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

Kalina Danova1,*, Alessandra Bertoli2, Laura Pistelli3, Dimitar Dimitrov4 and Luisa Pistelli2

1 Department of Plant Physiology, Sofia University “St. Kliment Ohridski”, Bulgaria
2 Dipartimento di Scienze Farmaceutiche, Via Bonanno 33, 56100 Università di Pisa, Italy,
3 Dipartimento di Biologia delle Piante Agrarie, Via Mariscoglio 34, 56124, Università 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

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 acid 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 acid 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.
**Pulsatilla montana** (Hoppe) Rchb. – This European geoelement is distributed in meadows and pastures at altitudes from 0 to 1400 m 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-Carpatho-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 1400 m 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, occurring on humus-carbonate soils (rendizines) in Slavianka Mountain and South Pirin from 1000 to 1500 m above the sea level. It grows in herbaceous communities together with *Festuca dalmatica*, *Saxifraga strirnyi*, *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* (Störrck) Zámelis (*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 & Aseenov (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 664 nm and 647 nm and photosynthetic pigments determined after the formulae (Moran 1982):

\[
Ca = 12,64 \cdot A664 - 2,99 \cdot A647 \\
Cb = - 5,6 \cdot A664 + 23,26 \cdot A647 \\
Ct = 7,04 \cdot A664 + 20,27 \cdot A647 \\
Ca - chlorophyll a \\
Cb – chlorophyll b \\
Ct – total chlorophylls
\]
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 phenolics 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% Na₂CO₃ 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% NaNO₂ and 10% AlCl₃ 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
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.

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.
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 'overflow 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. *rhodopaca* 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.

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