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An efficient *in vitro* propagation protocol for snowdrop anemone (*Anemone sylvestris* L.)

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Abstract

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This study investigated *in vitro* production of diploid (AS2) and tetraploid (AS4) cytotypes of snowdrop anemone. The effect of plant growth regulators (PGRs) on *in vitro* shoot multiplication and rooting was investigated. The effect of activated charcoal (AC) on root induction was also studied. Ploidy level affected growth characteristics during multiplication and rooting. Shoot induction in AS4 was higher on medium supplemented with cytokinin (3.2–3.6), while the AS2 clone formed the most shoots on PGR-free medium (3.6). The highest rooting percentage was achieved on PGR-free medium in both genotypes (AS2 clone, 100% and AS4 clone, 93.3%). The addition of AC to the PGR media largely increased root induction and root length. Rooted plantlets were successfully acclimatised in the greenhouse with 100% survival. Thus, the described micropropagation protocol represents a rapid and effective *in vitro* propagation method for utilisation in horticulture and conservation programmes of snowdrop anemone.

Keywords: *Ranunculaceae*; tetraploid; rhizogenesis; shoot organogenesis; activated charcoal

The snowdrop anemone (*Anemone sylvestris* L.) is a perennial horticultural plant with attractive, fragrant, open cup-shaped white flowers and expressible golden yellow anthers. This perennial is often grown in small and rock gardens (Hejný, Slavík 1988) or in naturalised and woodland gardens due to spreading by root suckers. It is also grown as a potted plant (Benary 2014). Snowdrop anemone extract contains many biologically active substances, e.g., phenolic compounds and antioxidants, that have medical and pharmaceutical benefits (Maslennikov et al. 2014). The species is classified as threatened in the Czech Republic (Danihelka et al. 2012), France, Germany, Switzerland, Serbia and Belarus (Hoskovec 2007). Changes in dry grassland management and secondary succession of shrubs result in the destruction of *A. sylvestris* habitats (Hroneš 2009). Therefore, determination of optimal conditions for the propagation and development of these plants will be of benefit for the conservation of *Anemone* genetic resources and will facilitate the development of novel genotypes in breeding programmes.

Snowdrop anemones can be propagated by seeds or plant division (Vít et al. 2001). Wild plants propagate only by sexual reproduction. Denisow and Wrzesień (2015) reported the negative effects of shrub expansion on flowering and male function, as well as seed set in three natural populations.

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Propagation through stem cuttings is unsuitable for the snowdrop anemone due to its specific rosette plant morphology. Vegetative propagation of ornamentals is often accomplished by micropropagation. This technique has been proven to be successful for mass propagation, thereby reducing the time needed for the establishment of novel cultivars for the market (Hartmann et al. 2011).

The *in vitro* regeneration of *Anemone* sp. has primarily focused on *A. coronaria* via embryogenesis induced from anther cultures (Laura et al. 2006). Johansson and Eriksson (1977) reported inductions of various embryos from anther cultures in four *Anemone* species (*A. virginiana*, *A. canadensis, A. multifida* and *A. rupicula*). Information related to shoot organogenesis is scant for anemones. Ruffoni et al. (2005) reported shoot induction from the floral stalk of *A. coronaria* on an induction medium supplemented with 2iP (6-(γ,γ-dimethylallylamino) purine). A micropropagation protocol has not yet been developed for *A. sylvestris*. One of the major factors affecting the success of *in vitro* plant propagation is the choice of PGRs (Amoo, Van Staden 2013). Additionally, substances other than PGRs are also used. These substances, such as, e.g., AC, regulate growth processes and are often used in tissue culture to improve cell growth and development (Pan, Van Staden 1998). AC has the ability to modify the composition of the medium and is often added to media at different stages of tissue culture owing to its beneficial effects (Moshkov et al. 2008). AC is often used for *in vitro* rooting. The presence of AC in the medium can have a dual effect on rhizogenesis: altering the composition of the medium and reducing light at the explant base.

The objective of this study was to establish an efficient and reproducible *in vitro* propagation system for two genotypes of *A. sylvestris* that vary at the ploidy level as a step towards their commercialisation.

MATERIAL AND METHODS

Plant material and culture conditions. In all experiments, we used *in vitro* shoots of two genotypes: AS2 (diploid, $2n = 16$) and AS4 (tetraploid, $2n = 32$). The tetraploid culture was generated in previous experiments by *in vitro*-induced polyploidization of shoot explants using oryzalin treatment (Czech University of Life Sciences Prague, Faculty of Tropical AgriSciences, Plant Tissue Culture Laboratory). AS4 is a novel clone with a high potential for the flower market. Stock material of the snowdrop anemone (AS2 and AS4) was maintained and multiplied on full-strength solid MS culture medium including vitamins (Murashige, Skoog 1962; Duchefa Biochemie B.V., The Netherlands) supplemented with 20 g/l sucrose, 2.5 g/l Phytagel^{TM} (Sigma–Aldrich, USA) and 0.5 μM BA (N^6-benzyladenine, Fig. 1A). The pH was adjusted to 5.7 prior to autoclaving. Axillary and adventitious shoots were cultured in 100-ml Erlenmeyer flasks containing 25 ml of medium. For the experiments, three shoots were placed in each glass vessel. The cultures were maintained in a growth room under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 60 μmol/m^2/s provided by cool-white fluorescent tubes (Tungsram; General Electric Co., USA) at 22 ± 1°C.

Newly grown shoot clumps were divided into two or three parts by sub-culturing onto fresh medium (three clumps per vessel) every three to four weeks for one year. Prior to the multiplication experiments, intact clumps were transferred to the same medium but without PGRs for two weeks; then, single shoots were used as the initial explants in the experiments.

Shoot multiplication and rooting experiments were conducted in three replications with 15 shoots per treatment (a total of 45 explants), whereas 25 rooted plants per treatment (a total of 75 shoot explants) were used for the acclimatization experiment.

Shoot multiplication. To evaluate the effect of PGRs on the multiplication rate, single shoots (2–3 expanded leaves, ≥ 1.5 cm long) were excised from the stock cultures (see Plant material) and multiplied on MS medium including vitamins supplemented with different cytokinins (BA, zeatin or kinetin) at different concentrations (0 or 1 μM). The shoot numbers per explant and shoot lengths were evaluated after three weeks.

***In vitro* rooting of regenerated shoots**. Shoot clumps harvested on multiplication media with kinetin and zeatin were transferred to PGR-free medium for two weeks for plant strengthening. Then, they were separated into single shoots (4–5 expanded leaves, ≥ 3 cm long) and placed vertically into full-strength solid WPM including vitamins (Woody Plant Medium; Lloyd, McCown 1980; Duchefa Biochemie B.V., The Netherlands). Media were supplemented with AC (0 or 1 g/l) and the auxin indole-3-butyric acid (IBA) or α-naphthalene acetic acid (NAA) at con-

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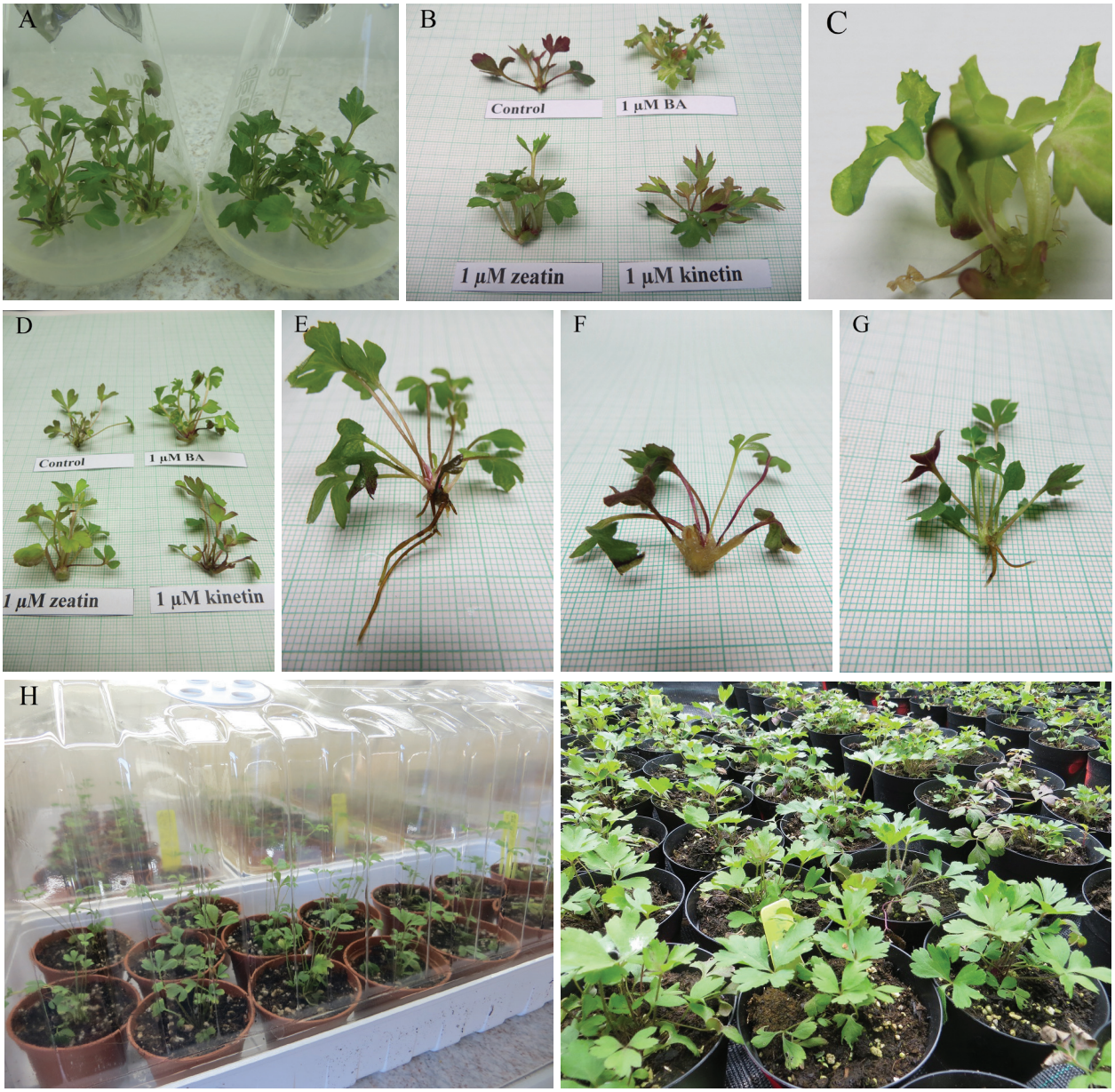

Fig. 1. Plant regeneration of snowdrop anemone. (**A**) bulking of stock material on PGR-free medium (left AS2 clone, right AS4 clone); (**B**) adventitious shoot regeneration on shoot induction media (MS medium with 0 or 1 µM BA, zeatin or kinetin) of the AS4 clone after three weeks; (**C**) detail of malformed leaves of the AS4 clone grown on medium with 1 µM BA; (**D**) adventitious shoot regeneration on shoot induction media (MS medium with 0 or 1 µM BA, zeatin or kinetin) in the AS2 clone after three weeks; (**E**) rooted shoot of the AS4 clone on PGR-free medium after three weeks; (**F**) callus induction in an unrooted shoot of the AS4 clone on medium supplemented with NAA after three weeks; (**G**) rooted shoot of the AS4 clone on medium supplemented with NAA and AC after three weeks; (**H**) acclimatisation of rooted plantlets in the culture room; (**I**) young plants in the cold frame

centrations of 0 or 3 µM alone or in combination with 0 or 0.2 µM BA for root induction. Rooting (expressed as the percentage of shoots producing roots, the number of roots per shoot and the root length) was measured using a binocular microscope (Carl Zeiss Jena, Germany) after three weeks.

Acclimatisation of rooted plants. For the acclimatisation experiments, we used rooted plantlets from media supplemented with auxin IBA (0 and 3 µM) and AC (0 and 1 g/l). Five days prior to plantlet transfer to *ex vitro* conditions, the aluminium foil caps of the Erlenmeyer flasks were perforated in

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four places (approximately 2-mm diameter holes) using forceps to promote better adaptation of the *in vitro*-rooted plantlets. Rooted plantlets were taken from the culture vessels, the phytagel medium was removed from the roots with tap water and they were transferred into plastic pots (Teku®; Pöppelmann, Germany, 6.0-cm diameter) containing steam peat-perlite substrate (1:1; *v*/*v*) and watered with tap water. The pots with plantlets were placed in boxes covered with clear plastic covers (low and high forms; FIMA Czech Republic) and transferred to a growth room with cool-white fluorescent tubes (Tungsram) and a PPFD of 60 μmol/m^2/s for a 16 h photoperiod at 24/19 ± 1°C (day/night) for four weeks. For the first two weeks, the plantlets were incubated under 100% humidity (boxes with a low cover, 47 × 20 × 10 cm); then, the humidity was reduced in exchange for high covers with a vent (47 × 20 × 20 cm, Fig. 1H). After four weeks in the boxes, the plants were replanted in larger pots (Teku® 8.0-cm diameter) with a peat substrate Remix-D (Rėkyva, Siauliai, Lithuania) and transferred to a greenhouse at 18/15°C (day/night). The plants were fertilised with 0.05% Kristalon® Blue every week. After six weeks in the greenhouse, the plants were replanted again in larger pots (Teku® 11.0 cm diameter) with the peat substrate and transferred to a cold frame. The plants were shadowed for two weeks and then exposed to the sun. Survival percentages of plantlets were recorded 10 weeks after the transfer from *in vitro* conditions.

Statistical analysis. A randomized complete block design was used for all experiments. Data were compared using multiple-factor analysis of variance (ANOVA). Mean comparisons among treatments were made using Duncan's test ($P \leq 0.05$). Additionally, the interaction between the factors for both shoot regeneration (genotype and PGRs) and root formation (genotype, PGRs and AC) was evaluated using Tukey's non-additivity test (Tukey 1949). Data were analysed using GLM in R (a language and environment for statistical computing; R Core Team 2015).

RESULTS AND DISCUSSION

Shoot multiplication

The effect of different cytokinins (BA, kinetin and zeatin) and PGR-free medium on shoot multiplication was examined. Shoot induction was observed on all media. The multiplication rate depended on the presence and the type of the cytokinin in the medium (Fig. 1B and D; Table 1). The AS4 clone produced significantly more new shoots on MS medium supplemented with a cytokinin compared to PGR-free medium. The highest number of shoots/explant was achieved on kinetin (3.6 shoots/explant) and the lowest number on PGR-free medium (2.5 shoots/explant). There were moderate differences in shoot induction among cytokinins (3.2–3.6 shoots/explant). In contrast, the AS2 clone formed the most shoots/explant on the PGR-free medium (3.6). Elongation of shoots in the AS2 clone was significantly supported by the addition of cytokinin to the medium. Zeatin was found to be the most effective cytokinin in promoting shoot elongation in both genotypes; the shoot length in the AS2 clone reached 26.2 mm and in the AS4 clone reached 22.9 mm.

The number of developed shoots was significantly dependent on the interaction of the genotype and PGRs ($F = 6.14$, $P = 0.001$). Additionally, the shoot length was also significantly dependent

Table 1. Effect of cytokinins (BA, kinetin and zeatin) on adventitious shoot regeneration of two snowdrop anemone clones (AS2 and AS4) after three weeks in culture

BA	Kinetin	Zeatin	AS2 clone			AS4 clone		
Plant growth regulator (μM)			No. shoots/explant	Shoot length (mm)	Callus	No. shoots/explant	Shoot length (mm)	Callus
0	0	0	3.6 ± 0.2^b	19.6 ± 0.7^a	N	2.5 ± 0.2^a	18.2 ± 0.6^a	N
1	0	0	3.2 ± 0.2^b	22.9 ± 0.7^b	N	3.4 ± 0.3^b	19.0 ± 0.5^a	C
0	0	1	2.6 ± 0.2^a	26.2 ± 0.9^c	C	3.2 ± 0.2^b	22.9 ± 0.7^b	C
0	1	0	3.0 ± 0.2^{ab}	22.3 ± 0.8^b	N	3.6 ± 0.2^b	18.3 ± 0.5^a	N

values are means ± standard error; significantly different treatment means are indicated using different letters within a column according to Duncan's test, at $P \leq 0.05$; N – no callus production; C – callus only formed at the base of shoot explant

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Table 2. Effect of different factors (genotype and medium composition) on shoot regeneration of snowdrop anemone

Source of variation	No. shoots/explant				Shoot length (mm)			
	df	Mean squares	*F*		df	Mean squares	*F*	
Genotype	1	0.711	0.33	ns	1	2,839.2	45.94	***
PGRs	3	4.256	1.99	ns	3	1,388.8	22.47	***
Genotype × PGRs	3	13.148	6.14	***	3	98.8	1.60	ns
Error	352	2.142			1122	61.8		

*, **, ***, ns – *F*-test significant at $P = 0.05$, $P = 0.01$, $P = 0.001$ or not significant; PGR – plant growth regulator

on the genotype ($F = 45.94$, $P = 0.001$) and PGRs ($F = 22.47$, $P = 0.001$) (Table 2) but not on the interaction of these variables.

Most species require supplementation of the medium with PGRs to induce shoot organogenesis. However, plant species in a few genera, such as *Populus* (Douglas 1984) and *Brunsvigia* (Rice et al. 2011), can form adventitious shoots on PGR-free medium. Growth regulator requirements can vary among species and even cultivars, but most species require a cytokinin, auxin, or both (Ďurkovič et al. 2012). Differences in the *in vitro* response between genotypes may be related to differences in endogenous plant growth regulator content (Šedivá et al. 2013a).

Supplementation with growth regulators is required by many members of the family Ranunculaceae; BA is the most common PGR added during the multiplication phase, often in combination with other growth regulators (Beruto, Debergh 2004; Šedivá, Kubištová 2008; Naumovski et al. 2009). However, a different demand for growth regulators was demonstrated in the present multiplication experiments in the snowdrop anemone. The quality of the snowdrop anemone shoots was determined by the type of cytokinin and the genotype. Shoots from media supplemented with BA were inferior to those supplemented with zeatin and kinetin. Hyperhydricity and malformed leaves were observed in the AS4 clone, although BA was applied at a relatively low level (1 μM) (Fig. 1C). Moreover, red leaves (under stress conditions) were observed in this clone when it was grown in PGR-free medium (Fig. 1B). Cytokinins have been shown to induce hyperhydricity in many species, usually in a concentration-dependent manner when other conditions in the culture system are not optimised (Ivanova, Van Staden 2008). The aromatic cytokinin BA remains the most widely used cytokinin in commercial micropropagation worldwide, but it may have several side effects, such as difficulty in rooting, toxicity, hyperhydricity, stunted shoots and callus formation (Magyar-Tábori et

Table 3. Effect of different factors (genotype and medium composition) on root regeneration of snowdrop anemone shoots

Source of variation	Rooting (%)				No. of roots per shoot				Mean root length (mm)			
	df	Mean squares	*F*		df	Mean squares	*F*		df	Mean squares	*F*	
Genotype	1	62.3	56.64	***	1	147.41	75.98	***	1	76.4	4.13	*
PGRs	5	88.4	80.36	***	5	74.77	38.54	***	5	135.9	7.35	***
AC	1	425.3	386.64	**	1	151.88	78.29	***	1	2493.1	134.76	***
Genotype × PGRs	5	4.6	4.18	**	5	8.75	4.51	***	5	41.2	2.23	*
PGRs × AC	5	81.4	74.0	***	5	65.94	33.99	***	5	186.4	10.08	***
Genotype × AC	1	3.1	2.82	ns	1	3.45	1.78	ns	1	11.7	0.63	ns
Genotype ×PGRs ×AC	5	4.6	4.18	**	5	18.95	9.77	***	5	5.5	0.30	ns
Error	48	1.1			1056	1.94			1708	18.5		

*, **, ***, ns – *F*-test significant at $P = 0.05$, $P = 0.01$, $P = 0.001$ or not significant; PGR – plant growth regulator; AC – aclivated charcoal

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Table 4. Effect of growth regulators (IBA, NAA and BA) and AC on root formation in shoots of snowdrop anemone (AS2 and AS4 clones) after three weeks in culture

IBA	NAA	BA	AC (0 g/l)				AC (1 g/l)			
Plant growth regulator (μM)			Rooting (%)	No. of roots/ shoot	Root length (mm)	Callus	Rooting (%)	No. of roots per shoot	Mean root length (mm)	Callus
AS2 clone										
0	0	0	100 ± 0.0^{d}	3.0 ± 0.2^{bc}	7.4 ± 0.4^{c}	N	91.1 ± 7.6^{a}	3.1 ± 0.3^{b}	8.1 ± 0.4^{a}	N
3	0	0	99.6 ± 2.2^{d}	3.7 ± 0.4^{c}	4.3 ± 0.2^{ab}	N	91.1 ± 7.6^{a}	2.6 ± 0.2^{b}	8.5 ± 0.5^{a}	N
0	3	0	24.4 ± 2.2^{a}	1.6 ± 0.2 ^{a}	2.8 ± 0.3^{a}	C	73.3 ±10.2^{a}	1.8 ± 0.2^{a}	7.3 ± 0.6^{a}	N
3	0	0.2	40.0 ± 3.8^{b}	2.2 ± 0.3^{ab}	3.7 ± 0.2^{a}	N	88.9 ± 0.0^{a}	3.0 ± 0.3^{b}	7.4 ± 0.4^{a}	N
0	3	0.2	24.4 ± 2.2^{a}	1.5 ± 0.3^{a}	3.0 ± 0.2^{a}	C	88.9 ± 6.7^{a}	3.3 ± 0.2^{b}	8.4 ± 0.4^{a}	N
0	0	0.2	62.2 ± 4.4^{c}	2.0 ± 0.3^{ab}	5.5 ± 0.4^{b}	N	91.1 ± 2.2^{a}	2.6 ± 0.3^{b}	7.7 ± 0.4^{a}	N
AS 4 clone										
0	0	0	93.3 ± 0.0^{d}	3.3 ± 0.3^{b}	8.2 ± 0.5^{b}	N	82.2 ± 2.2^{bc}	2.0 ± 0.2^{a}	8.2 ± 0.7^{ab}	N
3	0	0	77.8 ± 4.4^{c}	2.2 ± 0.3^{ab}	4.7 ± 0.3^{ab}	N	77.8 ± 4.4^{bc}	2.7 ± 0.2^{b}	8.7 ± 0.5^{b}	N
0	3	0	6.7 ± 0.0^{a}	1.3 ± 0.3^{a}	2.8 ± 0.5^{a}	C	88.9 ± 2.2^{c}	2.0 ± 0.2^{a}	7.3 ± 0.6^{ab}	N
3	0	0.2	35.6 ± 0.0^{b}	1.3 ± 0.1^{a}	4.5 ± 0.5^{ab}	N	73.3 ± 6.7^{ab}	1.9 ± 0.2^{a}	8.1 ± 0.6^{ab}	N
0	3	0.2	8.9 ± 2.2^{a}	1.5 ± 0.3^{a}	3.7 ± 0.4^{a}	C	60.0 ± 3.8^{a}	1.7 ± 0.2^{a}	6.6 ± 0.5^{a}	N
0	0	0.2	28.9 ± 6.7^{b}	1.2 ± 0.1^{a}	4.8 ± 0.7^{ab}	N	84.4 ± 5.9^{bc}	2.0 ± 0.2^{a}	7.4 ± 0.4^{ab}	N

values are means ± standard error; significantly different treatment means are indicated using different letters within a column according to Duncan's test, at $P \leq 0.05$. N – no callus production; C – callus production

al. 2010). Reducing or overcoming hyperhydricity can be achieved by either a change of the cytokinin type (Sharma, Mohan 2006), or the cytokinin concentration (Ivanova, Van Staden 2008) or the addition of auxin to the medium (Šedivá et al. 2013b). Callus induction was observed in both clones grown on the medium supplemented with zeatin and in the AS4 clone grown on the medium supplemented with BA (Table 1). However, callus formation was low and only observed at the base of the shoot.

Rooting and acclimatisation

All individual factors (genotype, PGRs and AC) had significant effects on all growth characteristics of root regeneration: rooting, number of roots per shoot and root length at different significance levels (Table 3). The effect of the interaction between two factors was found to be significant for genotype × PGRs and PGRs × AC for all growth characteristics. The effect of the interaction among all factors was found to be significant for rooting and the number of roots per shoot.

Root formation occurred in all experiments regardless of the presence of growth regulators in the WPM medium but varying rooting efficiencies were observed (Table 4). Rhizogenesis was observed even in the presence of cytokinin alone. The highest rooting percentage was achieved on PGR-free medium for both genotypes (100% for the AS2 clone and 93.3% for the AS4 clone) (Fig. 1E). Good root induction was also achieved with IBA (99.6% for the AS2 clone and 77.8% for the AS4 clone). In the AS4 clone, the highest root number and root length were recorded on the PGR-free medium (3.3 roots/shoot, 8.2 mm). In the AS2 clone, the highest root length was observed on the PGR-free medium (7.4 mm), but the highest root number was reached on the medium supplemented with IBA (3.7 roots).

For the two examined auxins, IBA promoted significantly more root induction relative to NAA. Supplementation of the medium with the cytokinin BA alone had a significantly more favourable effect on root induction (62.2% in the AS2 clone and 28.9% in the AS4 clone) compared to NAA (24.4% in the AS2 clone and 6.7% in the AS4 clone) or a combination of BA + NAA (24.4% in the AS2 clone

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and 8.9% in the AS4 clone). Overall, NAA alone or in combination with BA induced rooting at the lowest frequency and resulted in an increase of calluses (Fig. 1F; Table 4). Generally, the inclusion of PGRs (auxin, cytokinin or both) significantly reduced root induction, except on medium supplemented with IBA for the AS2 clone.

Most plants require the presence of auxins for efficient root regeneration (Pierik 1989). The *in vitro* rooting process is difficult in many species of the family Ranunculaceae, and the addition of auxin is necessary for *in vitro* root formation, e.g., in *Pulsatilla vernalis* (Šedivá 2012), *P. pratensis* (Naumovski et al. 2009) and *Ranunculus asiaticus* (Beruto, Debergh 2004), or for auxin-improved rooting efficiency e.g. in *Helleborus niger* (Seyring 2002) and *Pulsatilla koreana* (Lin et al. 2010). The choice of auxin depends on the Ranunculaceae species. For example, a high rooting ratio was achieved on medium supplemented with IBA in *H. niger* (Seyring 2002) and in *P. vernalis* (Šedivá 2012), and with NAA in *P. koreana* (Lin et al. 2010). The presence of NAA in the rooting medium of *A. sylvestris* had a negative impact, which is in accordance with the finding of Naumovski et al. (2009) in *P. pratensis,* where a very low percentage of rooting was observed on full-strength MS media supplemented with NAA regardless of the concentration. The synthetic auxin NAA is often converted to conjugates (mainly glucosyl esters) after uptake into plant tissues. This reversible conjugation may regulate the levels of the free active substance (Machackova et al. 2008). In contrast to NAA, IBA can be deactivated up to a point by oxidation (De Klerk al. 1999).

The duration of the inductive phase can vary. Seyring (2002) achieved the highest rooting percentage for the continuous cultivation of *H. niger* on rooting medium supplemented with 4.9 μM IBA compared to exposure of the shoots to a high level of IBA (75 μM) in the medium for 10 days, followed by transfer to PGR-free medium where the rooting was significant lower. Šedivá (2012) reported optimum rooting for incised shoots of *P. vernalis* grown on induction half-strength woody plant perlite medium with 20 mg/l IBA for three weeks, followed by transfer to an expressive medium.

However, the nature of the interaction between exogenous and endogenous PGRs remains unclear (Ruffoni, Savona 2013). Root formation in microcuttings is induced in medium with a high auxin-to-cytokinin concentration ratio or no cytokinins (Hartmann et al. 2011). Nevertheless, cytokinins can sometimes induce or promote root growth, e.g., in *Fraxinus excelsior* (Schoenweiss, Meier-Dinkel 2005). In snowdrop anemones, the frequency of root formation was higher in the presence of the cytokinin BA alone compared to some auxin treatments (Table 4).

When AC was added to the root medium, the rooting percentage ranged from 73.3% to 91.1% in the AS2 clone and 60–88.9% in the AS4 clone. In the AS2 clone, this same highest rooting percentage (91.1%) was achieved on PGR-free medium or on PGR media supplemented with IBA or BA. In the AS4 clone, the highest rooting percentage was recorded on medium supplemented with NAA (88.9%). Generally, the interaction of AC with PGRs mostly increased root formation, root number and root length compared to PGR treatments only. Furthermore, differences among treatments were smaller (Table 4). Supplementation of the medium with AC suppressed callusing of the shoots (Fig. 1G; Table 4). The main effects of AC are the adsorption of undesirable/inhibitory substances, growth regulators and other organic compounds and the release of growth promoting substances present in or adsorbed by AC. Its addition to culture medium may promote or inhibit *in vitro* growth depending on the species and tissues used (Pan, Van Staden 1998). The effect of AC on growth regulator uptake is still unclear (Thomas 2008).

Acclimatised snowdrop anemone plants (AS2 and AS4 clones) in the controlled environment room (Fig. 1H) showed 100% survival and normal growth and development after transfer to the greenhouse (Table 5) and the cold frame (Fig. 1I). Plants exhibited a 100% survival rate regardless of the treatment during the *in vitro* root stage. We acclimatised snowdrop anemone plantlets from treatments (0 or 3.0 μM IBA and 0 or 1.0 g/l AC for a total of 300 plants per clone) that resulted in a high percentage of rooting and high-quality roots. For easier transfer from *in vitro* to *ex vitro* conditions, the aluminium foil caps of the culture vessels containing the *in vitro* rooted plantlets were perforated. De Klerk (2002) reported that the conditions during the *in vitro* rooting treatment may have a tremendous effect on performance after *ex vitro* transfer.

In conclusion, a successful *in vitro* adventitious shoot regeneration, rooting, and plant acclimatisation protocol for *Anemone sylvestris* was devel-

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Table 5. Acclimatisation of plantlets of two snowdrop anemone clones (AS2 and AS4) subjected to various *in vitro* rooting conditions after 10 weeks in cultivation

Plant growth regulator (μM)	AS2 clone		AS4 clone	
IBA	AC (0 g/l)	AC (1 g/l)	AC (0 g/l)	AC (1 g/l)
Survival rate (%)				
0	100 ± 0.0[a]	100 ± 0.0[a]	100 ± 0.0[a]	100 ± 0.0[a]
3	100 ± 0.0[a]	100 ± 0.0[a]	100 ± 0.0[a]	100 ± 0.0[a]

means ± standard error; significantly different treatment means are indicated using different letters within a column according to Duncan's test, at $P \leq 0.05$

oped. The results showed that snowdrop anemones required no PGRs during root induction. The ploidy level affected the multiplication and rooting processes. This protocol will be used in future studies to evaluate the impact of ploidy on aesthetics, production features and secondary metabolite content, which are characteristics that are important for floriculture and pharmaceutical uses. Thus, based on the total acclimatisation of all plants, we can conclude that the micropropagation protocol described here was successful.

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