Botanica SERBICA 33 (2): 157-162 (2009)

Original Scientific Paper

In vitro culture of Balkan endemic and rare *Pulsatilla* species for conservational purposes and secondary metabolites production

Kalina Danova[1*], Alessandra Bertoli[2], Laura Pistelli[3], Dimitar Dimitrov[4] and Luisa Pistelli[2]

1 Department of Plant Physiology, Sofia University "St. Kliment Ohridski", Bulgaria

2 Dipartimento di Scienze Farmaceutiche, Via Bonanno 33, 56100 Universiá di Pisa, Italy,

3 Dipartimento di Biologia delle Piante Agrarie,Via Mariscoglio 34, 56124, Universitá di Pisa, Italy

4 Plant Section, National Natural History Museum, BAS, Sofia, Bulgaria

ABSTRACT: Shoot cultures of *Pulsatilla montana* ssp. *balcana, P. halleri* ssp. *rhodopaea* and *P. slaviankae* (Zimm.) Jordanov & Kozuharov were initiated from surface-sterilized seeds of the three species, collected at their natural habitats. Shoot cultures of the three species in Murashige and Skoog's culture medium (MS) exhibited a chlorophyll a/b (chl a/b) ratio ranging from 1, 86 to 3, 01. Moreover by using different concentrations of benzyladenine and 3-indole butyric acid the chl a/b ratio differed from species to species, showing a different adaptability of species to different media. The shoot cultures of the three *Pulsatilla* species were also analyzed as an *in vitro* source of polyphenolic antioxidant substances so they were screened for total phenolics, flavonoids and anthocyanidins content in three media variants, demonstrating the highest content for these substances in *P. montana* ssp. *balcana* and *P. halleri* ssp. *rhodopaea* in the growth regulator-free and 6-benzyladenine – supplemented medium, while in medium supplemented with auxin and cytokinin all three species produced commensurable levels of the studied metabolites.

Key words: *Pulsatilla montana* ssp. *balcana, P. halleri* ssp. *rhodopaea, P. slaviankae* (Zimm.) Jordanov & Kozuharov, shoot cultures, antioxidant phenolics, photosynthetic pigments

Received 08 Spetember 2009 Revision accepted 02 December 2009

UDK 582.675.1.085.2

INTRODUCTION

Plant species of the *Pulsatilla* genus are widely utilized in Eastern traditional medicine for treatment of enteritis and as anti-inflammatory, antispasmodic and antitumor remedies. Recent investigations revealed the phytochemical basis of the pharmacological properties of the species (Lee *et al.* 2001, Seong-Cheol 2005a, b; Duan *et al.* 2006;). Synonyms of the plant's name are Pasque Flower, Wind Flower, Meadow Anemone, Passe Flower, Easter Flower. The fresh *Pulsatilla* sp. plants have strongly acrid taste and are being avoided by cows and horses but consumed by sheep and goat. The plant is included in the British Pharmacopoeia and was formerly included in the United States Pharmacopoeia. The fresh plant contains the glycoside ranunculin which on wounding of the plant is converted into the acrid volatile oil protoanemonin (determining the anti-infectious action). On drying protoanemonin is converted into anemonin. Anemonin is a crystal odorless substance with laxative and depressant properties. It causes skin irritation, allergic reactions and CNS depression. The triterpenoid saponins are the active ingredients determining the antitumor activities of the studied Asian *P. koreana*, *P. cernua* and *P. chinensis* species.

*correspondence: k_danova@abv.bg

***Pulsatilla montana* (Hoppe) Rchb.** – this European geoelement is distributed in meadows and pastures at altitudes from 0 to 1400m above the sea level. In Bulgaria it occurs at the Black Sea region, Northeast Bulgaria, Danubian Plain, Predbalkan, Stara Planina, Sofia region, Znepole region, Vitosha, Sredna Gora, Rhodopes, Thracian Lowlands and Strandja. It grows at open spaces and bushes, in glades in mixed deciduous forests of *Quercus pubescens, Quercus cerris, Quercus dalechampii, Carpinus orientalis, Carpinus betulus* and *Betula pendula*. It occurs in herbaceous communities of *Festuca valesiaca*, *Poa bulbosa* and *Stipa capillata*. It occurs on both limy and silica rocky soils. *Pulsatilla montana* ssp. *balcana* is a Balkan endemic species.

***Pulsatilla halleri* (All.) Willd.** – is an Alpo-Carpato-Balkan element. Xerophyte and calciphile. The species occurs on dry rocky and grassy sites and bushes in Eastern Stara Planina (Sliven Balkan) and Central Rhodopes from 300 to 1400m above the sea level. The species is protected by the Law and listed in the Red Data Book. *Pulsatilla halleri* ssp. *rhodopaea* is endemic to the Balkan region.

***Pulsatilla slaviankae* (Zimm.) Jordanov & Kozuharov –** Bulgarian endemic species, listed in the Red Data Book. It is an obligatory calciphile, occuring on humus-carbonate soils (rendizines) in Slavianka Mountain and South Pirin from 1000 to 1500m above the sea level. It grows in herbaceous communities together with *Festuca dalmatica, Centaurea parilica, Saxifraga stribrnyi, Sideritis scardica, Cerastium decalvans* etc.

So far *in vitro* cultures of *Pulsatilla* species have been focused mainly on the conservation of rare or threatened representatives of the genus, as reported for micropropagation of *P. patens* Klavina *et al.* (2004). The species was characterized by medium to low multiplication potential in aseptic conditions which was considerably improved by the supplementation of low cytokinin concentration to the culture medium. Also, plant regeneration through stem tip, root segment and bud of *Pulsatilla chinensis* has been reported by Zhang *et al.* (2004). Plant regeneration of *Pulsatilla koreana* Nakai was achieved by adventitious shoot formation indirectly from callus and directly from adventitious root segments (Jung *et al.* 2007). *In vitro* propagation protocol of the rare, endangered, ornamental and medicinal *Pulsatilla pratensis* (L.) Miller ssp. *nigricans* (Störck) Zämelis (*Ranunculaceae*) was quite recently reported by Naumovski *et al.* (2009). Except the above mentioned *Pulsatilla* species, no data on tissue culture of any of the Balkan endemic representatives of the genus are found. Moreover, to the best of the authors' knowledge there are no reports in literature concerning the secondary metabolites production of the *in vitro* cultured *Pulsatilla* species. Unlike the widely studied wild growing Asian species, little information is available on the phytochemical composition of the Balkan *Pulsatilla* representatives. A chemotaxonomically focused study of Nikolova *et al.* (1998) reports for the first time on the presence of astragalin, isoquercitrin, quercetin, kaempferol and caffeic acid in the aerial parts of *P. slaviankae* and in a further research Nikolova & Asenov (2006) report on the presence of external isorhamnetin in the aerial parts of *P. montana* collected in Bulgaria.

The three *Pulsatilla* species studied by us, were introduced *in vitro* and preliminary investigations confirmed the presence of total phenolics and flavonoid compounds in shoot cultures in basal Murashige and Skoog's culture medium (MS) (Danova *et al*. 2008). The aim of the present study was to estimate the adaptability of the *Pulsatilla* species to different media by comparing the total content and ratio of photosynthetic pigments and to assess the potential of the species to produce antioxidant polyphenolics *in vitro.*

MATERIALS AND METHODS

Plant material and tissue culture. Intact plant material of the three species was collected from the sites of their natural distribution and herbarium specimens were deposited at the Institute of Botany, Bulgarian Academy of Sciences, Sofia - *Pulsatilla montana* ssp. *balcana* (SOM 163 521), *Pulsatilla halleri* ssp. *rhodopaea* (SOM 163 523) and *Pulsatilla slaviankae* (SOM 163 522).

Shoot cultures were induced from sterile germinated seeds of the three species (Danova *et al.* 2008). Shoot cultures were grown for 8 weeks on three media variants: MS – basic Murashige and Skoog's culture medium without growth regulators (Murashige & Skoog 1962), HP2 – 0.2 mg/l BA (6-benzyladenine) supplemented MS medium and HP3 – 0.2 mg/l BA + 0.1 mg/l IBA (3-indole butyric acid) supplemented MS medium.

Photosynthetic pigments determination. 100 mg FW (fresh weight) of the shoot cultures from the three media variants were cut and immersed in 5 ml DMFA (N,N-Dimethylformamide) at 4°C for 24 hours (until discoloration of the plant material). Absorption of the supernatant was measured at wavelengths 664nm and 647nm and photosynthetic pigments determined after the formulae (Moran 1982):

$$Ca = 12{,}64 \cdot A664 - 2{,}99 \cdot A647$$
$$Cb = -5{,}6 \cdot A664 + 23{,}26 \cdot A647$$
$$Ct = 7{,}04 \cdot A\,664 + 20{,}27 \cdot A647$$

Ca - chlorophyll a
Cb – chlorophyll b
Ct – total chlorophylls

For the calculation of DW (air dry weight), aliquots of the fresh plant material of the respective samples were dried at 60°C until constant weight.

Total phenoics determination. Total phenolics were determined by the Folin & Ciocalteu's colorimetric method of Singleton *et al.* (1999), modified. 100mg DW of the samples were extracted with hot ethanol, an aliquot of the extract was placed in test-tube and distilled water, 1:1 Folin & Ciocalteu's reagent and 20% Na_2CO_3 were added. The absorption was measured at 730nm and the TPL (total phenolics levels) were calculated by means of a calibration curve of chlorogenic acid and expressed as milligrams of chlorogenic acid equivalent per 1 gram of DW of the sample.

Total flavonoids determination. Total flavonoids content of leaf samples of the plant was measured using a colorimetric assay in accordance to a modification of the method of Zhishen *et al.* (1999). 100 mg of DW of the samples was extracted with hot ethanol and an aliquot of the extract were placed in test-tube and distilled water, 5% $NaNO_2$ and 10% $AlCl_3$ were added. After the addition of 1N NaOH and distilled water, the absorption at 510 nm was measured and the concentration was calculated by means of a calibration curve of (+)catechin. The TFL (total flavonoids levels) of leaf samples of the plants were expressed in milligrams of (+)catechin equivalent per 1 gram of DW of the sample.

Total anthocyanidins determination. 250mg of the fresh plant material was crushed with acidified ethanol (HCl/EtOH – 15:85) and after addition of distilled water and chloroform the mixture was centrifuged at 4000rpm for 20 minutes (Lohachoompol *et al.* 2004). The absorption of the supernatant was measured at 535nm and the TAL (total anthocyanidins levels) of the samples were expressed as relative measure of absorption units per 1 gram of DW of the sample. The DW was calculated as described above.

RESULTS AND DISCUSSION

Photosynthetic pigments contents and chl a/b ratio in the three culture media variants for the three *Pulsatilla* species are presented in Figure 1. *P. slaviankae* grown in a regulators-free medium (MS) exhibited the highest levels of total chlorophylls in comparison to the two other species (Figure 1-a). This is, however, due to highest chlorophyll b content, as it is seen in Figure 1-d. The highest chl a/b ratio in MS medium variant is observed for *P. montana.* The differences, observed between the three species in HP2 medium are significant only for *P. montana,* which displays the highest total chlorophyll content (Figure 1-b). In HP3 medium the relations between total chlorophylls (Figure 1-c and 1-f) are reverse to the ones in growth regulator-free medium.

Secondary metabolite levels in the three culture media variants for the three *Pulsatilla* species are illustrated in Figure 2. In the basal MS medium, without the influence

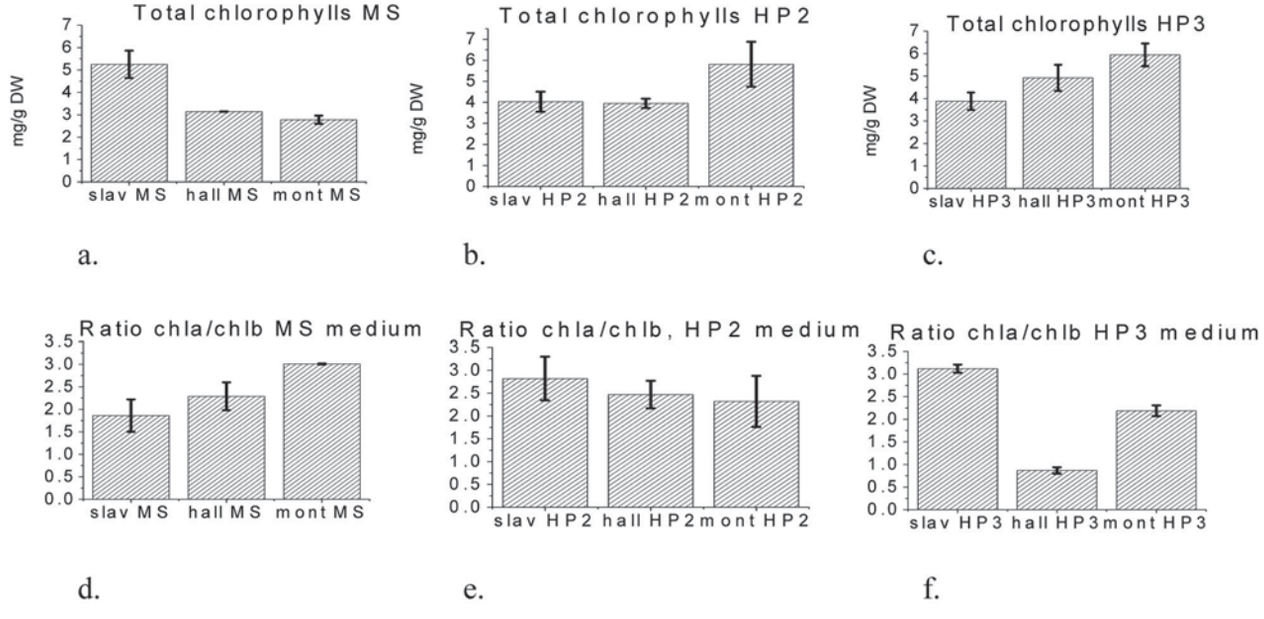

Fig. 1. Total chlorophyll and chl a/b ratio of the three species in the three media variants.

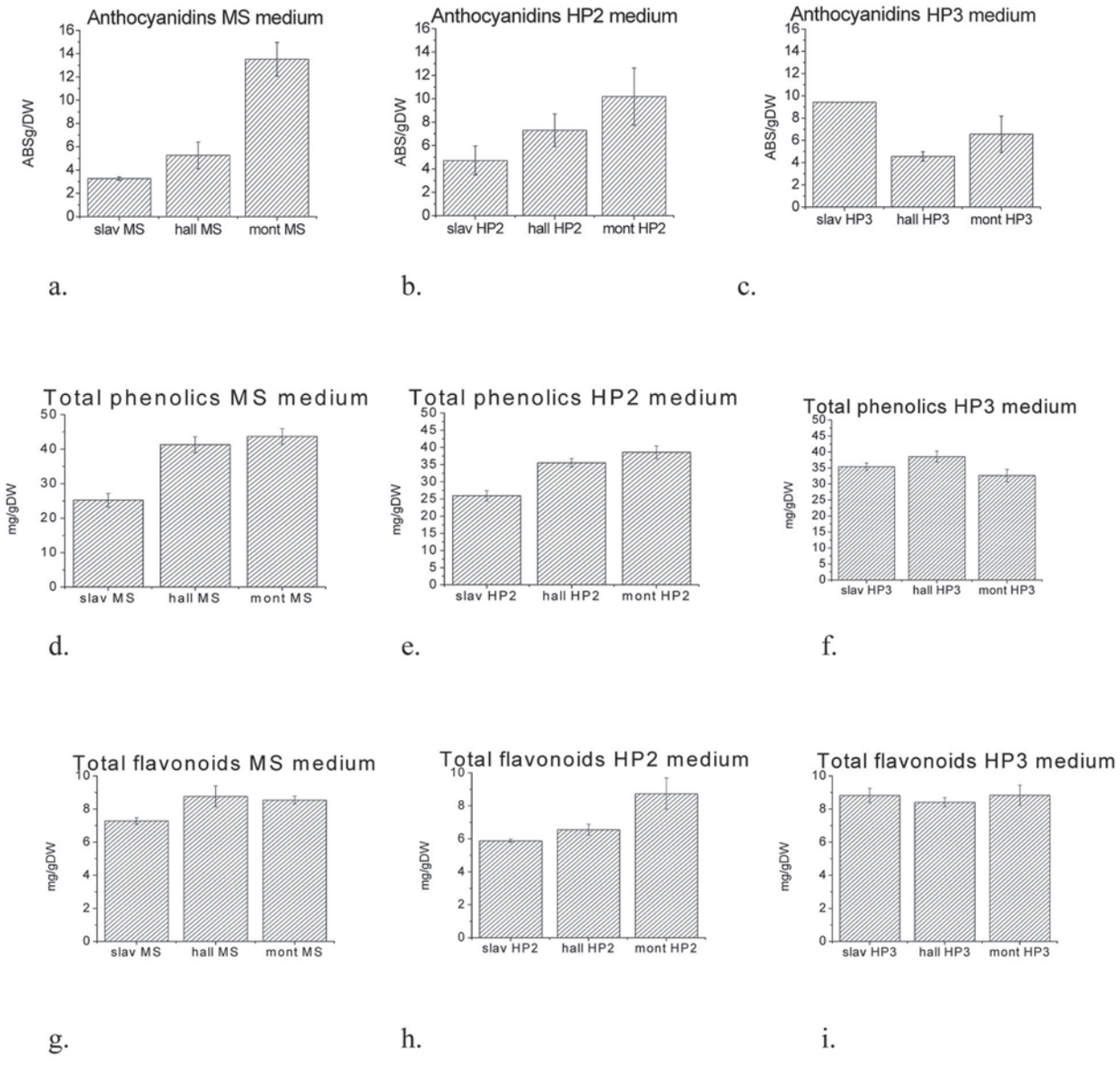

Fig. 2. Secondary metabolites levels in shoots cultures of the three species in the three media variants. TAL in basal MS (a), HP2 (b) and HP3 (c) media variants. TPL in basal MS (d), HP2 (e) and HP3 (f) media variants; and TFL in basal MS (g), HP2 (h) and HP3 (i) media variants.

of growth regulators, the levels of antocyanidins, total phenolics and flavonoids compounds were significantly higher for *P. halleri* and *P. montana* in comparison to *P. slaviankae* (Figure 2-a, d and g). The same ratio is observed also after the addition of BA to the culture medium (HP2 variant) (Figures 2-b, e and h).

Interestingly, after the addition of auxin + cytokinin (HP3 variant) to the culture medium, no significant differences have been generally detected between the three species regarding TPL and TFL, with only increased anthocyanidins in *P. slaviankae* in comparison to the other two species (Figure 2-c, f and i).

When comparing the TFL for the same species in the different media variants, it can be seen that for *P. halleri* and *P. slaviankae* there is a drop in the levels measured in BA – supplemented medium variant (HP2) in comparison to the growth regulator-free medium (MS) and medium supplemented with BA and IBA (HP3). These differences are not significant for *P. montana.* Interestingly when comparing the TAL for *P. slaviankae* and *P. halleri* the reverse relations are observed – in HP2 media variant the levels of total anthocyanidins exceed the ones for MS medium for *P. slaviankae* and for MS and HP3 media for *P. halleri,* the same is observed in *P. montana* for HP3 medium variant.

An attempt has also been made to establish a relationship between the adaptability of each species to the different media and the respective levels of secondary metabolites measured for each species/medium-defined variant.

As it is seen in Figures 1-d, e and f, the chl a/b ratio for *P. slaviankae* is highest in HP3 medium, implying a better photosynthesis process in this medium with the consequence of the best adaptability and optimal physiological conditions. For *P. halleri* this parameter has its highest value in both MS and HP2 media with no significant difference between the two variants. For *P. montana* the highest chl a/b ratio was also observed in both MS and HP2 medium variants without significant difference between the two values.

Secondary metabolite production determined in *P. slaviankae* appeared clearly indicated that the highest levels for TAL, TPL and TFL are measured in HP3 medium variant (Figure 2). However in other species, as *P. halleri*, TAL are highest in HP2 medium in comparison to the other two media variants. TPL and TFL have the highest value in MS medium variant. For *P. montana* TAL and TPL are highest for MS medium variant and TFL do not show significant differences in any of the studied variants.

For many carbon-based metabolites, several hypotheses have been developed to explain phenotypic and evolutionary patterns in the distribution of plant secondary metabolites (Heyworth *et al.* 1998). For example, according to the 'over flow metabolism' concept, when carbon production exceeds the carbon demand associated with plant growth, the excess carbon is channeled into biosynthesis of secondary metabolites (Matsuki 1996; Mosaleeyanon *et al.* 2005).

Our overall observations show that there is a connection between the chl a/b ratio and the levels of secondary metabolites measured in the *in vitro* cultured *Pulsatilla* species. For each species the levels of secondary metabolites are generally increased in media variants with an increased chl a/b ratio in comparison to the other variants. The high ratio of chl a/b clearly indicates that plants have good fitness, and can photosynthetize in the *in vitro* conditions environment, in spite of sugar addition in the vessels.

Other authors also investigate the content of substances with phenolic structure in wild growing representatives of the genus. Two cinnamic acid derivatives were isolated from the roots of *P. cernua* and shown to inhibit the growth of pathogenic bacteria such as *C. perfringens* and *E. coli*, while not having any adverse effects toward the growth of beneficial bacteria such as *bifidobacteria* and *L. acidophilus*; this finding supposed to be an indication of at least one of the pharmacological actions of *P. cernua* root (Lee *et al.* 2001). Our findings show that *Pulsatilla* species display a potential for *in vitro* production of antioxidant substances with phenolic structure, *P. montana* and *P. halleri* demonstrating higher levels than *P. slaviankae* for the investigated metabolites. There also is influence of growth regulators on secondary metabolites production for the three studied *Pulsatilla* species *in vitro*. Therefore the species are a favorable candidate for biotechnological research of secondary metabolites production in order to optimize them as a system for *in vitro* production of valuable substances with phenolic structure.

CONCLUSIONS

As far as the authors are aware this is the first report on *in vitro* culture and study of the impact of growth regulators on secondary metabolites production in *P. slaviankae, P. halleri* ssp. *rhodopaea* and *P. montana* ssp. *balcana. In vitro* culture of the three species is important from conservational point of view due to the endemic and threatened status of the species for the region of the Balkans and also as a potential source for secondary metabolites production in laboratory conditions. The biosynthetic capacity of the three species *in vitro* is susceptible to the effect of growth regulators in a species-dependent manner. Further phytochemical research is needed in order to investigate the qualitative content of secondary metabolites produced by the studied *Pulsatilla* species in culture.

Acknowledgements – This work is supported by the ERASMUS Lifelong Learning Program and the Operational Programme "Human resources development" co-financed by the European Union through the European Social Fund ISUN BG051P0001-3.3.04/42.

REFERENCES

Danova K, Markovska Y, Dimitrov D & Kapchina-Toteva V. 2008. *In vitro* culture initiation and phenol and flavonoid determination of some medicinal plants, endemic to the Balkan flora In: Proceedings book of International Scientific Conference Stara Zagora June 2007, vol. 1 "Plant breeding", pp 222 - 229.

Duan H, Zhang Y, Xu J, Qiao J, Suo Z, Hu G & Mu X. 2006. Effect of anemonin on NO, ET-1 and ICAM-1 production in rat intestinal microvascular endothelial cells. *J Ethnopharmacol*. **3:** 362-6.

Jung SJ, Jeong JH, Yoon ES & Choi YE. 2007. Plant Regeneration from Callus and Adventitious Root Segments of Pulsatilla koreana Nakai. *J. Plant Biotech*. **34**: 153-159.

Heyworth CJ, Iason GR, Temperton V, Jarvis PG & Duncan AJ. 1998. The effect of elevated CO_2 concentration and nutrient supply on carbon based plant secondary metabolites in *Pinus sylvestris* L. *Oecologia* **115:** 344-350.

Klavina D, Gailite A, Jakobsone G, Nečajeva J & Gavrilova G. 2004. Tissue culture technology in conservation of threatened plant species of Latvia. *Acta Universitatis Latviensis, ser. Biology* **676:** 183-188.

Lee HS, Beon MS & Kim MK. 2001. Selective growth inhibitor toward human intestinal bacteria derived from Pulsatilla cernua root. *J Agric Food Chem*. **49:** 4656-4661.

Lohachoompol V, Srzednicki G & Craske J. 2004. The Change of Total Anthocyanins in Blueberries and Their Antioxidant Effect After Drying and Freezing. *J. Biomed. Biotech*. **5:** 248-252.

Matsuki M. 1996. Regulation of plant phenolic synthesis: from biochemistry to ecology and evolution. *Aust. J. Bot.* **44:** 613-634.

Moran R. 1982. Formulae for determination of chlorophyllous pigments extracted with N,N-Dimethylformamide, *Plant Physiol.* **69**: 1376-1381.

Mosaleeyanon K, Zobayed SMA, Afreen F & Kozai F. 2005. Relationships between net photosynthetic rate and secondary metabolite contents in St. John's wort. *Plant Sci.* **169**: 523-531.

Murashige T & Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15:** 473-497.

Naumovski D, Radić S & Pevalek-Kozlina B. 2009. *In vitro* micropropagation of *Pulsatilla pratensis* (L.) Miller ssp. *nigricans* (Störck) Zämelis. *Propagation of Ornamental Plants*. **9**: 16-20.

Nikolova M & Asenov A. 2006. Surface flavonoid aglycones in newly studied plant species. *Natural Products Research* **20**: 103-106.

Nikolova M, Nikolov S & Koeva Y. 1998. Flavonoids in the overground part of *Pulsatilla slaviankae* (Rumm.) D. Yord et. Kož (Ranunculaceae). *Phytol. Balc.* **4:** 165.

Seong-Cheol B, Kim Y, Lee JH & Ahn BZ. 2005a. Triterpenoid Saponins from the Roots of *Pulsatilla koreana*. *J. Nat. Prod.* **68:** 268-272.

Seong-Cheol B, Lee JH, Song GY, Kim DH, Yoon MY & Ahn BZ**.** 2005b. Antitumor activity of *Pulsatilla koreana* saponins and their structure – activity relationship. *Chem. Pharm. Bull.* **53:** 1451—1454.

Singleton VL, Orthofer R & Lamuela-Raventós RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Meth. Enzymol*. **299:** 152-178**.**

Zhang ZX, Ding WQ, Tang Y, Shi WJ & Ye WC. 2004. Study of tissue culture of pasqueflower. *Zhongguo Zhong Yao Za Zhi.* **29:** 215-218 (in Chinese).

Zhishen J, Mengcheng T & Jianming W. 1999. The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* **64:** 555-559.

REZIME

In vitro culture retkih i endemičnih Balkanskih biljaka roda *Pulsatilla* u cilju zaštite i produkcije sekundarnih metabolita

Kalina Danova, Alessandra Bertoli, Laura Pistelli,
Dimitar Dimitrov, Luisa Pistelli

Kultura izdanaka *P. montana* ssp. *balcana, P. halleri* ssp. *rhodopaea* i *P. slaviankae* (Zimm.) Jordanov & Kozuharov je uspostavljena iz površinski sterilisanih semena tri vrste, sakupljenim u prirodi. Kultura izdanaka u "Murashige i Skoog" medijumu iskazuje odnos hlorofila a/b od 1,86 do 3,01. Ako se pak koriste različite koncentracije benziladeninan i 3-indol-buterne kiseline odnos hlorofila a/b varira od vrste do vrste ukazujući na različitu adaptabilnost vrsta na različitim medijumima. Kultura izdanaka tri vrste *Pulsatilla* analizirana je kao *in vitro* izvor za polifenolne antioksidante, pa su skenirani sadržaji fenola, flavonoida i antocijanidina na različitim medijumima. Najviši nivo ovih supstanci zabeležen je kod *P. montana* ssp. *balcana* i *P. halleri* ssp. *rhodopaea* koje su rasle na medijumima bez regulatora rastenja ali sa 6-benziladenina dok na medijumima sa dodatim auksinima i citokininima sve tri vrste proizvode srazmerno manje nivo istraživanih metabolita.

Ključne reči: *Pulsatilla montana* ssp. *balcana, P. halleri* ssp. *rhodopaea, P. slaviankae* (Zimm.) Jordanov & Kozuharov, kultura izdanaka, antioksidativni fenoli, fotosintetski pigmenti