measurements. The influence of the applied media (Tab. 1) on the evaluated characters was analyzed. The shoot growth of clone A on medium with BA (4.44 μM) and 2,4-D (1.13 μM) was significantly more intensive than that of clone B (Tab. 3). On the same medium variant, clone B had better leaf length increase than clone A. The remaining three medium variants, namely with BA (3.08 μM, 2.22 μM or 0.88 μM) and 2,4-D (1.13 μM), seemed better for the increase in leaf length and shoot weight of clone A. On medium with BA (0.88 μM) and 2,4-D (1.13 μM), for clone B the increase in leaf length and the number of multiplied shoots were lowest, whereas the increase in leaf length of clone A was highest (Tab. 3). The largest number of regenerated shoots of clone A (Fig. 1) was obtained on MS medium with the addition of BA (4.44 μM) and 2,4-D (1.13 μM). These results were confirmed by statistical analysis. One-way ANOVA indicated that type of medium significantly influenced the number of regenerated shoots for clone A. Multiple comparisons with Tukey's test showed that the number of shoots regenerated on MS medium with BA (4.44 μM) and 2,4-D (1.13 μM) was significantly higher than on the other three medium variants. For clone B, no statistically significant influence of the tested media on the number of regenerated shoots was found (Tab. 4).

Initially, leaves had diverse lengths and shoots had diverse weights. It may be suggested that beside the influence of different media, the initial shoot weight and leaf length (concomitant variables) could also influence the increase in the studied traits. ANCOVA showed that for clone A the concomitant variables affected the increase in leaf length and shoot weight after 4 weeks, whereas in clone B only initial shoot weight exerted a statistically significant influence on its increase. After eliminating the influence of the concomitant variables, we found that the differences in the average increases in leaf length and shoot weight for the four types of media tested were not statistically significant (Tab. 4).

All single shoots from multishoots subcultured on MS medium supplemented with indole-3-butyric acid (IBA; 2.45 μM) and on MS medium without phytohormones rooted. The obtained plantlets had well-shaped rosettes with several leaves and normally developed rootlets when the medium was supplemented with IBA, while those developed on medium without growth regulators had thin and elongated rootlets. Borodulina et al. (2001) and Mizuhiro et al. (2001) report on the rooting of several *Primula* species on 1/2 MS medium also without growth regulators. According to Coumans et al. (1979), Borodulina et al. (2001) and Schween and Schwenkel (2002), the use of auxins (IBA and NAA) promotes normal rooting of different *Primula* species, and this is supported by our results for *P. veris*.

In our study, in vitro culture lasted 8–10 weeks from the moment the seeds were placed on medium, and included seed germination, proliferation, and growth until shoot rooting. *P. veris* plantlets transferred to pots with soil showed further growth. After acclimation at room temperature, the plants transplanted to the soil started to flower and set seeds (Fig. 2).

Phytochemical analysis of fractionated extracts from leaves of *P. veris* by two-dimensional thin-layer chromatography confirmed the presence of flavonoids in them. Spots with intense blue fluorescence were observed on chromatograms of chloroform fractions. Chromatograms of butanol fractions showed numerous brown spots, changing to yellow fluorescent after visualization by $AlCl_3$ solution and to yellow or orange after spraying with NA. These results indicate the presence of flavonoid compounds. Chromatograms of water fractions showed trace amounts of phenolic compounds. The qualitative composition of those compounds in the material from in vitro cultures (Fig. 4) was similar to that in plant material from cultivation (Fig. 3), but quantitative differences may appear. Detailed phytochemical analyses are continuing.

Our in vitro studies on *P. veris* have been in progress since 2001 (Wesołowska et al., 2002; Wesołowska et al., 2003). We have encountered difficulties in seed sterilization and in obtaining a larger number of seedlings. In the applied conditions, in vitro clonal propagation has proved effective for the studied species. The developed method of in vitro clonal propagation from shoot tips can be used for culture of cowslip, a protected species of medicinal importance. Plants especially rich in biologically active compounds can be obtained as a result of clone selection. In vitro culture of *P. veris* enables active protection of this species.

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