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Abstract: Flow cytometry measurement confirmed the genetic stability of *Primula veris* L. (cowslip) plants obtained during the micropropagation process. The mean 2C DNA content was 0.97 pg/2C and *P. veris* can be classified in the group of plants of very small genomes. Comparative morphological analysis of cowslip plants regenerated in vitro and derived from seeds was carried out in three subsequent years under common garden conditions, which limited the effects of environmental factors. The origin of specimens significantly influenced most of the morphological traits examined. Plants that originated from seeds were characterized by more intensive development rate and higher total seed production compared to micropropagated plants. Significant differences between the two groups of specimens were observed for the number of flower stalks, flowers, fruits, and seeds per plant. No interseasonal variation within the reproductive traits such as number of seeds per flower stalk and per plant was found for plants derived from in vitro cultures. That allows prediction of seed production from in vitro regenerated plants after their successful acclimatization to field conditions. Importantly, plants derived from in vitro cultures set significantly bigger seeds. Our results are promising for broad uses of the regenerated in vitro cowslip plants in ornamental horticulture and the pharmaceutical industry.

Key words: Cowslip, flow cytometry, fruit and seed-set, genome size, seed number, seed size, tissue culture

1. Introduction

Primula veris L. (Primulaceae, also called cowslip) is a distylous outcrossing grassland herb characterized by declining number and size of populations in recent years. This has caused an increased interest in the species, with the goal of saving its contribution to biological diversity (Kéry et al., 2000; Brys and Jacquemyn, 2009; Berisha et al., 2015). One of the reasons causing the reduced number of cowslip populations may be very low level of genetic variation found in natural populations (Morozowska and Krzakowa, 2003). That is inconsistent with the mating system of cowslip and it might be partly explained by the presence of an imbalance of flower morphs, leading to a lack of compatible pollen and resulting in significantly reduced reproduction (Kéry et al., 2000; Morozowska and Urbański, 2000). Seed production of naturally growing cowslip plants amounts from 35.8 to 43.8 seed per capsule (Morozowska and Urbański, 2000), while for cultivated plants the mean seed number per capsule ranges from 46.2 to 51.7 (Morozowska, 2004a). Cowslip is a popular ornamental plant widely cultivated

throughout Europe. Besides the typical species, hybrids between *Primula veris* and *P. vulgaris* or *P. elatior* are also often cultivated in gardens and flower beds. Cowslip plants used in ornamental horticulture are regenerated from seeds in plant nurseries. *P. veris* is also a well-known, valuable medicinal herb that provides rhizomes and roots (*Primulae rhizoma cum radicibus*) with expectorant activity associated mainly with triterpene saponins, and flowers of similar medicinal use (*Primulae flos*) that contain a number of flavonol glycosides (Hegnauer, 1990) and several methoxyflavones (Huck et al., 2000; Stecher et al., 2003). Saponin content in rhizomes and roots of cultivated cowslip plants was found to be higher in comparison with plants from natural populations, which also favors conserving natural stands (Morozowska and Krzakowa, 2003). The leaves of cowslip also produce phenolic compounds (Hegnauer, 1990). Budzianowski et al. (2005) reported the presence of 10 lipophilic flavones in the leaves of both cultivated and in vitro regenerated *P. veris* plants. Some of these compounds have great potential antimutagenic and cytostatic activities (Tokalov

et al., 2004) or anticancer properties (Lee and Safe, 2000). Since nowadays cowslip is less abundant in continental Europe than in previous years, sustainable supply of the source material has become more difficult (Kéry et al., 2000; Thiem et al., 2008). An alternative to use of natural plants is cultivation of plants reproduced either from seeds or obtained from in vitro cultures; however, in the case of micropropagated plants, it is necessary to compare the genetic fidelity of mother plants with progenies. As reported by Antrobus and Lack (1993) *P. veris*, despite its heterostylous outcrossing system and a wide distribution in Europe, has unusually little variation. Four subspecies (subsp. *veris*, subsp. *columnae*, subsp. *canescens*, and subsp. *macrocalyx*) have been recognized in the species *P. veris* (Richards, 2002). The basic chromosome number is $x = 11$ (Clapham et al., 1987) and 2C DNA content was reported as 949 Mbp (Siljak-Yakovlev et al., 2010).

Flow cytometry (FCM) is a fast and accurate method for estimating nuclear DNA content (Doležel and Bartos, 2005; Ducar et al., 2018), and has been successfully applied in multiple studies of genome size and ploidy stability of ornamental and medicinal plants obtained during in vitro cultures (Thiem and Sliwinska, 2003; Sliwinska and Thiem, 2007). Regenerated in vitro plants of confirmed genetic stability may also serve as a source of good quality seeds for further multiplication for horticultural or pharmaceutical purposes.

The first objective of this study was to verify genetic stability of *Primula veris* plants derived from in vitro cultures by (1) estimating the nuclear DNA content of cowslip and (2) verifying the stability of the genome size of cowslip plants regenerated in vitro compared to seeds and seed-derived plants. The second objective was field evaluation of micropropagated plants and seed-derived plants in common garden conditions. We hypothesized that (1) the origin of the first generation of specimens does not influence their morphological characteristics, nor the size and weight of their seeds and (2) there is no relationship between the origin of individuals and interseasonal variation in their morphological traits.

2. Materials and methods
The plants used for flow cytometric and morphological analyses were of two different origins: (1) in vitro regenerated plants and (2) plants regenerated from seeds, hereafter referred to as in vitro origin and seed origin. Cultivation of in vitro and seed origin plants was started with the use of *P. veris* seeds collected in a natural population located in the Wielkopolska region, in Lednicki Landscape Park (52.526400N; 17.378492E) (Morozowska and Urbański, 2000).

2.1. In vitro plant regeneration
Regeneration of cowslip plants in tissue cultures was conducted according to the protocol described by Morozowska and Wesołowska (2004). Sterilization of seeds was done with the use of 70% ethanol for 30 s and then with 30% solution of Clorox commercial bleach with three drops of Tween 80 for 25–30 min. After that seeds were rinsed with sterile water several times and laid out for germination on solid MS medium with gibberellic acid (GA₃; 2.90 μM). After germinating seeds in vitro the seedling tips were used as explants for further multiplication of cowslip plants. Seedling explants were cultured on three different MS medium variants supplemented with BA (2.22 μM) and NAA (0.27 μM) (MS-1), KIN (9.30 μM) and IAA (11.42 μM) (MS-2), and BA (4.44 μM) and 2,4-D (1.13 μM) (MS-3). Multishoots arising from one explant (seedling tip) were considered the source of one clone culture. Regenerated multishoots were divided into single shoots, subcultured for 4–5 weeks on the same medium, and after several passages the plantlets were rooted in vitro and acclimated at room temperature for 3 weeks. The seedlings obtained, representing three different clone cultures, were used as the planting stock to start the cultivation of cowslip plants of in vitro origin.

2.2. Generative plant regeneration
Cultivation of seed origin plants was started with the use of seedlings obtained from seeds germinated under greenhouse conditions according to ISTA standards (ISTA, 2015). After 10 weeks in the greenhouse, plants were moved to a shade house and allowed to acclimatize to field planting conditions.

2.3. Field establishment of micropropagated and seed-derived plants
Primula veris seedlings of both origins were planted in experimental plots of the Department of Botany at Poznan University of Life Sciences (52.2727N; 16.5431E). Seedlings were transplanted at a spacing of 40 cm × 30 cm. A total of 150 individual plants of each origin were transplanted into experimental plots containing 50 ± 1 plants each. The fields were irrigated once per week to aid establishment and manual removal of weeds was applied. Cultivated plants were in good condition, with yearly flowering and seed production. Voucher specimens and seeds are deposited in the Herbarium of the Department of Botany at Poznan University of Life Sciences (POZNB).

2.4. FCM analysis
The first part of the study concerned evaluation of the genetic stability of in vitro derived cowslip plants. Plant material for flow cytometric analyses consisted of young leaves collected from plants of both origins. The representative plants derived from different cowslip clones obtained from in vitro cultures were examined by FCM separately. Flow cytometry analyses also included a sample of cowslip seeds used to start the in vitro cultures and plants regenerated generatively. Before the genome size measurements the

test for the presence of PI-staining (propidium iodide) inhibitors in plant tissues was performed according to the protocol reported by Price et al. (2000). Samples for nuclear DNA content estimation were prepared according to the procedure described by Galbraith et al. (1983), with some modifications. Plant tissues of the target species and of the internal standard (*Petunia hybrida* P×Pc6; 2C = 2.85 pg; Marie and Brown, 1993) were chopped with a sharp razor blade in a plastic petri dish containing 1 mL of Galbraith's buffer (Galbraith et al., 1983) supplemented with propidium iodide (PI; 50 µg/mL), ribonuclease A (RNase A; 50 µg/mL), and additionally an antioxidant of 1.5% (w/v) polyvinylpyrrolidone (PVP-10, Sigma-Aldrich, Poland) to obtain high resolution histograms and avoid a stoichiometric error in the DNA content caused by PI-staining inhibitors. For genome size estimation five whole seeds (with seed coats) or approximately 0.5–1 cm^2 of the leaf fragments of the target species and about 0.5–1 cm^2 of the leaf blade fragments of the internal standard were used. The nuclei suspension was passed through a 50-µm mesh nylon filter. For each sample, 7000–10,000 nuclei were measured using a Partec CyFlow SL Green flow cytometer (Partec GmbH, Münster, Germany), equipped with a high-grade, solid-state laser with green light emission at 532 nm and side (SSC) and forward (FSC) scatters. Analyses were performed on five replicates for each type of plant tissue. Leaf samples were analyzed after 5 to 10 min of incubation on ice and samples prepared from seeds, after 15 to 20 minu. The histograms obtained were analyzed using FloMax software (Partec, Münster, Germany). Means and standard deviations (SD) of the nuclear DNA content (pg/2C) and nuclear DNA fluorescence index (DI = 2C of *P. veris*/2C of *P. hybrida*) of each *P. veris* tissue type were calculated. The 2C DNA value was calculated using the linear relationship between the ratio of the 2C peak positions of the studied species and the internal standards on the histogram of fluorescence intensities. The 2C genome sizes were obtained after the conversions of values in picograms into base-pair numbers using the factor 1 pg = 978 Mbp (Doležel and Bartos, 2005).

2.5. Morphological analysis

Comparative morphological analyses of plants from two different origins made up the second part of our study. They were conducted in three consecutive years (2011–2013). Since the DNA ploidy stability of cowslip plants representing three different clone cultures was confirmed by FCM analysis, the morphological observations were performed without clone differentiation using 30 randomly selected plants in each plot, in three replications. Biometric measurements were taken at four plant developmental stages each year: at the end of vegetative phase, and during flowering, fructification, and seed maturation. The following characteristics were

evaluated: plant rosette diameter (RD; cm), number of flower stalks (scapes) per plant (STpl), height of the flower stalk (STH; cm), number of flowers on the flower stalk (FLst), number of flowers per plant (FLpl), number of fruits on the flower stalk (FRst), number of fruits per plant (FRpl), number of seeds per capsule (SEca), number of seeds per flower stalk (SEst), and number of seeds per plant (SEpl). Rosette diameter was calculated as an average from two perpendicular measurements, while height of the scape and number of flowers and fruits on the scape were measured or calculated for all or three scapes on each plant, if there were more than three. Number of flowers and fruits per plant as well as number of seeds per flower stalk and per plant were calculated as average values from the available data.

2.6. Seed analysis

Measurements were carried out on ripe and fully developed seeds collected in each of the three years of the study. In total, 1576 randomly selected seeds were examined to obtain morphological features and 776 seeds were weighed. The biometrical traits of seeds were analyzed using the WinSeedle 2003a software (Regent Instruments Inc., Quebec, Canada; http://regentinstruments.com). We measured the following seed characteristics: length (mm) and width (mm), surface area (mm^2), projected area (mm^2), volume (mm^3), and projected perimeter (mm). Surface area means total area of the surface of a three-dimensional seed object, projected area is two-dimensional area measurement of a three-dimensional seed by projecting its shape onto an arbitrary plane, and projected perimeter means perimeter of seed projected area. Seed width to length ratio (W/L) was calculated. We also measured individual seed weight (with accuracy of 0.00001 g) and we counted the number of seeds per gram and calculated 1000-seed weight according to the International Seed Testing Association rules (ISTA, 2015).

2.7. Statistical analysis

The results of nuclear DNA content were analyzed using one-way analysis of variance (ANOVA) and Duncan's test. Prior to statistical analyses of ten morphological traits and eight seed characteristics, the distributions of variables were checked with the Kolmogorov–Smirnov and Lillefors tests. The homogeneity of variance was tested using Bartlett's and Levene's tests. Nine of ten morphological traits were log-transformed to meet the assumptions of normality and homogeneity of variance. Two factor analysis of variance (two-way ANOVA) was used to examine the influence of plant origin and year of study on morphology and seed characters. Moreover, for ten morphological traits, one-way ANOVA was used to test the significance of differences in morphological features over a three-year period, independently for plants of in vitro origin and seed origin. Pearson correlation

coefficients were calculated to check if the relationships between particular morphological traits were similar between the two groups of specimens of different origin. In correlation analyses all measurements of each feature from three subsequent years were tested together as one group (N = 90). Statistical analyses were performed using JMP Pro 13.0.0 (SAS Institute Inc. Cary, NC, USA; http://www.jmp.com) and Statistica v. 11 (StatSoft, Poland).

3. Results

3.1. FCM analysis

The 2C DNA contents of all studied *P. veris* plants were very similar and ranged from 0.97 pg/2C to 0.98 pg/2C, which corresponds to 949 and 958 Mbp, respectively (Table 1). For plants regenerated in vitro on MS-1 and MS-2 and for seeds, the mean nuclear DNA content was 0.97 pg/2C, while for plants regenerated in vitro and cultured on MS-3, as well as plants of natural origin, the genome size was 0.98 pg/2C. Statistical analyses indicated no significant differences in genome size among all plant tissue types studied. The nuclear DNA fluorescence index among all samples ranged from 0.340 to 0.343 (Table 1). The coefficients of variation (CVs) for all *P. veris* plants ranged from 3.59% (seeds) to 4.89% (cultivated plants). The histograms contained two peaks where the first distinct peak corresponded to nuclei arrested in the G_0/G_1 phase of the cell cycle and a very small, or even lack of, the second peak that corresponded to the G_2 phase (Figures 1a and 1b). For seeds, an additional peak of the endosperm (3C) was observed (Figure 1c).

3.2. Morphological analysis of plants

Plant origin influenced five morphological characteristics significantly. Plants obtained from seeds had higher flower stalks (STH), higher number of stalks per plant (STpl), and higher numbers of flowers, fruits, and seeds per plant (FLpl, FRpl, SEpl) as compared to specimens obtained from in vitro cultures (Table 2; Figure 2). Year of study

affected all of the features with the exception of number of seeds per capsule (SEca). The combined effects of origin and year significantly influenced all characteristics except for the number of stalks per plant (STpl) and number of flowers per plant (FLst). For cowslips of in vitro origin, in three subsequent years, characteristics such as number of seeds per flower stalk (SEst) and number of seeds per plant (SEpl) did not differ significantly (one-way ANOVA; P > 0.05). Specimens of seed origin differed significantly in terms of all morphological features during a three-year growth period.

We found that the size of leaf rosette (RD) was strongly correlated with the number of flower stalks (STpl) in both groups of plants (0.51 < r < 0.61; P < 0.001; see Table 3). The correlation between size of leaf rosette (RD) and inflorescence stalk length (STH) was very strong (r = 0.72; P < 0.001) for individuals regenerated generatively and moderate (r = 0.44; P < 0.001) for individuals derived from in vitro cultures. The correlation between the number of flowers and fruits was almost perfect irrespective of plant origin (r > 0.95; P < 0.001). Furthermore, both groups of plants presented quite similar, very strong and strong relationships between number of flowers and fruits per plant (FLpl, FRpl) and number of seeds per plant (SEpl) (0.83 > r > 0.68; P < 0.001). In turn, the correlation between number of flowers and fruits per flower stalk (FLst, FRst) and number of seeds per flower stalk (SEst) were clearly stronger for individuals germinated from seeds (r > 0.64; P < 0.001) as compared to individuals derived from in vitro cultures (r < 0.16; P > 0.13).

3.3. Seed analysis

We found statistically significant differences in most of the morphological traits between seeds collected from individuals derived from in vitro cultures and individuals obtained from seeds (Table 4). Moreover, we found no influence of the year of seed collection on their morphological features. Therefore, the differences

Table 1 Nuclear DNA content of *Primula veris* in vitro regenerants, cultivated plants of seed origin and seeds (see description in text).

Plant material	DNA index ± SD	Nuclear DNA content		CV (%)
		2C (pg ± SD)	2C (Mbp)	
In vitro clone no. 1	0.340 ± 0.0007	0.97 ± 0.002	949	4.65
In vitro clone no. 2	0.341 ± 0.0012	0.97 ± 0.004	949	4.68
In vitro clone no. 3	0.343 ± 0.0022	0.98 ± 0.007	958	4.71
Plants of seed origin	0.343 ± 0.0035	0.98 ± 0.011	958	4.89
Seeds	0.340 ± 0.0011	0.97 ± 0.003	949	3.59

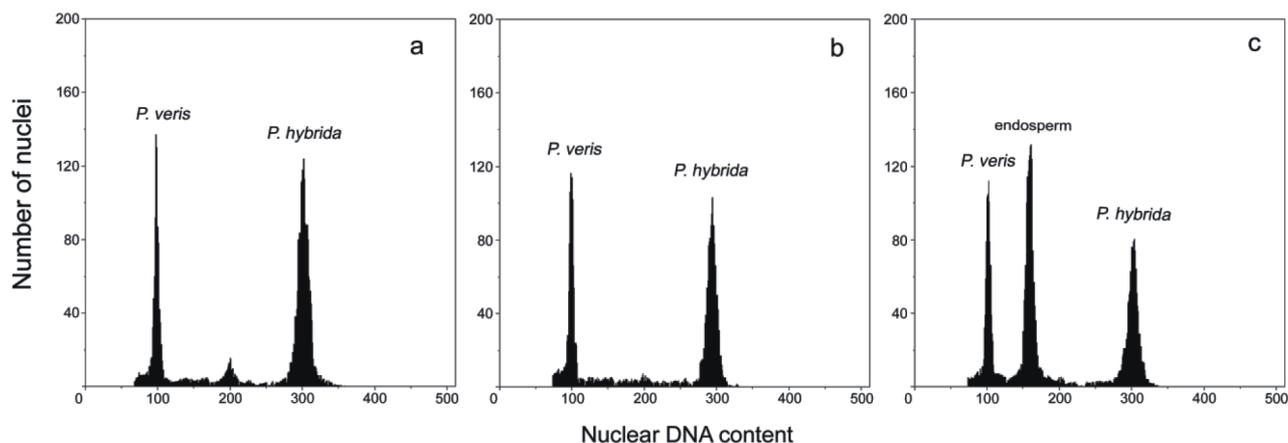Means were not significantly different (Duncan's test at P < 0.05)

Figure 1. Histograms of nuclear DNA content obtained after simultaneous analysis of nuclei isolated from *Petunia hybrida* (internal standard) and *Primula veris*: a) leaf, b) in vitro regenerants, c) seed with endosperm (3C).

between particular morphological traits of seeds from in vitro cultures and from plants regenerated generatively were not dependent on variability in weather conditions during the three consecutive growing seasons of the study. We found that seeds collected from in vitro plants were significantly larger in size in comparison to plants obtained from seeds. For example, mean seed projected area was 9.4% higher for in vitro plants and length was 4.3%, width 4.2%, volume 14.2%, surface area 8.9%, and projected perimeter 4.6% higher. However, we found no significant differences between the two groups of plants in seed W/L ratio. Although seeds collected from in vitro plants were significantly larger in comparison with seeds obtained from plants regenerated from seeds, there were no significant differences in individual seed biomass among the groups of plants (Table 4). However, the weight of 1000 seeds was greater in the case of plants originated from in vitro cultures (0.960 g) compared to plants of seed origin (0.803 g), while the number of seeds per gram was higher for specimens regenerated generatively (1180 seeds/g) than for plants regenerated in tissue cultures (1021 seeds/g).

4. Discussion

The mean 2C DNA content for all the samples studied was 0.97 pg/2C and was the same as that reported by Siljak-Yakovlev et al. (2010). According to Soltis et al. (2003), *P. veris* can be classified in the group of plants with very small genomes. It is generally known that genome size of in vitro cultures can be altered through changes either in the chromosome number or in the ploidy level. Such changes may result from oxidative stress damage caused to the plant during preparation of explants (Cassells and Curry, 2001), the prolonged culture period (Gantait et al., 2010), or due to many other conditions such as

the influence of different growth regulator treatments applied during micropropagation (Thiem and Sliwińska, 2003). The plant material analyzed was multiplicated via buds from existing meristems, which is the preferred method for maintaining genetic stability (Bajaj et al., 1988; Cassells and Curry, 2001). The mean nuclear DNA fluorescence index of all samples studied was nearly the same, meaning that all samples had the same ploidy level (all samples were diploid). No changes in nuclear DNA content have been found in several species cultured in vitro, like *Vaccinium* sp. and *Rubus* sp. (Gajdošová et al., 2006), *Rubus chamaemorus*, *Oenothera paradoxa*, *Inula verbascifolia*, *Solidago virgaurea*, and *S. graminifolia* (Thiem and Sliwinska, 2003; Sliwinska and Thiem, 2007). Some of these species are important medicinal or valuable rare, locally threatened plants. Maintaining genetic stability during in vitro cultures is especially important for such plants if they are used as a source of planting stock, along with seeds, for restoring wild populations of rare, endangered, or protected plants and for commercial cultivation to produce genetically stable plants for use either as ornamental plants or to obtain good quality pharmaceutical raw material (Thiem et al., 2008). Flow cytometric analyses also indicated that despite the presence of secondary metabolites in the leaf cytosol it was possible to obtain a high-resolution histogram if the nuclei isolation buffer was modified. The tested antioxidant has been successfully used in other species that contain phenolic compounds (Sliwinska et al., 2005; Rewers and Jedrzejczyk, 2016). We used whole seeds since halved seeds did not improve the quality of histograms (unpublished observation). The embryo nuclei were easy to distinguish and the endosperm peak did not overlap the G_0/G_1 peak of the internal standard. Additionally, the embryo in cowslip seeds is straight, small, and coated by endosperm, and so it

Table 2. Morphometric characteristics of *Primula veris*. Two-way ANOVAs were performed to determine the effect of plant origin (O) and year of seed collection (Y) and interaction among the variables studied (O × Y) on particular features: RD – plant rosette diameter, STpl – number of flower stalks per plant, STH – height of the flower stalk, FLst – number of flowers on the flower stalk, FLpl – number of flowers per plant, FRst – number of fruits on the flower stalk, FRpl – number of fruits per plant, SEca – number of seeds per capsule, SEst – number of seeds per flower stalk, SEpl – number of seeds per plant. Levels of statistical significance: * P < 0.05; ** P < 0.01; *** P < 0.001.

Feature	Year	Plants from in vitro cultures			Plants from seeds			ANOVA (F, P-value)		
		Mean	SE	CV	Mean	SE	CV	Origin	Year	O × Y
RD	1	23.28	0.85	20.0	18.53	0.89	26.2			
	2	15.85	0.87	29.9	15.03	0.88	32.0	0.09	59.29***	14.16***
	3	22.65	1.09	26.4	28.87	0.93	17.7			
STpl	1	4.53	0.34	40.9	9.23	0.53	31.2			
	2	3.07	0.30	54.1	8.00	0.76	52.2	147.67***	14.35***	1.50
	3	5.20	0.68	72.0	12.30	0.74	33.1			
STH	1	17.72	0.80	24.6	20.66	0.64	16.9			
	2	16.28	1.04	34.9	17.78	0.60	18.4	27.55***	2.56***	3.32*
	3	19.86	0.73	20.2	25.26	0.70	15.2			
FLst	1	14.40	0.92	35.0	12.37	0.76	33.7			
	2	9.18	0.51	30.5	8.43	0.45	29.2	1.37	3.37***	1.95
	3	11.32	0.47	22.7	12.08	0.53	24.2			
FLpl	1	66.83	7.36	60.3	114.63	9.49	45.3			
	2	29.70	3.65	67.3	68.87	8.16	64.9	71.13***	23.60***	4.51*
	3	61.50	9.44	84.1	148.03	10.49	38.8			
FRst	1	13.59	0.92	37.2	11.87	0.70	32.3			
	2	8.71	0.52	33.0	8.03	0.42	28.4	0.71	27.56***	3.07*
	3	10.44	0.37	19.6	11.61	0.42	19.6			
FRpl	1	62.74	7.05	61.6	110.45	8.81	43.7			
	2	28.53	3.64	69.9	65.92	7.85	65.2	87.31***	28.15***	4.52*
	3	56.62	8.27	80.0	142.84	9.45	36.2			
SEst	1	353.30	41.97	65.1	401.93	34.00	46.3			
	2	352.77	36.31	56.4	190.70	16.83	48.3	0.04	14.11***	15.36***
	3	335.25	26.03	42.5	481.42	28.68	32.6			
SEpl	1	1553.14	222.57	78.5	3775.27	424.18	61.5			
	2	1010.25	116.89	63.4	1503.28	197.52	72.0	78.12***	29.30***	8.26***
	3	1817.39	291.71	87.9	5807.54	443.52	41.8			
SEca	1	32.86	6.07	101.2	34.07	2.31	37.2			
	2	41.83	3.96	51.9	24.39	1.81	40.7	0.74	0.57	8.23***
	3	31.82	1.92	33.1	40.94	1.70	22.8			

was difficult to isolate just the embryo (Morozowska, 2002; Morozowska et al., 2011).

Morphological analysis of cultivated *P. veris* plants of different origin proved that generatively propagated plants are characterized by more vigorous development and two to three times higher seed production in comparison to plants regenerated vegetatively from tissue cultures. However, the results obtained also showed the permanent morphological stability of in vitro regenerated plants compared to plants obtained from seeds, which were highly variable among the three years of the study for all features analyzed. Importantly, the stability of plants of
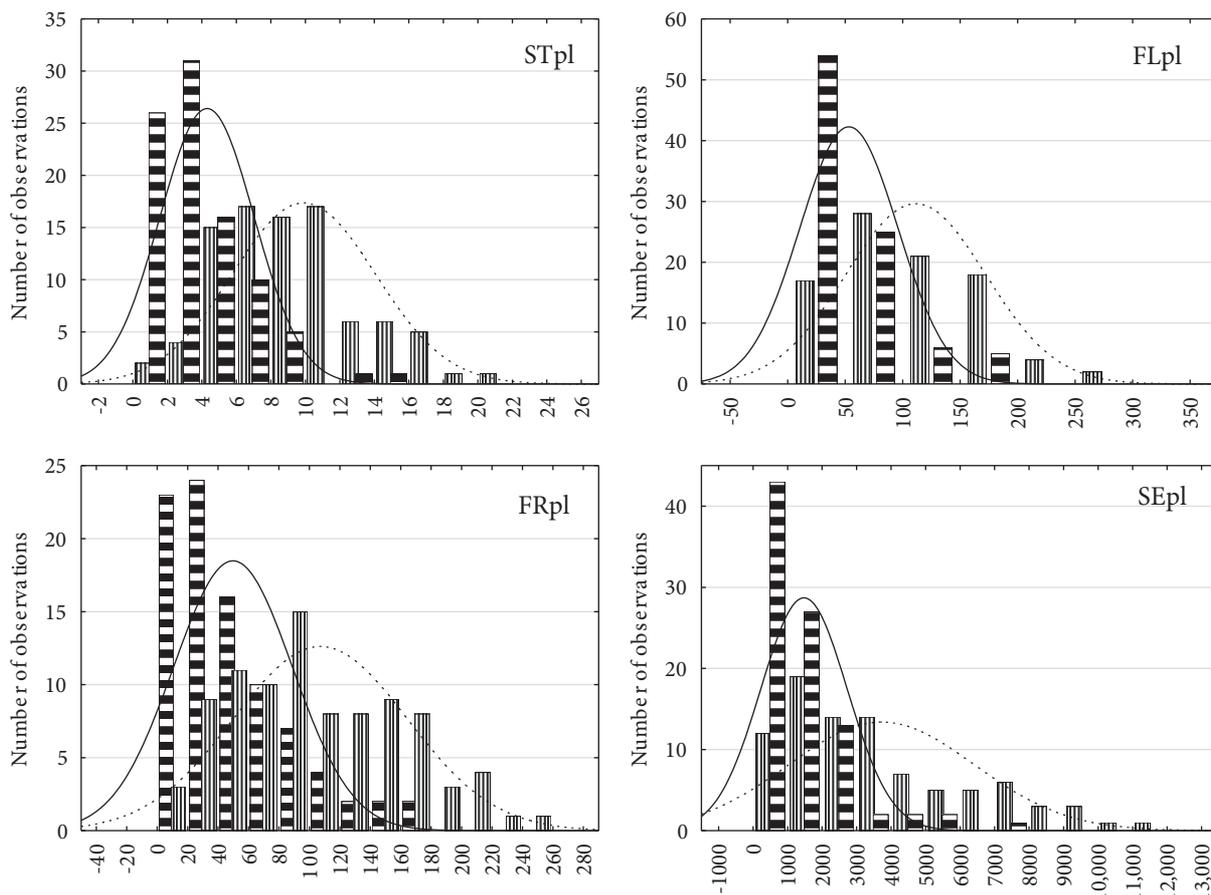
Figure 2. Histograms of the number of flower stalks per plant (STpl), number of flowers per plant (FLpl), number of fruits per plant (FRpl), and the number of seeds per plant (SEpl) in *Primula veris* specimens of in vitro origin (horizontally striped bars) and seed origin (vertical striped bars).

in vitro origin was a response to reproductive traits such as the number of seeds per flower stalk and per plant, which did not differ significantly among the years of the study. With reference to parameters characterizing the vegetative growth of plants Bhatia and Ashwath (2004) found that seed-grown tomato plants produced higher numbers of leaves and branches than tissue-cultured plants. In turn, Gantait and Sinniah (2011) proved that in vitro generated *Anthurium* plants exhibited comparable vegetative growth when compared to plants propagated through seeds.

Low seasonal variation in traits that determine reproductive success allows the expectation of predictable seed production, and seems to be stable for in vitro regenerated plants after successful acclimatization to field conditions. During the vegetative phase of development, plants of both origins examined did not differ significantly with respect to the size of leaf rosette. Although its diameter was highly variable within subsequent years of study, it was strongly correlated with the number and height of flowering stalks, as well as with number of flowers, fruits, and seeds present on the sampled plants. Dependency of seed production on plant size was noted in earlier studies on cultivated cowslip plants as well as for wild populations of *P. modesta* (Morozowska, 2004b; Shimono and Washitani, 2007).

Plants regenerated in vitro had significantly fewer flower stalks per individual, but average number of flowers per inflorescence did not differ between groups of specimens and no significant correlation was found between the number of flower stalks per plant and the number of flowers per inflorescence. However, for plants of both origins number of flowers per plant was strongly correlated with number of flower stalks per plant. That is in agreement with similar observations in natural cowslip populations, in which doubling or tripling of the number of inflorescence stalks per plant also increased the total number of flowers per plant about two to three times. However, no straight dependence between the number of flower stalks per plant and the number of flowers per

Table 3. Correlation matrix for the morphological traits analyzed in specimens of in vitro and seed origin. Abbreviations: RD – plant rosette diameter, STpl – number of flower stalks per plant, STH – height of the flower stalk, FLst – number of flowers on the flower stalk, FLpl – number of flowers per plant, FRst – number of fruits on the flower stalk, FRpl – number of fruits per plant, SEca – number of seeds per capsule, SEst – number of seeds per flower stalk, SEpl – number of seeds per plant. Levels of statistical significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

		Seed derived specimens											
		RD	STpl	STH	FLst	FLpl	FRst	FRpl	SEst	SEpl	SEca		
In vitro derived specimens	RD	-----	0.60***	0.72***	0.27	0.57***	0.33**	0.61***	0.42***	0.61***	0.32**	RD	Seed derived specimens
	STpl	0.52***	-----	0.40***	0.17	0.79***	0.21*	0.83***	0.22*	0.66***	0.15	STpl	
	STH	0.44***	0.31**	-----	0.44***	0.52***	0.49***	0.56***	0.56***	0.62***	0.40***	STH	
	FLst	0.41***	0.30**	0.17	-----	0.70***	0.97***	0.65***	0.65***	0.53***	0.21	FLst	
	FLpl	0.55***	0.90***	0.25*	0.64***	-----	0.70***	0.98***	0.53***	0.80***	0.22*	FLpl	
	FRst	0.39***	0.26	0.13	0.96***	0.58***	-----	0.69***	0.69***	0.58***	0.23*	FRst	
	FRpl	0.55***	0.89***	0.24*	0.64***	0.99***	0.62***	-----	0.54***	0.82***	0.24*	FRpl	
	SEst	0.26*	-0.04	0.35***	0.14	0.01	0.15	0.01	-----	0.84***	0.84***	SEst	
	SEpl	0.53***	0.75***	0.44***	0.29**	0.69***	0.26*	0.69***	0.53***	-----	0.70***	SEpl	
	SEca	–0.02	–0.21*	0.10	–0.35***	–0.29**	–0.38***	–0.32**	0.74***	0.24*	-----	SEca	
		RD	STpl	STH	FLst	FLpl	FRst	FRpl	SEst	SEpl	SEca		
		In vitro derived specimens											

inflorescence was found among wild growing plants (Tamm, 1972; Morozowska and Urbański, 2000).

Plants regenerated in vitro set fruits in 92.0% to 95.9% of flowers and plants derived from seeds set fruits in 95.7% to 96.5% of flowers. According to earlier results concerning cultivated cowslip plants regenerated from seeds, the intensity of fruiting was 84.8%–93.7% (Morozowska, 2003). In turn, results from long-term observations in natural *Primula veris* populations showed much lower fruiting intensity, which ranged from 9.0% to 52.3%, depending on the population (Morozowska and Urbański, 2000). Fruiting efficiency and seed set are important parameters characterizing plant fecundity, which is an essential component of fitness (Baker et al., 1994). The very high fruiting efficiency observed in the present study suggests strong reproductive potential of cultivated cowslip plants either regenerated vegetatively from tissue cultures or propagated generatively from seeds. Besides the seed number, seed size is a very important trait characterizing plant reproductive potential. The trade-off between seed size and seed number has received special attention, because seed quality and seed number affect plant reproductive success to a great extent.

Our results proved that although cowslip plants produced through tissue cultures set lower total numbers of seeds, they set seeds significantly bigger in comparison with plants regenerated generatively from seeds. Surprisingly, the individual seed biomass did not change significantly with species origin. On the other hand, when 1000-seed weight was taken into account, a trade-off between the seed weight and seed number was observed. Although the results were obtained under cultivation conditions, it cannot be excluded that studies based on large samples of seeds generate higher seed weight variation, as was found in natural populations (Wang et al., 2014).

Compensation of reduced seed size by higher seed number was already reported for *P. veris* in previous studies (Kéry et al., 2000; Morozowska, 2004b), and for other *Primula* species by Tremayne and Richards (2000). Baker et al. (1994) showed that *P. farinosa* tends to show a reduction in seed weight with increasing seed number per capsule; however, the authors found that it was clearly shown only for capsules containing more than 65 seeds. The same authors have also discovered a positive relationship between seed number per capsule and capsule number per inflorescence for *P. farinosa*. In the authors' opinion such dependence may indirectly influence the reproductive success of this species, as inflorescences with few flowers set the fewest seeds per capsule. In the present study no such correlation was found, but we agree with Baker et al. (1994) on the fact that the positive correlation between the increase of number of seeds per fruit and number of fruits per inflorescence may result in competition for limited maternal resources among seeds, and reduction in seed size may be a consequence of such a relationship. Furthermore, according to Boyd et al. (1990),

Table 4. Biometrical characteristics of *Primula veris* seeds. Two-way ANOVAs were performed for each seed morphological feature and seed weight to determine the effect of origin (O), year of seed collection (Y) and interaction among the variables studied (O × Y). N – number of seeds measured; W/L ratio – seed width/length ratio. Levels of statistical significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Feature	Year	Plants from in vitro cultures				Plants from seeds				ANOVA (F, P-value)		
		N	Mean	SE	CV	N	Mean	SE	CV	Origin	Year	O × Y
Seed weight (g)	1	130	0.000881	0.000023	29.1	130	0.000848	0.000026	34.6	1.106	2.512	1.637
	2	126	0.000848	0.000025	33.5	130	0.000875	0.000022	29.1			
	3	130	0.000845	0.000022	30.3	130	0.000792	0.000018	25.9			
Projected area (mm²)	1	407	1.4985	0.0190	25.6	367	1.3374	0.0200	28.6	27.950***	0.420	5.622**
	2	125	1.4135	0.0362	28.7	240	1.4102	0.0211	23.2			
	3	251	1.5179	0.0290	30.2	186	1.3528	0.0219	22.1			
Length (mm)	1	407	1.5991	0.0103	13.0	367	1.5084	0.0107	13.6	26.017***	0.095	6.800**
	2	125	1.5545	0.0183	13.1	240	1.5592	0.0113	11.2			
	3	251	1.6001	0.0151	14.9	186	1.5177	0.0115	10.3			
Width (mm)	1	407	1.2960	0.0092	14.3	367	1.2156	0.0097	15.3	15.851***	0.399	7.854**
	2	125	1.2456	0.0169	15.2	240	1.2608	0.0108	13.2			
	3	251	1.2911	0.0133	16.3	186	1.2370	0.0105	11.6			
Volume (mm³)	1	407	0.3429	0.0067	39.5	367	0.2907	0.0068	44.9	24.268***	0.499	5.786**
	2	125	0.3109	0.0125	45.1	240	0.3131	0.0072	35.6			
	3	251	0.3504	0.0106	47.8	186	0.2931	0.0073	33.8			
Surface area (mm²)	1	407	3.6341	0.0452	25.1	367	3.2478	0.0476	28.1	24.734***	0.408	6.828**
	2	125	3.4053	0.0846	27.8	240	3.4371	0.0506	22.8			
	3	251	3.6686	0.0689	29.8	186	3.2881	0.0501	20.8			
W/L ratio	1	407	0.8133	0.0041	10.2	367	0.8072	0.0038	9.1	0.374	0.747	1.362
	2	125	0.8030	0.0071	9.9	240	0.8098	0.0046	8.8			
	3	251	0.8098	0.0052	10.3	186	0.8168	0.0049	8.1			
Projected perimeter (mm)	1	407	4.5910	0.0306	13.4	367	4.3260	0.0334	14.8	25.448***	0.347	6.152**
	2	125	4.4390	0.0564	14.2	240	4.4466	0.0341	11.9			
	3	251	4.6020	0.0443	15.2	186	4.3568	0.0343	10.7			

who examined the population ecology of heterostyle and homostyle *Primula vulgaris* in field populations, seeds compete for limited maternal resources, which results in a trade-off between seed size and seed number. Baker et al. (1994) have shown that seed size and seedling performance were positively associated in *P. farinosa*. Such a relationship was also found for *P. vulgaris* and other *Primula* species for which the larger seeds might lead to more successful individuals than those arising from small seeds (Tremayne and Richrads, 2000; Lehtilä and Ehrlén, 2005). Many earlier studies have also shown that large seeds are associated with high seedling survival rate and vigorous growth and establishment, as well as the higher seed yield per plant (Baskin and Baskin, 2014; Ambika et al., 2014). However, according to Lehtilä and Ehrlén (2005), the reproductive success of *P. veris* depends both

on offspring number and quality; larger seed size does not always result in beneficial changes in seed quality, and it is important to know the source of size variation.

In natural populations of heterostylous perennials like primulas seed set and seed size depend on many factors such as population size, presence and reciprocal proportion of plants with different flower morphs, pollinator availability, presence of pathogens and herbivores, and other environmental or genetic factors (Baker et al., 1994; Kéry et al., 2000; Matsumura and Washitani, 2000; Lehtilä and Ehrlén, 2005).

Due to the application of common garden conditions we have been able to exclude most of the above-mentioned environmental factors. We acknowledge the proven genetic stability of *P. veris* plants derived from in vitro cultures as the most important finding concerning

practical use of this species as an ornamental plant and in the pharmaceutical industry. The hypotheses formulated concerning morphology were only partially positively verified. The origin of the specimens as well as the year of study significantly affected most of the examined morphological traits. No interseasonal variation in seed size was observed. Even though the total seed production of plants derived from in vitro cultures was much smaller compared to plants reproduced generatively, this was caused only by the smaller number of flower stalks per plant. In vitro regenerated plants set seeds significantly bigger and heavier according to 1000-seed weight. Such results may suggest that seeds produced by micropropagated plants would be advantageous for commercial cultivation for either horticultural or pharmaceutical purposes. However, since the differentiation of individual seed weight based on plant origin was not proven, further studies are needed to find out whether the observed advantages concerning seed size and probable seed weight are accompanied by increased seedling performance and higher quality of the offspring. Based on the results obtained we may advise using cowslip plants regenerated in vitro for both pharmaceutical research and horticultural purposes, but the final certification of such a standpoint needs further studies on quality of seeds and seedlings derived from tissue-cultured plants.

Acknowledgments

References

Ambika S, Manonmani V, Somasundaram G (2014). Review on effect of seed size on seedling vigour and seed yield. Research Journal of Seed Science 7: 31-38.

Antrobus S, Lack AJ (1993). Genetics of colonizing and established populations of *Primula veris*. Heredity 71: 252-258.

Bajaj YPS, Furmanova M, Olszowska O (1988). Biotechnology of the micropropagation of medicinal and aromatic plants. In: Bajaj YPS, editor. Biotechnology in Agriculture and Forestry, Medicinal and Aromatic Plants, Vol. 4. 1st ed. Berlin, Germany: Springer-Verlag, pp. 60-103.

Baker K, Richards AJ, Tremayne M (1994). Fitness constraints on flower number, seed number and seed size in the dimorphic species *Primula farinosa* L. and *Armeria maritima* (Miller) Wild. New Phytol 128: 563-570.

Baskin CC, Baskin JM (2014). Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination. 2nd ed. San Diego, CA, USA: Academic Press.

Berisha N, Millaku F, Gashi B, Krasniqi E, Novak J (2015). Initial determination of DNA polymorphism of some *Primula veris* L. populations from Kosovo and Austria. Physiol Mol Biol Pla 21: 117-122.

Bhatia P, Ashwath N (2004). Comparative performance of micropropagated and seed-grown tomato plants. Biol Plantarum 48: 625-628.

Boyd M, Silvertown J, Tucker C (1990). Population ecology of heterostyled and homostyle *Primula vulgaris*: growth, survival and reproduction in field populations. J Ecol 78: 799-813.

Brys R, Jacquemyn H (2009). Biological flora of the British Isles: *Primula veris* L. J Ecol 97: 581-600.

Budzianowski J, Morozowska M, Wesołowska M (2005). Lipophilic flavones of *Primula veris* L. from field cultivation and *in vitro* cultures. Phytochemistry 66: 1033-1039.

Cassells AG, Curry RF (2001). Oxidative stress and physiological epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. Plant Cell Tiss Org 64: 145-157.

Clapham AR, Tutin TG, Moore DM (1987). Flora of the British Isles. 3rd ed. Cambridge, UK: Cambridge University Press.

Doležel J, Bartos J (2005). Plant DNA flow cytometry and estimation of nuclear genome size. Ann Bot – London 95: 99-110.

Ducar E, Rewers M, Jedrzejczyk I, Martonfi P, Sliwinska E (2018). Comparison of the genome size, endoreduplication and ISSR markers polymorphism in eight *Lotus* (Fabaceae) species. Turk J Bot 42: 1-14.

Gajdošová A, Ostrolucká MG, Libiaková G, Ondrušková E, Šima D (2006). Microclonal propagation of *Vaccinium* sp. and *Rubus* sp. and detection of genetic variability in culture in vitro. Journal of Fruit and Ornamental Plant Research 14: 103-119.

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983). Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220: 1049-1051.

Gantait S, Mandal N, Bhattacharyya S, Das PK (2010). Sustainable in vitro propagation and clonal fidelity in strawberry. International Journal of Plant Developmental Biology 4: 19-25.

Gantait S, Sinniah UR (2011). Morphology, flow cytometry and molecular assessment of ex-vitro grown micropropagated *Anthurium* in comparison with seed germinated plants. Afr J Biotechnol 10: 13991-13998.

Hegnauer R (1990). Chemotaxonomie Der Pflanzen Band 9. Basel, Switzerland: Birkhaüser, pp. 288-293.

Huck CW, Huber CG, Ongania KH, Bonn GK (2000). Isolation and characterization of methoxylated flavones in the flowers of *Primula veris* by liquid chromatography and mass spectrometry. J Chromatogr A 870: 453–462.

ISTA (2015). International Rules for Seed Testing. Vol. 2015, pp. 1-276.

Kéry M, Matthies D, Spillmann HH (2000). Reduced fecundity and offspring performance in small populations of the declining grassland plants *Primula veris* and *Gentiana lutea*. J Ecol 88: 17-30.

Lee JE, Safe S (2000). 3',4'-Dimethoxyflavone as an aryl hydrocarbon receptor antagonist in human breast cancer cells. Toxicol Sci 58: 235-242.

Lehtilä K, Ehrlén J (2005). Seed size as an indicator of seed quality: a case study of *Primula veris*. Acta Oecol 28: 207-212.

Lehtilä K, Syrjänen K, Leimu R, Garcia MB, Ehrlén J (2005). Habitat change and demography of *Primula veris*: identification and management targets. Conserv Biol 29: 833-843.

Marie D, Brown C (1993). A cytometric exercise in plant histograms, with 2C values for 70 species. Biol Cell 78: 41-51.

Matsumura Ch, Washitani I (2000). Effects of population size and pollinator limitation on seed-set of *Primula sieboldii* populations in a fragmented landscape. Ecol Res 15: 307-322.

Morozowska M (2002). Some factors affecting viability, dormancy and germination of *Primula veris* (L.) seeds. Folia Horticulturae 14: 243-253.

Morozowska M (2003). Flowering, fruiting and seed set of *Primula veris* L. plants in field cultivation. Folia Horticulturae 2003/2: 80-82 (article in Polish with an abstract in English).

Morozowska M (2004a). Studies on some seed traits of *Primula veris* L. plants from cultivated populations. Herba Pol 50: 137-144.

Morozowska M (2004b). Vegetative development, flowering, fruiting and saponin content in cultivated cowslip (*Primula veris* L.) plants. Herba Pol 50: 28-35.

Morozowska M, Czarna A, Kujawa M, Jagodziński AM (2011). Seed morphology and endosperm structure of selected species of Primulaceae, Myrsinaceae, and Theophrastaceae and their systematic importance. Plant Syst Evol 291: 159-172.

Morozowka M, Krzakowa M (2003). Genetic variation of *Primula veris* in natural and cultivated populations. Acta Biol Cracov Bot 45: 177-182.

Morozowska M, Urbański Ł (2000). Characteristics of flowering and fructifying and the evaluation of the seed production of cowslip plants (*Primula veris* L.) from populations located on the Wielkopolska Lowland. Roczniki Akademii Rolniczej w Poznaniu Seria Botanika 3: 175-184 (article in Polish with an abstract in English).

Morozowska M, Wesołowska M (2004). *In vitro* clonal propagation of *Primula veris* L. and preliminary phytochemical analysis. Acta Biol Cracov Bot 46: 169-175.

Price HJ, Hodnett G, Johnston JS (2000). Sunflower (*Helianthus annuus*) leaves contain compounds that reduce nuclear propidium iodide fluorescence. Ann Bot 86: 929-934.

Rewers M, Jedrzejczyk I (2016). Genetic characterization of *Ocimum* genus using flow cytometry and inter-simple sequence repeat markers. Ind Crop Prod 91: 142-151.

Richards AJ (2002). Primula. 2nd ed. London, UK: BT Batsford.

Shimono A, Washitani I (2007). Factors affecting variation in seed production in the heterostylous herb *Primula modesta*. Plant Spec Biol 22: 65-76.

Siljak-Yakovlev S, Pustahija F, Solic EM, Bogunic F, Muratovic E, Basic N, Catrice O, Brown SC (2010). Towards a genome size and chromosome number database of Balkan flora: C-values in 343 taxa with novel values for 242. Adv Sci Lett 3: 190-213.

Sliwinska E, Thiem B (2007). Genome size stability in six medicinal plant species propagated in vitro. Biol Plantarum 51: 556-558.

Sliwinska E, Zielińska E, Jędrzejczyk I (2005). Are seeds suitable for flow cytometric estimation of plant genome size? Cytom Part A 64A: 72-79.

Soltis DE, Soltis PS, Bennett MD, Leitch IJ (2003). Evolution of genome size in the angiosperms. Am J Bot 90: 1596-1603.

Stecher G, Huck CW, Stöggl WM, Bonn GK (2003). Phytoanalysis: a challenge in phytomics. Trends in Analytical Chemistry 22: 1-14.

Tamm CO (1972). Survival and flowering of perennial herbs. III. The behavior of *Primula veris* on permanent plots. Oikos 23: 159-166.

Thiem B, Budzianowski J, Wesołowska M, Ratajczak L, Morozowska M, Skrzypczak L (2008). Secondary metabolites of in vitro cultures of selected Polish rare and endangered plants. Herba Pol 54: 158-167.

Thiem B, Sliwinska E (2003). Flow cytometric analysis of nuclear DNA content in cloudberry (*Rubus chamaemorus* L.) in vitro cultures. Plant Sci 164: 129-134.

Tokalov SV, Kind B, Wollenweber E, Gutzeit HO (2004). Biological effects of epicuticular flavonoids from *Primula denticulate* on human leukaemia cells. J Agri Food Chem 52: 239-245.

Tremayne A, Richards AJ (2000). Seed weight and seed number affect subsequent fitness in outcrossing and selfing *Primula* species. New Phytol 148: 127-142.

Wang KJ, Li XH, Yan MF (2014). Genetic differentiation in relation to seed weights in wild soybean species (*Glycine soja* Sieb. & Zucc.). Plant Syst Evol 300: 1729-1739.