Genotype‑dependent mass somatic embryogenesis: a chance to recover extinct populations of Pulsatilla vulgaris Mill.

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Abstract

The paper presents a technique for micropropagation of endangered in Europe and extinct in Poland Pulsatilla vulgaris for ex situ conservation of the genetic resources. Genotype-dependent induction of somatic embryogenesis and rooting was revealed in series of two experiments (I and II) performed under the same experimental conditions. Shoot tips of seedlings were the best explants in both experiments and Murashige and Skoog (MS) medium supplemented with 0.25 or 0.5 mg L⁻¹ BAP was suitable for induction of somatic embryos (SE) and adventitious shoots. Mass SE was obtained in experiment I after explants transfer on ½ MS (2% sucrose) + 0.45 mg L⁻¹ B1 and extending culture to 2–3 months without passages. Rooting of adventitious shoots was a critical point. Out of seven rooting media used in experiment I, only two, ½ MS hormone free (2% sucrose) + 0.45 mg L⁻¹ B1 or MS + 5 mg L⁻¹ NAA + 3.76 mg L⁻¹ B2 resulted in altogether 36.4% rooted shoots. In experiment II, somatic embryogenesis, rooting and acclimatization of adventitious shoots failed. Regenerated plantlets and seedlings converted from SE from experiment I were acclimatized to ex vitro conditions. Both genome size, determined by flow cytometry, and genetic diversity analyzed by ISSR markers, confirmed the compatibility of regenerants from experiment I with P. vulgaris initial seedlings and commercial cultivar. Regenerants obtained in experiment II differed genetically from the regenerants of experiment I and cultivar. Propagated in vitro tissues/organs (SE, adventitious shoots) of P. vulgaris could be a source of material for cryopreservation, artificial seed production and/or for acclimatization of regenerated plantlets and could be used for restoration of the extinct populations.

Key Message

The micropropagation technique via organogenesis and somatic embryogenesis of endangered in Europe pasqueflower was developed as a tool for species recovery. The critical point is that somatic embryogenesis is genotype-dependent, which affects the repeatability of the experiments and also imposes applying molecular techniques to confirm the genetic fidelity of the regenerants with the initial material and other genotypes.

Keywords In vitro micropropagation · Genome size · ISSR markers · Ex situ conservation

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Introduction

The preservation of endangered and/or threatened plant species by biotechnological methods such as in vitro culture is one of the ex situ conservation pathways. 42% of threatened European species are in ex situ collections (Sharrock and Jones 2011), which prevents loss of plant biodiversity and allows to recover genetic diversity from ex situ preserved material. Although the final step, translocation of the focal species or population from botanical gardens to the nature, is highly challenging task, 60% of cases of ex situ conservation were successful or partially successful (Abeli et al. 2020, 2021).

Nine occurring in Europe Pulsatilla species are rare, endangered and/or endemic, occupy small populations on local, light habitats acting as refugia (Akeroyd 1993). Overall species decline through Europe has been principally driven by the competition with grasses in the absence of appropriate management regimes, reduced grazing pressure, enhanced nitrogen deposition with acid rains and illegal transfer to gardens (Walker and Pinches 2011). In Poland main threat is a secondary succession from grassland through light, semi-open pine forest to shady, dense pine or pine-oak forest, maybe also flower picking and collecting herbarium specimens (Zarzycki et al. 2014).

Pulsatilla vulgaris Mill. of Ranunculaceae (2n = 2x = 16 or 2n = 4x = 32, Sramkó et al. 2019) was widespread throughout calcareous grasslands and open pine forests in western and central Europe, but now its populations are strongly declined (DiLeo et al. 2017). In Poland the species is extinct, its natural occurrence has not been confirmed in formerly known locations since 1930s (Zarzycki et al. 2014). Ex situ living collections from several Polish historical sites are cultivated in botanical gardens (Galera et al. 1999).

The effective technique to preserve genetic resources of Pulsatilla species is ex situ conservation using in vitro culture for plant multiplication via organogenesis and/or somatic embryogenesis (SE) (Šedivá 2002, 2012; Rybczyński and Mikula 2006; Danova et al. 2009; Naumovski et al. 2009; Sauliene and Brinkyte 2009; Lin et al. 2011; Priede and Kļaviņa 2011; Šedivá and Žlebčík 2012). Development of synthetic seeds through encapsulation system (enclosing totipotent plant parts e.g., somatic embryos) provides a proficient method for continuous distribution and short/medium-term conservation of the germplasms (Gantait et al. 2015). In twenty-first century the germplasm of increasing number of endangered species is propagated via SE and protected as synthetic seeds or cryopreserved (e.g., Moon et al. 2013; Kang et al. 2014).

We aimed to: (1) develop an efficient micropropagation technique of P. vulgaris, extinct in Poland, using as an initial material indigenous plants from ex situ collection of botanical garden; (2) confirm genetic fidelity of regenerants with initial material by molecular markers; (3) estimate genome size of the source material and regenerants by flow cytometry. For practical purpose, the protocol could be used for mass micropropagation of P. vulgaris for restoration of Polish extinct populations.

Materials and methods

Plant material

The following material was used: (1) Pulsatilla vulgaris plants (Fig. 1a) from the collection of the Botanical Garden of the Polish Academy of Science – Center for Biological Diversity Conservation in Powsin (Powsin BG) as a source material; (2) seeds collected from these plants in two seasons (2019 for the in vitro experiment I and 2020 for the experiment II); (3) P. vulgaris cultivar commercially obtained from Łobżów Garden in Kraków (Poland) as a reference for plants from Powsin BG (P. vulgaris in Poland does not grow in nature); (4) P. pratensis growing close to Skołczanka nature reserve (Kraków, Poland) collected with the permission of the Regional Directorate for Environmental Protection. It was necessary to include this species in the study because in the vicinity of P. vulgaris in Powsin BG, specimens of P. pratensis grew and cross-pollination resulting in possible hybrid origin of the collected seeds could not have been excluded.
Media and culture conditions

Media inducing somatic embryogenesis and organogenesis

Seeds harvested in 2019 and 2020 in Powsin BG were sterilized in 70% ethanol for 30 s and then in 50% commercial bleach Ace with sodium hypochlorite for 15 min, washed thrice with autoclaved distilled water. After 5 weeks of culture on ½ MS medium (Murashige and Skoog 1962) with 2% sucrose (w/v), solidified with 0.8% agar (w/v), medium pH 5.7–5.8, cotyledons, hypocotyls with roots and shoot tips were excised from the seedlings and used as explants. All explants were placed on inducing media solidified with 0.8% agar (w/v): MS + 0.25 or 0.5 mg L\(^{-1}\) BAP (according to the protocol of organogenesis in *P. patens*, Priede and Kļaviņa 2011) in Petri dishes. The first passage on the same fresh media was after 3 weeks from the culture establishment, next after 6 weeks.

In the experiment II (2020) for SE and organogenesis the same conditions and media were used based on the results of experiment I.

Rooting media

In the experiment I adventitious shoots or clusters of adventitious shoots (shoots’ separation from callus and their split into separate shoot was difficult and causing their damage) developing on morphogenic callus were transferred on 7 rooting media after next 4–6 weeks: (1) ½ MS (2% sucrose) + 0.45 mg L\(^{-1}\) B1; (2) MS + 5 mg L\(^{-1}\) NAA + 3.76 mg L\(^{-1}\) B2 (5 or 7 days in darkness according to Priede and Kļaviņa 2011); (3) ½ MS (2% sucrose) + 2 mg L\(^{-1}\) IBA + 0.45 mg L\(^{-1}\) B1; (4) MS (2% sucrose) + 2.5 mg L\(^{-1}\) NAA + 0.45 mg L\(^{-1}\) B1; (5) ½ MS + 2 mg L\(^{-1}\) IBA; (6) ½ MS + 0.5 mg L\(^{-1}\) IBA; (7) MS + 10 mg L\(^{-1}\) NAA. Half of shoots on media with NAA (nos. 4, 7) after 5 or 7 days was transferred on hormone free medium, the rest of explants were maintained on medium with NAA. All cultures were maintained in a growth chamber at 25 ± 3 °C under a 16 h photoperiod (cool-white fluorescent lamps, 60–90 μmol m\(^{-2}\) s\(^{-1}\)).

In the experiment II for shoot rooting ½ MS (2% sucrose) + 0.45 mg L\(^{-1}\) B1 was used based on the results of experiment I.

Acclimatization to ex vitro conditions

Plants developed from somatic embryos in experiment I and rooted adventitious shoots obtained in the experiment I and II were transferred into sterile plastic containers filled with sterile perlite, perlite or sand mixed with garden soil (1:1) and maintained in a growth chamber for several days. Then the containers were opened in non-sterile conditions for ex vitro acclimatization.

Histological analysis for the confirmation of somatic embryogenesis and organogenesis

Paraffin method was used for histological analysis. Morphogenic/embryogenic callus produced on explants in the experiment I was fixed in the mixture of glacial acetic acid and 96% ethanol (1/3, v/v) dehydrated in an ethanol series, embedded in paraffin, and sectioned in 10–12 μm slices on a rotary microtome (Adamas Instrumenten BV, HM 340E). Sections were stained with Ehrlich’s hematoxylin (FLUKA, Switzerland) and alcian blue (FLUKA, Switzerland) and mounted in Entellan (Sigma-Aldrich, USA).

Genome size estimation

Leaves of seedlings of *P. vulgaris* obtained from seeds collected in 2019 in Powsin BG, adventitious shoots obtained in experiments I and II, leaves of plants of *P. vulgaris* cultivar, and of *P. pratensis* were used to establish genome size by flow cytometry (FCM), using buffer for nuclei isolation developed by Marie and Brown (1993); propidium iodide (50 μg/mL) was applied for DNA staining, and *Secale cereale* (2C = 16.19 pg; Doležel et al. 1992) served as an internal standard (for details of sample preparation and analysis see Żabicki et al. 2019). CV of the G0/G1 peak of *Pulsatilla* ranged between 2.51 and 4.58%. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of *Pulsatilla* /internal standard on a histogram of fluorescence intensities.

Genetic diversity estimation by ISSR markers

DNA isolation

Leaves of three *P. vulgaris* plants from Powsin BG, six of cultivar, 12 seedlings of both experiments, 10 adventitious shoots obtained in experiment I and four in experiment II, three of *P. pratensis* collected for molecular analysis were dried in silica gel (silicon dioxide; F.H.U. “DOR-CHEM”, Poland). DNA was isolated using CTAB extraction method (Gavel and Jarret 1991). Quality of DNA was tested on 1% agarose gel.

ISSR markers

Six primers of ISSR markers were selected (Gupta et al. 1994; Stepansky et al. 1999) (Table 1). The analysis with ISSR markers was based on protocols developed by Zabicka et al. (2020); the same reagents and laboratory equipment were used. The PCR products were separated in 1% agarose
gel with 1X TBE and SimplySafe (EURx Sp. z o.o. Gdansk, Poland) for about 90 min at 120 V. Band patterns of 38 samples were captured with a MultiDoc-It™ Imaging System with VisionWorks® LS Analysis Software (UVP, Upland, CA, USA). PCR reaction cycle were repeated on selected samples to test reproducibility of obtained results.

Split phylogenetic network (NeighborNet) was constructed in SplitsTree v. 4.6 (Huson and Bryant 2006) based on Dice coefficient to evaluate the relationships between examined individuals, bootstrap was calculated on 1000 replicates.

Statistics

To evaluate the significance of media composition influence on the culture results of the experiment II, ANOVA followed by Tukey HSD test post hoc (P = 0.05) was used. All calculations were performed in Statistica 13.3 software (TIBCO Software Inc., Palo Alto, California, USA).

Statistical analysis of genome size was performed using ANOVA followed by Tukey HSD test post hoc for different N (P = 0.05) in Statistica 13.3 software (TIBCO Software Inc., Palo Alto, California, USA).

Results

Somatic embryogenesis and organogenesis induction, regenerants acclimatization

In the experiment I, due to poor seed germination, only several seedlings were used as explants. Dissected shoot tips of seedlings were the best explants and MS + 0.25 mg L\(^{-1}\) BAP the most suitable inducing medium. Cotyledons, hypocotyls + roots poorly responded to in vitro conditions, becoming brownish and finally degenerated (Fig. 1b–d). Mass indirect (via callus) and direct somatic embryos (SE) development was obtained from shoot tips after callus or explants transfer from inducing medium (MS + 0.25 mg L\(^{-1}\) BAP) onto ½ MS (2% sucrose) + 0.45 mg L\(^{-1}\) B1 and extending the culture to 2–3 months without passage. In indirect pathway, callus induction and proliferation on shoot tips started after two weeks of culture (Fig. 1e). On one explant dozens, uncountable SE developed that converted into seedlings (Fig. 1e, g–j). Simultaneously with SE induction on the inducing medium, adventitious shoots were formed on the same explants (Fig. 1g). Both SE (Fig. 2a–c) and organogenesis (Fig. 2d–f) were confirmed on histological sections.

From the mass of seedlings converted from SE, 121 differing in size and organ development stage were separated, and 55 of the largest were moved to ex vitro conditions (Fig. 1k). The smallest seedlings were transferred to hormone-free MS supplemented with 2% sucrose to grow before being moved to ex vitro conditions, but their survivability was close to zero.

The rate of individual shoots and clusters of adventitious shoots rooting on ½ MS hormone free, 2% sucrose + 0.45 mg L\(^{-1}\) B1 (40 of 108) or MS + 5 mg L\(^{-1}\) NAA + 3.76 mg L\(^{-1}\) B2 (3 of 10) reached altogether 36.4% (43 of 118). Finally, 35 the most vital rooted shoots were transferred to non-sterile conditions for gradual acclimatization. On the remaining five media, none adventitious shoots had formed roots.

Based on the results of the experiment I, the experiment II was performed using more initial explants in several repetitions (Suppl. Table 1). Although for adventitious shoots and SE induction the same, most efficient media, as in the experiment I (MS + 0.25 or 0.5 mg L\(^{-1}\) BAP), the same type of explants (shoot tips, cotyledons, hypocotyls + roots) were applied under the same culture conditions as in experiment I, the results were different. Among explants used, shoot tips produced the highest frequency of adventitious shoots (97.2%), hypocotyls + roots and cotyledons responded with lower frequency (21.8% and 8.8%, respectively). There were no differences between media, the differences were evident between type of explants. The shoot tips responded with the highest frequency to the in vitro culture. Somatic embryos were not induced on any of the explant and medium (Suppl. Table 1). Out of 146 individual shoots and clusters of uncountable adventitious shoots transferred to rooting medium (½ MS hormone free, 2% sucrose + 0.45 mg L\(^{-1}\) B1), only five shoots (3.42%) formed roots. Their acclimatization to ex vitro conditions filed.

Genome size-based uniformity of regenerants and initial seedlings

Flow cytometry revealed a difference in genome size between P. vulgaris cultivar (2C = 25.94 pg) and P. pratensis (2C = 12.80 pg) that corresponds well with their ploidy level (2n = 4x = 32 and 2n = 2x = 16, respectively) (Table 2). Genome size of regenerants obtained in the experiment I (2C = 25.72 pg) did not differ statistically from 2C-value established for P. vulgaris seedlings obtained from seeds collected in Powsin BG and for the commercial cultivar but differed from the value established for regenerants obtained in the experiment II (2C = 25.14 pg). All adventitious shoots regenerated in the experiment II differed also in their genome size from the cultivar but not from initial seedlings from Powsin BG.
Neighbornet of 21 *Pulsatilla vulgaris* sensu lato (three plants from Powsin BG—P; 12 seedlings from seeds of experiments I and II—NP and 2NP, respectively; six plants of cultivar from Łobzów Garden—L), 14 regenerants (10 from the experiment I—REG; four from the experiment II—2Reg) and three *P. pratensis* (Pr) had shown significant divergence of *P. pratensis* specimens, with 94.2 branch support (Fig. 3). All regenerants from the experiment I (REG) clustered together with *P. vulgaris* specimens, including live collected plants (L, P) and seedlings (NP). Specimens regenerated during the experiment II (2Reg) formed a third significantly distinct group with 88.4 branch support (Fig. 3).

### Discussion

The main conclusion from the studies is that it was possible to achieve mass somatic embryogenesis in *P. vulgaris* but the developed protocol was not reproducible. Although the initial material for in vitro culture (seedlings) in experiment I and II originated from seeds produced by plants of the same clump of *P. vulgaris* growing in the Powsin BG, mass somatic embryogenesis was obtained exclusively in experiment I.

### Is somatic embryogenesis in *P. vulgaris* genotype-dependent?

Somatic embryogenesis is a multi-step, molecular and biochemical process of embryo formation based on cell totipotency in which the vegetative cells acquire embryogenic competence under stress conditions (Pérez-Núñez et al. 2009; Thorpe 2012; Gulzar et al. 2020). This process is regulated by a set of genes of which only a few have been extensively studied, and chromatin remodeling by different mechanisms regulates these genes activity (reviewed by Gulzar et al. 2020).

As the stress induced by plant growth regulators, nutrient, oxygenic, desiccation and other signalling elements during in vitro culture lead to vegetative cells transformation into embryos via activation/deactivation of genes and transcriptional networks (Feher 2015), different species/genotype/explant reaction is easy to explain. Genotype-dependent somatic embryogenesis was described in several ornamental and economically important species (e.g., Chengalrayan et al. 1998; Mishra and Khurana 2003; Fiuk and Rybczyński 2008; Santos et al. 2018; Sánchez-Romero 2021). In olive several different protocols for somatic embryogenesis were developed with varied efficiency, depending on the genotype, limiting standardization and the applicability (Sánchez-Romero 2021).

The question arises whether different respond of *P. vulgaris* explants in experiment I and II could be the case of genotype-dependent? Could individuals representing genetic variation resulting from intraspecific crossing be considered as distinct genotypes? In living collection of Powsin BG *P. vulgaris* clumps (clones) grew close to *P. pratensis*, but the occurrence of other *Pulsatilla* species in garden collection is also possible. Hence the seeds could have originated from cross-pollination within *P. vulgaris* but their hybrid origin cannot be also excluded. Based on ISSR molecular markers, initial material (seedlings) used for in vitro culture in experiment I and II originating from seeds collected in two seasons, was genetically close to *P. vulgaris* living collection in Powsin BG and cultivar from Łobzów Garden and distinct from *P. pratensis* (Fig. 3). This eliminates hybrid origin of seeds and *P. pratensis* as one putative parent.

*Pulsatilla vulgaris* is predominately self-incompatible species, cross-pollination increases genetic diversity. In German populations high within-population variability (84.4%) and a weak, but significant, differentiation among populations were detected (Hensen et al. 2005). Genetic diversity of pasqueflower seems to be strongly affected by demographic changes i.e., decline, expansion and fragmentation (Walker and Pinches 2011). We could thus conclude that seedlings of *P. vulgaris* used in two experiments were different genotypes resulted from cross-pollination.

### Genome size as an indication of somaclonal variation leading to genome multiplication/reduction under in vitro culture

Somaclonal variation is an important drawback of organogenesis/somatic embryogenesis, providing genetic variation in regenerated plantlets as a consequence of the change of chromosome number, chromosomal aberrations, genetic alterations, or epigenetic modification (Larkin and Scowcroft 1981). Regenerants converted from SE and adventitious shoots of *P. vulgaris* obtained in experiments I possessed the same genome size as initial seedlings and cultivar from...
Fig. 2  Somatic embryogenesis (SE) and adventitious shoot formation on histological sections of *Pulsatilla vulgaris* callus in experiment I.  
*a* Globular SE (arrowhead) and meristematic center (arrow), visible callus cells differing in shape and size;  
b Heart-stage SE;  
c Torpedo SE with embryonic root (arrow) and cotyledon (asterisk);  
d Multiple meristematic centers (arrows) in callus located along the cells differentiating into vascular tissue (arrowhead);  
e, f – Different stages of adventitious shoots development (arrows). Bar = 100 µm (a–c, e, f); 200 µm (d)

Table 2  Genome size (2C DNA) of *Pulsatilla* species

| Species/regenerants | Chromosome number | Material origin                                                                 | 2C DNA (pg) | Mean ± SD |
|---------------------|-------------------|--------------------------------------------------------------------------------|--------------|-----------|
| *P. vulgaris* Mill  | 16, 32^A          | Cultivar from Łobzów Garden (L); N = 9                                          | 25.94^ab     | 0.31      |
|                     |                   | Seedlings from seeds collected in Powsin BG in 2019 (NP); N = 2                 | 25.83^c      | 0.16      |
| Regenerants         |                   | Plants regenerated from shoot tips of seedlings obtained from seeds collected in Powsin BG in experiment I (REG); N = 21 | 25.72^ab     | 0.21      |
|                     |                   | Plants regenerated from shoot tips of seedlings obtained from seeds collected in Powsin BG in experiment II (2Reg); N = 9 | 25.14^c      | 0.36      |
| *P. pratensis* L    | 16^B, 32^C        | Plants collected close to Skołczanka Nature Reserve; S Poland (Pr); N = 10       | 12.80        | 0.07      |

^A^Many authors (cited from Bolkovskikh et al. 1969)

^B^From Marhold et al. (2005)

^C^From Böcher 1954 (cited from Bolkovskikh et al. 1969)

Mean values followed by different letters differ significantly at P < 0.05 in one-way ANOVA followed by Tukey HSD test post hoc for different N (number of replications). *P. pratensis* samples were excluded from statistical analysis.
Łobzów Garden. They possessed genome size of tetraploid cytotype of *P. vulgaris* (2C = 25.72 pg; 2n = 4x = 32), which was twice as large as in diploid *P. pratensis* (2C = 12.80 pg; 2n = 2x = 16). Genome size of adventitious shoots produced in experiment II (2C = 25.14 pg), although significantly differed from regenerated shoots of experiment I, possessed mean 2C DNA content similar to tetraploid *P. vulgaris* and not to diploid *P. pratensis*. Based on this characteristic they could be considered as regenerants of *P. vulgaris* and thus could be reintroduced to the nature.

**ISSR molecular markers separated regenerants of experiment II from *P. vulgaris***

Combined results of genome size value and ISSR markers clearly indicated that regeneration via organogenesis and SE successfully achieved in experiment I is suitable procedure for obtaining plants for reintroduction. Surprisingly, regenerated shoots of experiment II although representing genome size similar to *P. vulgaris*, genetically formed a separate, strongly (88.4) supported branch (Fig. 3). Their origin is difficult to reconstruct—genetic variation is too high to explain it as genome alteration influenced by culture conditions (somaclonal variation). The genetic distance from *P. vulgaris* and *P. pratensis* is very long what eliminates these two species as putative parents of the seeds. It could be hypothesized that the initial seedlings for the experiment II (not used for ISSR analysis) originated from hybrid seeds of *P. vulgaris* with other tetraploid *Pulsatilla* species growing in Powsin BG. In *Pulsatilla* hybridization and introgression played a crucial role in the evolutionary history of the genus, and interspecific hybrids or species of hybrid origin occur in nature. Hybrids are fertile due to weak genetic isolation mechanisms (Szczechińska et al. 2017; Torzewski 2018; Li et al. 2019).

**Ex situ conservation of *P. vulgaris* using seed collections is highly disputable**

In natural populations and in ex situ collections seed set may be pollen-limited (Jonsson et al. 1991). *P. vulgaris* as an early flowering plant may face disadvantages of low pollinator abundances and unfavorable weather conditions influencing seed set. Pollinator exclusion, self-pollination and wind pollination only resulted in a very low percentage of seed set (Kehrberger and Holzschuh 2019). The limitation is also low seed germination frequency of *P. vulgaris* from Powsin BG on sterile media (MS, filter paper, lignin) which did not exceed 20% and was slightly higher (~ 30%) in non-sterile garden soil (present studies; data not shown). Field experiments have indicated that increasing the sizes of local populations of *P. vulgaris* by transplanting ex situ grown seedlings or just by sowing seeds was hardly ever successful in Switzerland (Pfeifer et al. 2002) but successful in three English populations (Gargiulo et al. 2019).

Rare and endangered with extinction species are effectively propagated via SE and could be protected as artificial seeds or cryopreserved (e.g., Rybczyński and Mikula 2006; Moon et al. 2013; Kang et al. 2014; Streczynski et al. 2019). Among *Pulsatilla* species, only one species, specifically *Pulsatilla koreana* has been propagated via SE so far (Lin et al. 2011). In the recent paper we have revealed that SE is also possible for *P. vulgaris* however usage of seeds from living collections should be done with cautious.

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**Fig. 3** NeigborNet of *Pulsatilla vulgaris* sensu lato (Powsin BG—P; seedlings from seeds of I and II experiments—NP and 2NP, respectively; plants of cultivar from Łobzów Garden—L), regenerants (from the experiment I—REG and experiment II—2Reg) and *P. pratensis* (Pr) constructed on 93 ISSR loci using Dice distance. Branch support values are based on bootstrap analysis with 1000 replicates.
Conclusion

(1) Mass somatic embryogenesis, rooting of adventitious shoots and regenerants acclimatization of *P. vulgaris* was strongly genotype-dependent; the developed protocol was not reproducible, although the same culture conditions, explants, plant growth regulators, media were used. The critical points demonstrated here will be helpful in further studies on the micropropagation of *P. vulgaris* aiming to save germplasm of this endangered in Europe species.

(2) For restoration of extinct *P. vulgaris* populations it is important to confirm genetic compatibility of regenerated plantlets with the initial material by molecular markers to avoid introducing new variability to natural populations.

(3) Genome size alone cannot be used for identification of genome qualitative alteration; regenerants with similar genome size might be genetically highly different as was shown in *P. vulgaris* regenerants in experiment II.

(4) The study highlights the necessity to be cautious when using material from botanical gardens. In the case of *P. vulgaris* it was not possible to obtain different material from natural, local genetic resources as this species is extinct in Poland.

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Author contributions JŻ and PŻ: planned and carried out the in vitro experiments, data analysis and interpretation of the data. AS: conception and design, performance of experiments, critical reading of the manuscript. MJ-K and TN: material collection, result discussion. ES: genome size analysis and interpretation of the data. AK: seeds and encapsulation agent and matrix. JŻ and PŻ: planned and carried out the in vitro culture of Balkan endemic and rare *Pulsatilla* species for conservation purposes and secondary metabolites production. Bot Serbica 33:157–162

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