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Regeneration of different *Cyclamen* species via somatic embryogenesis from callus, suspension cultures and protoplasts

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ABSTRACT

The present study is the first report of the establishment of embryogenic callus cultures from seedling tissue, the regeneration of plants via somatic embryogenesis and the development of a regeneration system from protoplast to plant, using three wild species of *Cyclamen*, *Cyclamen graecum* Link, *Cyclamen mirabile* Hildebrand, *Cyclamen trochopteranthum* Schwarz (syn. *Cyclamen alpinum* hort. Dammann ex Sprenger). The ability to form embryogenic callus and to regenerate via somatic embryogenesis was strongly genotype-dependent for each species. From 0.5 g callus, up to 1461 somatic embryos were formed in the case of *C. mirabile*. Culture media with different concentrations of plant growth regulators, $CaCl_2$ and activated charcoal significantly influenced embryo formation in this species. Up to 1.4×10^6 protoplasts were isolated from 1 g of *C. graecum* cell suspension. Diverse growth responses of the protoplasts in two embedding agents, agarose and alginate, were observed for the different *Cyclamen* species. These specific growth characteristics could be used as a selection marker for future fusion experiments. From both protoplast culture systems, somatic embryos were regenerated, grown to plantlets and acclimatised to greenhouse conditions.

1. Introduction

The genus *Cyclamen* (Myrsinaceae) consists of about 22 species (Grey-Wilson, 2003) that are mainly distributed along the Mediterranean basin and surrounding countries. Some species have been cultivated in Western European countries since the 18th century, but the only species that has gained worldwide economic importance is *Cyclamen persicum* Miller. With some exceptions, such as *C. coum* Miller and *C. hederifolium* Aiton, these species are rarely cultivated because they grow slowly and have widely unknown cultivation requirements. *Cyclamen graecum* Link is found in parts of Greece and its isles. *Cyclamen mirabile* Hildebrand as well as *Cyclamen trochopteranthum* Schwarz (syn. *Cyclamen alpinum* hort. Dammann ex Sprenger) are endemic to South Western Anatolia, Turkey (Grey-Wilson, 2003). The three species were chosen for some valuable traits, which are not found in *C. persicum* cultivars. *C. graecum* has a high variance in leaf shapes, sizes and patterns and has been proven to bear a high disease resistance (Grey-Wilson, 2003; Ishizaka, 2008). *C. mirabile* has characteristic pink leaf patterns (Grey-Wilson, 2003), while *C. alpinum* flowers are outstanding by a unique flower shape. All three species are characterized by a better cold hardiness compared to *C. persicum* (Ishizaka, 2008). All these characteristics would be desirable traits for *Cyclamen* breeders for the improvement of the existing and the development of new ornamental crops.

Yesson and Culham (2006) presented a phyloclimatic study that showed the high impact of global climate change on the natural habitat of these species. According to the authors' calculations, the natural habitat of cyclamen will decline and almost all of the wild species, such as *C. mirabile*, *C. graecum* and *C. alpinum*, will be endangered in about 40 years. Conservation of these species could be accomplished by seed storage, but the establishment of in vitro culture methods may also help to preserve these species and to utilise their gene pools for *Cyclamen* breeding. Thus, the increasing interest of scientists and professionals for these species might inform the public about the value of this beautiful genus and its habitat. First approaches have been made in propagating other wild cyclamen species than *C. persicum* in vitro via shoot regeneration and somatic embryogenesis (Prange et al., 2008; Seyring et al., 2009).

In *C. persicum*, classical breeding methods have led to horticulturally valuable and popular varieties. Breeding efforts have not been as extensive with the other species from this genus. Nevertheless, they have desirable and valuable properties, such as cold hardiness, flower fragrance and attractive leaf shapes. Currently,

Abbreviations: 2iP, 6-(γ,γ-dimethylallylamino)purine; 2,4-D, 2,4-dichlorophenoxyacetic acid; pcv, packed cell volume; PGR, plant growth regulator.

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the attempts to crossbreed *C. persicum* with other *Cyclamen* species by classical breeding techniques have not been successful. Since 1990, hybrids between *C. persicum* and up to 10 *Cyclamen* wild species have been produced via embryo rescue (Ishizaka, 2008). However, in some cross-combinations, like *C. persicum* × *C. coum* and *C. persicum* × *C. alpinum*, no hybrid embryos were obtained so far (A. Ewald, personal communication).

Cross-incompatibility can be overcome by somatic hybridisation using protoplast fusion. Moreover, it may be interesting to study maternal inheritance of somatic hybrids (e.g., *C. persicum* × *C. graecum* and *C. persicum* × *C. mirabile*) and to compare them to hybrids obtained by embryo rescue. Due to the combination of two complete diploid genomes, the likelihood of obtaining fertile hybrids may also be enhanced in somatic hybrids. For protoplast fusion, the regeneration of plants from protoplasts is often one of the bottlenecks of the system. Winkelmann et al. (2006) and Prange et al. (2010) described protoplast to plant regeneration systems for *C. persicum* and *C. coum*, respectively, using somatic embryogenesis. The establishment of in vitro culture techniques will enable systematic micro-propagation and preservation of these putatively endangered species. Moreover, it will allow to integrate these species in breeding programs with *C. persicum*, especially if advanced breeding techniques like protoplast fusion are aspired.

In this study, we adapted these protocols for the three species *C. graecum*, *C. mirabile* and *C. alpinum*. In detail, the aims were (i) to establish callus cultures of *C. graecum*, *C. mirabile* and *C. alpinum*, (ii) to adapt a regeneration protocol via somatic embryogenesis for these three species, and (iii) to establish and optimise a protoplast to plant regeneration system as a prerequisite for somatic hybridisation.

2. Materials and methods

2.1. Establishment of callus cultures

To establish the embryogenic callus cultures, seeds of *C. graecum*, *C. mirabile* and *C. alpinum* (provided by the Cyclamen Society and the Leibniz-Institute of Vegetable and Ornamental Crops Grossbeeren/Erfurt, Germany) were sterilised with sodium hypochlorite (1%) and germinated under sterile conditions as described previously (Prange et al., 2008). Ten seeds (to encompass different genotypes) per species were used. Seedlings of about 1.5 cm in size were separated into the cotyledon, tuber and roots, cut into 2–3 mm explants and incubated on 2.1.S medium in the dark at 24 ± 1 °C. This medium was identical to the half strength MS (Murashige and Skoog, 1962)-based callus induction medium described by Winkelmann et al. (1998a,b) and Prange et al. (2008), which contained 2.0 mg L^{-1} 2,4-D and 0.8 mg L^{-1} 2iP as plant growth regulators (PGRs) and 3.7 g L^{-1} Gelrite. Explants and developing calluses were transferred to fresh medium twice after 8 weeks and then incubated without subculturing until the formation of embryogenic cultures or somatic embryos were observed. In some experiments, the callus of a *C. persicum* cultivar ('Maxora light purple', Varinova, The Netherlands) was used as a reference. This callus was obtained from ovules using the protocol of Schwenkel and Winkelmann (1998).

2.2. Callus growth

Callus growth was determined according to Prange et al. (2010) by culturing 0.5 g of embryogenic culture on 2.1.S or 2.31.S (which was identical to 2.1.S but contained 1.0 mg L^{-1} 2,4-D and 0.4 mg L^{-1} 2iP) media for 4 weeks in the dark at 24 ± 1 °C and measuring the increase in the fresh weight during this period. The experiment was performed twice with four replicates (Petri dishes) per variant.

2.3. Establishment and growth of embryogenic suspension cultures

The induced callus cultures were used to start embryogenic suspension cultures as described by Schwenkel and Winkelmann (1998) and Winkelmann et al. (1998a) using the 2.1.L and 2.31.L (identical to the 2.1.S and 2.31.S media but without Gelrite) media. In stably growing suspensions, the packed cell volume (pcv) was determined every 2–3 days in scaled 1.5 mL reaction tubes (Winkelmann et al., 1998a) over a period of 28 days. The growth rates (μ) were calculated based on the linear phase of the growth curves and calculated as the slope of the linear regression with μ [Δpcv/d] (according to Winkelmann et al. (2001)). The experiment was performed with four replicates per variant and one repetition.

2.4. Regeneration and germination of somatic embryos

Callus cultures of *C. graecum* and *C. persicum* were maintained on 2.1.S solid medium for more than 12 months and the callus cultures of *C. alpinum* and *C. mirabile* were maintained for only about 4 months, when the experiments were performed. The induction of somatic embryos from embryogenic calluses was optimised using six different solid media based on 2.1.S medium (callus induction medium after Schwenkel and Winkelmann, 1998) without PGRs (=2.2.S). The tested culture media differed in the concentrations of PGRs, calcium, activated charcoal and ammonium nitrate: 2.2.S, 2.25.S (2× $MgSO_4$, 2× $CaCl_2$ and 2× microelements; 3.0 g L^{-1} Gelrite for solidification), 2.16.S (0.8 mg L^{-1} kinetin), 2.40.S (0.25× NH_4NO_3), 2.41.S (3× $CaCl_2$; 1 g L^{-1} activated charcoal), and 2.42.S (1 g L^{-1} activated charcoal; detailed composition for these media in Prange et al. (2010)). Three different callus lines, i.e., callus cultures of the same genotype cultivated in different vessels, were ground and divided into six 0.5-g portions and plated onto the six different media. The number of embryos that differentiated from 0.5 g of callus was counted after 8 weeks. One hundred single embryos per variant were transferred to fresh medium with the same respective composition to test the ability to germinate. After 8 weeks of cultivation in the dark at 22–24 °C, germinated embryos (embryos with elongation of the cotyledon of about 0.8–1.0 cm) were counted. Embryos were separated and 10–20 somatic embryos were cultivated per vessel until the largest embryo reached a cotyledonal length of 1.5 cm. They were then transferred from darkness to a light/dark cycle of 16/8 h with cool white fluorescent light at a photon flux density of 25–40 μmol m^{-2} s^{-1}. Plantlets with at least one leaf were acclimatised to greenhouse conditions: After washing off the culture medium they were transferred to pricking substrate and preventively sprayed once with a fungicide. For 14 days they were kept at high humidity under foil, before they were cultivated in a greenhouse at 18/16 °C day and night temperature. After about 2 months the young plants were potted in 10 cm pots.

2.5. Protoplast isolation and regeneration to plants

Suspension cultures were established as described above and used for protoplast isolations 8–12 days after subculture. Protoplast isolation and culture was performed as described by Prange et al. (2010). Two enzyme solutions and two different amounts of initial material were compared to optimise the protoplast yield: 0.5 or 1.0 g of cell suspension was incubated in 5 mL of enzyme solution E1 (2.0% Cellulase R-10 and 0.5% Macerozyme R-10; Duchefa, The Netherlands) or E2 (3.0% Cellulase R-10 and 1.0%

Table 1
Genotypes with formation of friable and embryogenic callus in the three different *Cyclamen* species.

Species	No. of genotypes (out of 10)	Explant types with (+) and without (−) formation of embryogenic and friable callus			Callus morphology
		Cotyledon	Tuber	Roots	
C. mirabile	1	+	−	−	Brown, friable topped with somatic embryos
C. alpinum	2	+	+	+	Brown, friable topped with somatic embryos
C. graecum	1	+	+	+	White-yellow, more creamy than friable, no formation of embryos or other differentiation

Macerozyme R-10). Two to seven protoplast isolations with one to seven repetitions per variant were evaluated. The number of independent isolation experiments (n) is indicated in the figures and tables.

Protoplast sizes were evaluated by measuring the diameters of 50 protoplasts from 2 independent isolations by light microscopy immediately after isolation in isolation buffer.

Protoplasts were cultivated at a final density of 1.5×10^5 cells per mL. Their culture conditions were optimised by comparing two different embedding matrices (alginate and agarose) and two different culture media (8pmC.1 and 8pmC.2). These culture media had the same composition as 8pm7 medium [Winkelmann and Grunewaldt (1995), which was modified KM8p from Kao and Michayluk (1975)], except that the concentrations of PGRs were 1 mg L^{-1} 2,4-D and 0.4 mg L^{-1} 2iP (8pmC.1) and 0.5 mg L^{-1} 2,4-D and 0.2 mg L^{-1} 2iP (8pmC.2). To embed the protoplasts in alginate films, 2.3% sodium alginate was mixed in a 1:1 ratio with 0.5 M mannitol containing the protoplasts, polymerised on $CaCl_2$ agar [0.02 M $CaCl_2$, 0.48 M mannitol, 16 g L^{-1} Plant Agar (Duchefa, The Netherlands)] and cultured in 8pmC.1 or 8pmC.2 medium, respectively. To embed the protoplasts in agarose lenses, 8pmC.1 or 8pmC.2 medium containing the protoplasts was mixed with 3% LM agarose (Biozym Scientific GmbH, Germany) heated to 50 °C in a 2:1 ratio. This mixture was pipetted in lenses in 5.5 cm Petri dishes according to Binding et al. (1988), solidified for about 1 h and cultivated in the dark.

Cell division frequencies were determined 7 and 14 days after protoplast isolation by counting an average of 150 protoplasts per Petri dish in different areas under the microscope. Two to three different isolation experiments with one to six repetitions per variant were evaluated. Proliferating calluses with about 1 mm diameters were transferred from the protoplast films/lenses onto 2.31.S medium for further callus growth. After 8 weeks, calluses with diameters of at least 3 mm were transferred to 2.25.S medium in plastic containers with a total volume of 250 mL for differentiation and further development of the somatic embryos. Embryos of about 2–5 mm in size were separated and 10–20 somatic embryos were cultivated per vessel until the largest embryo reached a cotyledonal length of 1.5 cm. Transfer to a light/dark cycle and acclimatisation to greenhouse conditions were performed as described for somatic embryos (see above).

2.6. Statistics

Data were analysed using the analysis of variance (ANOVA) followed by the Tukey test, if several treatments were compared (Sahai and Ageel, 2000). The arcsine-transformation was applied to the percentage data to stabilise variance, and the log-transformation was used when the data were not normally distributed. When the number of replicates differed among variants, generalised linear models with logit-link and the quasibinomial family were estimated, followed by an analysis of deviance (McCullagh and Nelder, 1989) and pairwise comparisons as described by Hothorn et al. (2008). All statistical analyses were performed using R-2.8.1 (R Development Core Team, 2008).

3. Results

3.1. Establishment of embryogenic callus cultures from seedling tissue

In all three species, most of the seedling explants showed callus growth or enlargement of the explants within the first 8 weeks. The developing calluses were mostly hard and compact and showed no formation of somatic embryos after transfer to 2.2.S medium (medium without PGRs). After about 4 months, many explants had died or turned brown. However, some explants from all three species not only formed hard, but also friable embryogenic calluses and in some cases somatic embryos (Table 1, Fig. 1). Formation of these callus types under the tested conditions was strongly genotype-dependent and found in only 1 or 2 out of the 10 genotypes per species (Table 1). The calluses were continuously cultivated on 2.1.S and 2.31.S media and subcultured monthly.

3.2. Growth of embryogenic callus and suspension cultures

The use of the smallest possible amount of plant growth regulators needed for proper development reduces costs and the likelihood that mutations will accumulate during in vitro culture (von Arnold, 2008). Therefore, the possibility of decreasing the concentration of plant growth regulators from the standard protocol described by Winkelmann et al. (1998a,b) without introducing drawbacks, e.g., early differentiation during culture or slowing of growth, was tested.

Calluses of *C. alpinum* were excluded from the experiment because they showed a high rate of differentiation of somatic embryos in the established callus cultures on both media. In contrast, callus cultures of *C. mirabile* and *C. graecum* retained friable consistencies on both media and did not begin to differentiate during the callus culture. The increases in fresh mass in the callus cultures of *C. mirabile* were equal on both media (2.28 ± 0.27 and 2.15 ± 0.33 g, respectively, Table 2), while callus cultures of *C. persicum* and *C. graecum* showed enhanced growth on 2.31.S medium with the lower concentration of the plant growth regulators (Table 2). However, the difference was only significant for *C. persicum* (ANOVA, $p = 0.0343$).

Suspension cultures were established from the undifferentiated parts of the *C. alpinum* callus. These suspensions partly formed clusters with diameters of about 3 mm that resembled globular

Table 2
Callus growth given as the increase in fresh mass [g] in 4 weeks on two media differing in their content of 2iP and 2,4-D.

	2.1.S (2.0 mg L^{-1} 2,4-D, 0.8 mg L^{-1} 2iP)	2.31.S (1.0 mg L^{-1} 2,4-D, 0.4 mg L^{-1} 2iP)
C. mirabile	2.28 ± 0.27 a	2.15 ± 0.33 a
C. graecum	2.67 ± 0.67 a	3.77 ± 0.53 a
C. persicum	1.48 ± 0.35 a	2.22 ± 0.63 b

Mean indicated by the same letters did not differ significantly according to ANOVA (comparisons only within one species).

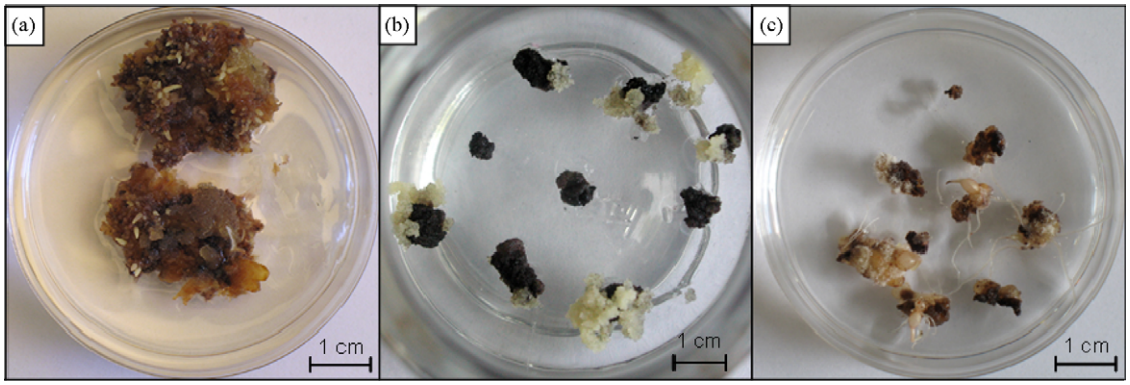

Fig. 1. Callus induction from different seedling explant types of the three *Cyclamen* species. (a) Cotyledon explants of *C. mirabile* after about 6 months with formation of friable callus and somatic embryos. (b) Tuber explants of *C. graecum* after about 4 months with friable and embryogenic yellow callus. (c) Root explants of *C. alpinum* after about 6 months with formation of brown embryogenic callus and somatic embryos.

embryos and turned a red-brown colour in some culture stages. These cultures could be used without restrictions for protoplast isolation, but evaluation of the pcv did not give reproducible results. From callus cultures of *C. mirabile* and *C. graecum*, very fine, stably growing suspensions were established. Statistical analysis by *t*-test revealed no significant differences in the growth rates between the two media (2.31.L and 2.1.L). Suspension cultures of *C. mirabile* showed growth rates of $\mu = 0.30\ \Delta$pcv/d (2.1.L medium) and $\mu = 0.37\ \Delta$pcv/d (2.31.L medium), and the growth rates of *C. graecum* were comparable ($\mu = 0.40\ \Delta$pcv/d for 2.1.L and $\mu = 0.36\ \Delta$pcv/d for 2.31.L).

3.3. Regeneration and germination of somatic embryos

The somatic embryogenic capacity of the established callus cultures and their further development was tested on six different media (Table 3). The six media tested had been designed in order to avoid malformations which had previously been observed in other *Cyclamen* species, like predominant root formation or soft tissues. Therefore, the effects of additional calcium, activated charcoal and kinetin were evaluated in this experiment.

On average, in all of the media, 971 embryos formed per 0.5 g embryogenic callus in *C. mirabile*, which was almost 2 times higher than in *C. alpinum*, which formed 528 embryos, and 4 times higher than in *C. persicum*, which formed 254 embryos per 0.5 g embryogenic callus. During long-term culture over more than 12 months, the embryogenity of *C. graecum* calluses decreased to only 4 embryos per 0.5 g embryogenic callus under these experimental conditions. The embryo formation rates on the different media were compared by pairwise chi-square tests with Holms continuity correction. For *C. graecum* and *C. alpinum*, no significant differences between the embryo formation rates on the six different media were found. For *C. mirabile* and *C. persicum*, the pairwise comparisons of the 2.2.S medium (standard medium) with some of the other media displayed significance (Table 3). For *C. mirabile*, a statistically significant improvement in the embryo formation was achieved on the charcoal-containing media (2.41.S and 2.42.S) and the medium supplemented with kinetin (2.16.S), whereas the reduced NH_4NO_3 content (2.40.S) significantly reduced the number of somatic embryos. In *C. persicum*, embryo formation was significantly enhanced on media with additional $CaCl_2$ (2.25.S and 2.41.S) and negatively affected on medium supplemented with kinetin (2.16.S).

After embryo differentiation, germination of somatic embryos was evaluated. Due to the poor embryo differentiation in *C. graecum*, the germination rates were not determined. In *C. alpinum*, only a few embryos (0–2%) germinated and about 77% of the singularised somatic embryos showed proliferative secondary embryogenesis. The germination rates ranged between 3 and 40% in *C. mirabile* and between 11 and 60% in *C. persicum*. Both species showed the highest germination rates on 2.41.S medium with activated charcoal without additional plant growth regulators (Table 3).

In conclusion, in all three species, somatic embryos were obtained, although their qualities were different. While *C. mirabile* produced well-shaped embryos without malformations that developed normally, many embryos of *C. graecum* turned brown and showed symptoms of hyperhydricity. In *C. alpinum*, the main problem was secondary embryogenesis even after several subcultures on PGR-free medium.

For *C. mirabile*, 169 plants were transferred to the greenhouse and 148 (88%) survived.

Table 3
Comparison of embryo formation and germination rate on six different solid media. Given are mean and standard deviations of 3 replicates.

Species	Culture medium	Embryo formation embryos per 0.5 g fresh mass	Germination rate [%]
C. mirabile	2.2.S	874 ± 91	3
	2.25.S	1140 ± 199 n.s.	6
	2.16.S	643 ± 136*	31
	2.40.S	382 ± 96*	17
	2.41.S	1461 ± 155*	40
	2.42.S	1328 ± 178*	27
C. alpinum	2.2.S	532 ± 84	0
	2.25.S	390 ± 112 n.s.	0
	2.16.S	573 ± 67 n.s.	2
	2.40.S	318 ± 115 n.s.	0
	2.41.S	749 ± 102 n.s.	0
	2.42.S	610 ± 53 n.s.	0
C. graecum	2.2.S	5 ± 4 n.s.	Not determined
	2.25.S	7 ± 6 n.s.	
	2.16.S	0 ± 0 n.s.	
	2.40.S	0 ± 0 n.s.	
	2.41.S	4 ± 2 n.s.	
	2.42.S	5 ± 3 n.s.	
C. persicum	2.2.S	276 ± 98	35
	2.25.S	394 ± 129*	60
	2.16.S	153 ± 67*	11
	2.40.S	52 ± 18 n.s.	21
	2.41.S	402 ± 164*	60
	2.42.S	248 ± 63 n.s.	28

Only the pairwise comparisons with medium 2.2.S are indicated: data marked with n.s. (not significant) did not differ significantly at $p > 0.05$, data marked with * differed significantly from medium 2.2.S at $p < 0.05$. 2.2.S, standard differentiation medium after Schwenkel and Winkelmann (1998); 2.25.S, 2× $CaCl_2$, 2× $MgSO_4$, 2× microelements; 2.16.S, +0.8 mg L^{-1} kinetin; 2.40.S, 0.25× NH_4NO_3; 2.41.S, 3× $CaCl_2$ + 1 g L^{-1} activated charcoal; 2.42.S, +1 g L^{-1} activated charcoal.

Table 4
Protoplast yields from suspension cultures of three different *Cyclamen* species. Comparison of different initial weights and enzyme solutions used for isolation. All isolations were incubated 15–18 h in the respective enzyme solution. "*n*" is the number of independent isolations. E1 = enzyme solution 1 (2.0% Cellulase R-10, 0.5% Macerozyme R-10), E2 = enzyme solution 2 (3.0% Cellulase R-10, 1.0% Macerozyme R-10).

Enzyme solution	Initial cell weight per isolation [g]	Protoplasts × 10^5 per gram fresh mass		
		C. mirabile (*n*)	*C. alpinum* (*n*)	*C. graecum* (*n*)
E1	0.5	8.01 ± 2.05 (5)*	3.73 ± 1.27 (4)	6.50 ± 5.97 (5)
E1	1.0	4.42 ± 1.28 (5)*	4.24 ± 1.05 (4)	13.56 ± 7.99 (4)
E2	0.5	11.44 ± 4.09 (5)*	6.33 ± 2.16 (3)	–
E2	1.0	5.46 ± 1.47 (5)*	2.93 ± 0.97 (5)	–

The ANOVA and *F*-test revealed that the amount of initial weight and the enzyme solution did not differ significantly, except for *C. mirabile* where the amount of initial weight differs significantly at the 5% significance level (*$p = 0.0268$).

3.4. Protoplast isolation

Viable protoplasts were isolated from suspension cultures of all species with mean diameters ranging between 30 and 37 μm. Cell walls were digested completely with both enzyme solutions (E1 and E2), which was tested randomly by Calcofluor White staining (after Hahne et al., 1983) and showed no fluorescence. For *C. graecum*, only enzyme solution E1 was used because enzyme solution E2 resulted in mostly damaged and non-viable protoplasts.

The protoplast yields and the qualities of protoplasts showed high variation between the different isolation experiments (Table 4). The average protoplast yields ranged between 2.9 × 10^5 (*C. alpinum*) and 13.6 × 10^5 (*C. graecum*). Statistical analysis (Tukey test, applied for combinations of the three species) revealed no significant differences between the protoplast yields of the three species under the same isolation conditions. The effects of the different enzyme solutions and variations in the initial weight for each species were not significant for *C. alpinum* and *C. graecum* according to ANOVA and *F*-test. In *C. mirabile*, protoplast yields decreased significantly when twice the initial weight was used (Table 4).

3.5. Protoplast culture

In *C. mirabile*, *C. alpinum* and *C. graecum*, the first cell divisions were monitored 24–48 h after isolation. All three species showed significantly different growth responses in 8pmC.2 medium, when cultivated in alginate or agarose (Tukey test): *C. graecum* showed the highest division frequency, followed by *C. mirabile*.

The embedding matrix affected the division frequency as well as the morphology of the cultivated protoplasts (Figs. 2 and 3). In *C. mirabile*, statistical analysis revealed a significant effect of the embedding agent (Fig. 2). Use of the embedding agent agarose resulted in significantly enhanced division frequencies in 8pmC.1 but reduced division frequencies in 8pmC.2. In both cases, misshaped cells developed (Fig. 3d and f) in comparison to culture in alginate.

In *C. alpinum*, cell division frequencies were significantly higher in 8pmC.1 medium than in 8pmC.2 medium, in which almost no cell division was observed (Fig. 2). In addition, the embedding agent had a significant effect on the division frequency. In alginate, cells showed normal development (Fig. 3a), so division could be easily evaluated and the development of microcalluses was visible 1 or 2 weeks after isolation. In agarose, cells were elongated and showed bulged surfaces (Fig. 3b). However, after 6–10 weeks of culture in both embedding media, microcalluses formed and somatic embryos were regenerated.

In *C. graecum*, the sensitivity to plant growth regulators was shown in former studies (data not shown); therefore, only the lower amount of plant growth regulators was used. No negative effect of the embedding agent on cell division and no malformations were observed. In both embedding agents, protoplasts were spherical at the beginning of development and showed regular divisions and a high formation of microcalluses after a few days.

In controls of *C. persicum* protoplasts, development in alginate was good, but in agarose malformation of protoplasts was pronounced and almost no cell division was found. Regeneration of microcalluses and somatic embryos in this embedding agent was not possible (data not shown).

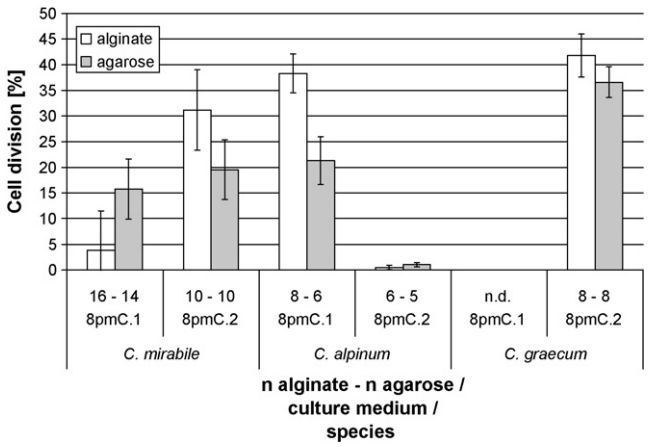

Fig. 2. Influence of the culture medium and the embedding agent on the cell division of *C. mirabile*, *C. alpinum* and *C. graecum* protoplasts after 2 weeks of culture (I = standard deviation, *n* = number of replications per variant), n.d. = not determined.

3.6. Protoplast-derived callus growth and regeneration

When microcalluses of all species reached diameters of about 1 mm in the alginate films/agarose lenses, they were transferred to solid 2.31.S medium for further callus growth. Calluses were grown for about 8–16 weeks until they reached at least 3 mm in diameter. These calluses were transferred to 2.25.S medium for the differentiation of somatic embryos, which formed after an additional 8–16 weeks of culture and were separated and continuously grown. Despite the diverse development in the two protoplast culture systems, regeneration from protoplasts to plants in all three species was possible with both culture systems. Somatic embryos regenerated from protoplasts and showed similar development to those regenerated from embryogenic callus cultures (see above).

When somatic embryos reached cotyledonal lengths of about 1.5 cm, they were transferred to light and grown to plantlets (Fig. 4a–c). In this stage, 3, 9 and 187 plantlets of the species *C. graecum*, *C. alpinum*, and *C. mirabile*, respectively, were transferred to the greenhouse (Fig. 4d–f), of which 3, 5, and 107 were successfully acclimatised. These plants will be screened for somaclonal variations in future experiments. Currently, morphological aberrations in comparison to the plants regenerated from calluses have not been observed.

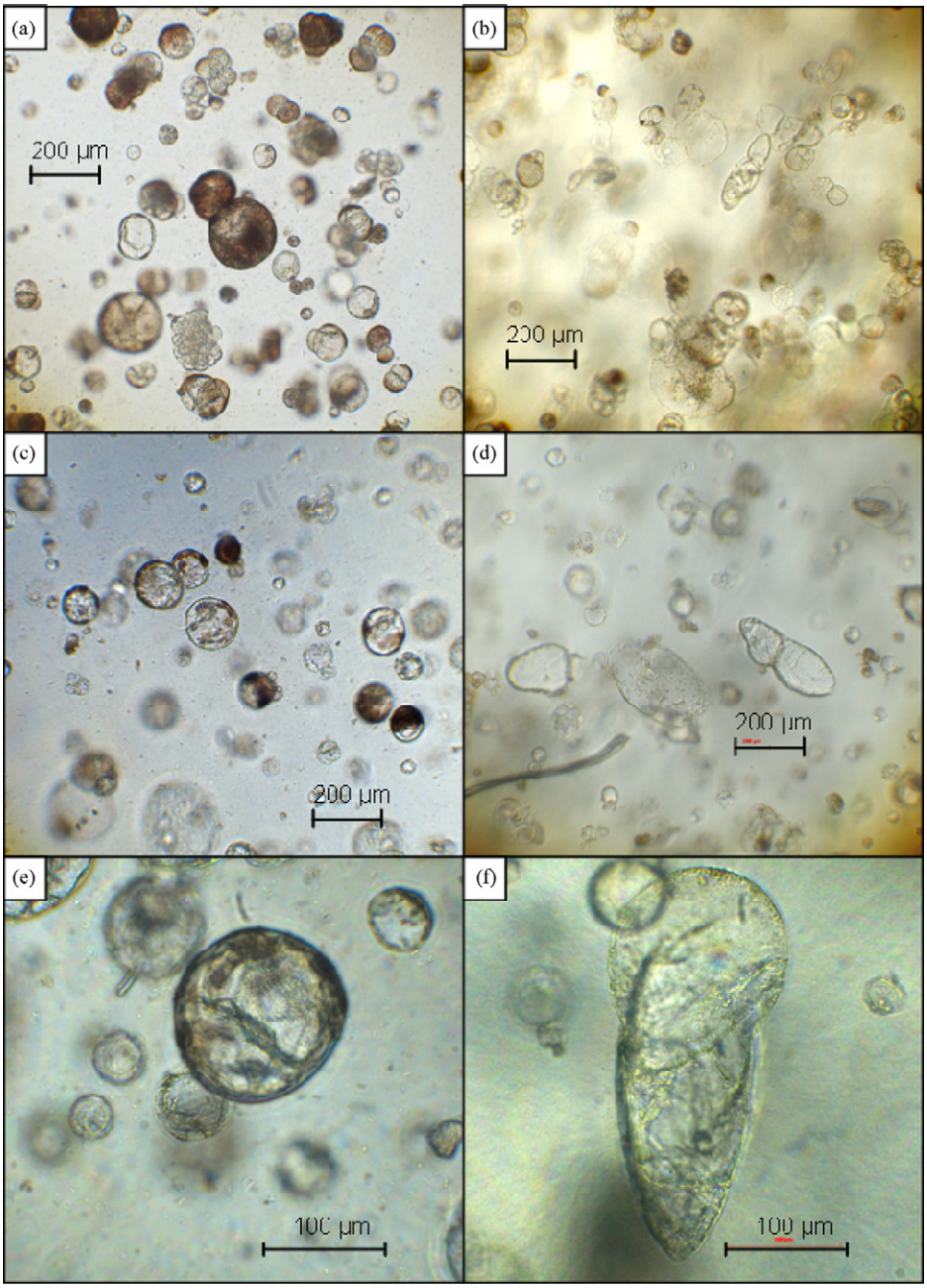

Fig. 3. Comparison of the embedded protoplast cultures after 2 weeks. (a) Protoplasts of *C. alpinum* embedded in alginate. (b) Protoplasts of *C. alpinum* embedded in agarose. (c) and (e) Protoplasts of *C. mirabile* embedded in alginate. (d) and (f) Protoplasts of *C. mirabile* embedded in agarose.

4. Discussion

4.1. Callus induction from seedling tissue of C. mirabile, C. alpinum and C. graecum

In this study, we demonstrated that the culture medium that Schwenkel and Winkelmann (1998) developed for the induction of embryogenic calluses from ovules of *C. persicum* can also be successfully applied to the induction of embryogenic calluses from seedling tissue of other *Cyclamen* species, e.g., *C. alpinum*, *C. graecum*, and *C. mirabile*, using a modified protocol. Schwenkel and Winkelmann (1998) obtained embryogenic calluses after about 8 weeks of culture, whereas callus induction in our study took about 4–6 months. Stress, caused by aging of the cultures without subcultivation as well as reduced availability of water, nutrients and plant growth regulators in the culture medium, is the likely reason for callus induction and induction of somatic embryos in our protocol. Dudits et al. (1995) described hormone- or stress-induced activation of a signal transduction system and the resulting molecular changes that lead to the formation of non-differentiated calluses or somatic embryos. We observed the development of undifferentiated calluses as well as the formation of somatic embryos in aging cultures.

Embryogenic cultures were obtained from seedling tissues of 1–2 out of 10 tested genotypes of *C. mirabile*, *C. alpinum* and *C. graecum*. Like in many other species [e.g., soybean (Parrott et al., 1989) and cotton (Trolinder and Xhixian, 1989); for a general overview, see Henry et al., 1994] and in *C. persicum* (Takamura and Miyajima, 1996; Takamura and Tanaka, 1996; Schwenkel and Winkelmann, 1998; Winkelmann and Serek, 2005), the ability to regenerate in

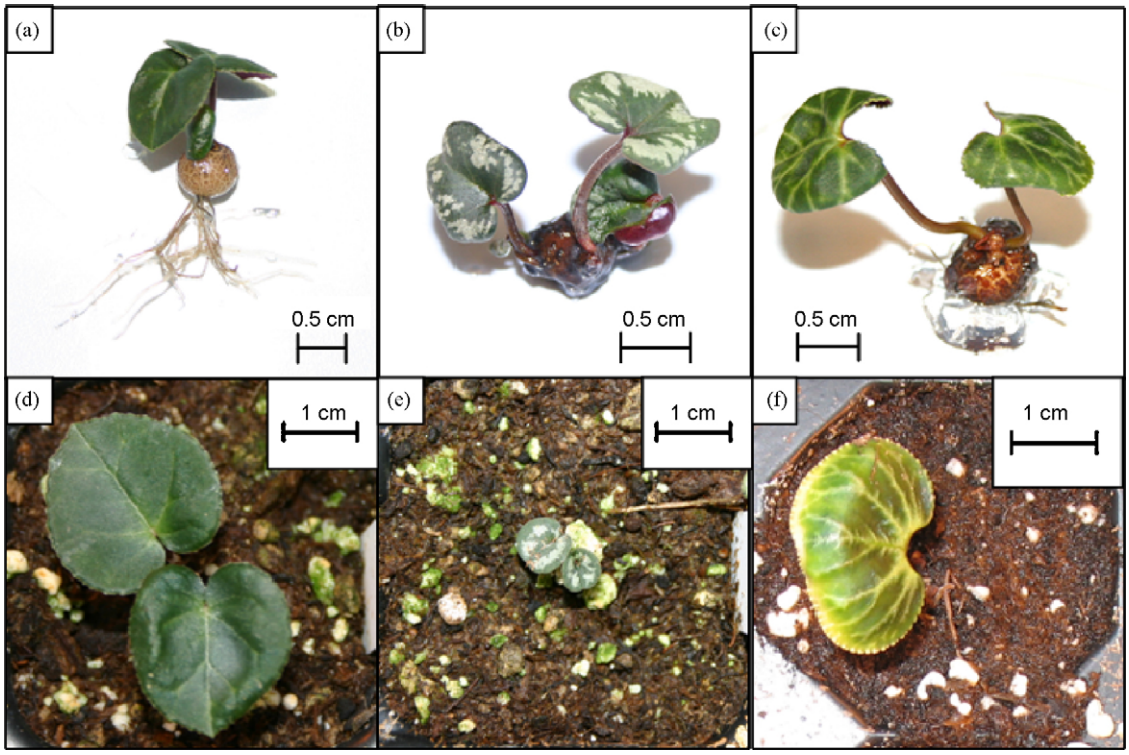

Fig. 4. *C. mirabile* (a), *C. alpinum* (b) and *C. graecum* (c) plants regenerated from protoplasts prior to transfer to the greenhouse. *C. mirabile* (d), *C. alpinum* (e) and *C. graecum* (f) plants regenerated from protoplasts about 2.5 weeks after transfer to the greenhouse.

vitro via somatic embryogenesis is genotype-dependent. Takamura and Miyajima (1997) induced embryogenic calluses and embryoids from aseptic seedling tissue and were able to induce callus and/or embryos from 4 out of 13 *C. persicum* cultivars (31%). In contrast to these results, Schwenkel and Winkelmann (1998) were able to induce embryogenic calluses from ovules in almost all genotypes of *C. persicum* (29 of 30 genotypes tested, 97%). These differences might have originated in the tissue (aseptic seedlings versus ovules) or the media used for the experiments, whereas the callus induction medium from Schwenkel and Winkelmann (1998) and the 2.1.S medium in this study were identical. Another explanation was given by Püschel et al. (2003), who postulated that the ability to regenerate via somatic embryogenesis was genetically controlled by two major genes (in *C. persicum*). Media and tissue are important factors, but according to observations of these authors and our own experience, the genetic background is likely to be crucial for success of the experiment. A closer relationship between the cultivars of *C. persicum* might explain the high percentage of plants with the ability to undergo somatic embryogenesis.

The strong dependence on the genotype will however be limiting for in vitro regeneration to be used for the preservation of the germplasm of these species.

4.2. Callus growth and morphology

In our studies with different *Cyclamen* species, mainly grey, dark yellow and brown calluses were induced. A correlation between the embryogenity and colour was not observed.

For long-term maintenance of all of our callus cultures, we attempted to minimise the decrease in the embryogenic potential, which was more or less observed in all cultures after a certain time and was very pronounced in *C. graecum*. The negative effect of 2,4-D and other PGRs on the formation of somatic embryos has already been described in the literature and explained by the accumulation of PGRs (Negrutiu et al., 1979). Therefore, we optimised our culture conditions in terms of reductions of the plant growth regulators for each species. The increase in the fresh mass on solid medium and the growth rates in liquid medium were not compromised when the amount of plant growth regulators was reduced from 2.0 mg L^{-1} 2,4-D and 0.8 mg L^{-1} 2iP (2.1 media) to 1.0 mg L^{-1} 2,4-D and 0.4 mg L^{-1} 2iP (2.31 media). In some cases, enhanced growth on 2.31.S medium was observed and was not accompanied by unwanted early differentiation events. In addition, in *C. coum*, Prange et al. (2010) observed significantly enhanced growth rates in suspension cultures when 2.31.L medium was used instead of 2.1.L. An inhibitory effect of 2,4-D on protoplast cultures has also been described by Shimizu et al. (1996). The authors observed suppression of cell division and colony formation on protoplast cultures of *Iris germanica* cultivated in increasing concentrations of 2,4-D (higher than 1.0 mg L^{-1}). Other factors like density of the culture, temperature, sugar content and the use of different culture media have to be tested in future experiments to optimise the in vitro culture conditions. Cryopreservation of callus or suspension cultures, which has already been successfully tested in *C. persicum* (Winkelmann et al., 2004), might be a reasonable tool for PGR-sensitive callus cultures to maintain them in their "embryogenic state".

4.3. Regeneration via somatic embryogenesis

Plants from all three species were regenerated via somatic embryogenesis on all six media tested (Table 3). The somatic embryos in *Cyclamen* species can be easily distinguished from adventitious shoots, because they do not have any connection to the callus, are white instead of pink to reddish in colour and develop well-shaped tubers.

The compositions of the six media tested for the regeneration of somatic embryos did not have significant effects on embryo formation of *C. graecum* and *C. alpinum*. For *C. mirabile*, the two media containing activated charcoal (2.41.S and 2.42.S) had a significant positive effect on the regeneration of somatic embryos. In medium 2.41.S, additionally the amount of $CaCl_2$ was three times increased,

but the higher calcium content did not further improve differentiation. Activated charcoal can positively or negatively affect in vitro cultivated material (for review, see Pan and van Staden, 1998). In our study, the positive effect might be due to the binding of PGRs. Endogenous (natural or artificial) PGRs that are released in some cultures into the surrounding medium (Pan and van Staden, 1998 and references) can be absorbed by the activated charcoal, and the absolute concentration of PGRs in the culture system is consequently lowered. The development of somatic embryos, which in *Cyclamen* normally occurs in PGR-free medium after a certain PGR treatment, can thereby be promoted.

The solidification agent Gelrite has not only many advantages (Huang et al., 1995), but also comprises a high binding capacity for ions that are necessary for solidification and have a high impact on gel strength (Huang et al., 1995; Cameron, 2001). The availability of magnesium, calcium, zinc and manganese for the cultured plants/explants has been proven to be reduced in Gelrite-solidified culture media (Van Winkle et al., 2003). Therefore, these compounds were added in higher amounts to media 2.25.S and 2.41.S leading to enhanced somatic embryogenesis in *C. persicum*. For very vigorously growing (mainly rooted) plants, liquefaction of the medium has often been observed, indicating that (rooted) plants might be able to extract calcium and magnesium ions from the medium. Adjusting the calcium and magnesium content has to be carefully combined with a reduction of the Gelrite content, because additional calcium and magnesium ions result in stronger gels.

4.4. Protoplast isolation

Protoplasts isolated from *C. coum* suspension cultures (Prange et al., 2010) with an average size of 60 μm were bigger than protoplasts isolated in this study from suspension cultures of *C. persicum*, *C. mirabile*, *C. alpinum* and *C. graecum*, which were on average 30–37 μm in diameter.

The high variation in the protoplast yields between different experiments or internal repetitions is a commonly described problem in protoplast studies (Meyer et al., 2009). Therefore, optimisation of the isolation conditions was supported by statistical significance in only a few cases. The significant decrease in protoplast yield in *C. mirabile* using an initial weight of 1.0 g for the isolation experiments compared to 0.5 g can be explained by saturation and inhibition of the enzymes with substrate. This process might be reinforced by sedimentation of the cells to the bottom of the vessel and insufficient access to plant material during the digestion process.

4.5. Protoplast culture and regeneration to plants via somatic embryogenesis

As previously shown for *C. graecum* (data not shown), the reduction in plant growth regulators from 1.0 mg L^{-1} 2,4-D and 0.4 mg L^{-1} 2iP (8pmC.1) to 0.5 mg L^{-1} 2,4-D and 0.2 mg L^{-1} 2iP (8pmC.2) did not result in reduced protoplast division frequencies. However, division frequencies were significantly reduced for *C. alpinum*.

In *C. mirabile* and *C. alpinum*, protoplast development differed morphologically using the two embedding methods. In agarose, the protoplasts were misshaped with irregular cell wall strengths and enlarged compared to the spherical protoplasts embedded in alginate. This was partly reflected in the reduced division frequencies of *C. mirabile* and *C. alpinum* protoplast cultures when agarose was used as the embedding agent. In *C. graecum*, the division frequencies and protoplast morphologies in both embedding agents were similar and comparable to the morphology observed in alginate in the other two species. These parameters were also examined in former studies of *C. persicum* and *C. coum* protoplasts and showed different reactions in the two embedding methods: protoplasts of *C. persicum* cultivars were only cultivated effectively in alginate films (T. Winkelmann and A. Prange, unpublished results), while protoplasts of *C. coum* showed good growth (Prange et al., 2010) and no morphological malformations in both embedding systems. There are some distinctions between the two embedding protocols in addition to the embedding agent. The protoplasts are exposed to slight heat shocks (about 30–35 °C) during embedding in agarose. The protoplasts are suspended in different "carrier" media prior to embedding and are therefore incubated under different osmotic conditions before the culture medium is added. In addition, the form of the gelling agent (lenses versus films) is different during culture. To find the clear causal factors in our culture systems, the osmolarity, culture form and aeration need to be standardised. These differences have been described several times in the literature for many species under more standardised conditions. Therefore, we assume that the differences are most likely not caused by technical factors but by the embedding agent. Winkelmann (1994) observed malformations in protoplasts of *Saintpaulia ionantha* and an interruption in development when they were cultivated in agarose lenses. Culture in alginate led to good development, microcallus formation and regeneration to plants. We observed the cell wall development in detail and found uneven cell wall formation in protoplasts embedded in agarose, whereas in alginate the developing cell wall was evenly distributed around the cell during culture. For *Arabidopsis thaliana* (Damm and Willmitzer, 1988), the use of only alginate as an embedding agent has been shown to lead to successful regeneration of plants. Detailed studies at the histological level in sunflower protoplasts (Fischer and Hahne, 1992) indicated that degenerative multicellular structures are formed when this species is cultivated in agarose, in contrast to the more vital callus-like structures that developed in alginate. Different studies have also described differences in the physiological response of embedded cells that are dependent on the embedding agent, e.g., pectin production in protoplast-derived cells of *Linum usitatissimum* (David et al., 1995) or alkaloid production of *Catharanthus roseus* protoplasts (Aoyagi et al., 1998). Another study on *L. usitatissimum* protoplasts (Roger et al., 1996) showed a high induction of ionically bound cell wall proteins in protoplasts cultivated in alginate, which were absent in agarose-embedded cultures. The authors suggested that these plant cells perceive and respond directly to the adjacent extracellular matrix. In summary, alginate (or its impurities) and agarose should not be viewed as inert structural agents but as interactive additives that have structural and/or chemical influence.

In *C. persicum*, it was not possible to regenerate microcalluses from protoplasts cultured in agarose (T. Winkelmann and A. Prange, unpublished results). This might be a helpful tool for later fusion experiments to eliminate the non-fused protoplasts of one parental line.

In conclusion, in this study we described for the first time the regeneration of plants via somatic embryogenesis and the use of embryogenic cultures as a source for protoplasts of *C. alpinum*, *C. graecum* and *C. mirabile*. Plants were regenerated from protoplasts in all three species but in different efficiencies due to limitations on the differentiation of somatic embryos. These systems are prerequisites for protoplast fusion or direct transformation and introduce new breeding approaches via somatic hybridisation in *Cyclamen*.

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References

Aoyagi, H., Sakamoto, Y., Asada, M., Tanaka, H., 1998. Indole alkaloids production by *Catharanthus roseus* protoplasts with artificial cell walls containing of glucuronic acid rich alginate gel. J. Ferment. Bioeng. 85 (3), 306–311.

Binding, H., Bünning, D., Görschen, E., Jörgensen, J., Kollmann, R., Krumbiegel-Schroeren, G., Ling, H.Q., Monzer, J., Mordhorst, G., Rudnick, J., Sauer, A., Witt, D., Zuba, M., 1988. Uniparental fusant and chimeric plants regenerated from protoplasts after streak plating in agarose gels. Plant Cell Tissue Organ Cult. 12, 133–135.

Cameron, S.I., 2001. Use of a prototype gel hardiness tester to demonstrate the effect of variable calcium concentration on gel rigidity. In Vitro Cell. Dev. Biol.: Plant 37, 419–424.

Damm, B., Willmitzer, L., 1988. Regeneration of fertile plants from protoplasts of different *Arabidopsis thaliana* genotypes. Mol. Gen. Genet. 213, 15–20.

David, H., Bade, P., David, A., Savy, C., Demazy, C., Van Cutsem, P., 1995. Pectins in walls of protoplast-derived cells imbedded in agarose and alginate beads. Protoplasma 186, 122–130.

Dudits, D., Gyorgyey, J., Bogre, L., Bako, L., 1995. Molecular biology of somatic embryogenesis. In: Thorpe, T.A. (Ed.), In Vitro Embryogenesis in Plants. Kluwer Academic Publishers, Dordrecht, Boston, London, pp. 267–308.

Fischer, C., Hahne, G., 1992. Structural analysis of colonies derived from sunflower (*Helianthus annuus* L.) protoplasts cultured in liquid and semi solid media. Protoplasma 169, 130–138.

Grey-Wilson, C., 2003. Cyclamen: A Guide for Gardeners, Horticulturists and Botanists, New edition. Batsford, London.

Hahne, G., Herth, W., Hoffmann, F., 1983. Wall formation and cell division in fluorescence labelled plant protoplasts. Protoplasma 115, 217–221.

Henry, Y., Vain, P., De Buyser, J., 1994. Genetic analysis of in vitro plant tissue culture responses and regeneration capacities. Euphytica 79, 45–58.

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biometrical J. 50, 346–363.

Huang, L.C., Kohashi, C., Vangundi, R., Murashige, T., 1995. Effect of common components on hardiness of culture media prepared with Gelrite™. In Vitro Cell. Dev. Biol.: Plant 31, 84–89.

Ishizaka, H., 2008. Interspecific hybridisation by embryo rescue in the genus *Cyclamen*. Plant Biotechnol. 25, 511–519.

Kao, K.N., Michayluk, M.R., 1975. Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126, 105–110.

McCullagh, P., Nelder, J.A., 1989. Generalized Linear Models, 2nd edition. CRC, Chapman & Hall.

Meyer, L., Serek, M., Winkelmann, T., 2009. Protoplast isolation and plant regeneration of different genotypes of *Petunia* and *Calibrachoa*. Plant Cell Tissue Organ Cult. 99, 27–34.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15, 473–497.

Negrutiu, I., Jacobs, M., Gaspar, T., 1979. Leaf formation and peroxidases from *Arabidopsis* callus. Z. Pflanzenphysiol. 91, 119–126.

Pan, M.J., van Staden, J., 1998. The use of charcoal in *in vitro* culture—a review. Plant. Growth Reg. 26, 155–163.

Parrott, W.A., Williams, E.G., Hildebrand, D.F., Collins, G.B., 1989. Effect of genotype on somatic embryogenesis from immature cotyledons of soybean. Plant Cell Tissue Organ Cult. 16, 15–21.

Prange, A.N.S., Serek, M., Winkelmann, T., 2008. Vegetative propagation of different *Cyclamen* species via adventitious shoot formation from seedling tissue. Propag. Ornam. Plant 8 (4), 204–209.

Prange, A.N.S., Serek, M., Bartsch, M., Winkelmann, T., 2010. Efficient and stable regeneration from protoplasts of *Cyclamen coum* Miller via somatic embryogenesis. Plant Cell Tissue Organ Cult. 101, 171–182.

Püschel, A.K., Schwenkel, H.G., Winkelmann, T., 2003. Inheritance of the ability for regeneration via somatic embryogenesis in *Cyclamen persicum*. Plant Cell Tissue Organ Cult. 72, 43–51.

R Development Core Team, 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, http://www.R-project.org.

Roger, D., David, A., David, H., 1996. Immobilisation of flax protoplasts in agarose and alginate beads—correlation between ionically bound cell-wall proteins and morphogenetic response. Plant Physiol. 112, 1191–1199.

Sahai, H., Ageel, M.I., 2000. The Analysis of Variances. Birkhäuser, Boston.

Schwenkel, H.G., Winkelmann, T., 1998. Plant regeneration via somatic embryogenesis from ovules of *Cyclamen persicum* Mill. Plant Tissue Cult. Biotechnol. 4 (1), 28–34.

Seyring, M., Ewald, A., Mueller, A., Haensch, K.T., 2009. Screening for propagation suitability in vitro of different *Cyclamen* species. Electron. J. Biotechnol. 12 (4).

Shimizu, K., Yabuya, T., Adachi, T., 1996. Plant regeneration from protoplasts of *Iris germanica* L. Euphytica 89, 223–227.

Takamura, T., Miyajima, I., 1996. Embryo development in crosses between diploid and tetraploid cyclamen. J. Japan Soc. Hort. Sci. 65 (1), 113–120.

Takamura, T., Miyajima, I., 1997. Micropropagation of *Cyclamen persicum* Mill. In: Bajaj, Y.P.S. (Ed.), Biotechnology in Agriculture and Forestry, Vol. 40. High Tech and Micropropagation IV. Springer, Berlin Heidelberg, pp. 96–112.

Takamura, T., Tanaka, M., 1996. Somatic embryogenesis from the etiolated petiole of cyclamen (*Cyclamen persicum* Mill.). Plant Tissue Cult. Lett. 13, 43–48.

Trolinder, N.L., Xhixian, C., 1989. Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Rep. 8, 133–136.

Van Winkle, S.C., Johnson, S., Pullman, G.S., 2003. The impact of Gelrite and activated carbon on the elemental composition of two conifer embryogenic tissue initiation media. Plant Cell Rep. 21, 1175–1182.

von Arnold, S., 2008. Somatic embryogenesis. In: George, E.F., Hall, M.A., DeKlerk, G.J. (Eds.), Plant Propagation by Tissue Culture, 3rd Edition—Volume 1. The Background. Springer, Dordrecht, pp. 335–354.

Winkelmann, T., 1994. Zur Transformation, Protoplastenregeneration und -fusion bei *Saintpaulia ionantha* und *Episcia* spec. Dissertation, University of Hanover (in German).

Winkelmann, T., Grunewaldt, J., 1995. Genotypic variability for protoplast regeneration in *Saintpaulia ionantha* (H. Wendl.). Plant Cell Rep. 14, 704–707.

Winkelmann, T., Hohe, A., Schwenkel, H.G., 1998a. Establishing embryogenic suspension cultures in *Cyclamen persicum* 'Purple Flamed'. Adv. Hort. Sci. 12, 25–30.

Winkelmann, T., Sangwan, R.S., Schwenkel, H.G., 1998b. Flow cytometric analyses in embryogenic and non embryogenic callus lines of *Cyclamen persicum* Mill.: relation between ploidy level and competence for somatic embryogenesis. Plant Cell Rep. 17, 400–404.

Winkelmann, T., Hohe, A., Schwenkel, H.G., 2001. Embryogenic suspension cultures in *Cyclamen persicum* Mill. established from somatic embryos or callus. In: Schwenkel, H.G. (Ed.), Reproduction of *Cyclamen persicum* Mill. through Somatic Embryogenesis Using Suspension Culture Systems. European Commission, pp. 35–45.

Winkelmann, T., Mussmann, V., Serek, M., 2004. Cryopreservation of embryogenic suspension cultures of *Cyclamen persicum* Mill. Plant Cell Rep. 23, 1–8.

Winkelmann, T., Serek, M., 2005. Genotypic differences in callus formation and regeneration of somatic embryos in *Cyclamen persicum* Mill. Euphytica 144, 109–117.

Winkelmann, T., Specht, J., Serek, M., 2006. Efficient plant regeneration from protoplasts isolated from embryogenic suspension cultures of *Cyclamen persicum* Mill. Plant Cell Tissue Organ. Cult. 86, 337–347.

Yesson, C., Culham, A., 2006. A phyloclimatic study of *Cyclamen*. BMC Evol. Biol 6, 72, http://www.biomedcentral.com/1471-2148/6/72.