Plant Cell Tiss Organ Cult (2018) 132:317–327
DOI 10.1007/s11240-017-1331-3

ORIGINAL ARTICLE

Morphological, physiological and genomic comparisons between diploids and induced tetraploids in *Anemone sylvestris* L.

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Received: 15 June 2017 / Accepted: 20 October 2017 / Published online: 2 November 2017

Abstract The objective of this research was to induce mitotic chromosome doubling in *Anemone sylvestris* L. The mitosis inhibitor oryzalin was directly added to the induction medium at 1, 2, 5, 10 and 15 µM for 8, 10 or 12 weeks of cultivation. Three tetraploid plants (2n = 4x = 32), 0.8% (polyploidization efficiency), were obtained from diploid plants (2n = 2x = 16) in three treatments (1 µM for 10 weeks, 5 µM for 8 weeks and 8 µM for 10 weeks). Ploidy level was confirmed by flow cytometry. Morphological characteristics (e.g. flower diameter, total plant height, leaf area) and chlorophyll content differences between diploid and tetraploid *A. sylvestris* were observed together with polyphenol content and antioxidant activity. The inter primer binding sites markers were used for evaluation of polymorphism. New genotypes with different morphological and biological characteristics were obtained through somatic polyploidization. The tetraploid plants were stronger, more vigorous and had an early flowering, which is essential for its use as an ornamental plant. The iPBS analysis showed unique amplicons that can be used for the purposes of molecular identification of tetraploid plants of *A. sylvestris* in the future. The results demonstrate the first report of in vitro induction of tetraploids of *A. sylvestris*.

Keywords Antioxidant activity · Chlorophyll content · iPBS · Oryzalin · Polyphenol content · Ranunculaceae

Communicated by Sergio J. Ochatt.

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Introduction

Snowdrop windflower (*Anemone sylvestris* L.) of the family Ranunculaceae is a perennial herbaceous plant with use in gardens due to its decorative appearance (Hejný and Slavík 1988; Bényei Himmer et al. 1997) and it is protected in many countries (Hoskovec 2015; Kwiatkowska-Falinska and Falinski 2007; Bényei Himmer et al. 1997). It inhabits Eurasian grasslands, woodland margins and forest-steppe communities (Hejný and Slavík 1988). The fresh plant is poisonous because of its content of glucoside ranunculin whose aglycone is a toxic protoanemonine (Hejný and Slavík 1988).

Induced chromosome doubling in vitro is an old but still used method of plant breeding applied to a wide range of ornamental plants and crop species, including *R. asiaticus* (Dhooghe et al. 2009a), *Phlox subulata* (Zhang et al. 2008), *Punica granatum* (Shao et al. 2003), *Humulus lupulus* (Trojak-Goluch and Skomra 2013), *Solanum*

tuberosum (Greplova et al. 2009). However, there is a lack of literature concerning on breeding of *Anemone* ssp. Antimitotic agents such as colchicine, oryzalin and trifluralin are commonly used for chromosome doubling. Polyploid plants have often increased organ size, prolonged flowering period or increased resistance to abiotic stresses, diseases and pests (Dhooghe et al. 2011). Flower size, compactness of plant or flowering period are the features that could increase the ornamental value of *A. sylvestris*. Content of effective compounds e.g. chlorophyll (the part of the photosynthetic apparatus) in polyploid plants were also altered in some plant species (Madani et al. 2015; Mathura et al. 2006). For assessing plant genetic diversity different molecular techniques are commonly used (Ovesná et al. 2013), but the molecular studies in the genus *Anenome* are limited due to the lack of known genus specific molecular markers (Sun et al. 2015). Kalendar et al. (2010) described a method that serves as a general marker system for plant species—inter-primer binding site (iPBS). It is based on retrotransposon sequences, which are ubiquitous in plant genomes, and thus usable as markers.

The objective of the study was to obtain tetraploid plants (2n = 4x = 32) from diploid plants (2n = 2x = 16) by in vitro induced chromosome doubling using oryzalin as mitosis inhibitor for horticultural purposes with possible different morphological and biological characteristics.

Materials and methods

Plant material and culture establishment

Seeds of *A. sylvestris* L. were obtained via Index Seminum (Hortus Botanicus Tallinnensis) from natural locality Pimestiku, Ida-Viru Country, Estonia.

Seeds were surface disinfested by immersing in 70% (v/v) ethanol for 1 min, then 1% (v/v) solution of sodium hypochlorite (NaClO, commercial bleach) containing two drops of Tween 20 for 20 min followed by three rinses in sterile distilled water. They were placed to germinate on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose, 0.8% agar (w/v) and 2.9 µM GA_3 (gibberellic acid) for four weeks. The pH of the medium was adjusted to 5.7 ± 0.1 before autoclaving at 1.1 kg cm^{-2} for 20 min.

Seedlings were multiplied in 100 ml Erlenmeyer flasks (one plantlet per flask) containing 20 ml of MS medium supplemented with 1.5 µM GA_3 and 0.5 µM BAP (6-benzylaminopurine). All plants were subcultivated every six weeks. All cultures were maintained at 25/20 ± 0.3 °C under 16/8 h light regime in a cultivation box (POL-EKO ILW350/350 STD) provided by cool-white fluorescent lamps (40.5 µmol m^{-2} s^{-1}).

Chromosome doubling

Plants with a length of 2–3 cm were cultured on multiplication media containing 1, 2, 5, 10 or 15 µM of oryzalin for 8, 10 or 12 weeks of cultivation. Control plants were cultured on oryzalin-free medium. Five explants per flask in five replications were used for each treatment. Oryzalin was dissolved in 1 M solution of sodium hydroxide (NaOH), according to Dhooghe et al. (2009a), filter sterilized (0.22 µm) and added to the medium after autoclaving. Plants were rinsed three times in sterile distilled water after cultivation time, transferred to fresh medium without antimitotic agent for six weeks before the determination of ploidy level by flow cytometry.

Flow cytometry

Leaves from each plant were used for flow cytometry analysis. Fresh leaf samples of 0.5 cm^2 were chopped using a razor blade in 1 ml 0.1 M citric acid monohydrate and 0.5% Tween 20 in Petri dishes. They were filtered through 50 µm nylon mesh and 1 ml 0.4 M $Na_2HPO_4 \cdot 12H_2O$ (sodium phosphate dibasic dodecahydrate) and 4 mg l^{-1} DAPI (4′,6-diamidino-2-phenylindole) was added. The tested samples were analysed for relative fluorescence intensity using a flow cytometer (Partec CyFlow, Münster, Germany). Diploid plants were used as an external standard for flow cytometer calibration. More than 1500 nuclei per sample were measured during each analysis. Histograms of DNA content were evaluated using the Flomax software package. The stability of ploidy level was retested after 9 months.

Plant production

Stock material of *A. sylvestris* (diploid and tetraploid plants) was maintained and propagated on full-strength solid MS culture medium including vitamins (Duchefa Biochemie B.V., Haarlem, The Netherlands) supplemented with 2% sucrose, 0.25% Phytagel™ (Sigma-Aldrich, St. Louis, MO). Plant regulators were omitted. Separated single shoots (4–5 expanded leaves, ≥ 3 cm long) were placed vertically into full-strength solid WPM including vitamins (Woody Plant Medium; Lloyd and McCown 1980; Duchefa Biochemie B.V., Haarlem, The Netherlands) and supplemented with 1 g l^{-1} activated charcoal for root induction. In vitro rooted plantlets were transferred into plastic pots containing peat-perlite substrate and were gradually acclimatized. Plants were produced according to the protocol Šedivá et al. (2017).

Morphological characteristics analysis

After the diploid (control) and tetraploid plants had been transferred to the pots for 4 months, the morphological characteristics were observed and evaluated. During the summer 120 plants of each group were transplanted into the field.

The width and length of the longest leaves (corresponding to total plant height) and the length and diameter of central parts of their petioles were measured using a digital caliper during the first year. Twenty plants of each ploidy level were measured. In the second year flower diameter, carpel diameter, number of petals, peduncle length and diameter and total plant height were measured using a digital caliper.

Chlorophyll content

Chlorophyll content was estimated by taking SPAD (soil plant analysis development) measurements (SPAD-502, Minolta, Japan) in the flag leaves of *A. sylvestris* under field conditions. The measurements were randomly carried out in 12 plants of each group (diploid and tetraploid). The relationship of SPAD measurements with leaf chlorophyll content was calculated using a general relation from different crops species according to Zhu et al. (2012).

Total polyphenol content and antioxidant activity

Leaf tissue of diploid (ASD) and tetraploid (ASP) plants were dried and coarsely powered by mortar and pestle to determine the polyphenol content and antioxidant activity. The plant material was placed in a dark bottle and extracted by maceration technique by using solvents with crescent polarity, *n*-hexane (H), $CHCl_3$ (C), $CHCl_3$:MeOH 9:1 (CM) and MeOH (M) at room temperature. Each leaf sample was extracted three times with the same solvent (1:8 w/v). The mixtures were filtered by cellulose filter paper (17–25 µm) and the combined liquids were evaporated to dryness under reduced pressure by using a rotary evaporator. MeOH extracts were partitioned by BuOH (M/B) and water (M/W). The extracts and fractions were kept in the dark at room temperature until their use.

The total polyphenolic content (TPC) was determined by the Folin–Ciocalteu reagent (Russo et al. 2012). Briefly, 75 µl of diluted extract and 425 µl of distilled water were added to 500 µl Folin–Ciocalteu reagent and 500 µl of Na_2CO_3 (10% w/v). The mixture was mixed and incubated for 1 h in the dark at room temperature. After incubation, the mixture was measured at 723 nm using a UV–Vis spectrophotometer (Spectrostar-Nano). The total phenolic content was expressed as mg gallic acid equivalent (GAE) g^{-1} of extract. The experiment was carried out in triplicate.

The radical-scavenging activity was evaluated by using DPPH (2,2-diphenyl-1-picrylhydrazyl) test (Russo et al. 2015a). Briefly, 50 µl of plant extracts were added to 200 µl of DPPH solution in a 96 wells-plate and left in the dark. After 30 min the absorbance was monitored at 515 nm by using a spectrophotometer (Spectrostar-Nano, BMG LABTECH, Germany). Trolox was used as standard and results were expressed as mg Trolox Equivalent·g^{-1} of extract (mgTE·g^{-1}). Experiments were carried out in triplicate.

The reducing power of the extracts was determined by ferric reducing ability power (FRAP) test (Russo et al. 2015b). The stock solution included 300 mM acetate buffer pH 3.6, 10 mM TPTZ [2,4,6-tris(2-tripyridyl)-s-triazine] solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The working solution was prepared by mixing acetate buffer, TPTZ and $FeCl_3 \cdot 6H_2O$ (10:1:1). Plant extracts (25 µl) were allowed to react with 225 µl of the FRAP solution for 40 min at 37 °C. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. Trolox was used as standard and results were expressed in mg TE·g^{-1} of sample. Experiments were carried out in triplicate.

The lipid peroxidation inhibition was carried out by the β-carotene bleaching assay (Dekdouk et al. 2015). The β-carotene solution (0.2 mg of β-carotene dissolved in 0.2 ml of chloroform), linoleic acid (20 mg) and Tween 20 (200 mg) were mixed. Chloroform was removed by using a rotary evaporator at 37 °C. Then, distilled water (50 ml) was added. The emulsion (950 µl) was transferred into different tubes containing 50 µl of extract or ethanol as control. BHT (butylated hydroxytoluene) was used as positive control. 250 µl of emulsion were transferred into individual wells of a plate. The plate was placed at 50 °C for 3 h. The absorbance was measured at 470 nm using a spectrophotometer (Spectrostar Nano, BMG LABTECH, Germany). The results, performed in triplicate, were expressed as percentage antioxidant activity (% AA).

iPBS analysis

Total RNA was extracted by GeneJET Plant RNA Purification Mini Kit (ThermoScientific) following the instructions of the manufacturer. RNA quality and concentration were quantified by spectrophotometer (Implen Nanophotometer® P-Class). Reverse transcription was performed with the Tetro cDNA Synthesis Kit (ThermoScientific) using 4000 ng of total RNA extracted from young leaves, according to the manufacturer's recommendations and under the temperature and time profile listed in the manufacturer's protocol. iPBS primers were used for the analysis according to Žiarovská et al. (2013). PCR conditions were as follows: 95 °C 5 min (95 °C 1 min; 55 °C 1 min; 72 °C 2 min) 45x;

72 °C 10 min. Amplification was performed using a BIO-RAD C1000™ Thermal Cycler. In total, nine different iPBS primers were used (1838, 1854, 1899, 2270, 2274, 2279, 1886, 1897 and 2374), all of them with the sequences as described by Kalendar et al. (2010). Amplified products were separated in 6% PAGE gels and evaluated by GelAnalyzer software.

Statistical analyses

All statistical analyses were performed using the SPSS 20.0 statistical software package (SPSS Inc, Chicago, IL, USA). The Kolmogorov–Smirnov test was used to check the normal distribution of the data. The independent-samples student-t test (Levene's test to verify homogeneity of variance) and the Mann–Whitney U test were used to check for differences in leaf and flower characteristics between diploid and tetraploid plants.

The experiments of antioxidant activity and total polyphenol content were carried out in triplicate and results were compared by one-way ANOVA and Tukey's test. A difference was considered statistically significant if $p < 0.05$.

Results and discussion

Chromosome doubling

A total 375 plants were exposed to oryzalin across five concentrations and three treatment times. Oryzalin was used as an antimitotic agent, because several studies indicate it is more effective than colchicine or trifluralin in chromosome doubling (Dhooghe et al. 2009a; Sakhanokho et al. 2009; Viehmannová et al. 2009). The results obtained are summarized in the Table 1. The number of regenerated tetraploids was very low; three tetraploids (0.8%) were regenerated (Table 1; Fig. 1). One tetraploid plant was obtained for each treatment—1 µM and 10 weeks of cultivation, 5 µM and 8 weeks and 10 µM and 8 weeks (Table 1). The results report for the first time the in vitro induction of polyploids in *A. sylvestris*. The effect of oryzalin for chromosome doubling was also determined in other ornamental species of the Ranunculaceae family such as *R. asiaticus* (Dhooghe et al. 2009a), *Helleborus niger* and *H. orientalis* (Dhooghe et al. 2009b), equally in other species of different botanical families: *Rosa persica*, Rosaceae (Khosravi et al. 2008), *Hedychium muluense*, Zingiberaceae (Sakhanokho et al. 2009); *Tulipa gesneriana*, Liliaceae (Podwyszynska 2012); *Escallonia* spp., Escalloniaceae (Denaeghel et al. 2015). Plant rate viability was lower at higher concentrations of the antimitotic agent and during longer treatment time (Table 1). Oryzalin was more effective for chromosome doubling than colchicine in the case of *R. asiaticus* (32.5% for oryzalin compared to 23.3% for colchicine) (Dhooghe et al. 2009a) and less toxic than colchicine (Viehmannová et al. 2009).

Morphological characteristics

The differences between morphological characteristics for diploid and tetraploid plants growing in pots and field conditions were evaluated. Tetraploids had significantly larger leaves in all measured parameters ($P < 0.05$) (Table 2).

Table 1 Induced polyploidy of *Anemone sylvestris* by oryzalin treatment

Concentration (µM)	Time (weeks)	Number of plants	Survival rate (%)	Diploid plants	Mixoploid plants	Tetraploid plants	Efficiency rate (%)
1	8	25	80	20	0	0	0
	10	25	76	18	0	1	4
	12	25	52	13	0	0	0
2	8	25	80	20	0	0	0
	10	25	94	24	0	0	0
	12	25	68	17	0	0	0
5	8	25	80	19	0	1	4
	10	25	48	12	0	0	0
	12	25	16	4	0	0	0
10	8	25	64	15	0	1	4
	10	25	60	5	0	0	0
	12	25	52	13	0	0	0
15	8	25	68	17	0	0	0
	10	25	68	17	0	0	0
	12	25	0	0	0	0	0

There was a significant increase in leaf length (122%), leaf width (106%), petiole length (124%), petiole width (72%) and leaf area (385%) in the tetraploids in comparison with diploids. Leaf index was increased (leaf length/leaf width) from 0.84 to 0.9 contrary to reports by Zhang et al. (2008), Tang et al. (2010), Allum et al. (2007), Kaensaksiri et al. (2011) and Gantait et al. (2011) indicating that tetraploidization usually influences leaf width more than length. However, the results showed that *A. sylvestris* leaf length was enhanced slightly before width similarly to observation of Rêgo et al. (2011) in *Passiflora edulis* and Pansuksan et al. (2014) in one tetraploid line of *Mitracarpus hirtus*.

The flower morphology was also evaluated in diploid and tetraploid plants cultivated in pots and in the field. Tetraploid plants started flowering one week earlier than diploids. From a total of 45 diploid plants cultivated in pots, only 9 (20%) flowered and all of them had five petals. 87% of tetraploid plants (26 of 30 plants) flowered of which four plants (13%) had flowers with seven petals and nine

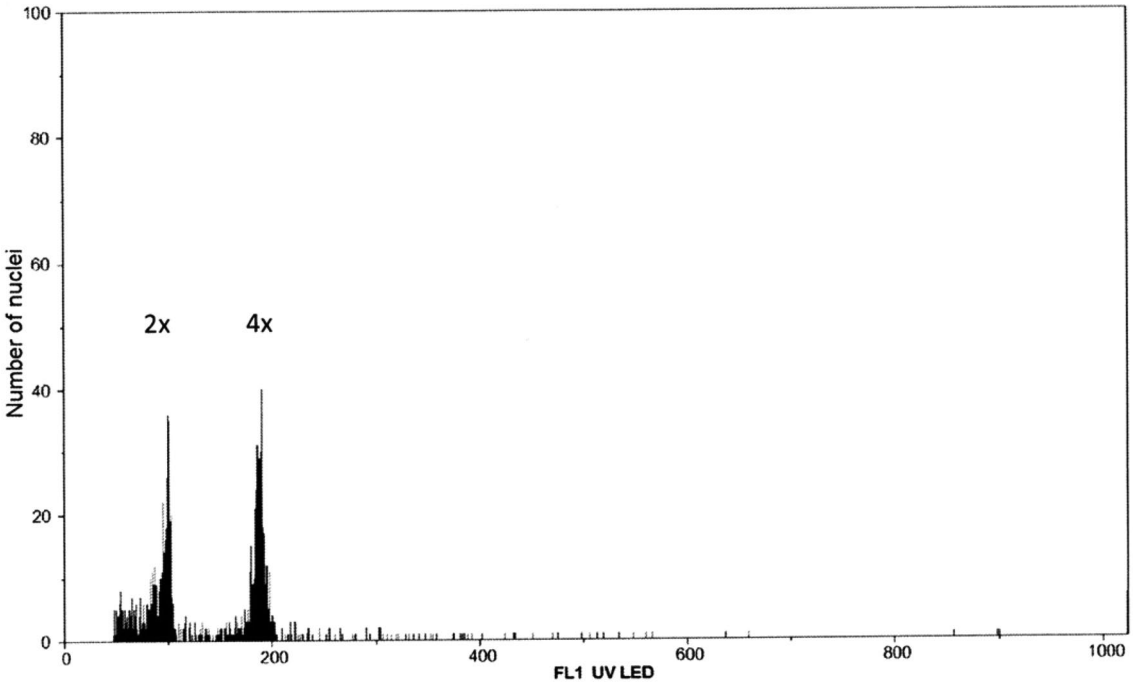

Fig. 1 Flow cytometry analysis of oryzalin-treated and control *A. sylvestris*: histogram of control diploid (2×) and tetraploid (4×) plants

Table 2 Morphological characteristics of leaves of diploid and tetraploid plants cultivated in pots

Plants tested	Leaf length (mm)	Leaf width (mm)	Leaf index (length/width)	Petiole length (mm)	Petiole width (mm)	Leaf area (cm^2)
Diploid	29.91 ± 5.33^{a}	35.89 ± 6.25^{a}	0.84 ± 0.07^{a}	31.57 ± 5.73^{a}	0.68 ± 0.07^{a}	5.52 ± 1.77^{a}
Tetraploid	$66.47 \pm 12.44^{b,**}$	$74.27 \pm 13.75^{b,**}$	$0.90 \pm 0.06^{b,*}$	$70.76 \pm 10.64^{b,**}$	$1.17 \pm 0.21^{b,**}$	$26.76 \pm 9.35^{b,**}$

Data are shown as mean ± SD (standard deviation)

Different superscript letters within the same column differ significantly (*$p < 0.01$; **$p < 0.001$)

Table 3 Morphological characteristics of flowers of diploid and tetraploid plants cultivated in pots

Plants tested	Flower diameter (mm)	Carpel diameter (mm)	Peduncle length (mm)	Peduncle diameter (mm)	Total plant height (mm)
Diploid	47.56 ± 8.41^{a}	5.04 ± 0.78^{a}	100.51 ± 27.31^{a}	1.35 ± 0.22^{a}	173.98 ± 33.52^{a}
Tetraploid	$56.68 \pm 9.34^{b,*}$	5.77 ± 0.96^{a}	$154.91 \pm 27.49^{b,**}$	$1.72 \pm 0.22^{b,**}$	$233.19 \pm 46.14^{b,**}$

Data are shown as mean ± SD (standard deviation)

Different superscript letters within the same column differ significantly (*$p < 0.05$; **$p < 0.01$)

plants (30%) had flowers with six petals. The tetraploids showed a significant increase ($P < 0.05$) in the flower diameter (19%), peduncle length (54%) and peduncle diameter (27%) (Table 3).

Under field conditions 240 plants were cultivated, 120 were diploids and 120 tetraploids. The tetraploids had a 100% survival rate in the next year, while the diploids only 58% (70 plants). Of a total of 120 tetraploids, 76 (63%) plants flowered, and of those 21 plants had flowers with six petals and one plant with flowers with seven and eight petals. Of the 70 diploid plants, only 15 (8%) plants flowered; all of them consist of five petals. As for plants grown in pots, tetraploid plants compared with diploids showed a significant increase ($P < 0.05$) in flower diameter (27%), peduncle length (72%), peduncle diameter (57%) (Table 4; Figs. 2, 3).

In general, the tetraploid plants in comparison with the diploids, flowered earlier, both in pots and under the field conditions. They also showed better regeneration, were more resistant to the conditions of cultivation, were more vigorous, bigger (greater growth of biomass) and the flowers were bigger, in some cases with greater number of petals and stronger stamens. Similar results were obtained in *Mecardonia tenella* (Escandón et al. 2007), *Gerbera jamesonii* (Gantait et al. 2011), *Crocosmia aurea* (Hannweg et al. 2013) and *Dendrobium nobile* (Vichiato et al. 2014).

Chlorophyll content

Chlorophyll loss is associated with environmental stress and may be a good indicator of stress in plants (Hendry and Price 1993). We did not find significant differences between diploid and polyploid plants ($t = -0.90$; $p = 0.378$) in the chlorophyll content. However, the values obtained from polyploid plants tended to be higher than those obtained from diploid plants (Table 5) and could indicate a higher photosynthetic efficiency as has been reported in other species (Liao et al. 2016; Ewald et al. 2009).

Total polyphenol content and antioxidant activity

Secondary metabolites are widely distributed in nature and they have a strong and often positive impact on human health. Comparative studies of secondary metabolites in diploid and polyploid genotypes have been conducted on numerous medicinal plants; it was reported that polyploidy often appears to result in increased expression of secondary metabolites, but not always and the effects of polyploidy are not predictable (Sanwal et al. 2010). All

Table 4 Morphological characteristics of flowers of diploid and tetraploid plants cultivated under field conditions

Plants tested	Flower diameter (mm)	Carpel diameter (mm)	Peduncle lenght (mm)	Peduncle diameter (mm)	Total plant height (mm)
Diploid	50.43 ± 8.14^{a}	3.90 ± 0.82^{a}	74.44 ± 24.92^{a}	1.19 ± 0.21^{a}	113.21 ± 29.32^{a}
Tetraploid	$64.45 \pm 5.00^{b,**}$	$4.69 \pm 0.66^{b,*}$	$127.79 \pm 28.48^{b,**}$	$1.87 \pm 0.18^{b,**}$	$190.37 \pm 32.54^{b,**}$

Data are shown as mean ± SD (standard deviation)

Different superscript letters within the same column differ significantly (*$p < 0.05$; **$p < 0.001$)

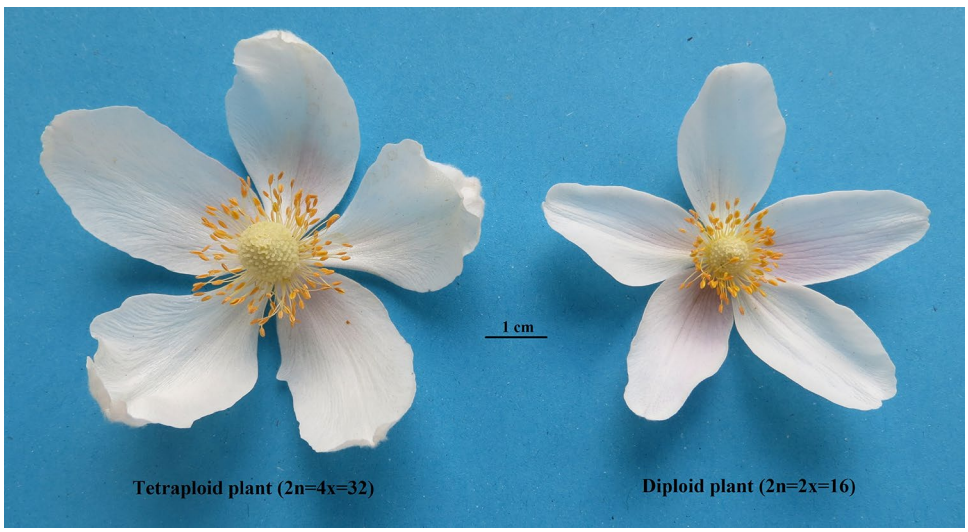

Fig. 2 Morphological variation between diploid (control) and tetraploid flowers of *A. sylvestris* cultivated under field conditions

Fig. 3 Morphological variation between diploid (**a**) and tetraploid (**b**) plants of *A. sylvestris* cultivated under field conditions

Table 5 Comparison between chlorophyll content of diploid and tetraploid plants

Ploidy	Chlorophyll content (SPAD units)	Leaf chlorophyll content (μg cm^{-2})
Diploid	40.68 ± 6.27^{a}	0.44 ± 0.11^{a}
Tetraploid	42.90 ± 5.79^{a}	0.48 ± 0.10^{a}

Data are shown as mean ± SD (standard deviation)

Different superscript letters within the same column differ significantly ($p < 0.05$)

extracts and fractions obtained by maceration and partition from diploid and tetraploid *A. sylvestris* plants were tested to measure the content of polyphenols and their antioxidant activity.

The content of polyphenols was determined by Folin–Ciocalteu assay. Results (Table 6) showed quantitative differences which ranged from 67.1 ± 8.5 to 118.4 ± 2.57 mg GAE g^{-1} dry weight and from 62.7 ± 5.9 to 131.7 ± 13.5 mg GAE g^{-1} dry weight in diploid and tetraploid plants, respectively. Among all extracts and fractions, BuOH and aqueous fractions, derived from methanol extracts, facilitated the extraction of more polyphenolic compounds than others. Comparing the fractions it was observed that the tetraploid plants were richer of polyphenols than the diploid ones.

Generally phenolic compounds are responsible for antioxidant capacity in plants (Condelli et al. 2015). In this study antioxidant properties were investigated by using three different assays. Reducing power was measured using the FRAP assay and results (Table 6) ranged from 25.5 ± 4.0 to 192.3 ± 2.8 mg TE g^{-1} for tetraploid plants and from 21.0 ± 3.1 to 159.4 ± 10.0 mg TE g^{-1} for diploid plants. The BuOH and aqueous fractions had the highest reducing power for both genotypes, but the aqueous fraction derived from tetraploid plants had the highest activity.

Similar results were observed by DPPH radical scavenging activity. Aqueous fractions showed the highest radical-scavenging activity, showing similar values of 116.1 ± 17.6 and 115.3 ± 10.5 mg TE g^{-1} for polyploids and diploids respectively (Table 6). Further, BuOH fractions, reported higher activity than the less polar fractions; and the tetraploid plants reported higher DPPH-scavenging activity than diploid plants.

Lipid peroxidation is a free radical chain reaction that occurs in biological membranes. In this study, lipid peroxidation inhibition was assessed by evaluating the bleaching of β-carotene molecules in the β-carotene linoleate model system. Peroxylradical (LOO·) formed by linoleic acid in the presence of oxygen during incubation at 50 °C reacted with β-carotene to form a stable β-carotene radical. BHT was used as reference standard and only MeOH extracts and their fractions acted to inhibit the lipid peroxidation at the final tested concentration (0.13 mg ml^{-1}). The results were expressed as % AA (Table 6) and the tetraploid MeOH extract was the most efficient in LOO· ($AA = 76.5 \pm 4.1\%$). To compare different results obtained from different antioxidant methods, the relative antioxidant activity index (RACI) was calculated. It is based on calculation assigning equal weight to all antioxidant activity tests together with those obtained from TPC, as it is widely reported as TPCs are strictly related to extract's antioxidant capacity (Fernandes et al. 2013). Results, presented in the histogram (Fig. 4), indicating that the aqueous fractions, followed by the BuOH fractions were the most biologically active. A comparison between the same fractions the investigated plants, revealed that tetraploid plants had higher RACI values than diploid plants. Other studies explored the variability between diploid and polyploid species as reported for the root of *Platycodon grandiflorum* (Boo et al. 2013). In particular, the tetraploid root of *P. grandiflorum* demonstrated higher DPPH-radical scavenging and antioxidant enzyme activity than diploid. Moreover, induced polyploidy can influence the content of phytochemicals in plant; it was demonstrated that in *Zingiber officinale*,

Table 6 Comparison between total polyphenol content (TPC), reducing power (FRAP) and radical scavenging of extracts between diploid and tetraploid *A. sylvestris*

Samples	TPC (mg GAE g^{-1})	FRAP (mg TE g^{-1})	DPPH (mgTE g^{-1})	BCB % AA (0.13 mg ml^{-1})
ASPE	62.7 ± 5.9^{a}	$25.5 \pm 4.0^{a,f}$	$30.1 \pm 4.0^{a,b,f}$	–
ASPC	72.4 ± 8.1^{a}	$51.0 \pm 3.9^{b,d}$	$57.1 \pm 3.5^{b,c}$	–
ASPCM	$85.7 \pm 9.1^{a,b}$	$25.2 \pm 4.0^{a,f}$	$11.1 \pm 0.8^{a,f}$	–
ASPM	65.1 ± 7.1^{a}	$69.2 \pm 8.2^{b,c}$	$54.1 \pm 2.5^{b,c,d}$	76.5 ± 4.1^{a}
ASPM/B (2)	131.7 ± 13.5^{c}	81.5 ± 17.0^{c}	96.3 ± 22.3^{e}	49.0 ± 4.6^{b}
ASPM/W (2)	$116.3 \pm 9.9^{c,d}$	192.3 ± 2.8^{e}	116.1 ± 17.6^{e}	30.7 ± 3.4^{c}
ASDE	67.0 ± 8.5^{a}	$46.9 \pm 3.1^{f,b}$	$16.6 \pm 0.5^{a,d,f}$	–
ASDC	$100.1 \pm 9.2^{b,d,e,f}$	$31.8 \pm 2.7^{a,d,f}$	8.1 ± 0.9^{f}	–
ASDCM	71.0 ± 12.3^{a}	21.0 ± 3.1^{a}	$13.7 \pm 2.7^{a,f}$	–
ASDM	$78.1 \pm 8.1^{a,e}$	137.2 ± 7.6^{g}	$48.8 \pm 7.2^{a,b,c}$	9.5 ± 0.6^{d}
ASDM/B (2)	$118.4 \pm 2.6^{c,f}$	156.2 ± 10.6^{g}	$78.8 \pm 31.4^{c,e}$	–
ASDW (1)	$87.7 \pm 4.5^{a,e}$	159.4 ± 10.0^{g}	115.3 ± 10.5^{e}	71.5 ± 7.6^{a}

Results are expressed as the mean (standard deviation) of three determinations

Different superscript letters denote statistically significant differences in the same column ($p < 0.05$)

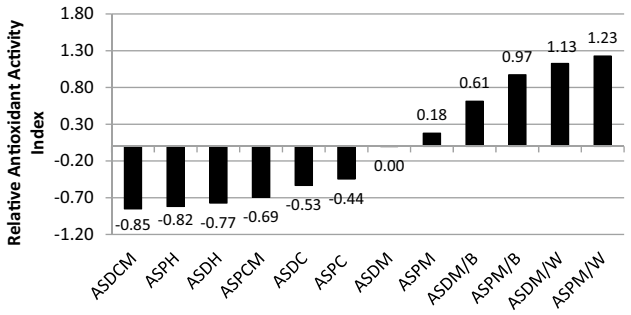

Fig. 4 Comparison of RACI of investigated extracts and fractions of diploid and polyploid *A. sylvestris*. Leaf tissue of diploid (ASD) and tetraploid (ASP) plants extracted with: *n*-hexane (H), $CHCl_3$ (C), $CHCl_3$/MeOH 9:1 (CM) and MeOH (M) BuOH (M/B) and water (M/W)

Table 7 Amplification characteristics of the primers used in the study

Primer name	Number of amplified loci in diploid plant	Number of amplified loci in tetraploid plant
1838	22	17
1854	17	17
1899	20	24
1886	20	17
1897	28	25
2374	17	17
2270	22	22
2274	15	15
2279	18	18

the polyploid plants had the highest content of some phytochemicals and phenolic compounds than diploids (Boo et al. 2013; Sanwal et al. 2010).

Molecular variability analysis based on iPBS marker

To date, no specific DNA markers have been reported for *A. sylvestris* and thus, utilization of the nonspecific markers can be used for the analysis of its genome variability. Here, the variability of the retrotransposons insertion pattern was analysed for the diploid and tetraploid plants of *A. sylvestris* by iPBS. In the past, other nonspecific markers such as RAPD random (amplification of polymorphic DNA) or inter-simple sequence repeat (ISSR) were used for the analysis of genome stability (Tiwari et al. 2013; Žiarovská et al. 2014; Saha et al. 2016). Here, iPBS was applied for testing of the genome stability of induced polyploid plants for the first time. The number of amplified fragment levels obtained by individual primers ranged from 17 to 28 and 45% of them were markers that provide different amplification patterns for diploid and tetraploid plants (Table 7).

The levels of amplified fragments that were achieved in the study are in concordance with the variability of the iPBS fragment amplification that is typical for these techniques. The iPBS method produces on average 15–50 bands from 100 to 5000 bp in length that can be detected on agarose gels (Kalendar et al. 2010). Not all retrotransposons are a part of individual genomes of plants and thus not all the primer binding sites that were generated by Kalendar et al. (2010) can be utilized by iPBS primers to produce amplification patterns. Lapiņa et al. (2012) analysed the genetic variability in Latvian populations of alfalfa. Among 29 used iPBS markers, ten gave monomorphic products and one marker resulted in no amplification. Four of the primers used in the study generated different amplification patterns for diploid and tetraploid plants—1886,

Table 8 Comparison between numbers of specific insertions of retrotransposon sequences detected for diploid and tetraploid plants of *A. sylvestris*

Primer name	Number of unique insertions in diploid plant/length of amplicons	Number of unique insertions in tetraploid plant/length of amplicons
1886	2/780 bp; 450 bp	1/1800 bp
1897	2/560 bp; 320 bp	17
1838	3/990 bp; 620 bp; 530 bp	3/1100 bp; 960 bp; 170 bp
1899	0	2/1500 bp; 1100 bp

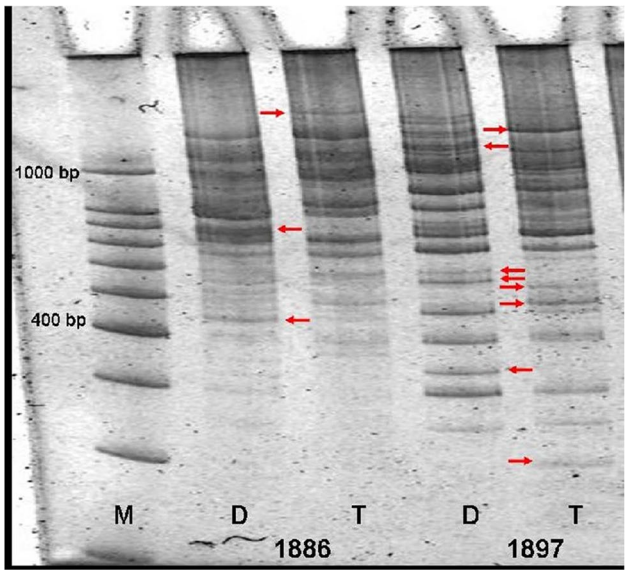

Fig. 5 Electrophoreogram of the iPBS profiles of 1886 and 1897 markers. Regions with the unique bands are marked by arrow. *M* marker, *D* diploid plant, *T* tetraploid plant

1897, 1838 and 1899. Specific unique insertions of different lengths were obtained (Table 8; Fig. 5) and in silico alignment analysis was performed for them. All of them were BLASTed against Ranunculaceae (taxid:3440) in the National Center for Biotechnology Information (NCBI) (Zhang et al. 2000). Different types of transposable element sequences were returned as matched with the used iPBS markers (Table 9).

Here, the amplification pattern of retrotransposons was analysed in *A. sylvestris* under the stress of polyploidization. Transcriptional activation of retrotransposons has been observed in many plant species under different abiotic or biotic stresses. Retrotransposons Tnt1 and Tto1 in tobacco are activated by both abiotic as well as biotic stress (Takeda et al. 1998). In *Solanaceae*, the effect of stress conditions on transcriptional regulation was also reported for the Tnt1 retrotransposon (Grandbastien et al. 2005). Some retrotransposons are active during the developmental stages, as the maize Prem-2 element (Turcich et al. 1996). Voronova et al. (2011) reported the transcriptional activation of many non-specific retrotransposon-like sequences in *Pinus sylvestris* after exposure to heat stress.

The positive amplification of iPBS on cDNA is a result of the non-specific nature of the PBS primers (Kalendar et al. 2010). Transcriptional activation was verified for the groups of retrotransposons with the sequences of primer binding sites that mached the iPBS markers 1886, 1897, 1838 and 1899. In all of them unique amplicons were generated that can be used for the purposes of molecular identification of tetraploid plants of *A. sylvestris* in the future.

In conclusion, a new genotype (tetraploid plants) of *A. sylvestris* with different morphological and biological characteristics was created by in vitro induced chromosome doubling using oryzalin. The new genetic material seems to be a valuable ornamental crop, therefore further experiments proving its horticultural qualities will be carried out. The use of molecular markers will accelerate the breeding progress in this plant.

Table 9 In silico analysis of the iPBS primers that provided different amplification profiles for diploid and tetraploid plants of *Anenome sylvestris*

Primer name	Retrotransposon(-like) sequences matched in the alignment	Species matched in the alignment
1886	Stowaway MITE; transposons Caspar, XJ, Angela, Ophelia2, Jodi, Fred; retrotransposon FL12; Ty1-copia retrotransposon RLC_Lu6	*Aegilops tauschii; Linum usitatissimum*
1897	None	None
1838	Retrotransposon FL1; retrotransposon Cassandra	*L. usitatissimum*
1899	Retrotransposon FL1	*L. usitatissimum*

Acknowledgements This study was supported by Project Nos. 20175009 and 20115003 of University Internal Grant Agency, Czech University of Life Sciences Prague, by European Community under Project Nos. 26220220180 Building Research Centre "AgroBioTech", by Project Nos. 17-25536Y of the Czech Science Foundation and by Silva Tarouca Research Institute for Landscape and Ornamental Gardening (the institutional support VUKOZ-IP-00027073). The authors thank to prof. Jaroslav Doležel and Jana Čížková, Ph.D. from Institute of Experimental Botany, The Czech Academy of Sciences, the Czech Republic, for their help in the flow-cytometric analyses.

Author contributions PZ and EF established in vitro culture, realized induced polyploidization, flow cytometry analyses and morphological observations in vivo. JŠ transferred plants in ex vitro conditions and realized morphological analyses. JŽ was responsible for iPBS analyses. JLRS and DMF assessed chlorophyll content and made statistical analyses. DR and LM assessed chemical analysis and antioxidant activity. EF and LM designed the workflow of the experiments.

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