# Somatic hybridisation in different *Cyclamen* species

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Dipl.-Biol. Anika Nadja Sabine Prange

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| Referentin: | Prof. Dr. Traud Winkelmann |
| Korreferentin: | Prof. Dr. Margrethe Serek |
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## Abstract

*Cyclamen persicum* Miller is an economically important ornamental plant worldwide, in contrast to other species of the genus *Cyclamen*. Due to the breeding achievements made in *C. persicum* and the numerous interesting and valuable traits of the other *Cyclamen* species, a hybridisation of these species with resulting fertile hybrids is desirable. Sterile seedlings of *C. mirabile* Hildebrand, *C. coum* Miller, *C. graecum* Link and *C. hederifolium* Aiton were used as a starting material for adventitious shoot induction on two different culture media. Shoot cultures were obtained from all species, grown to plantlets and acclimatised to the greenhouse. Mature leaves of greenhouse-grown plants and sterile seedlings served as the initial material for establishment of embryogenic cultures of *C. coum*, *C. graecum*, *C. mirabile* and *C. alpinum* Schwarz. The ability to form adventitious shoots, to form embryogenic callus and to regenerate by somatic embryogenesis was strongly genotype-dependent for each species. Embryogenic callus cultures exhibited a high regeneration capacity of up to 2,922 somatic embryos per gram of fresh mass in *C. mirabile*. Somatic embryogenesis in some species was affected by decreasing embryogenic capacity, browning, hyperhydricity and continuous secondary somatic embryogenesis, but plants from all species were regenerated and acclimatised to greenhouse conditions with high efficiency in some species (*C. coum* and *C. mirabile*). Protoplasts were successfully isolated from adventitious shoots (*C. graecum*), somatic embryos (*C. coum*) and embryogenic suspension cultures (*C. alpinum*, *C. coum*, *C. graecum* and *C. mirabile*). The protoplast culture conditions were optimised regarding embedding agents (agarose and alginate) and different concentrations of plant growth regulators (PGRs) with the different *Cyclamen* species showing different culture condition preferences. Protoplasts from adventitious shoots were not regenerated to plants, whereas protoplasts derived from both somatic embryos and embryogenic suspension cultures were successfully cultured *in vitro*, regenerated into plants by somatic embryogenesis and acclimatised to the greenhouse. Plants regenerated from *C. coum* protoplasts were analysed for somaclonal aberrations using phenotypic analyses and flow cytometric measurements revealing the occurrence of tetraploid plants. Protoplasts of *C. coum* and *C. graecum* were fused with protoplasts of *C. persicum* by applying the technique of polyethylene glycol (PEG)-mediated protoplast fusion. Heterofusion frequencies were detected by double fluorescent staining with fluorescein diacetate and scopoletin. Based on these staining methods, the selection of heterofusion products using Cell Finder Slides, Fluorescence Activated Cell Sorting (FACS) and a microfluidic chip was tested. The first somatic hybrids were obtained between *C. persicum* and *C. coum* fusion combinations but not for *C. persicum* and *C. graecum* fusion combinations. Hybrid plants were characterised by a partly intermediate phenotype with greater resemblance to *C. coum*. Flow cytometric measurements revealed an additive DNA content of both parental species in the hybrids. By the application of the developed species specific marker and RAPD PCR (randomly amplified polymorphic DNA polymerase chain reaction), however, the *C. persicum* genome was barely detected indicating aneuploidy or chromosome recombination in the hybrid plants.

(*key words*: *Cyclamen*, somatic embryogenesis, protoplast fusion, molecular marker)

**Kurzfassung**

*Cyclamen persicum* Miller ist im Gegensatz zu anderen Arten der Gattung *Cyclamen* weltweit eine wirtschaftlich bedeutende Zierpflanze. In Anbetracht der Züchtungserfolge in *C. persicum* und den zahlreichen interessanten und wertvollen Eigenschaften der anderen *Cyclamen* Arten ist eine Hybridisierung dieser Arten mit resultierenden fruchtbaren Hybriden wünschenswert. Sterile Sämlinge von *C. mirabile* Hildebrand, *C. coum* Müller, *C. graecum* Link und *C. hederifolium* Aiton wurden als Ausgangsmaterial für die Induktion von Adventivsprossen auf zwei verschiedenen Kulturmedien verwendet. Adventivsprosse wurden bei allen Arten erhalten, zu Pflanzen angezogen und ins Gewächshaus überführt. Blätter von Gewächshauspflanzen und sterilen Sämlingen dienten als Ausgangsmaterial für die Etablierung von embryogenen Kulturen von *C. coum*, *C. graecum*, *C. mirabile* und *C. alpinum* Schwarz. Die Fähigkeit Adventivsprosse und embryogenen Kallus zu bilden und durch somatische Embryogenese zu regenerieren war für jede Art stark Genotyp abhängig. Embryogene Kalluskulturen verfügten über eine hohe Regenerationsfähigkeit von bis zu 2922 somatischen Embryonen pro Gramm Frischmasse in *C. mirabile*. Die Entwicklung der somatischen Embryonen war in einigen Arten beeinträchtigt durch die Abnahme der Fähigkeit Embryonen zu bilden, Verbräunung, Hyperhydritzität und durch anhaltende sekundäre Embryogenese. Dennoch wurden bei allen Arten Pflanzen regeneriert und ins Gewächshaus überführt; bei einigen Arten mit sehr hoher Effizienz (*C. coum* und *C. mirabile*). Protoplasten wurden erfolgreich aus Adventivsprossen (*C. graecum*), aus somatischen Embryonen (*C. coum*) und embryogenen Suspensionskulturen (*C. alpinum, C. coum, C. graecum* und *C. mirabile*) isoliert. Die Kulturbedingungen der Protoplasten wurden bezüglich der Einbettungsmedien (Agarose und Alginat) und verschiedenen Konzentrationen von pflanzlichen Wachstumsregulatoren mit den verschiedenen *Cyclamen* Arten optimiert, wobei die verschiedenen *Cyclamen* Arten unterschiedliche Wachstumsbedingungen bevorzugten. Protoplasten von Adventivsprossen wurden nicht zu Pflanzen regeneriert. Sowohl aus somatischen Embryonen als auch aus embryogenen Suspensionskulturen isolierte Protoplasten wurden erfolgreich *in vitro* kultiviert, zu Pflanzen regeneriert und ins Gewächshaus überführt. Aus *C. coum* Protoplasten regenerierte Pflanzen wurden mittels phänotypischer Untersuchungen und durchflusszytometrischer Messungen auf somaklonale Variation untersucht, die das Vorhandensein von tetraploiden Pflanzen zeigten. Protoplasten von *C. coum* und *C. graecum* wurden mit Protoplasten von *C. persicum* mit Hilfe der Polyethylen-Glykol (PEG) vermittelten Protoplasten-Fusion fusioniert. Heterofusionsfrequenzen wurden durch die doppelte Fluoreszenzfärbung mit Fluoresceindiacetat und Scopoletin detektiert. Basierend auf diesen Färbemethoden, wurde die Selektion von Heterofusionsprodukten mittels „Cell Finder Slides“, „Fluorescence Activated Cell Sorting“ (FACS) und mikrofluidischen Chips geprüft. Erstmalig wurden somatische Hybriden aus *C. persicum* und *C. coum* Fusionen erhalten, jedoch nicht aus Fusionen von *C. persicum* und *C. graecum*. Hybride Pflanzen zeigten einen teils intermediären Phänotyp, mit größerer Ähnlichkeit zu *C. coum*. Die durchflusszytometrischen Messungen zeigten einen addierten DNA-Gehalt von beiden elterlichen Arten in den Hybriden. Bei der Anwendung der entwickelten Artspezifischen Marker und der RAPD PCR, war das *C. persicum* Genom nur schwer nachweisbar, was auf Aneuploidie oder Chromosomen-Rekombination in den hybriden Pflanzen hinweist.

(*Schlüsselwörter: Cyclamen*, somatische Embryogenese, Protoplastenfusion, molekularer Marker)

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Annotation: Chapter 3 of this thesis is a compilation of already published (3.1-3.4) or submitted (3.4.) scientific publications forming the ‘basis’ of this cumulative PhD thesis. For easy reading, cross-references within this thesis refer only to the corresponding citation and not to the respective chapters; citations included in chapter 3 are printed in bold letters in the thesis.

## List of Abbreviations

| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| 2iP | 6-γ,γ-(dimethylallylamino)-purine |
| AGO | Argonaut |
| ANOVA | Analysis of variance |
| ABA | Abscisic acid |
| AFLP | Amplified fragment length polymorphism |
| AGP | Arabinogalactan protein |
| BA | 6-benzylaminopurine |
| cDNA | complementary DNA |
| DAPI | 4',6-Diamidino-2-phenylindol |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EST | Expressed sequence tag |
| FACS | Fluorescence activated cell sorting |
| FDA | Fluorescein diacetate |
| FISH | Fluorescence *in situ* hybridisation |
| FSC | Forward Scatter |
| $GA_3$ | Gibberellic acid |
| GISH | Genomic *in situ* hybridisation |
| GST | Glutathione S-transferase |
| HSP | Heat shock protein |
| IAA | Indole-3-acetic acid |
| ITS | Internal transcribed spacer |
| LEA | Late embryogenic abundant |
| MS | Nutrient medium composition after MURASHIGE AND SKOOG (1962) |
| NAA | α-naphthylacetic acetic acid |
| NCBI | National Center for Biotechnology Information |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| PGR | Plant growth regulator |
| PI | Propidium iodide |
| PP2A | Protein phosphatase 2A |
| RAPD | Randomly amplified polymorphic DNA |
| RBITC | Rhodamine-B-isothiocyanate |

| | |
|---|---|
| RFLP | Restriction fragment length polymorphism |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| ROS | Reactive oxygen species |
| SCAR | Sequenced characterised amplified region marker |
| SERK | Somatic embryogenesis receptor-like kinase |
| SOD | Superoxide dismutase |
| SSC | Side Scatter |
| TRITC | Tetramethylrhodamine-5-(and 6)-isothiocyanate |
| TDZ | Thidiazuron |
| XET | Xyloglucan endotransglycosylase |
| XTH | Xyloglucan endotransglycosyltransferase |

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# 1 Introduction

## 1.1 The genus *Cyclamen*

The name *Cyclamen* has its origin in the Greek word “cyclos” (= circle, disc), which is most likely a tribute to the circular tubers of these plants. It can also refer to the -in some species- round shaped leaves or the often curling peduncles during seed ripening (Grey-Wilson, 2003). The name is mentioned very early in the antiquity and was certainly known by Theophrastus (371-287 BC) and following scientists (Grey-Wilson, 2003).

### 1.1.1 Morphology

*Cyclamen* are herbaceous perennials with over-ground or subterranean tubers. Although *Cyclamen* belong to the dicotyledonous plants, only one cotyledon is developing during germination. The tuber is formed by the compressed hypocotyl. The mostly fine, in some species fleshy, root system originates often species specific from the bottom, the lower part or the whole tuber. Leaves and flowers have long peduncles and petioles originating mainly at the top of the tuber or from secondary meristems. There are many leaf varieties -such as the heart- or kidney-shaped *C. coum* or the ivy-shaped *C. hederifolium*- and their margins may be denticulated. Some wild species are scented (*C. purpurascens*, *C. persicum* wild forms) and some have auriculated corolla lobes (“auricles”) (*C. hederifolium*, *C. graecum*) (www.cyclamen.org; Grey-Wilson, 2003).

The flower colour in *Cyclamen* is limited to white, pink, and their intermediates, sometimes with a dark blotch at the corolla lobes (the “eye”). The main pigments in (purple-pink) *Cyclamen (C. persicum)* flowers are anthocyanins (Karrer & Widmer, 1927): mainly malvidin 3-glucosides and peonidin glycosides (Karrer & Widmer, 1927; Robinson & Robinson, 1934; Van Bragt, 1962; Webby & Boase, 1999). Anthocyanin synthesis in petals is mainly controlled by one major gene (W) and homozygous recessive plants (ww) are therefore white (Seyffert, 1955). Blue and deep yellow petal colours are completely absent in this genus. Miyajima *et al*. (1991) selected a *C. persicum* cultivar with very faint yellow coloured petals caused by an accumulation of chalcone glycosides. The globose fruits of most wild species (except for *C. persicum*) have decorative fruit pedicels that coil very tightly close to the ground during ripening. In *C. persicum* they remain almost completely uncoiled. About 5 to 40 seeds, depending on the species, are developing per

fruit. The fruit capsule splits at the apex for releasing the seeds, which are, due to their sweet coating, distributed by ants (myrmecochory) and other insects (Grey-Wilson, 2003).

### 1.1.2 Taxonomy

Today -depending on the author- about 20 *Cyclamen* species are known (Clennett, 2002; Debussche & Thompson, 2002; Grey-Wilson, 2003; Compton *et al*., 2004). *Cyclamen* has long been a genus in the Primulaceae family until Kallersjö *et al.* (2000) realigned it in the family Myrsinaceae (order Ericales) based on three chloroplast DNA sequences.

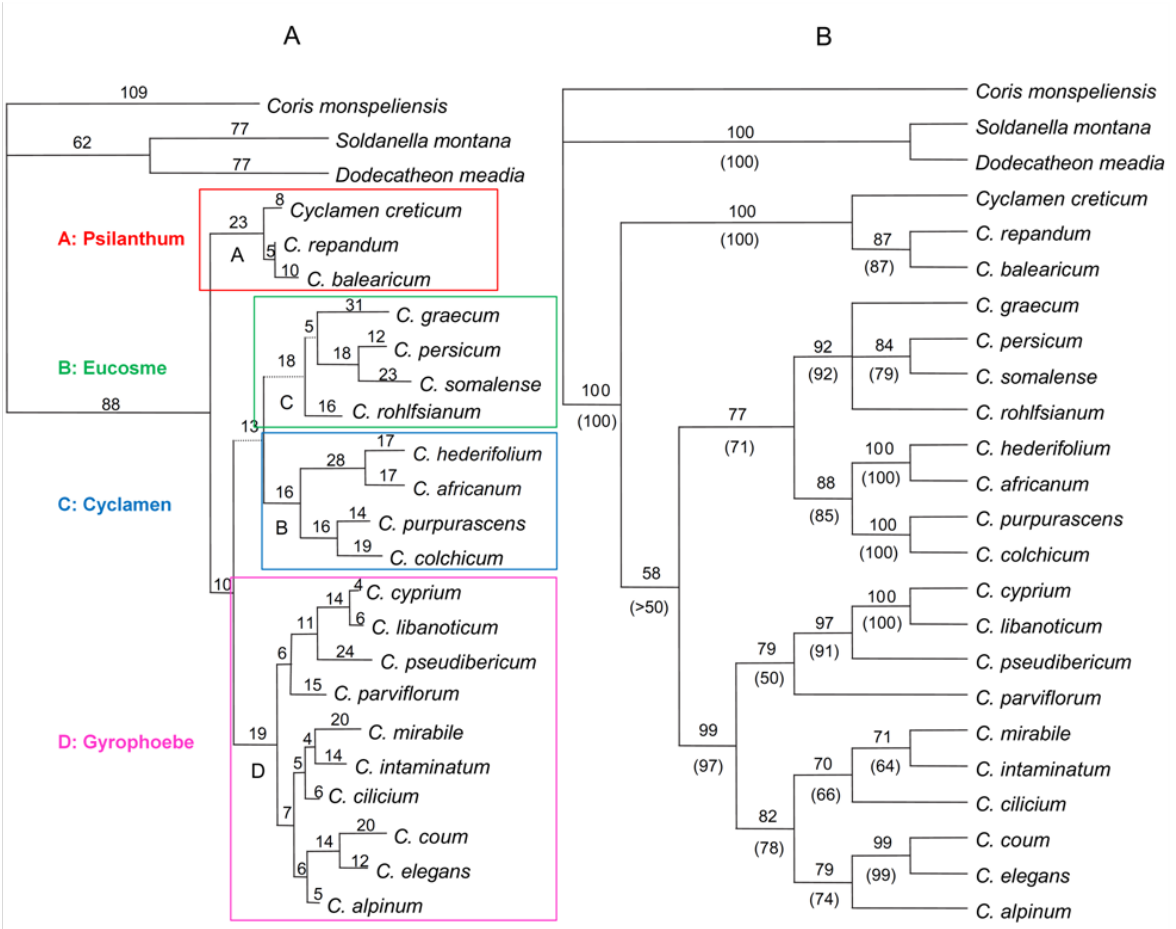


**Figure 4.** Maximum parsimony analysis of nrDNA ITS and cpDNA *trn*L intron sequence data including coded indels combined with morphological data. A, one of two MPTs represented as a phylogram. Dotted lines are those absent in the strict consensus. Numbers indicate branch lengths, letters indicate clades mentioned in the text. B, bootstrap tree showing support values of the analysis of data including coded indels above lines and support values for the same data without indels in parentheses below lines.

**Figure 1:** Cladistic tree of the genus *Cyclamen* generated by combining molecular (nrDNA ITS and cpDNA *trn*L intron) and morphological data (Compton *et al*. 2004). The capital letters A, B, C and D indicate the clades. A = Psilanthum, B = Cyclamen, C = Eucosme, D = Gyrophoebe already described by Anderberg *et al*. 2000 (Figure adopted from Compton *et al*. 2004, with modifications)**.**

The internal classification of the genus has been altered more than 7 times in the last 70 years (Compton *et al*., 2004). The grouping of the different species was performed by many authors using morphological, cytological and molecular data (e.g., Schwarz, 1955, 1964; Grey-Wilson, 2003, Anderberg, 1994; Anderberg *et al*., 2000; Clennett, 2002) resulting in a very inconsistent internal classification. Compton *et al.* (2004) generated a cladistic tree by combining the molecular data of the nrDNA ITS published by Anderberg *et al.* (2000) (subjoining additional nrDNA ITS data that were not included by Anderberg *et al.*, 2000) and own data of the the cpDNA *trn*L intron regions and morphological data. This combined data analysis resulted in the cladistic tree shown above (Fig. 1).

### 1.1.3 Distribution, Habitat and Phenological Adaption

The most recently described species is *C. somalense* Thulin & Warfa which was discovered in 1989 in North-Eastern Somalia. Characterised by a tropic climate this distribution area is quite unusual for a member of the genus *Cyclamen* and its next relative is found 2800 km north in Israel (*C. persicum*). *C. somalense* is most likely a relict species from the Tertiary (Thulin & Warfa, 1989) when *Cyclamen* (or their ancestors) most likely had a much bigger and closed distribution area. Today, the other *Cyclamen* species are found in most cases in relatively small distribution areas around the Mediterranean basin, most of the surrounding countries (western Asia and parts of Northern Africa) and Central Europe except for Morocco, Egypt and the Spanish mainland (Grey-Wilson, 2003; Yesson & Culham, 2006; Fig. 2). Some species have overlapping and wider distribution areas (e.g., *C. purpurascens*, *C. coum*). Depending on the species *Cyclamen* are found in various habitats such as open sites and in shady brushwood of woodlands, in very humus-laden soils but also in very rocky almost soil-less areas (Grey-Wilson, 2003).

Their live-cycles are confined in their main Mediterranean habitat by drought in summer and cold humidity in the winter season. Most *Cyclamen* species are adapted to these conditions by annual periods of dormancy and development. The occurrence in Northern (e.g., Poland, Southern Germany) and mountainous regions of their dispersal area goes along with different grades of hardiness of the different species. *C. hederifolium* and *C. coum* are among the most hardy ones, whereas *C. africanum*, *C. rohlfsianum* and some others do not tolerate any frost. However, different authors describe different temperature ranges for these species (cf. Grey-Wilson, 2003 and references therein). *Cyclamen* are flowering from August to May (Debussche *et al*., 2004) and are often

grouped as spring-flowering (in general synanthous leaves) and autumn-flowering (hysteranthous or synanthous leaves).

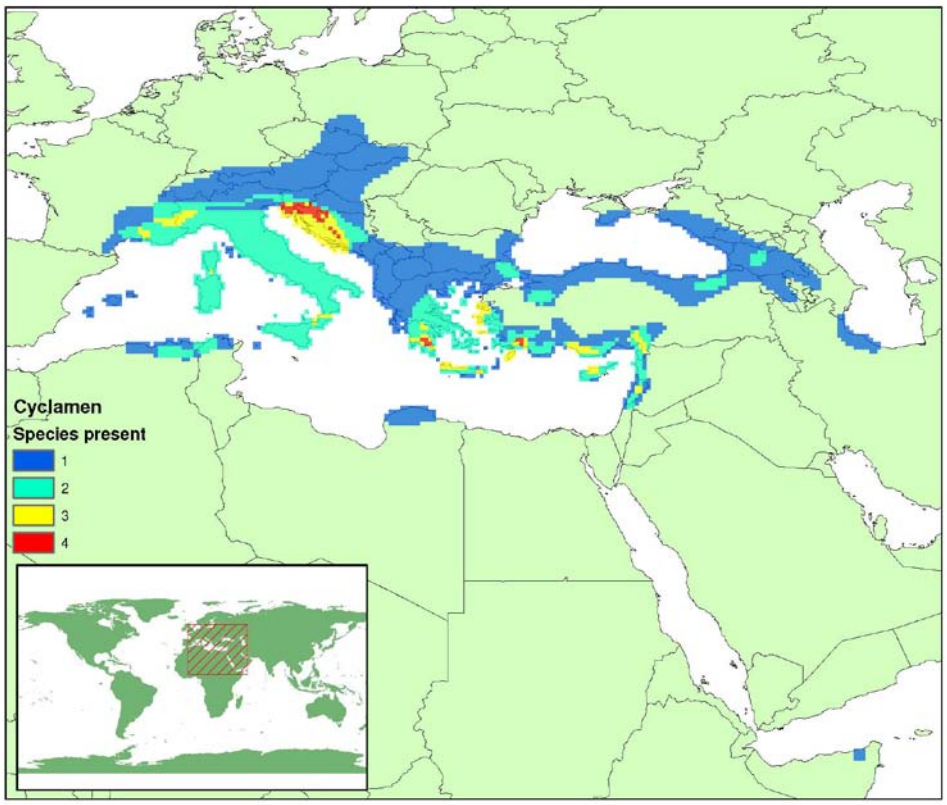


**Figure 2:** *Cyclamen* diversity. Illustration of the *Cyclamen* distribution, based on the distribution maps of Grey-Wilson (2003); Distribution area of: one species: blue, two species: green, three species: yellow, four species: red. (Figure entirely adopted from Yesson & Culham (2006)).

The characteristic hysteranthous/synanthous leaves can, for *C. coum* and *C. hederifolium* wild species and others, be eliminated by artificial constant culture conditions (e.g., in the greenhouse or indoors). Spring- and autumn-flowering and synanthous and hysteranthous leaves are often regarded as species characterising traits. In Israel a spring-flowering synanthous and an autumn-flowering hysteranthous population of *C. persicum* is known (Grey-Wilson, 2003; Schwartz-Tzachor *et al.*, 2006) although this species is (in other areas) only spring-flowering with synanthous leaves. Culturing tests showed retention of the autonomous blooming rhythm even after transplantation which indicates a genetic determination of this character (Schwartz-Tzachor *et al.*, 2008). The extent of genetic or epigenetic-determination and temporary adaptations to the climatic conditions are not fully understood and are under investigation for many *Cyclamen* species and other geophytes of the Mediterranean region (Debussche *et al.*, 2004).

## 1.2 *Cyclamen persicum* Miller (cultivated cyclamen)

*Cyclamen persicum* Miller is a major commercial plant, especially in Europe and Japan. Referring to cyclamen as a pot plant, one mostly means *C. persicum*. In Germany C*. persicum* is also produced and marketed as cut flower (Bongartz, 1999). No other species in the genus *Cyclamen* has been cultivated and bred for such a long time and gained worldwide economic importance. In Germany *C. persicum* has a market share of 8% (i.e. 8% of 1.200 billion € per year) in the category "flowering potted plants" [market share calculated by the retail prize in 2008 by the ZMP, Zentrale Preisberichtsstelle GmbH, Bonn, Germany (in liquidation)].

### 1.2.1 Cultivation

*C. persicum* is mainly propagated via seeds, with an optimal germination temperature of 15 - 20°C (in the dark) (Horn, 1996) yielding a germination after about 3 - 4 weeks (Maatsch, 1971). A finished product for sale is grown in about 25 - 27 weeks in mini type varieties (mini and micro) and 30 - 32 weeks in topical giganteum type varieties such as the ones used in this thesis (www.varinova.nl). The time from sowing to the appearance of the first flowers takes about 23 weeks (mini and micro varieties) to 27 weeks (giganteum varieties) (www.varinova.nl). *C. persicum* should be cultivated in a porous, water drainable substrate such as a mixture of peat-moss, peat dust, Perlite and clay (45:32:15:4) with an addition of lime (www.varinova.nl). The use of fertilizer has to be carefully adjusted according to the many cultivation recommendations existing. Too little or excessive supply of potassium can affect the plant quality and the flowering time negatively (Horn, 1996).

During the cultivation of *C. persicum* the temperature plays an important role for the plant development and the resulting quality of the final product. For germination 15 - 20°C (in the dark) and for the vegetative growth 16 - 22°C are described as optimal (Horn, 1996). Experienced breeders recommend 16 - 14 °C (day and night temperature) as optimal growth conditions for the plant production (www.varinova.nl), resulting in a high quality product that is *inter alia* characterised by a compact growth, with long upright peduncles and flowers. Temperatures above 25°C may lead (under humid conditions) to excessive leaf growth (Horn, 1996; www.varinova.nl), the arrest of flower formation and the arrest of the peduncle elongation. This results in low quality plants with loose growth and short flowers or even "flowering below the leaves" (Horn, 1996). High temperature in

combination with aridity resembles the conditions in the natural habitat, where this combination (high temperature and summer drought) induces the beginning of the summer dormancy period (Horn, 1996; Grey-Wilson, 2003). In general, *C. persicum* is very sensitive to high temperature with variations between the different varieties. High temperature often leads to a reduced shelf life in heated living rooms or to the development of unsightly plants with few or no flowers and a loose growth.

For the use as cut flower in general, giganteum varieties are cultivated preferably with long flower stalks (peduncle length above 26 cm or 30 cm) (Horn, 1996; Bongartz, 1999). They are cut about 4 days after flourishing to obtain an optimal vase life of about 16 - 18 days (Horn, 1996). The shelf life of the flowers on the plant is comparable with about 18 days (Horn, 1996). According to Horn (1996) the vase life of cyclamen cut flowers can even be enhanced to 26 days by loading the flowers with a 15% sucrose solution and other measures for the preservation of the vase life. In contrast to these recommendations, various cut flower foods and other additives tested on *Cyclamen* cut flowers by Neumaier *et al.* (2009) influenced the vase life negatively, especially the ones containing sugar or cellulose cards containing aluminium sulphate. Investigations regarding the influence of the harvest time during the flowering season (autumn/winter 2007/2008) revealed a lower flower quality combined with a shorter vase life the later the flowers were harvested (Neumaier *et al.*, 2009).

### 1.2.2 Breeding

Systematic breeding of *C. persicum* started in the 18$^{th}$ century in Europe. First reports about selection of different varieties in flower colour were reported by Van Kampen in 1739 in the Netherlands (Grey-Wilson, 2003). Henceforward, many commercially valuable characteristics of wild *C. persicum* were improved, but unfortunately the fragrance of the wild form was lost during this process (Grey-Wilson, 2003; Ishizaka, 2008). Today some smaller varieties, developed in the 20$^{th}$ century by crossing with wild *C. persicum* (Wellensiek, 1961), are scented (Bongartz, 1999). The time from sowing to flowering was shortened from 3 to 4 years to 15 months mainly by eliminating the resting period in the summer (Grey-Wilson, 2003; Takamura, 2007) and today even “fast cropping” protocols shortened the culturing time to 8 months (Widmer, 1976). The petal length was enhanced up to 10 cm and different growth types (mini, midi, maxi, giganteum) were developed (Grey-Wilson, 2003; Takamura, 2007; Ishizaka, 2008). Single, double, crested and partly or completely fringed flower forms (the latter one also with flexed corolla lobes) were

developed and the flower colour is now ranging from white over very pale yellow (Miyajima *et al.*, 1991) to purple and true deep red and also flamed varieties are available. Disease resistance against many bacterial infrections such as the bacterial disease caused by *Erwinia herbicola* pv. *cyclamenae*, *Pseudomonas marginalis* pv. *marginalis* (Ishizaka, 1996) or *Fusarium oxysporum* SCHL. f. *cyclaminis* (Gerlach, 1954 in Ewald *et al.*, 2000a) does not occur in *C. persicum*, but is found in wild *Cyclamen* species. Hardiness, variation in leaf shapes and a broad range of flower colours including blue, violet-blue and deep yellow and orange are not available in any *C. persicum* variety today (Takamura, 2007). Tetraploid *C. persicum* cultivars were also developed, but today about 70% diploid F1 hybrids are sold (Bongartz, 1999), which show greater uniformity, speed up production and allow a greater range of varieties by exploiting the genetic diversity (Grey-Wilson, 2003). No other *Cyclamen* species have been involved in this breeding process.

### 1.2.3 Tissue Culture

*C. persicum* is almost exclusively propagated via seeds for breeding purposes and plant production. For seed production *Cyclamen* have to be cross pollinated due to inbreeding depression in self-pollinated progenies (Wellensiek, 1959). The development and maintenance of constant quality breeding lines for F1 hybrid production is laborious and therefore expensive (Wellensiek, 1959; Pueschel *et al.*, 2003). Maintenance of breeding lines by crossing of selected plants of similar appearance often yields inhomogeneous plant material (Rout *et al.*, 2006). Therefore, vegetative propagation is often reported as an important tool for the multiplication and maintenance of breeding lines (Pueschel *et al*., 2003; Winkelmann & Serek, 2005). Also applications for the mass propagation of single elite plants (Pueschel *et al*. 2003; Winkelmann & Serek, 2005), especially in cultivars with complex inheritance of their specific characters (‘Victoria’ *Cyclamen*; Zimmer & Buchwald, 1989) or for sterile interspecies hybrids (e.g., ‘Odorella’; Winkelmann *et al*., 2006a) are described. Vegetative propagation of *C. persicum* is only possible in efficient amounts if tissue culture techniques are used.

#### 1.2.3.1 Organogenesis

Since 1956 about 20 studies dealt with organogenesis in *C. persicum* cultivars using mature tuber tissue (Mayer, 1956; Stichel, 1959; Loewenberg, 1969; Okumoto & Takabayashi, 1969; Pierik, 1975 in Geier *et al.*, 1979; Geier 1977), petioles (Morel, 1975;

Wicart *et al.*, 1984; Schwenkel & Grunewaldt, 1988), etiolated petioles (Ando & Murasaki, 1983; Murasaki & Tsurushima, 1988), anthers (Geier, 1977; Geier *et al*., 1979), ovaries (Wicart *et al*., 1984) and leaf blades (Geier, 1977; Wicart *et al*,. 1984; Hoffmann & Preil, 1987; Schwenkel & Grunewaldt, 1988; Dohm *et al.*, 1991; Schwenkel & Grunewaldt, 1991). The first report about organogenesis described organ formation e.g., from the tuber tissue (Mayer, 1956; Stichel, 1959). In some cases whole plant were regenerated (Mayer, 1956; Stichel, 1959). In other studies, organ formation was reported to be poor and the regeneration of whole plants was not successful (Okumoto & Takabayashi, 1969; Loewenberg, 1969; Pierik, 1975 in Wainwright & Harwood, 1985). In tuber tissue contamination with endogenous microorganisms was often observed (Stichel, 1959; Okumoto & Takabayashi, 1969; Pierik 1975 in Geier *et al*., 1979; Geier, 1977) and the induction of shoots and roots at the same explants turned out to be problematic (Stichel, 1959). *Cyclamen* seedlings were already assigned to have regenerative ability in older publication (Hildebrand, 1898; Winkler, 1902; Goebel, 1908; Hill, 1920 in Geier *et al.,* 1990) that are absent in mature plants. The good regenerative ability of juvenile tissue was used by Dillen *et al*., (1996) for organogenesis from young leaf tissue. The combination of the advantages of highest possible sterility and juvenility for organogenesis was the usage of sterile seedling tissue as explant source, which was easily obtained by surface sterilisation of seeds (Wainwright & Harwood, 1985; Hawkes & Wainwright, 1987; Fukui *et al*., 1988; Zimmer & Buchwald, 1989; Bach *et al*., 1998). This technique was used in this thesis for the induction of shoot cultures and the regeneration of plants from wild *Cyclamen* species (**Prange *et al*., 2008**). Karam & Al Majathoub (2000a, b) were able to induce shoots from seedling and from mature tissue in wild *C. persicum* species.

#### 1.2.3.2 Somatic Embryogenesis

Somatic embryogenesis describes a process in which somatic cells develop into somatic embryos which resemble zygotic embryos morphologically (von Arnold, 2008). Due to their origin, somatic embryos are clones of the originating plant and, therefore, this is a way of vegetative propagation. This process occurs naturally within ovules (e.g., in *Paeonia*) and on leaves (e.g., in *Kalanchoe*) (von Arnold, 2008). In tissue culture, this process has been observed for the first time in cell suspension cultures of *Daucus carota* (Steward *et al*., 1958; Reinert, 1958) and has henceforward been described for a wide range of species in the whole plant kingdom. The temporary exposition of explants to auxin-containing media is one of the main pre-treatments leading to the development of embryogenic callus or somatic embryos. The choice of the explant tissue is often crucial

for experimental success (von Arnold, 2008) but also genotype dependent reactions are frequently described (e.g., Meijer & Brown, 1987; Pueschel *et al*., 2003).

Somatic embryogenesis was described for the first time in *C. persicum* by Wicart *et al.* (1984). The authors induced somatic embryos using leaf blades, petioles, and ovaries via a callus phase on 2,4-D containing media. Somatic embryogenesis most likely can be induced from microspores, ovules, embryos, and seedlings (von Arnold, 2008) and therefore, many authors despite Wicart *et al.* (1984) achieved somatic embryogenesis in *C. persicum* on seedling tissue (Fukui *et al*., 1988; Kreuger *et al*., 1995; Takamura *et al*., 1995; Bach *et al*., 1998), anthers, ovaries, and zygotic embryos (Kiviharju *et al.*, 1992) but also on young leaf blades (Otani & Shimada, 1991). One efficient and transferable protocol was developed by Schwenkel & Winkelmann (1998) using ovules as explant material. Somatic embryogenesis has turned out to be a very efficient way of *in vitro* propagation in *C. persicum* (Wicart *et al*., 1984; Fukui *et al*., 1988; Otani & Shimada, 1991; Kiviharju *et al*., 1992; Kreuger *et al.*, 1995; Takamura, *et al*. 1995; Schwenkel & Winkelmann, 1998; Hohe *et al*., 2001; Winkelmann *et al*., 2006b) which is evading problems with tuber formation which may occur in plant regeneration via shoot induction and rooting in this genus. In wild *C. persicum* somatic embryogenesis (microtuberisation) has been described by Karam & Al Majathoub (2000b).

#### 1.2.3.3 Protoplast culture

Two reports of protoplast culture and regeneration are described for *C. persicum* (Morgan, 1999; Winkelmann *et al*., 2006b). Morgan (1999) used adventitious shoots as material for protoplast isolation with further cultivation of protoplasts in agarose lenses. He was able to grow calluses from which three showed shoot development (Morgan, 1999). Unfortunately the author described his results as not reproducible. Winkelmann *et al*. (2006b) developed an efficient regeneration protocol applicable to different genotypes which uses embryogenic suspension cultures of *C. persicum* cultivars as starting material for the protoplast isolation. Plant regeneration was performed via a callus phase by somatic embryogenesis.

## 1.3 *Cyclamen* ”Wild Species“ (Wild Cyclamen)

Other species in the genus *Cyclamen* are rarely cultivated compared to *C. persicum*, mainly because of their slower cultivation time and their often more delicate and unknown

cultivation requirements. For detailed monographs of each species see Grey-Wilson (2003). For detailed overview on the ploidy and chromosome number, dormant period, flowering period and habit and useful characteristics the reader is referred to (Ishizaka, 2008); Table 1 is adopted from this review article.

In this study, *Cyclamen* species (besides *C. persicum*) are called “wild species” because breeding has -except of *C. coum* Miller and *C. hederifolium* Aiton- not been performed with economic importance. *Cyclamen* seeds were used, which were not modified by professional breeding. They were provided by the *Cyclamen* Society and the Leibniz-Institute of Vegetable and Ornamental Crops Grossbeeren/Erfurt, Germany. The *Cyclamen* Society is a global society of *Cyclamen* followers and is organising many activities and services about *Cyclamen*. It was approved by the International Society for Horticultural Science (ISHS) for the registration of all *Cyclamen* cultivars other than *C. persicum* (www.cyclamen.org).

A phyloclimatic study (Yesson & Culham, 2006) shows the endangerment of almost the whole genus *Cyclamen* by global climate change in about 40 years. Global warming will reduce the habitat of *Cyclamen* and, thereby, their dispersal areas (Yesson & Culham, 2006). In the future, it will be important to enthuse the public for these plants and draw public attention to their preservation. Professional collection of wild *Cyclamen* tubers in their native countries and their purchase by “*Cyclamen* fanciers” has decreased the occurrence of these species in the past (Grey-Wilson, 2003). For this reason the exact location of *C. somalense* has been kept secret (Thulin & Warfa, 1989). This might truly be necessary to protect this rare species which would be a highly interesting research object for scientist and breeders: (1) It is cladistically grouped close to *C. persicum*, which makes it a good (perhaps the best) putative hybridisation partner. (2) Due to its tropic distribution area it might bear a high temperature resistance which is an interesting trait to enhance shelf life in *Cyclamen* as indoor plants.

**Table 1:** Chromosome number and horticultural characteristics of *Cyclamen* species. (Table adopted from Ishizaka (2008)).

| Species | Ploidy level and chromosome number ($2n$) | Dormant period[a] | Flowering period[a] | Flowering habit[b] | Useful characteristics for improving *C. persicum* cultivars | References |
|---|---|---|---|---|---|---|
| *C. repandum* | $2x=20$ | May–Aug. | Mar.–Apr. | L | Cold hardiness | Greilhuber (1989), Legro (1959), Grey-Wilson (2002) |
| *C. balearicum* | $2x=20$ | May–Aug. | Mar.–Apr. | L | | Greilhuber (1989), Legro (1959), Grey-Wilson (2002) |
| *C. peloponnesiacum* | $2x=20$ | May–Aug. | Mar.–Apr. | L | | Grey-Wilson (2002) |
| *C. creticum* | $2x=20, 22$ | May–Aug. | Mar.–Apr. | L | | Greilhuber (1989), Legro (1959), Grey-Wilson (2002) |
| *C. coum* | $2x=30$ | May–Sept. | Feb.–Mar. | L | Cold hardiness | Grey-Wilson (2002), Legro (1959) |
| *C. elegans* | $2x=30$ | May–Sept. | Feb.–Mar. | L | | Grey-Wilson (2002), Legro (1959) |
| *C. alpinum* | $2x=30$ | May–Sept. | Feb.–Mar. | L | Cold hardiness | Grey-Wilson (2002), Legro (1959) |
| *C. parviflorum* | $2x=30$ | Absent | Feb. | L | Cold hardiness | Grey-Wilson (2002), Legro (1959) |
| *C. intaminatum* | $2x=30$ | May–Sept. | Oct. | L | Cold hardiness | Grey-Wilson (2002), Legro (1959) |
| *C. cilicium* | $2x=30$ | May–Sept. | Oct. | L | Cold hardiness | Grey-Wilson (2002), Legro (1959) |
| C. mirabile | $2x=30$ | May–Sept. | Oct. | L | Cold hardiness | Grey-Wilson (2002), Legro (1959) |
| *C. libanoticum* | $2x=30$ | May–Sept. | Feb.–Mar. | L | | Grey-Wilson (2002), Legro (1959) |
| *C. cyprium* | $2x=30$ | May–Sept. | Jan.–Feb. | L | | Grey-Wilson (2002), Legro (1959) |
| *C. pseudibericum* | $2x=30$ | May–Sept. | Feb.- Mar. | L | Cold hardiness | Grey-Wilson (2002), Legro (1959) |
| *C. colchicum* | $2x=34$ | Absent | Jul.–Oct. | L | Cold hardiness, Flower fragrance | Grey-Wilson (2002), Hravětová et al. (1983) |
| *C. purpurascens* | $2x=34$ | Absent | Aug.–Mar. | L | Cold hardiness, Flower fragrance | Hravětová et al. (1983), Legro (1959) |
| *C. purpurascens* | $4x=68$ | Absent | Aug.–Mar. | L | Cold hardiness, Flower fragrance | Ishizaka and Kondo (2004) |
| *C. hederifolium* | $2x=34$ | May–Sept. | Oct. | F | Cold hardiness, Attractive leaves | Bennett and Grimshaw(1991), Hravětová et al.(1983) |
| *C. hederifolium* | $3x=51$ | —[c] | — | — | | Bennett and Grimshaw (1991) |
| *C. hederifolium* | $4x=68$ | May–Sept. | Oct. | F | Cold hardiness, Attractive leaves | Bennett and Grimshaw (1991), Grey-Wilson (2002) |
| *C. africanum* | $2x=34$ | May–Sept. | Oct. | F | Attractive leaves | Bennett and Grimshaw (1991) |
| *C. africanum* | $4x=68$ | May–Sept. | Oct. | F | Attractive leaves | Bennett and Grimshaw (1991), Legro (1959) |
| *C. persicum* wild plant | $2x=48$ | May–Sept. | Feb.–Mar. | L | | Legro (1959), Pavliček et al. (2008) |
| *C. persicum* cultivar | $2x=48$ | Absent | Oct.–Feb. | L | | De Haan and Doorenbos (1951), Legro (1959) |
| *C. persicum* cultivar | $3x=72$ | — | — | — | | Takamura and Miyajima (1996a,b,c,1999) |
| *C. persicum* cultivar | $4x=96$ | Absent | Oct.–Feb. | L | | De Haan and Doorenbos (1951), Legro (1959) |
| *C. persicum* cultivar | $4x=90, 92, 94, 95$ | — | — | — | | Legro (1959) |
| *C. somalense* | — | — | — | — | | Grey-Wilson (2002), Thulin and Warfa (1989) |
| *C. graecum* | $4x=84$ | May–Sept. | Sep.–Oct. | F | Cold hardiness, Disease resistance | Ishizaka (1996) |
| *C. rohlfsianum* | $4x=96$ | May–Sept. | Sep.–Oct. | F | Attractive leaves | Ishizaka et al. (2002a), Shibusawa and Ogawa (1997) |
| *C. rohlfsianum* | $6x=144$ | May–Sept. | Sep.–Oct. | F | Attractive leaves | Ishizaka et al. (2002a) |

[a] Dormant period and flowering period were examined in Saitama Prefecture, Japan.
[b] L, flowers appear after leaf appearance leaves; F, flowers appear before leaf appearance leaves.
[c] Not examined.

Despite seed production, which requires much time and experience, the propagation by tissue culture can be an important tool to preserve these species and satisfy the demand for plant material.

*Cyclamen* wild species bear many properties and benefits which *C. persicum* wild forms and cultivars lack. This makes the wild species an important genetic resource for *Cyclamen* breeders. They cannot directly widen the range of flower colours but some species of this genus have different leaf shapes and sizes, different flower forms, disease resistances (Ewald *et al*., 2000a,b; Ishizaka, 2008) and are scented. Some *Cyclamen* species are very hardy (e.g., *C. coum*, *C. purpurascens*, *C. hederifolium*) which is an important property for breeders in the temperate zone for the development of *Cyclamen* as a garden perennial. On the other hand, some species might be able to enhance the temperature tolerance in heated living rooms by a possibly enhanced higher temperature tolerance (e.g., *C. somalense*).

Many experiments were performed for vegetative propagation of *C. persicum* cultivars (cf. Chapter 1.2.2), but tissue culture studies dealing with vegetative propagation in wild *Cyclamen* species are limited to very few publications except the ones presented in this thesis (**Prange *et al*., 2008, 2010a,b**). Karam & Al-Majathoub (2000a,b) regenerated shoots and microtubers of wild *C. persicum* collected in Jordan. The authors tested different MS (Murashige & Skoog, 1962) based media and regenerated shoots from mature tissue (petiole, petal and peduncle explants) and shoots and microtubers from seedling tissue (wounded blades, blades with midrib, blades without midrib, central lamina, and petiolated lamina of *in vitro* leaves). Furukawa *et al.* (2002) used aseptic seedling tissue (sectioned petiole and leaves) for the induction of shoots (*C. africanum*, *C. hederifolium* var. album) and somatic embryos (*C. africanum*, *C. cilicium*, *C. persicum*, *C. rohlfsianum*, *C. trochopteranthum*) on modified MS (Murashige & Skoog, 1962) media. Seyring *et al.* (2009) used different explants (placentas with ovules, leaves, petioles and peduncles) for the propagation of five different *Cyclamen* species (*C. africanum* Boiss. and Reut., *C. cilicium* Boiss. and Heldr., *C. coum* Mill., *C. hederifolium* Ait., *C. persicum* Mill., *C. purpurascens* Mill.) via shoot regeneration or embryo like structures on modified N69-medium (Nitsch & Nitsch, 1969).

The induction of shoot cultures and the regeneration of plantlets in four different *Cyclamen* wild species (*C. coum, C. graecum, C. hederifolium, C. mirabile*) is described in this thesis (**Prange *et al*., 2008**). The induction of embryogenic cultures (callus and suspension cultures) and the regeneration via somatic embryogenesis is described for four different *Cyclamen* wild species (*C. alpinum, C. coum, C. graecum, C. mirabile*) (**Prange *et al*., 2010 a,b**). Based on the protocol of Winkelmann *et al.* (2006b) protoplast isolation and

regeneration to plants of these species were established within this thesis (**Prange *et al*., 2010 b,c**).

## 1.4 Interspecific Hybrids in *Cyclamen*

Hybridisation by classical breeding inside the genus *Cyclamen* has been rarely reported due to the disjunction of the genus into different subgenera with different chromosome numbers (Glasau, 1939; Grey-Wilson, 2003; Ishizaka, 2008). Even though some classical crossings between *Cyclamen* species are described in literature (Grey-Wilson, 2003) hybridisation with *C. persicum* as one hybridisation partner by classical methods is unsuccessful as far as it is published by now because of incompatibility reactions (Takamura, 2007; Ishizaka, 2008; Grey-Wilson, 2003) *inter alia* arising in abortion of embryos in the ovules.

**Table 2:** Interspecific hybridisation between *Cyclamen persicum* cultivars and other wild species. (Table adopted from Ishizaka (2008)).

| Cross combination | Methods[a] | Fertility of hybrids | Chromosome number of hybrids ($2n$) | References |
|---|---|---|---|---|
| *C. persicum* ($2n=2x=48$)×*C. repandum* ($2n=2x=20$) | ER | Sterile | 34 | Ishizaka and Uematsu (1990) |
| *C. persicum* ($2n=2x=48$)×*C. libanoticum* ($2n=2x=30$) | ER | Sterile | 39 | Shibusawa and Ogawa (1997) |
| *C. persicum* ($2n=2x=48$)×*C. cyprium* ($2n=2x=30$) | ER | Sterile | 39 | Shibusawa (personal communication) |
| *C. persicum* ($2n=2x=48$)×*C. mirabile* ($2n=2x=30$) | ER | —[b] | — | Yamashita and Takamura (2007) |
| *C. persicum* ($2n=2x=48$)×*C. hederifolium* ($2n=2x=34$) | ER | Sterile | 41 | Ishizaka and Uematsu (1992) |
| *C. persicum* ($2n=2x=48$)×*C. hederifolium* ($2n=2x=34$) | ER, CD | Fertile | 82 | Ishizaka and Uematsu (1994) |
| *C. hederifolium* ($2n=2x=34$)×*C. persicum* ($2n=2x=48$) | ER | Sterile | 41 | Ishizaka and Uematsu (1994) |
| *C. hederifolium* ($2n=2x=34$)×*C. persicum* ($2n=2x=48$) | ER, CD | Fertile | 82 | Ishizaka and Uematsu (1994) |
| *C. persicum* ($2n=4x=96$)×*C. hederifolium* ($2n=2x=34$) | ER | Sterile | 64 | Ishizaka and Uematsu (1992) |
| *C. persicum* ($2n=4x=96$)×*C. hederifolium* ($2n=4x=68$) | ER | Sterile | — | Ishizaka (unpublished) |
| *C. persicum* ($2n=2x=48$)×*C. africanum* ($2n=2x=34$) | ER | Sterile | — | Takamura et al. (2002) |
| *C. persicum* ($2n=2x=48$)×C. colchicum ($2n=2x=34$) | ER | Sterile | — | Yamashita and Takamura 2007 |
| *C. persicum* ($2n=2x=48$)×*C. purpurascens* ($2n=2x=34$) | ER | Sterile | 41 | Ishizaka and Uematsu (1995a), Ewald (1996) |
| *C. persicum* ($2n=2x=48$)×*C. purpurascens* ($2n=2x=34$) | ER, CD | Fertile | 82 | Ishizaka and Uematsu (1995b) |
| *C. persicum* ($2n=4x=96$)×*C. purpurascens* ($2n=2x=34$) | ER | Sterile | 65 | Ishizaka and Uematsu (1995a) |
| *C. persicum* ($2n=4x=96$)×*C. purpurascens* ($2n=4x=68$) | ER | Fertile | 82 | Ishizaka and Kondo (2004) |
| *C. persicum* ($2n=2x=48$)×*C. graecum* ($2n=4x=84$) | ER | Sterile | 66 | Ishizaka (1996, 2003) |
| *C. persicum* ($2n=4x=96$)×*C. graecum* ($2n=4x=84$) | ER | Fertile | 90 | Ishizaka (1996, 2003) |
| *C. graecum* ($2n=4x=84$)×*C. persicum* ($2n=4x=96$) | ER | Fertile | 90 | Ishizaka (unpublished) |
| *C. persicum* ($2n=2x=48$)×*C. rohlfsianum* ($2n=4x=96$) | ER | Sterile | — | Ishizaka (unpublished) |
| *C. persicum* ($2n=2x=48$)×*C. rohlfsianum* ($2n=6x=144$) | ER | Sterile | — | Ishizaka (unpublished) |
| *C. persicum* ($2n=4x=96$)×*C. rohlfsianum* ($2n=4x=96$) | ER | Fertile | — | Shibusawa and Ogawa (1997), Ishizaka (unpublished) |
| *C. persicum* ($2n=4x=96$)×*C. rohlfsianum* ($2n=6x=144$) | ER | Fertile | — | Ishizaka (unpublished) |
| *C. rohlfsianum* ($2n=6x=144$)×*C. persicum* ($2n=4x=96$) | ER | Fertile | — | Ishizaka (unpublished) |

[a] ER, hybrids were produced by embryo rescue. CD, amphidiploids were produced by chromosome doubling.
[b] Not examined.

The incompatibility barriers limiting hybridisation by classical breeding have been overcome using the embryo rescue technique. For the application of this tissue culture

method (for review, see Sharma *et al*. (1996) and references therein) the plants are classically crossed and after fertilization the developing embryo is cultivated *in vitro* to prevent its abortion. Ten different interspecific crosses in the genus *Cyclamen* have been produced using this method (Ishizaka & Uematsu, 1992; 1994; 1995; Ishizaka 1996; 2003; Ewald, 1996; Ishizaka, 2008). Table 2 gives a detailed overview on interspecific hybridisations between *C. persicum* and other *Cyclamen* species described in the literature so far. Some of the *Cyclamen* hybrids were introduced into the market as new ornamental plants [e.g., 'Odorella', (Ewald, 1996) and 'Kaori-no mai' (Ishizaka, 2008)]. The obtained allodiploid hybrids from these studies were in most cases infertile due to abnormal meiotic chromosome behaviour (Ishizaka, 2008) and could not be used in breeding programs. Pretreatment like polyploidization or the usage of naturally occurring allotetraploid wild species partly yielded fertile plants (Ishizaka, 2008). Although no extraordinary flower colour is known from any wild *Cyclamen* species Kondo *et al*. (2009) obtained flower colour mutants in a hybrid between *C. persicum* and *C. purpurascens* (cultivar 'Kaori-no mai') by irradiation. The obtained plants developed (already existing) red-purple flowers and contained delphinidin 3,5-diglucoside instead of malvidin 3,5-diglucoside, which has not been proven to occur in *Cyclamen* so far and is therefore an important step to a wider range of flower colours in *Cyclamen* cultivars.

Somatic hybridisation, which is discussed in the following chapter, has -to the authors best knowledge- not been applied for the improvement of the existing or the development of new *Cyclamen* species.

## 1.5 Somatic Hybridisation

For the hybridisation of different species via gametic hybridisation using classical breeding or embryo rescue successful fertilisation is necessary. Prerequisite for the success is a sexual compatibility and thereby a close relationship of the two species.

Somatic hybridisation via protoplast fusion combines two somatic cells and therefore, avoids pollination and fertilization and, thereby, the pre- and post-fertilization barriers separating two sexually incompatible species. There are even reports of successful fusion of animal with plant cells: Makoonkawkeyoon *et al.* (1995) reported the successful fusion of tobacco mesophyll protoplasts with mouse spleen cells from which mouse immunoglobuline producing plants were derived. The fusion of cells has no phylogenetic limitations. But the regeneration of organisms with two combined genomes is usually

limited to related species Johnson & Veilleux (2001). In plant sciences, this method has been used to combine many species including two distantly related species such as soybean (*Fabaceae*) and tobacco (*Solanaceae*) or carrot (*Apiaceae*) and *Petunia* (*Solanaceae*) resulting in hybrid cell lines which could not be regenerated to plants due to genetic incompatibility (Evans, 1983). In contrast to this, the assembly of the two Solanaceae tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum*), resulted in hybrid plants with intermediate phenotype (Melchers *et al*., 1978). These hybrids are often fertile, e.g., *Brassica napus* and *Arabidopsis thaliana* hybrids (Forsberg *et al*., 1994). However, also reports about sterile hybrids are numerous, e.g., *Lycopersicon esculentum* and *Solanum tuberosum* hybrids (Schiller *et al.*, 1982), precluding the incorporation into breeding programs without further modifications as polyploidization, e.g., by colchizine treatment (Ishizaka & Uematsu, 1994). Protoplast fusion is an important tool for the developments of new crops in some species [e.g., Brassicaceae and Solanaceae (Millam *et al*., 1995; Waara & Glimelius, 1995)]. Fusion of diploid somatic cells (and their nuclei) leads to tetraploid fusion products in contrast to gametic hybridisation where haploid gametes are combined. The combination of whole somatic cells combines not only two genomes with parts of the maternal plasmon as in gametic hybrids; it combines two entire plasmons or parts of them (Binding *et al*.,1986). Symmetric fusion generally refers to the fusion of two entire cells, whereas asymmetric fusion refers to the fusion of an entire protoplast with a protoplast part as obtained after gamma irradiation (Gleba *et al*., 1988) or irradiation with UV light. The genetic variation in somatic hybrids includes segregation and, thereby, new combinations of mixed organelles, cytoplasmic and nuclear recombination (Evans, 1983). Somaclonal variation that can occur in high amounts during protoplast culture and fusion (Winkelmann *et al*. 2008), as in any other tissue culture, can be utilized to uncover the natural variability and to develop new crops (Evans *et al.* 1984; Evans & Sharp, 1986).

In contrast to target specific genetic modification, somatic hybridisation does not require foreign marker genes (e.g., herbicide or antibiotic resistance genes) and the transfer of unidentified genes or polygenic traits is possible. In Germany, plants obtained via protoplast fusion are not necessarily classified as genetically modified (“Gesetze zur Regelung der Gentechnik §3”).

### 1.5.1 Methods of Somatic Hybridisation

For somatic hybridisation in plants the separation and removal of the cell wall is prerequisite and is done since 1960 by enzymatic digestion (Cocking, 1960) which was optimised by other authors [e.g. Takebe *et al.* (1968)]. These enzymes are multi-component enzyme mixtures (e.g., Cellulase R-10, Macerozyme R-10) of fungal origin bearing Cellulase, Pectinase, Hemicellulase and other enzyme activities (Beldmann *et al*., 1985) of which mainly the pectinases are digesting the middle lamella and the cellulases the cell wall. Due to the high intracellular osmotic pressure the wall-less cells ("protoplasts") have to be kept in iso-osmotic conditions according to the plant species, which is adjusted with mannitol, sorbitol, sucrose or other compounds of high osmolarity. For the protoplast fusion technology (somatic hybridisation) the isolation of protoplasts in high quantities and ability of these cells to regenerate to whole plants (totipotency) is essential (Waara & Glimelius, 1995). The first report of successful plant regeneration from protoplast was dated in 1971 (Nagata & Takebe, 1971; Takebe *et al*., 1971). Since then, plant regeneration for more than 320 higher plants from various genera has been described (Waara & Glimelius, 1995). For *C. persicum* an efficient protoplast isolation protocol and protoplast to plant system via somatic exists (Winkelmann *et al*., 2006b); cf. sub-chapter 1.2.2). In this study, protoplast isolation and regeneration via somatic embryogenesis for *C. coum* (**Prange *et al.* 2010a**) *C. alpinum*, *C. graecum* and *C. mirabile* (**Prange *et al.* 2010b**) was successfully established.

The removal of the cell wall leaves cells with naked plasma membranes, which bear a negative charge due to the phosphate groups of the phospholipid bilayer. Protoplast fusion itself has to be mediated by different chemical or physical triggers, which are often combined. The two main methods are electrofusion and chemical fusion, which are often varied in their experimental setup and their chemicals. For electrofusion the cells are attaching to each other in an alternating electric field (in a special fusion chamber) and fusion is induced by a short electric pulse of direct current (e.g., Watts & King, 1984; Bates & Hasenkampf, 1985; Koop & Schweiger, 1985; Puite *et al*., 1986; Fish *et al*., 1988; Guo & Deng, 1999; Durieu & Ochatt, 2000). For chemical fusion the agglutination of protoplasts is mainly mediated by polyethylene glycol (PEG). The fusion is induced after dilution of PEG with a solution containing calcium and a high pH (Kao & Michayluk, 1974; Kao, 1975; Waara & Glimelius, 1995) and no special equipment such as a fusion chamber is necessary. Fusion has also successfully been performed with one step fusion agents combining PEG, calcium and high pH [e.g., Menczel & Wolfe (1984)]. PEG is often

combined with different adjuvants such as glycine (Kao, 1975) or dimethylsulfioxide (DMSO) (Haydu *et al*., 1977; Menczel & Wolfe, 1984; Terada *et al*., 1987), or is even replaced completely by glycine (Durieu & Ochatt, 2000).

The developments of protoplast fusion started in 1970 when Power *et al*. first conducted fusion experiments by pushing protoplasts together with micro needles and observed highest fusion in solutions supplemented with sodium nitrate. In 1973, Keller & Melchers described the fusogenic effect of calcium and high pH on tobacco leaf protoplasts and one year later Kao & Michayluk (1974) combined and improved these methods by using PEG as mediator for protoplast agglutination. In the following years PEG-mediated fusion was one of the most successfully applied and often modified protocols (Waara & Glimelius, 1995). Adjuvants such as calcium (Kao, 1975), glycine (Kao, 1975), dimethylsulfoxide (DMSO) (Menczel & Wolfe, 1984) or other compounds were added to the fusion solutions. High pH is also often, but not always used to induce fusions. Fusions were also induced by high pH or calcium directly in the fusion solution (Menczel & Wolfe, 1984; Pelletier *et al*., 1983) or in succeeding dilution steps (Waara & Glimelius, 1995). Olivares-Fuster *et al*. (2002) combined PEG-mediated chemical fusion and electrical fusion in *Citrus*. For all fusion methods, heterofusion frequencies were ranging from about 1% to 20% depending on the plant material and the fusion conditions applied (Waara & Glimelius, 1995), but also much higher frequencies have been reported.

### 1.5.2 Selection of Heterofusion Products

Selection of heterofusion products is a crucial step. After protoplast fusion, the heterofusion products are still mixed with non-fused, homo-fused and multi-fused protoplasts. For selection and separation of heterofusion products numerous strategies have been evolved. The usage of auxotrophic (e.g., Sidorov *et al*., 1981a; Gupta *et al*., 1982; Sidorov & Maliga, 1982), chlorophyll deficient mutants (e.g., Gupta *et al*., 1984; Sidorov & Maliga, 1982) or resistant cell lines (e.g., Datta *et al*., 1990; Binding *et al*., 1982), which disable the parental cell line growth, has often been applied and partly combined with the visual identification of cells of different morphology in the protoplast culture (e.g., colour, protoplasmic strands, vacuolation, organelles) (Menczel & Wolfe, 1984; Kirti *et al*., 1992). Whereas special mutants have no general applicability, the utilization of different tissues for protoplast fusion for morphological selection can be applied to different plant systems. A prerequisite, however, is the regenerative capacity

and that the protoplasts yielded from these tissues are sufficient for protoplast fusion. Anyhow, these conditions are often the limiting factors in other species than *Solanum* and *Nicotiana* and many efforts were made for the development of tissue- and plant-independent selection strategies. Metabolic complementation using chemical compounds such as rhodamine 6-G, iodoacetate or iodacetamid (Sidorov *et al.*, 1981b; Menczel *et al*., 1982; Terada *et al*., 1987; Akagi *et al*., 1989; Böttcher *et al*., 1989; Ge *et al*., 2006) or irradiation (Sidorov *et al*., 1981b; Menczel *et al*., 1982; Akagi *et al*., 1989; Ge *et al*., 2006) has often been successfully used for the selection of heterofusion products. A second approach was the staining of protoplasts with fluorescence vital stains like fluoresceindiacetat (FDA), rhodamine-B-isothiocyanate (RBITC) or scopoletin often paired with chlorophyll autofluorescence (e.g., Kanchanapoom *et al.*, 1985; Durieu & Ochatt, 2000; Pattanavibool *et al*., 1998; Waara *et al*., 1998). This method requires, as the selection by morphological traits, the mechanical separation of the identified heterofusion products by micromanipulation (e.g., Hein *et al*., 1983; Puite *et al*., 1986; Sasamoto *et al*., 2000), other mechanical methods (e.g., Kao, 1977) or by cell sorting (e.g., Afonso *et al*., 1985; Hammatt *et al*., 1990; Waara *et al*., 1998). Another strategy to avoid any selection is the fusion of individual pairs of protoplasts, developed by Koop & Schweiger (1985). Although a broad range of approaches for the detection and separation of heterofusion products has been made, the application of these strategies is limited. Often special requirements and sensitivities of the plant species and their protoplasts are making the application of selection strategies impossible. Additionally, the optimisation of the described selection techniques is often troublesome (Waara & Glimelius, 1995).

The unselected cultivation and regeneration after protoplast fusion is always bearing the danger of the somatic hybrids to be overgrown and to remain undetected, if they are more fragile and less vigorous (e.g., Gleddie *et al.*, 1986) than the parental regenerants. However, many workgroups were successful in regenerating somatic hybrids without selection after fusion (Fish *et al*., 1987; Chaput *et al*., 1990; Sihachakr *et al*., 1989). Callus and shoots were identified by morphological differences between the somatic hybrids during later culture by morphological differences between the parental and the hybrid tissue (e.g., pigmentation, hairs) (e.g., Binding *et al*., 1982; Gleddie *et al*., 1986; Handley *et al*., 1986; Fish *et al*., 1987), by hybrid vigour of the hybrid tissue (e.g., Handley *et al*., 1986; Debnath & Wenzel, 1987; Chaput *et al*., 1990) or by efficient physiological or molecular biological screening methods (see below, chapter 1.5.3).

### 1.5.3 Identification and Characterisation of Somatic Hybrid Plants

Somatic hybrid plants produced from different species can often be identified by morphological characters of the developing callus, shoots or the whole plants, which are often intermediate between the parental species. Early detection of the hybrid status is desirable due to the often labour-intensive cultivation of a huge amount of putative hybrid calluses and plantlets. Many cytological, physiological and molecular methods were developed for these purpose, in the following some examples will be given.

For the establishment of physiological fingerprints isoenzyme patterns [e.g., alcohol dehydrogenase (EC 1.1.1.1.), aspartate aminotransferase (EC 2.6.1.1.), glutamate-oxaloacetate transaminase (EC 2.6.1.1.), 6 phosphogluconate dehydrogenase (EC 1.1.1.44), glucosephosphate isomerase (EC 5.3.1.9)] are a stable trait for the identification of species or varieties. This trait has been often used for somatic hybrid identification in the early years of research in this field (e.g., Wetter, 1977; Fish *et al*. 1988; Chaput *et al*., 1990; Rasmussen & Rasmussen, 1995).

After somatic hybridisation, the addition of the two chromosome sets can be detected by flow cytometry (e.g., Fahleson *et al*., 1988; Buitefeld *et al*., 1989; Chaput *et al*., 1990; Sundberg & Glimelius, 1991; Miranda *et al*., 1997), which is a fast and easy method for mass screenings, but putative chromosome losses (aneuploidy) have to be considered in the interpretation of the results and can lead to the oversight of hybrid plants. The direct counting of chromosomes is a very simple, but more time consuming method (Fahleson *et al*., 1988, Guo *et al*., 2002), which delivers more accurate results, especially if the chromosomes of the two species are of different size. These investigations are also important to confirm the true hybrid status and to exclude the existence of chimeras which can also occur as a result or by product in somatic hybridisation experiments (e.g., Sjödin & Glimelius, 1989).

Many molecular methods were developed in the last decades for hybrid identification, often with the focus of fast, easy and cost efficient methods. One of these methods is the PCR with arbitrary primers named RAPD (Random Amplified Polymorphic DNA) (Williams *et al*.. 1990b; McClelland & Welsh, 1990). In a simple PCR reaction a genomic fingerprint of the parental and the hybrid plant can be established and compared. It can be applied in all domains and no sequence information about the target organisms is necessary and has often been used for identification of somatic hybrids (e.g., Baird *et al*., 1992; Xu *et al*.,

1993; Rasmussen & Rasmussen, 1995, Rasmussen *et al*., 1997; Guo *et al*., 2002; Xia *et al*., 2003). This analysis has often a limited reproducibility, and, therefore, many repetitions are necessary to yield scorable band patterns. It has also taken into account that bands of similar size are not necessarily homologous, which might lead to misinterpretations (e.g., Quiros *et al*., 1995; Degani *et al.*, 1998).

Some more complex and work intensive methods for the establishment of genetic fingerprints from organisms with unknown sequence data are RFLP (Restriction Fragment Length Polymorphism) and AFLP (Amplified Fragment Length Polymorphism) which are combining PCR-reactions, digestion with restriction enzymes and other techniques (Weising *et al*., 2005) leading to a higher stability and reproducibility than RAPD screenings. RFLP (e.g., Williams *et al*., 1990a; Sundberg & Glimelius, 1991) and AFLP (e.g., Brewer *et al*., 1999; Guo *et al*., 2002; Ma *et al*., 2006) were successfully applied for somatic hybrid analysis. Furthermore, other molecular methods based on species specific repetitive DNA sequences (e.g., Fahleson *et al*., 1997) as well as Southern Hybridisation (Fish *et al.*, 1988; Sundberg & Glimelius, 1991; Miranda *et al.*, 1997) were applied.

A combination of molecular methods and hybrid chromosome visualisation are the FISH (Fluorescence In Situ Hybridisation) and the GISH (Genomic In Situ Hybridisation) techniques, which are more complex to establish. They provide information about the genetic composition of chromosomes and the distribution of genetic material originating from different species (Raina & Rani, 2001; Jiang & Gill, 2006). The parental chromosomes or chromosome parts are individually labelled and can be attributed to each of the two parental plants. Therefore, these methods can be used not only to discriminate parental chromosomes, but also to detect recombination (Buitefeld *et al*., 1998; Jacobsen *et al*., 1995; Xia *et al*., 2003; Ma *et al*., 2006).

# 2 AiF Cooperation Project "Development and Evaluation of Methods for the Creation of New *Cyclamen*"

## 2.1 Project Description

The thesis presented here was performed as a part of the cooperation project entitled “Development and Evaluation of Methods for the Creation of New *Cyclamen*” (“Entwicklung und Bewertung von Methoden zur Erstellung neuer *Cyclamen*”) which was financially supported by the German Federation of Industrial Research Association (AiF) within the program PRO INNO (Grant no. KF0054802MD5, 01.01.2006-30.12.2008).

The project was aiming to create a broad spectrum of completely new *Cyclamen* species and genotypes, respectively. These plants should be obtained by (a) gametic hybridisation using the embryo rescue technique accomplished at the Leibniz-Institute of Vegetable and Ornamental Crops (Großbeeren/Erfurt, Germany) and (b) by somatic hybridisation using protoplast fusion carried out at the Institute for Floriculture and Woody Plant Sciences, University of Hannover, and presented in this thesis. The propagation and cultivation techniques as well as breeding and horticultural properties of the obtained hybrids had to be evaluated and optimised by the other project partners. Figure 3 gives a visualisation of the project partners, their connections and contributions.

(1) Sub-Project “Somatic Hybridisation of Different *Cyclamen* Species”, Leibniz University of Hannover, Institute for Floriculture and Woody Plant Science, Prof. Dr. Traud Winkelmann (coordinator), Anika Prange

(2) Sub-Project “Gametic Hybridisation of Different *Cyclamen* Species”, Leibniz Institute of Vegetables and Ornamental Crops, Großbeeren/Erfurt, Dr. Aloma Ewald, Dr. Sandra Reinhardt

(3) Sub-Project “Propagation of Different *Cyclamen* Species”, Steva GmbH, Gerd Steverding, Dr. Mahir Girmen

(4) Sub-Project “Value Analysis for Breeding Properties”, Varinova B.V., Berkel en Rodenrjis, The Netherlands, Bart Kuijer

(5) Sub-Project “Cultivation Techniques and Horticultural Evaluation”, Blumen Liede, Manfred Roeßmann; ARKADIA - das grüne Zentrum, Arpke, Siegfried Müller, Renate Schmeikal

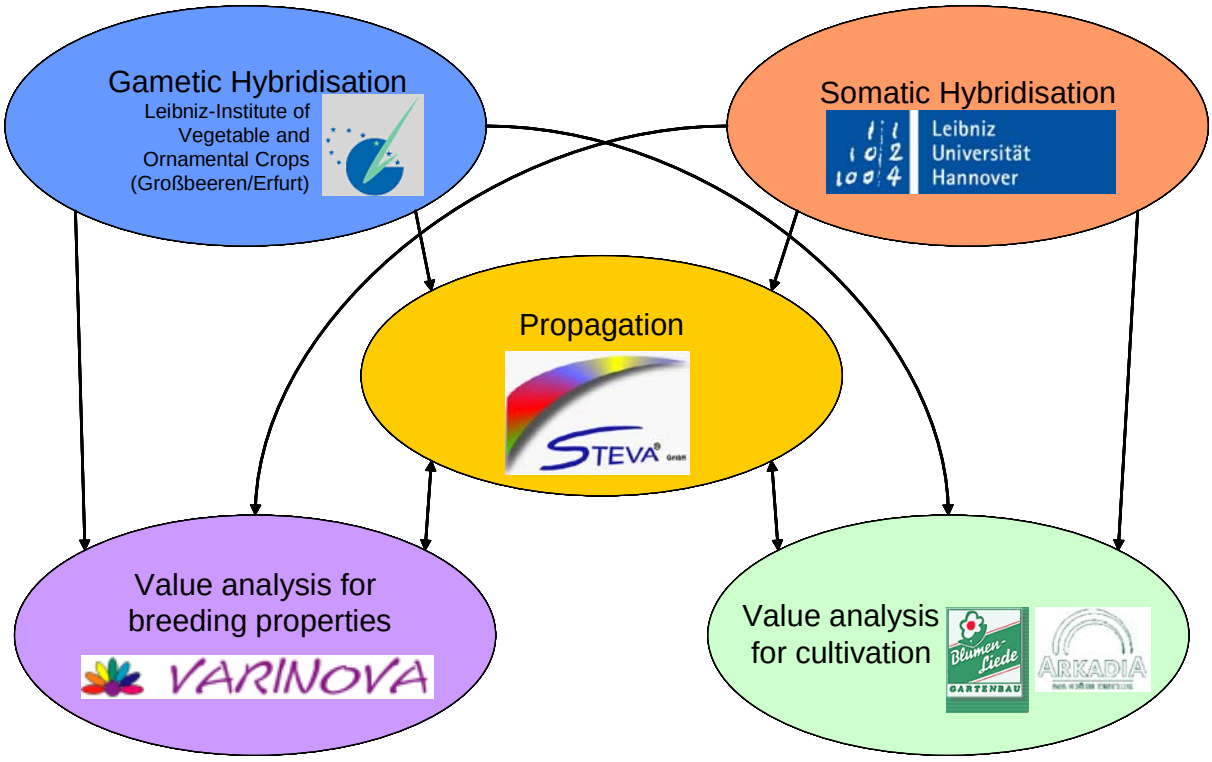


**Figure 3:** The five partners of the AiF cooperation project and their contributions.

## 2.2 Goals of this Thesis within the Project

In detail, the objectives of this thesis and the sub-project "Somatic Hybridisation of Different *Cyclamen* species" were:

i) Establishment of *in vitro* shoot cultures from different "wild" *Cyclamen* species as initial material for protoplast isolation (cf. chapter 3.1; **Prange *et al*., 2008**).

ii) Establishment of a regeneration system from protoplasts to plants via somatic embryogenesis in *Cyclamen coum* (cf. chapter 3.2; **Prange *et al*., 2010a**) and adaption and optimisation of the protoplast to plant system to other wild *Cyclamen* species (cf. chapter 3.3; **Prange *et al*., 2010b**).

iii) Somatic hybridisation of *C. persicum* cultivars and other *Cyclamen* species. Including, the development of a selection system for heterologous fusion products, the establishment of a chemical fusion method using polyethylene glycol (PEG), the fusion of protoplasts and regeneration of hybrid plants and the establishment of a molecular marker system and characterisation of the hybrid plants (cf. chapter 3.4; manuscript, submitted to *Plant Cell Reports*, **Prange *et al*., 2010c**).

# 3 Compilation of Scientific Publications

In sub-chapter 3.1, protocols for shoot regeneration from different wild *Cyclamen* genotypes (*C. graecum* Link, *C. hederifolium* Aiton, *C. mirabile* Hildebrand, *C. coum* Miller) using seedling tissue (cotyledon, tuber, root) were established for the first time (**Prange *et al*., 2008**).

In sub-chapter 3.2, the induction and characterisation of embryogenic callus and the establishment of embryogenic suspension cultures in *C. coum* are described for the first time. Plants were regenerated from this material via somatic embryogenesis, protoplasts were isolated from embryogenic suspension cultures and somatic embryos were regenerated to plants. The somaclonal variation and occurrence of polyploidisation was determined in these regenerants (**Prange *et al*., 2010a**).

In sub-chapter 3.3, the induction and characterisation of embryogenic callus and the establishment of embryogenic suspension cultures in *C. graecum*, *C. mirabile*, *C. graecum*, *C. trochopteranthum* Schwarz (Syn. *C. alpinum* hort. Dammann ex Sprenger) are shown for the first time. Plants were regenerated from this material via somatic embryogenesis, protoplasts were isolated from embryogenic suspension cultures and regenerated to plants (**Prange *et al*., 2010b**).

In sub-chapter 3.4, the protoplast fusion of two different Cyclamen species is reported for the first time. Somatic hybrids of *C. coum* and *C. persicum* were obtained via protoplast fusion and hybrid plants were regenerated via somatic embryogenesis. The hybrid status of the plants was verified via flow cytometry and molecular markers (**Prange *et al*., 2010c**).

Four annexe to sub-chapter 3.4 (**Prange *et al*., 2010c**) with supplementary material are included:

Annex I: Staining of Protoplasts

Annex II: Protoplast Fusion

Annex III: Selection of the Heterofusion Products
Cell Finder Slights, Fluorescence Activated Cell Sorting, Microfluidic Chips

Annex IV: Species-Specific Molecular Markers

## 3.1 Publication 1 (www.journal-pop.org/2008_8_4_204-209.html)

**Prange, A.N.S., Serek, M. & Winkelmann, T. (2008)**. Vegetative propagation of different Cyclamen species via adventitous shoot formation from seedling tissue. *Propagation of Ornamental Plants* **8**: 204-209.

### VEGETATIVE PROPAGATION OF DIFFERENT CYCLAMEN SPECIES VIA ADVENTITIOUS SHOOT FORMATION FROM SEEDLING TISSUE

**Anika Nadja Sabine Prange[1]*, Margrethe Serek[1], and Traud Winkelmann[2]**

[1]Gottfried Wilhelm Leibniz University of Hannover, Institute of Floriculture and Woody Plant Science, Floriculture Section, Herrenhaeuser Str. 2, D-30419 Hannover, Germany, *Fax: 0049 511 762 2654, *E-mail: prange@zier.uni-hannover.de
[2]University of Applied Sciences Weihenstephan and Research Station for Horticulture, Am Staudengarten 8, D-85354 Freising, Germany

**Abstract**
While *Cyclamen persicum* Miller has gained economic importance as an important ornamental plant worldwide, only a few of the other nineteen species of the same genus have commercial relevance although having a lot of interesting and valuable traits. To examine these traits under *in vitro* culture and greenhouse conditions an efficient *in vitro* propagation method of different *Cyclamen* species was established. Sterile seedlings of *Cyclamen mirabile* Hildebrand, *Cyclamen coum* Miller, *Cyclamen graecum* Link and *Cyclamen hederifolium* Aiton divided in cotyledon, tuber and roots were used as a starting material. Shoot induction was obtained on half-strength Murashige and Skoog based medium containing 0.5 mg $l^{-1}$ NAA and 1 mg $l^{-1}$ BAP and on medium with 0.5 mg $l^{-1}$ IAA, 1 mg $l^{-1}$ BAP, 1 mg $l^{-1}$ 2iP and 1 mg $l^{-1}$ kinetin as growth regulators. The most suitable tissues for shoot induction were tubers and/or cotyledons depending on the species. Less or no shoots were obtained from roots when used as starting tissue, but root formation was found in high frequency. There were pronounced genotypic differences in the reaction to the different media within one species. The medium with lower contents of cytokinins showed high formation of roots, possibly due to high endogenous auxin content in the tissue.

**Keywords:** adventitious shoot induction, cyclamen, *in vitro* propagation, organogenesis, ornamental plant, regeneration

Full paper available: **www.journal-pop.org/2008_8_4_204-209.html** (abstract)
Journal homepage: **www.journal-pop.org/index.php**
Electronic offprints (pdf file) of the publication can be ordered at:
www.journal-pop.org/subscribtion.php, follow “Order this journal” (pdf offprint: 40US$)

## 3.2 Publication 2 (www.springerlink.com/content/p0172m027727)

**Prange, A.N.S., Serek, M., Bartsch, M. & Winkelmann, T. (2010a)**. Somatic embryogenesis as suitable basis for efficient and stable regeneration from protoplast to plant in Cyclamen coum Miller. *Plant Cell, Tissue and Organ Culture* **101**: 171-182.

**SOMATIC EMBRYOGENESIS AS SUITABLE BASIS FOR EFFICIENT AND STABLE REGENERATION FROM PROTOPLAST TO PLANT IN CYCLAMEN COUM MILLER.**

**Anika Nadja Sabine Prange[1]*, Margrethe Serek[1], Melanie Bartsch1 and Traud Winkelmann[2]**

Institute of Floriculture and Woody Plant Science, Gottfried Wilhelm Leibniz University of Hanover, Herrenhaeuser Str. 2, 30419 Hannover, Germany
e-mail: traud.winkelmann@zier.uni-hannover.de

**Abstract**
Embryogenic cultures of *Cyclamen coum* were established on solid media and in suspension, and their growth characteristics in response to different concentrations of plant growth regulators (PGRs) were evaluated. Embryogenic cultures exhibited a high regeneration capacity of 876 somatic embryos per gram fresh mass. Up to $4.24 \times 10^5$ protoplasts per gram of fresh mass were isolated from somatic embryos and embryogenic suspension cultures. Protoplasts derived from both embryos and suspension cultures were successfully cultured in vitro and regenerated into plants via somatic embryogenesis. Phenotypic analyses and flow cytometric measurements revealed that some regenerated plants were tetraploid. About 20% of the protoplast-derived calluses used for regeneration were tetraploid, while tetraploidy was found in 0.9% of the plants regenerated from the embryogenic cultures.

**Keywords:** Flow cytometry - Ornamental plant - Ploidy stability - Protoplast isolation - Somaclonal variation - Vegetative propagation

Full paper available: **www.springerlink.com/content/p0172m027727**
Journal homepage: **www.springer.com/life+sciences/plant+sciences/journal/11240**
**DOI: 10.1007/s11240-010-9674-z**

## 3.3 Publication 3 (www.sciencedirect.com/science/journal/03044238)

**Prange, A.N.S., Bartsch, M., Serek, M. & Winkelmann, T. (2010b)**. Regeneration of different Cyclamen species via *somatic embryogenesis from callus, suspension cultures and protoplasts. Scientia* Horticulturae **125**: 442-450.

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

Anika Nadja Sabine Prange, Melanie Bartsch, Margrethe Serek, Traud Winkelmann*

*Gottfried Wilhelm Leibniz Universitaet Hannover, Institute of Floriculture and Woody Plant Science, Herrenhaeuser Str. 2, D-30419 Hannover, Germany*

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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 $1.4 \times 10^6$ 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.

* Corresponding author. Tel.: +49 511 762 3602; fax: +49 511 762 3608.
*E-mail address:* traud.winkelmann@zier.uni-hannover.de (T. Winkelmann).

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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 ($\mu$) were calculated based on the linear phase of the growth curves and calculated as the slope of the linear regression with $\mu$ [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 $1.5 \times 10^5$ 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 ($2.28 \pm 0.27$ and $2.15 \pm 0.33$ 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).

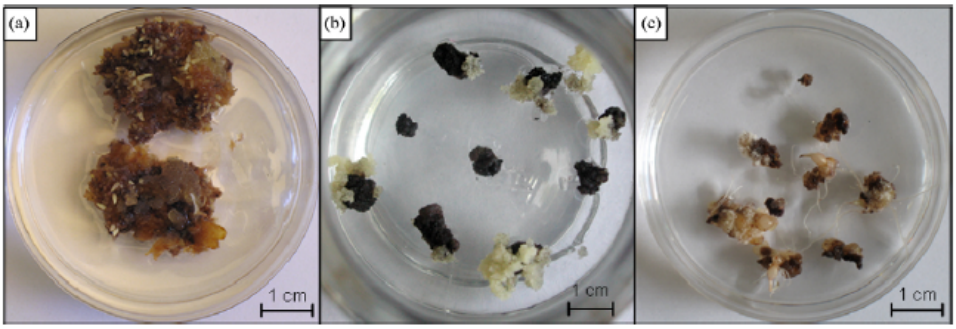


**Fig. 1.** Callus induction from different seedling explant types of the three *Cyclamen* species. (a) Cotyledon explants of *C. mirabile* after about 6 months with formation of friable callus and somatic embryos. (b) Tuber explants of *C. graecum* after about 4 months with friable and embryogenic yellow 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 $2.9 \times 10^5$ (*C. alpinum*) and $13.6 \times 10^5$ (*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.

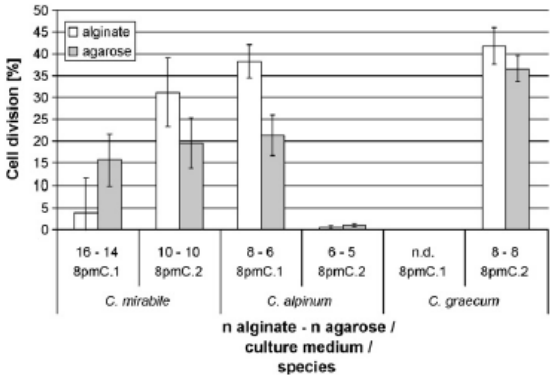


**Fig. 2.** Influence of the culture medium and the embedding agent on the cell division of *C. mirabile*, *C. alpinum* and *C. graecum* protoplasts after 2 weeks of culture (I – standard deviation, *n* – number of replications per variant), n.d. – not determined.

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).

### 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.

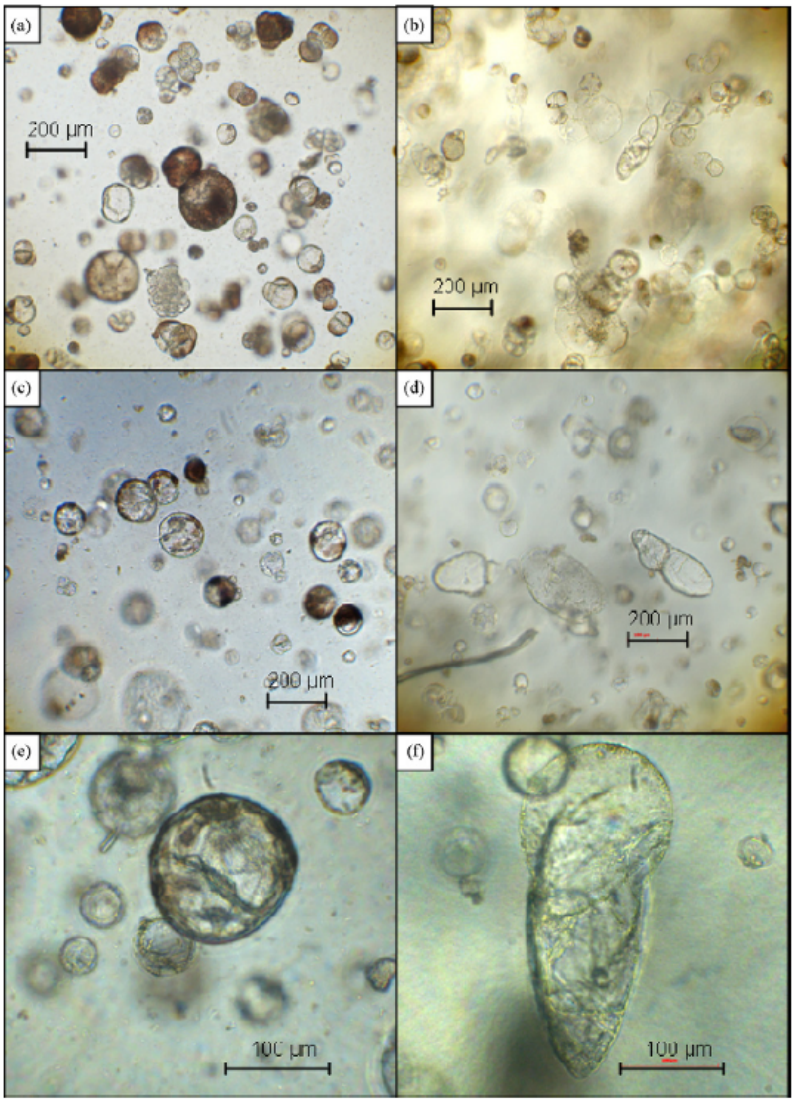


**Fig. 3.** Comparison of the embedded protoplast cultures after 2 weeks. (a) Protoplasts of *C. alpinum* embedded in alginate. (b) Protoplasts of *C. alpinum* embedded in agarose. (c) and (e) Protoplasts of *C. mirabile* embedded in alginate. (d) and (f) Protoplasts of *C. mirabile* embedded in agarose.

## 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

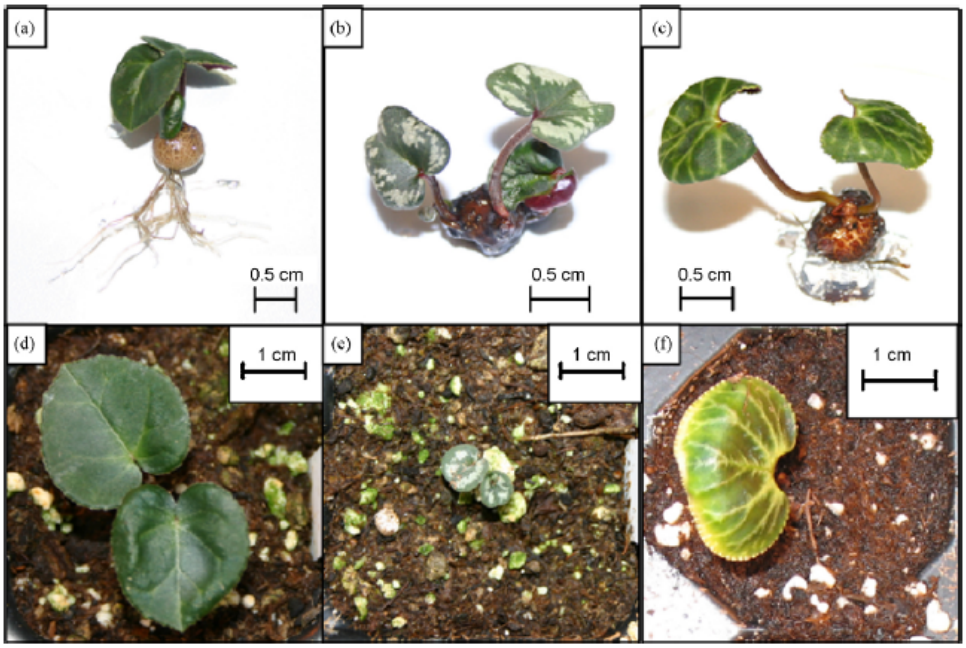


**Fig. 4.** *C. mirabile* (a), *C. alpinum* (b) and *C. graecum* (c) plants regenerated from protoplasts prior to transfer to the greenhouse. *C. mirabile* (d), *C. alpinum* (e) and *C. graecum* (f) plants regenerated from protoplasts about 2.5 weeks after transfer to the greenhouse.

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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## 3.4 Publication 4 (Manuscript for Review)

**Plant Cell Reports**

Manuscript for Review

# Interspecific somatic hybrids between Cyclamen persicum and C. coum, two sexually incompatible species



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| | |



# Interspecific somatic hybrids between *Cyclamen persicum* and *C. coum*, two sexually incompatible species

Anika Nadja Sabine Prange, Melanie Bartsch, Margrethe Serek, Traud Winkelmann

Gottfried Wilhelm Leibniz University Hannover, Institute of Floriculture and Woody Plant Science, Herrenhaeuser Str. 2, D-30419 Hannover, Germany,
Tel. +49 511 762 3602, Fax: 0049 511 762 3608, email:
traud.winkelmann@zier.uni-hannover.de

**Key words:** ornamental plant, protoplast fusion, species-specific molecular marker, staining, scopoletin, flow cytometry, DNA content

**Abstract**

By applying polyethylene glycol (PEG)-mediated protoplast fusion, the first somatic hybrids were obtained between *Cyclamen persicum* and *C. coum* – two species that cannot be combined by cross breeding. Heterofusion was detected by double fluorescent staining with fluorescein diacetate and scopoletin. The highest heterofusion frequencies (of about 5%) resulted from a protocol using a protoplast density of 1 x $10^6$ $ml^{-1}$ and 40% PEG. Somatic hybrids were identified by flow cytometry and PCR with species-specific DNA markers. The DNA content of *C. coum* was estimated for the first time by propidium iodide staining to be 14.7 pg/ 2C. This DNA amount was 4.6 times higher than that of *C. persicum*. Among 200 plants regenerated from fusion experiments, most resembled the *C. coum* parent, whereas only 5 plants showed typical *C. persicum* phenotypes. By flow cytometry, seven putative somatic hybrids were detected. A species-specific DNA marker was developed based on the sequence of the 5.8S gene in the ribosomal nuclear DNA and its flanking internal transcribed spacers ITS1 and ITS2. Out of the seven somatic hybrid plants identified by flow cytometry, the hybrid status of only one could be verified by the species specific DNA marker as well as sequencing of the amplification product. RAPD markers turned out to be less informative and applicable for hybrid identification, as no clear additivity of the two parental marker bands was observed.

**Abbreviations:** BA – 6-benzyladenine, 2iP – 6-(γ,γ-dimethylallylamino)purine, DAPI - 4′,6-diamidino-2-phenylindol, 2,4D – 2,4-dichlorophenoxyacetic acid, FDA – fluorescein diacetate, FISH - fluorescent-in-situ-hybridization, PEG - polyethylene glycol, PGR – plant growth regulator, RFLP - Restriction-fragment length-polymorphism

## Introduction

In recent years, scientists and breeders have endeavoured to include different *Cyclamen* species into breeding programs of *C. persicum* and to combine the valuable *C. persicum* qualities developed in decades of breeding with new traits. Hybrid inviability (post-fertilisation barriers) and cross incompatibility (pre-fertilisation barriers) have impeded hybridisation between *C. persicum* and other *Cyclamen* species both in nature and in classical breeding attempts (Ishizaka 2008). Hybrids of a number of species combinations have been obtained by using the embryo rescue technique (Ewald 1996, Ishizaka 2008 and references therein), which is able to overcome hybrid inviability by cultivating the hybrid embryos in vitro to prevent abortion. By using this method, new ornamentals have been developed that are already being introduced to the market (Ewald et al. 2000, Ishizaka 2008). However, these hybrids are often sterile, and therefore they cannot be directly included in breeding programs. In addition to hybridisation by fertilisation (gametic hybridisation), two plant species can be hybridised via protoplast fusion (somatic hybridisation), by which hybrid lethality and cross incompatibility can be overcome. Gametic hybridisation combines two haploid genomes, whereas somatic hybridisation assembles -entirely or partially- two diploid genomes and potentially all parental organelles. In interspecific crosses in the genus *Cyclamen*, which is subdivided into different subgenera differing in chromosome number (e.g., Glasau 1939, Grey-Wilson 2003, Ishizaka 2008), problems with chromosome pairing can occur during meiosis, leading to infertility. This might be overcome by combining two entire diploid genomes using somatic hybridisation. The probability of obtaining fertile hybrid plants is enhanced, and polyploidisation prior to subsequent classical breeding might be unnecessary. The possible transfer of all organelles and the possible exclusion of maternal effects is also an interesting feature of this hybridisation method.

After the first enzymatic isolation of plant protoplasts in 1960 (Cocking 1960), many techniques and protocols for protoplast isolation, protoplast regeneration and protoplast fusion were developed. A prerequisite for obtaining hybrid plants via somatic hybridisation is a protocol capable of regenerating plants from protoplasts. Plant regeneration from protoplasts via somatic embryogenesis has already been described for *C. persicum* and *C. coum*

(Winkelmann et al. 2006, Prange et al. 2010). Up to now, no *C. persicum* (2n=48) *C. coum* Miller (2n=30) interspecific hybrids have been obtained either by classical breeding or with the embryo rescue technique. *C. coum* is naturally a winter or spring flowering plant with high frost hardiness that even flowers in the snow (Grey-Wilson 2003). Therefore, the combination of the horticultural qualities of *C. persicum* with the frost hardiness and interesting leaf and flower shapes of *C. coum* promises the creation of a new perennial of high commercial value, especially for breeders and consumers in the temperate zone.

It should be mentioned that a hybrid between *C. coum* and *C. persicum* named *C. x atkinsii* has been described in the literature of the 19th century (Henfrey et al. 1852, Grey-Wilson 2003). However, neither the original plant (nor any progeny) currently exists. At that time, the hybrid status was only proven phenotypically, and it has not been scientifically accepted due to unreproducibility (Glasau 1939, Grey-Wilson 2003).

This study had the following objectives: i) the optimisation of a stable and non-destructive staining method for *Cyclamen* protoplasts to evaluate the heterofusion frequencies of different fusion protocols, ii) the development and comparison of different PEG-mediated protoplast fusion protocols, iii) the fusion of *C. persicum* and *C. coum* protoplasts and the regeneration of hybrid plants, and iv) the development of a system for the detection of somatic hybrids between *C. persicum* and *C. coum* using newly developed species-specific molecular markers and flow cytometry.

## Materials and methods

### *Establishing embryogenic cultures and standard protoplast isolation*

Embryogenic callus cultures of the *C. persicum* cultivar 'Maxora light purple' (Varinova B.V., The Netherlands) were obtained based on the protocol of Schwenkel and Winkelmann (1998) from single ovules of unpollinated flower buds. Embryogenic callus cultures of *C. coum* were induced on leaf explants from greenhouse grown plants (Prange et al. 2010). Embryogenic suspensions were established from these callus cultures of both species and used for protoplast isolation. The establishment and characterisation of embryogenic callus and suspension cultures (Winkelmann et al. 1998), optimisation of the protoplast isolation procedure and the regeneration of plants via somatic embryogenesis for both species have been previously described (Winkelmann et al. 2006, Prange et al. 2010).

For protoplast isolation, 6-12 d old cell material of *C. persicum* was incubated for 16 h in enzyme solution E1 containing 2.0% Cellulase R-10 and 0.5% Macerozyme R-10. For *C. coum*, the incubation time was increased to 24 h and the enzyme concentration was raised

to 3.0% Cellulase R-10 and 1.0% Macerozyme R-10. Protoplasts were isolated and cultivated as described in Prange et al. (2010) using medium 8pmC.1. This medium was based on the medium 8pm7 described by Winkelmann and Grunewald (1995) [based on KM8p, after Kao and Michayluk (1975)].

*Staining of protoplasts*

To monitor the process of protoplast fusion and to determine the heterofusion frequencies after protoplast fusion, protoplasts were stained with scopoletin and fluorescein diacetate (FDA).

For staining with scopoletin after Kanchanapoom et al. (1985), 0.2 or 0.4 mL of a scopoletin stock solution (1 mg scopoletin in 1 mL deionised water) per 25 mL of culture medium was added to the suspension cultures one day prior to protoplast isolation.

For staining with FDA (modified after Durieu and Ochatt 2000), the staining solution was prepared freshly by mixing 20 µL FDA stock solution (5 mg FDA per 1 mL acetone) with 1 mL protoplast buffer as described by Prange et al. (2010). Staining with FDA was performed during the first washing step of protoplast isolation by adding 25 µL staining solution per 1 mL of protoplast suspension. Protoplasts were pelleted as described in the standard protocol (Prange et al. 2010) and washed three times with isolation buffer after staining with FDA. Fluorescence was visualised under an inverted epifluorescence microscope (Axioskop; Zeiss, Germany) using an HBO® mercury short-arc lamp (Osram, Germany) and filter sets from AHF Analysentechnik AG (Germany). For FDA, the GFP Bright Line HC filter set F36-525 (520/35 nm excitation, 495 nm beam splitter, 472/30 nm emission) and for scopoletin a modified DAPI HC filter set F36-500 (386/23 nm excitation, 409 nm beam splitter, 377/50 nm emission) was used.

*Fusion of protoplasts*

Protoplasts were pelleted immediately after protoplast isolation and cell density was adjusted to a final density of 1 x $10^6$ or 4 x $10^6$ cells per mL in the respective solution for protoplast fusion. To optimise heterofusion frequency, two different protocols were tested with two different protoplast densities and different PEG concentrations.

Protocol I

After isolation, protoplasts were suspended in Kao solution I (500 mM glucose, 0.7 mM $KH_2PO_4$ x $H_2O$, 3.5 mM $CaCl_2$, pH 5.5) modified after Kao (1975). Protoplast suspension (300 µL) was pipetted in small droplets into 3-cm-Petri dishes. After settlement of the

protoplasts (at least 20 min), 600 µL PEG solution (30%, 35% or 40% PEG 6000, 10.5 mM $CaCl_2$ x 2 $H_2O$, 0.7 mM $KH_2PO_4$ x $H_2O$) was added and incubated for 20 to 30 min. After 20 min, the mixture was carefully diluted with 1 mL Kao solution I and 1 mL Kao solution II (50 mM glycine, 50 mM $CaCl_2$, 300 mM glucose, pH 9-10.5). After 20 min, the supernatant was removed and replaced by 0.5-1 mL culture medium (8pmC.1). Protoplasts were incubated for about 2-5 h in darkness for protoplast fusion. Controls were incubated in isolation buffer until the final embedding step.

Protocol II

For the second protocol (modified after Durieu and Ochatt (2000) and Menczel and Wolfe (1984)), the protoplasts were suspended in protoplast isolation buffer, and 300 µL of the protoplast suspension was pipetted in droplets into 3-cm Petri dishes. The PEG solution (30% or 35% PEG, 4% sucrose, 0.05 M $CaCl_2$) was added freshly prior to fusion with one drop of NaOH-glycine buffer (1 M NaOH, 0.4 M glycine) per 2 mL of PEG solution. After settlement of the protoplasts (at least 20 min), 450 µL supplemented PEG solution was added to the protoplasts. After 20-30 min, the supernatant was removed and the protoplasts were flooded with 0.5-1 mL culture medium (8pmC.1) and incubated 2-5 h for fusion as described above. After the final incubation in culture medium, the protoplasts were removed from the bottom of the Petri dish by gentle rotation. The protoplast suspension was mixed in a 5:3 ratio with preheated (about 50°C) low melting (LM) agarose solution [3% LM agarose (Biozym Scientific, Germany), 0.5 M Mannitol]. Protoplast cultures were embedded according to Binding et al. (1988). Fusion frequencies were determined about 12 h after embedding under an inverted epifluorescence microscope in the Petri dishes by counting about 90-100 protoplasts per repetition. In each viewing field, the single-stained protoplasts of each colour and the double-stained protoplasts were counted. Heterofusion frequencies were determined by dividing the number of double-stained protoplasts by the total number of protoplasts and multiplying by 100.

*Growth of protoplast-derived embryogenic cultures and plant regeneration*

About 1,000 to 1,500 protoplast-derived calluses (or more precisely, embryogenic cultures) per experiment from three independent fusion experiments were transferred to culture medium 2.31.S (solid medium containing 1 mg $L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.4 mg $L^{-1}$ 6-γ,γ-dimethylallylamino purine (2iP) according to Prange et al. (2010)) from the agarose lenses when they reached a size of about 1 mm in diameter and were cultivated for about 8 to 16 weeks. Somatic embryos and embryogenic cultures >5 mm in diameter were

transferred to medium 2.2.S (solid medium without plant growth regulators, according to Prange et al. (2010)) for development of somatic embryos. After 8 to 16 additional weeks, somatic embryos were singularised, cultivated in the dark to a cotyledonal length of about 1.5 cm and then transferred to a light/ dark cycle of 16/ 8 h with light supplied by cool white fluorescence lighting at 25 – 40 µmol $m^{-2}$ $s^{-1}$ photon flux density. Cotyledons, or preferably young secondary leaves, were used for flow cytometry and DNA isolation.

*Flow cytometry*

The absolute DNA content of *C. coum* was determined by propidium iodide staining using the CyStain PI absolute P- Kit (Partec GmbH, Germany). In four replications each, greenhouse-grown leaf material of *C. coum* was mixed with leaves of two reference plants, *Secale cereale* ssp. cereale (genebank Gatersleben acc. no. R737, 16.01 pg/ 2C) and *Vicia faba* cv. Tinova (genebank Gatersleben acc. no. FAB 602, 26.21 pg/ 2C), respectively. The absolute DNA content was estimated with a CyFlow®Ploidy Analyser (Partec GmbH, Germany) by counting at least 5,000 particles within the *C. coum* peak, applying a 488 nm laser and comparing the values of the G1 peak means to those of the internal reference standards. The relative DNA content of 46 potential hybrids with deviating morphology was compared with the parental *C. coum* and *C. persicum* plants (as controls) by flow cytometric measurements using 4′,6-diamidino-2-phenylindol (DAPI) (Table 1). Extraction and staining of nuclei were performed using the CyStain® UV precise P-Kit (Partec GmbH, Germany). About 0.5 $cm^2$ of leaf blades of in vitro grown cotyledons or preferably primary leaves were mixed with the same amount of in vitro leaf material from the *C. coum* (and sometimes also the *C. persicum*) parent. The relative DNA content of the sample was analysed with a CyFlow®Ploidy Analyser (Partec GmbH, Germany) by counting at least 2,000 particles within the main peak under UV excitation.

*DNA isolation*

DNA was isolated from in vitro grown plant material (cotyledons, leaves) using the DNeasy Plant Mini Kit (QIAGEN, Germany) or NucleoSpin® Plant II Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The plant material was pulverised in liquid nitrogen using a Mixer Mill MM 301 (Retsch, Germany) for 2 min (1500 $min^{-1}$).

*Species-specific PCR*

Species-specific PCR-based markers were developed for hybrid identification. Primer pairs were designed from the sequence data of the 5.8S gene in the ribosomal nuclear DNA and its flanking internal transcribed spacers ITS1 and ITS2 (Anderberg et al. 2000) for both *C. persicum* and *C. coum* (Table 2). Primers were selected in polymorphic regions of these sequences to ensure high species specificity. For the discrimination of mismatch amplifications, the resulting amplification products were chosen to have different lengths. To enhance the specificity of the primers for the *C. coum* DNA sequence and to minimise the binding sites in the *C. persicum* DNA-sequence, three nucleotides were mutated within the primer region of the *C. coum* primer pair (Table 2).

PCR reactions were performed in 25 µL volumes using 1x reaction buffer according to Williams et al. (1990) and containing 0.2 mM of each deoxyribonucleoside triphosphate, 10 pmol of each primer, 1 U Taq-polymerase (FIREPol® DNA Polymerase; AS Solis BioDyne, Estonia) and 10 ng DNA. Thermal cycling was conducted for 3 min of initial denaturation (95°C), 35 cycles of i) denaturation (30 s, 95°C), ii) annealing (30 s, 61 or 67°C depending on the primer pair (Table 2)) and iii) elongation (90 s, 72°C) and a final elongation step for 5 min (72°C). Reaction products were separated in 1% TAE agarose gels and visualised by ethidium bromide staining under UV light.

*C. persicum*-specific PCR fragments of the putative hybrid and a *C. persicum* control were excised from the agarose gel and purified using the Nucleo Spin® Extract II Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Purified PCR products were cloned into pJET1.2 using a CloneJET$^{TM}$ PCR Cloning Kit following the sticky-end cloning protocol (Fermentas, Germany). Sequencing of the cloned PCR products was performed by Eurofins MWG (Germany) using the pJET1.2 forward sequencing primer (5'-CGACTCACTATAGGGAGAGCGGC-3').

*RAPD analysis*

For RAPD (Random Amplified Polymorphic DNA)-PCR (Williams et al. 1990; McClelland and Welsh 1990), the decamer-primers A03, A08, A13, A15, A18 and C01-C05 (Carl Roth GmbH & Co KG, Germany) were used. At least two repetitions of each PCR reaction were performed in 25-µL volumes using 1x reaction buffer according to Williams et al. (1990) and containing 0.2 mM each of deoxyribonucleoside triphosphate, 10 pmol decamer primer, 1 U Taq-polymerase (FIREPol® DNA Polymerase; AS Solis BioDyne, Estonia) and 10 ng DNA. Thermal cycling was conducted by 5 min of initial denaturation (95°C), 40 cycles of i) denaturation (30 s, 95°C), ii) annealing (30 s, 39°C) and iii) elongation (90 s, 72°C) and a

final elongation step for 5 min (72°C). Reaction products were separated in 1% TAE agarose gels and visualised by ethidium bromide staining under UV light. For RAPD analysis, only reproducible amplification products scoreable in two replications were counted.

*Statistics*

Generalised linear models with were estimated by logit-link and quasibinomial family, followed by an analysis of deviance (McCullagh and Nelder 1989) and pairwise comparisons as described by Hothorn et al. (2008) when the number of replicates differed among variants. All statistical analyses were performed using R-2.9.2 (R Development Core Team, 2008).

## Results

*Protoplast staining*

Because protoplasts of *C. persicum* and *C. coum* could not be distinguished under the microscope, staining protocols for protoplasts of each parent had to be defined in order to be able to detect heterofusion. The goal was to select staining agents that are not harmful to the protoplasts, easy to detect, stable for at least some hours and that do not leach. We observed that FDA (or its solvent acetone) was highly destructive to protoplasts of *C. persicum* when applying the protocol of Durieu and Ochatt (2000), who in contrast did not find detrimental effects on protoplasts of *Latyrus sativus* L. The FDA staining caused agglutination and disruption of *C. persicum* protoplasts during the staining procedure and ongoing washing steps. Therefore, the FDA concentration and the staining protocol were modified, resulting in the present protocol.

For scopoletin, the concentration described by Kanchanapoom et al. (1985) proved to be appropriate, and no agglutination or reduced viability was observed during protoplast isolation. Additionally, the effects of the optimised FDA staining procedure and the scopoletin staining on cell division of *C. persicum* protoplast cultures were evaluated (Table S1 and S2). No significant influence of FDA, acetone or scopoletin on the division frequency of protoplasts was observed (Table S1 and S2). During protoplast cultivation, visual screening for microcallus formation revealed no influence of the different treatments. Protoplast-derived cultures of all treatments retained their capacity of somatic embryogenesis and somatic embryos matured and developed normally. FDA-stained protoplasts fluoresced light to bright green for 48 h up to four days (Fig. 1c, e). Scopoletin-stained protoplasts fluoresced bright blue (Fig. 1a, d). The blue fluorescence was found to have accumulated inside the vacuole as observed by Kanchanpoom et al. (1985) and Taguchi et al. (2000), and it was observable up to

24 - 48 h after protoplast isolation. Unstained protoplasts showed blue background fluorescence, which in some experiments was almost as intense as that emitted from the scopoletin-stained protoplasts. Due to fading of the two stains, heterofusion frequencies were determined 12 to 24 h after protoplast fusion.

*Protoplast fusion and determination of heterofusion frequencies.*

Both stains did not leach during protoplast fusion and the colour remained inside the protoplasts. The addition of the PEG fusion agent led to heavy aggregation and plasmolysis of the protoplasts, which formed characteristic "nets" (Figure 1a-c). After the addition of 8pmC.1 medium, most of the protoplasts retained their globular shape. The different protocols for protoplast fusion were evaluated by determining the heterofusion frequency of the stained protoplasts. In all variations of the two protoplast fusion methods, heterofusion products were observed (Fig 2), all of them leading to significantly higher heterofusion frequencies than in the controls (below 1% spontaneous fusion). The higher density of 4 x $10^6$ protoplasts $mL^{-1}$ resulted in reduced but less variable fusion frequencies (Fig. 2b versus Fig. 2a). Heterofusion frequencies ranged between 2.8% ± 2.3 (Kao protocol, 30% PEG) and 4.5% ± 4.3 (NaOH-glycine buffer protocol, 35% PEG). Although the different variations of the fusion methods and PEG concentrations did not affect the heterofusion rate significantly, the following tendencies were observed: (i) higher PEG concentrations often yielded higher fusion rates; (ii) the NaOH-Glycine protocol was in general more aggressive, and using 40% PEG in the fusion solution in this protocol was not tolerated by the protoplasts (data not shown); and (iii) increased stringency and fusion effectiveness were accompanied by increased protoplast deformation, agglutination and damage.

*Selection of heterofusion products*

The initial intention of selecting heterofusion products by detecting their position on Cell Finder Slides (POG Präzisionsoptik Gera GmbH, Germany), which are marked with a coordinate system, was difficult to realise. Due to the high friability of the developing microcalluses and the high cell densities during protoplast cultivation (which were obligatory for cultivation success after fusion), efficient separation of the selected heterofusion products was not possible. Therefore, we focused on the regeneration of unselected calluses from three independent protoplast isolations with all variations of the fusion methods.

Plantlets were regenerated from about 200 calluses obtained from the fusion experiments. The plants mostly resembled the *C. coum* parent, except for five plants that resembled the

*C. persicum* phenotype. Some plants displayed cotyledons and first leaves with striking morphologies, like malformations, pointed leaves, red-coloured or pink-patterned leaves and round connate leaves. However, these deviant characteristics often vanished with progressive development of the plants in vitro.

These deviating plants were preferably chosen for flow cytometric measurements.

*Estimation of the DNA content of* C. coum

Whereas the DNA content of *C. persicum* (2x = 48) had been previously estimated at 3.21 pg/2C (Borchert et al. 2007), no information was available for *C. coum* (2x = 30). Determination of *C. coum*'s absolute DNA content using propidium iodide staining revealed 14.71 pg/ 2C ± 0.13. The details of the measurements are included as supplementary material (Table S3). Hence, the DNA content of *C. coum* was about 4.6 times higher than that of *C. persicum*, enabling the flow cytometric detection of somatic hybrids.

*Flow cytometric analyses of putative somatic hybrids*

To detect somatic hybrids by flow cytometry, the sensitivity of the cytometer (the gain) was adjusted so that the diploid peak of *C. coum* appeared at about channel 100, and that of *C. persicum* at about 20 to 25. Nuclei of somatic hybrid plants (*C. coum* (2n=2x=30) + *C. persicum* (2n=2x=48) = presumably 78 chromosomes) were expected to have a fluorescence signal at about 120 to 125.

At first, several repeated measurements of different leaves of a single plant of *C. coum* revealed a variation of the peak position of about 20%. According to Prange et al. (2010), plants giving a fluorescence signal between about 80 and 120 can be classified as diploid and those with signals of 217 – 226 as tetraploid. Due to the variation in the flow cytometric measurements, each sample of the respective putative hybrid was co-chopped with *C. coum* leaf material as an internal standard, as recommended by Dolezel (1991), Dolezel and Bartos (2005) and Bennett et al. (2008).

Hybrid plants were identified by the detection of two separate peaks, indicating the measurement of two groups of nuclei distinguished by their DNA content. Six out of 46 morphologically deviating plants (19%) showed two distinct peaks (Table 1, Fig. 3), and three plants showed one very broad peak with two pronounced pinnacles. Therefore, DNA contents were assigned to these nine plants that corresponded to the sum of the DNA contents of both parental species. Flow cytometric measurements repeated after four months confirmed the

hybrid DNA content of seven of these plants, namely plant No. 5 out of the group with a broad peak and two pinnacles and all the plants with two distinct peaks (Table 2).

*Hybrid verification with a newly developed species-specific molecular marker*

The developed species-specific molecular marker was tested with DNA of the two parental species and an equal mixture of their DNA. As expected, the two primer pairs amplified two fragments of different lengths, which were exclusively observed in PCR preparations with the respective DNA of the *C. coum* (564 bp) and *C. persicum* (136 bp) target species and their mixture (Fig. 4).

DNA from the seven putative hybrid plants identified by flow cytometry, or in some cases plants originating from the same callus (and thus the same fusion event), was analysed using this marker system. One plant (No. 31) showed in both separate PCR reactions a species-specific amplification product of the expected length (Fig. 4). PCR reactions using DNA of this plant always yielded less intense bands in the *C. persicum*-specific amplification, whereas in reactions with the *C. coum*-specific primer pair, they were comparable to the control. This might partly be explained by the four to five times lower absolute DNA content of *C. persicum* in comparison to *C. coum* (see above). However, amplification of this fragment from mixed DNA in a 1:5 ratio (*C. persicum*: *C. coum*) did not reduce the amount of the amplification product to the same extent (data not shown). To ensure that this fragment was not a mismatch amplification, it was cloned and sequenced. Sequencing revealed 100% congruence of the fragments with the *C. persicum* sequence from the NCBI database (Fig. 5). The phenotype of this plant is depicted in Fig. 6: the leaf size and the growth type resemble *C. coum*, whereas the leaves are more heart-shaped, as in *C. persicum*. The leaf pattern deviates from both species. This hybrid plant originated from a variant of the Kao fusion protocol (Protocol I) (40% PEG).

In addition to the seven putative hybrids that came out of the flow cytometric analyses, 26 plants were randomly chosen among the regenerated plants and submitted for molecular characterisation with the species-specific primers. This attempt was undertaken in order to test for potential hybrids that might have been overlooked by only selecting morphologically deviating plants. In total, DNA was isolated from 33 plants regenerated from different calluses out of three different protoplast isolation procedures. However, no more putative hybrid plants were detected. The hybrid status of the putative hybrid plants No. 5, 23, 29, 30, 33, and 45 could thus not be confirmed with molecular methods.

*RAPD analysis of a somatic hybrid*

RAPD-PCR of DNA from putative hybrid plant No. 31 (Table 3) resulted in band-patterns that deviated from both parents. New bands were reproducibly observed, and some parental bands were absent (Table 3). The amplification of *C. persicum*-specific bands from this hybrid's DNA was reproducibly detected only for one case, and often only very weak amplification products were obtained.

## Discussion

*Scopoletin and FDA were suitable stains for detection of heterofusions*

Protoplasts isolated from embryogenic suspension cultures could not be visually distinguished and therefore could not be monitored during the fusion process. Thus, scopoletin and FDA were used to stain the protoplasts; neither stain interfered with the further development and growth of protoplasts. The blue background fluorescence of the unstained protoplasts observed in our study was also found by Waara et al. (1998) in potato protoplasts and ascribed to phenolic compounds and other secondary metabolites. Fluctuating intensities of this background fluorescence were observed between the different protoplast isolations. Due to the involvement of scopoletin and other coumarin compounds in the hypersensitive reaction of plants (Dorey et al. 1999), the formation of the fluorescing compounds might be related to stress factors during preculturing of the embryogenic suspension cultures, other factors that induce genes connected to the hypersensitive reaction or (more generally) to the synthesis of secondary metabolites. The conversion of scopoletin to scopolin, a 7-O-glucoside of scopoletin, and its active translocation to the vacuole (Kanchanapoom et al. 1985, Taguchi et al. 2000) is an advantage under protoplast fusion conditions. The stain is mainly enclosed in the plasma membrane and the tonoplast and does not leak from the cells when the outer membrane is perforated or disrupted during protoplast fusion. Leakage of the staining agent was observed to be problematic for the dye rhodamine-B-isothiocyanate (RBITC), leading to staining of all protoplasts of both parents (A. Prange, T. Winkelmann unpublished results). The establishment of a staining method for protoplasts is suggested by many authors as a prerequisite for the selection of heterofusion products (e. g., Kanchanapoom et al. 1985, Durieu and Ochatt 2000, Waara et al. 1998). Some workgroups have successfully separated heterofusion products by staining/ bleaching/ or autofluorescence followed by micromanipulation (e.g., Yarrow et al 1986, Mattheij and Puite 1992, Rasmussen et al. 1997) or by fluorescence-activated cell sorting (FACS) (Rasmussen et al. 1997, Waara et al 1998), which might be worth testing in future studies with *Cyclamen*.

*PEG induced heterofusions between* C. persicum *and* C. coum

The 1% to 5.6% heterofusion frequencies obtained in the *C. persicum* - *C. coum* fusion experiments were low in comparison to the 20% heterofusion rate described by Durieu and Ochatt (2000) but quite comparable to the 3% heterofusion frequencies obtained by Hein et al. (1983) applying PEG-mediated fusion. Kao et al. (1974) reported maximum heterofusion frequencies from 10% to 40%, depending on the species combination. In our experiments, the highest estimated heterofusion frequency in one Petri dish (35% PEG, NaOH protocol) was about 15% and therefore was also comparable to these results.

No statistical difference between the heterofusion frequencies of the different fusion methods was found. However, observation of the fusion process revealed gradual effects of the different fusion protocols and PEG concentrations. According to our observations, high quality protoplasts withstood needed more stringent fusion conditions than low quality protoplasts, which were often destroyed under the treatments yielding high fusion frequencies. Therefore, the lack of significant differences between the fusion protocols can most likely be explained by the changing quality of the protoplasts after protoplast isolation, which has also been observed by other authors (Durieu and Ochatt 2000, Meyer et al. 2009).

Among the 200 plants regenerated from the fusion experiments, the vast majority resembled the *C. coum* phenotype, whereas only a few showed *C. persicum* characteristics. Thus, either the fusion and culture conditions were better tolerated by *C. coum* protoplasts because of higher quality and stability, or (more likely) the embedding in agarose favoured the development of *C. coum* protoplasts as was reported earlier (Winkelmann et al. 2006, Prange et al. 2010). The regeneration of both parental types was a positive indication, because protoplasts of both species were able to survive the stressful fusion procedure.

*Flow cytometric identification of somatic hybrids*

The DNA content of *C. coum* (14.71 ± 0.13 pg/ 2C) was estimated for the first time in this study and was found to be 4.6 times that of *C. persicum* (3.21 ± 0.003 pg/ 2C; Borchert et al. 2007). This fact supports our own and earlier observations that *C. coum* has the largest chromosomes of the genus (Glasau 1939).

High fluctuation in the peak position of diploid *C. coum* leaves was observed in the current measurements as well as in previous reports using DAPI staining (Prange et al. 2010). One explanation for this could be that anthocyanins in the leaf tissue and phenolic substances interfere with the DNA staining, as reported by many authors (e.g., Bennett et al. 2008 and

references therein, Greilhuber 2008 and references therein) for propidium iodide staining. In samples mixed and prepared simultaneously (chopping and staining), the inhibiting influence of anthocyanins in *Euphorbia pulcherrima* affected the internal standard in the same manner as the tissue samples in the experiments of Bennett et al. (2008). We suggest that DAPI staining is affected by these substances in a similar manner as described by these authors, which makes internal standardisation obligatory. When this internal standard was used, reproducible results were obtained.

Although the peak positions shifted between different measurements, flow cytometric detection of the hybrid plants by two separate peaks was reproducible even after four months (data not shown).

*Species-specific marker supports hybrid status for one plant*

The species-specific molecular markers developed in this study were successfully applied to four other genotypes of both *C. persicum* and *C. coum* (results not shown), indicating their species-wide applicability.

When the seven putative somatic hybrids identified by flow cytometry were subjected to this molecular marker analysis, only one of them yielded a *C. persicum* amplification product, and it was relatively weak. This fragment however, was reproducibly amplified in independent reactions. Moreover, sequencing of the obtained fragment clearly indicated the presence of the *C. persicum* sequence in this somatic hybrid. The weak band as well as the low number of *C. persicum*-specific RAPD markers might on the one hand be due to the 4.6 times lower DNA content of *C. persicum* in comparison to *C. coum*. However, adding less *C. persicum* DNA to a DNA mix did not result in PCR amplification as weak as in hybrid No. 31. The amplification of non-specific DNA fragments from hybrid plant No. 31 using RAPD primers reflected the results obtained with the specific marker. Twenty-three amplification products of the same length as in the *C. coum* parent were obtained from this hybrid DNA. In contrast to this, the detection of *C. persicum*-specific amplification products was not reproducible, and often only very weak amplification products were observed. The reproducible occurrence of additional amplification products in the hybrid band pattern and the absence of some amplification products from both parental plants indicated the hybrid status of plant No. 31. In our opinion, RAPD markers have to be combined with other detection methods to substantiate the hybrid status of plants, and they are not suitable for fast screening methods, because many replications are necessary to obtain reliable results. In future experiments, AFLP markers or

microsatellites should be used in order to detect the relative DNA contributions of the two parents.

On the other hand, it is possible that *C. persicum* chromosomes are sorted during mitosis. In accordance with this, the elimination of chromosomes has often been described in somatic hybrids, especially for one hybrid partner (Kao 1977, Binding and Nehls 1978), Wetter and Kao 1980, Karp et al. 1982). These studies report such abnormalities in the mitosis of hybrid cells as chromosomes adhering to one another (Kao 1977), malformed chromosomes (ring, shortened or elongated chromosomes) and fragmented chromosomes. This cytological reorganisation can lead to the elimination of alleles, which results in weaker amplification of the target sequence. The phenotypic resemblance to the *C. coum* parent supports the assumption that parts of the *C. persicum* chromosomes were lost in the hybrid plants. However, these results are contradictory to the flow cytometric measurements, which showed a clearly detectable difference in the DNA content of the hybrids relative to the *C. coum* parent. This contradiction could not be conclusively resolved, but it appears to be common in many studies dealing with interspecific hybridisation, and in our opinion this question should be addressed in future publications. This contradiction clearly points to the fact that different methods need to be combined to verify and characterise somatic hybrid plants.

The copy numbers of the 5.8S gene in the *C. persicum* and *C. coum* genomes have (to our knowledge) never been examined. Typically, several hundred tandemly repeated copies of the rDNA genes and their spacer regions are present in eukaryotic genomes (Hillis and Dixon, 1991), but only one copy per genome (e.g., in a ciliate protozoa [Yao and Gall, 1977]) has been reported. The analysis of the distribution of rDNA genes using restriction fragment length polymorphisms (RFLPs) of rDNA spacers and fluorescent-in-situ-hybridisation (FISH) of an 18S gene in different somatic hybrids of *Medicago sativa* revealed various changes in the distribution of these genes (Cluster et al. 1996). In one hybrid (*M. sativa-coerulea*), genes of both parents were completely integrated, and in a second hybrid (*M. sativafalcata*), the genes were only partially integrated, and unequal rDNA gene recombination was observed between somaclonal plants (Cluster et al. 1996). In a third hybrid (*M. sativa-arborea*), half of the RFLPs of one parent were lost, and this was attributed by the authors to simple chromosome loss (Cluster et al. 1996). For our results, this could mean that the rDNA sequence repeats might have restructured and/ or were partly discarded from the hybrid plant genome (including most of the binding sites for the *C. persicum*-specific molecular marker).

Further species-specific markers derived from other gene sequences could help to further describe the genomic composition of the somatic hybrids.

In conclusion, the presence of both parental species-specific markers was shown, and sequencing of the weaker PCR fragment from plant No. 31 revealed the presence of the target DNA of the *C. persicum* parent. In conjunction with the flow cytometric results, this plant contains demonstrable DNA of both parents, and it must therefore be considered to be the first somatic hybrid between *C. coum* and *C. persicum*.

Detailed studies regarding its phenotype and stability, and further genetic characterisation (e.g., by chromosome counting and FISH/GISH) should now follow with fully grown hybrid plants.

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Table 1: Flow cytometric analyses of morphologically deviating plants regenerated from fusion experiments. Repetitions four months later confirmed these measurements (data not shown).

| No of plants | Main peak positions [min-max] | Corresponding species | Ploidy |
|---|---|---|---|
| 31 | One main peak: 76 - 122 | *C. coum* | 2x |
| 3 | One main peak: 217 - 226 | *C. coum* | 4x |
| 0 | not automatically detected | *C. persicum* | 4x |
| 6 | Two main peaks: 92-117 / 107-135 | *C. coum* x *C. persicum* | putative hybrid |
| 3 | One broad peak with two pinnacles each: 65-136 | *C. coum* x *C. persicum* | putative hybrid |

Table 2: Primers for the detection of the 5.8S gene in the ribosomal nuclear DNA and its flanking internal transcribed spacers ITS1 and ITS2 (Anderberg et al. 2000) in DNA of the two parental species *C. persicum* and *C. coum*. Bold letters indicate nucleotides that have been modified for better discrimination from the non target sequence.

| Primer name | Primer sequence | Expected fragment length [bp] | Annealing temperature [°C] |
|---|---|---|---|
| C_persicum_fw | 5′– GCA CGC GCG CGC CAG CCT AA –3′ | 136 | 67 |
| C_persicum_rev | 5′– CCG CTC GCG GCG GAC GTC CT –3′ | | |
| C_coum_fw | 5′– TAA TAA CTC GGG CGC G**G**T GTT C –3′ | 564 | 61 |
| C_coum_rev | 5′– CAC **A**C**T** CGA CGT CCG GGG TCA A –3′ | | |

Table 3: Analysis of the amplification products obtained by 10 RAPD-primers of the putative hybrid plant No 31. Only amplification products were taken into account, which were at least amplified in two independent PCR reactions.

| | Putative hybrid plant No 31. |
|---|---|
| *C. coum* amplification products | 23 |
| Missing *C. coum* amplification products | 4 |
| *C. persicum* amplification products | 1 |
| Missing *C. persicum* amplification products | 23 |
| Additional new amplification products | 4 |

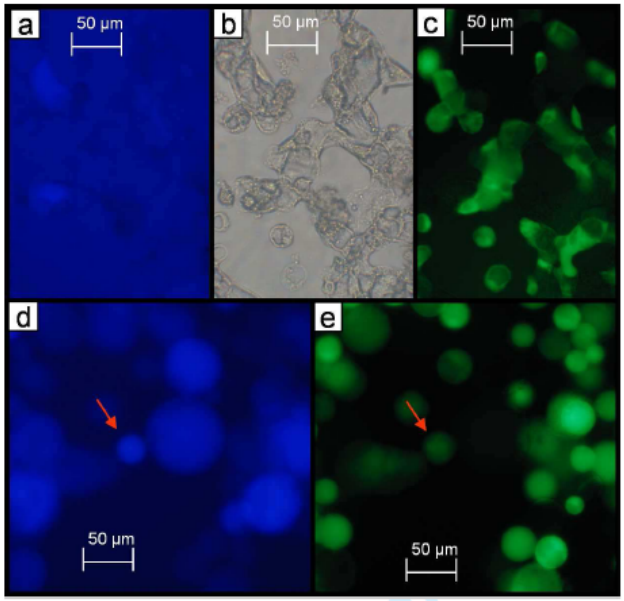



Fig 1: a)-c) Fusion experiment with *C. coum* protoplasts (scopoletin stained, blue fluorescence) and *C. persicum* protoplasts (FDA stained, green fluorescence) after addition of the PEG solution forming characteristical "nets". a) DAPI HC filter set, *C. coum* protoplasts are visible by their blue fluorescence b) transmitted light: all protoplasts are visible c) GFP Bright Line HC filter set: *C. persicum* protoplasts are visible by their green fluorescence d)-e) Protoplasts of *C. coum* and *C. persicum* after fusion in culture medium 8pmC.1. The red arrow indicates a fused/double stained protoplast d) DAPI HC filter set, e) GFP Bright Line HC filter set

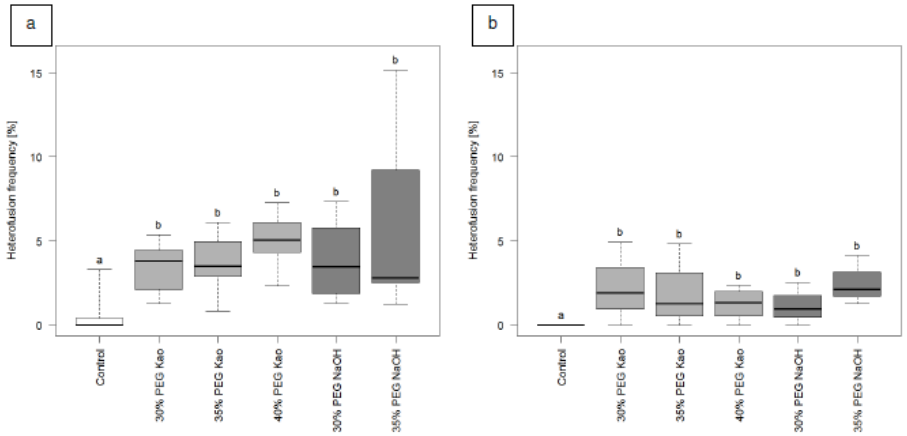



Fig. 2:
Heterofusion frequencies after different fusion treatments. a) Cell density 1 x $10^6$ protoplasts per mL; n = 8 to 9 repetitions per variant (data collected in four to five independent experiments) b) Cell density 4 x $10^6$ protoplasts per mL; box whiskers extend to the maximum (minimum) values within the 1.5 fold of the interquartile range; more extreme values are marked as dots; n = 3 to 4 repetitions per variant (data collected in two to three different experiments)

Two factorial ANOVA revealed significant treatment effects ($p<0.0001$) and significant differences between the two densities ($p<0.0001$), and absence of an interaction of both effects ($p=0.7202$). Pairwise comparisons of all treatments revealed a significant difference between the control and the five fusion treatments.
Each mean value (± standard deviation) followed by the same lower-case letter did not differ significantly at $p>0.05$.

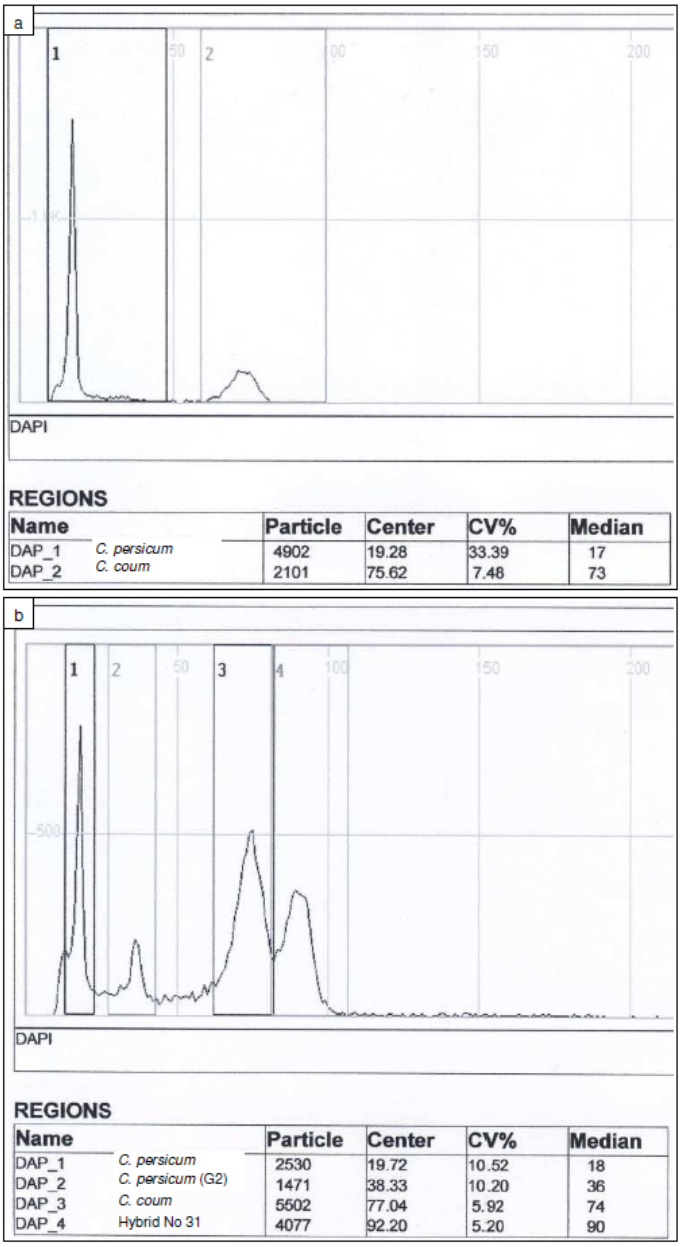



Fig. 3: Flow cytometric measurements of the two parental species and of a mixed sample of *C. coum*, *C. persicum* and the putative hybrid plant No 31.

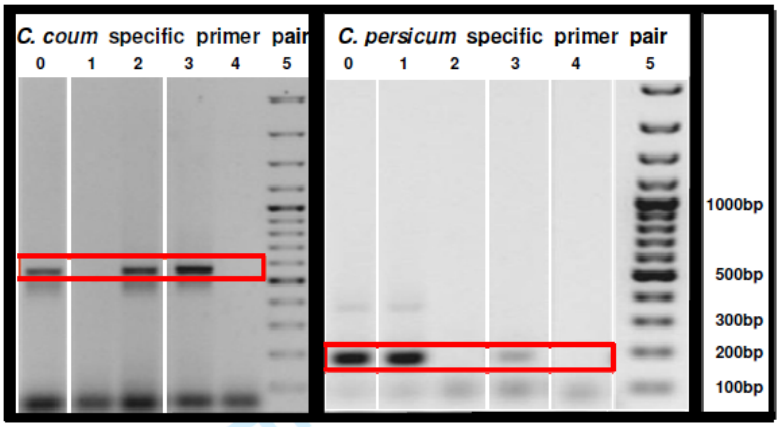



Fig. 4: Detection of *C. coum* specific amplification products (564 bp) and *C. persicum* specific amplification products (136 bp) using different DNA templates: 1=DNA mixture of *C. coum* and *C. persicum* (1:1); 2= *C. persicum*; 3= *C. coum*, 4= putative hybrid plant No 31, 5=negative control ($H_2O$), 6=100 bp DNA ladder

a) *C. persicum* > AACAGAAGCACCGGGCACCCGTGTCGCGGCAATTTGTGGTTGCCAAAC
b) Hybrid 31 > AACAGAAGCACCGGGCACCCGTGTCGCGGCAATTTGTGGTTGCCAAAC
c) *C. coum* > AATCGAAGCATCGGCCACTGGTGCCGCGGCAATTTGTGGTTGCCAAAC

a) *C. persicum* CGTCTCATCCTGCCGCGCCGGTGTCTCAACCGGTTGGCTTCCCGACCC <
b) Hybrid 31 CGTCTCATCCTGCCGCGCCGGTGTCTCAACCGGTTGGCTTCCCGACCC <
c) *C. coum* CGTCTCATCCTGCCGCGCCCGTGCCTCAACCGATTGGCTTCTTGACCC <

Fig. 5: Alignment of the sequenced target region of the *C. persicum* specific marker (without the flanking primer sequence). a) Sequenced region from the *C. persicum* parental plant. b) Sequenced region from the putative hybrid plant No 31. c) The corresponding region from *C. coum* (underlined and adopted from the NCBI-database Genbank No AF164001.1). This region is not amplified by the *C. persicum* specific primer pair.
The corresponding region of the *C. coum* sequence differs in twelve nucleotides (indicated with grey background) from the two identical sequences from the *C. persicum* parental plant and the putative hybrid plant No 31.

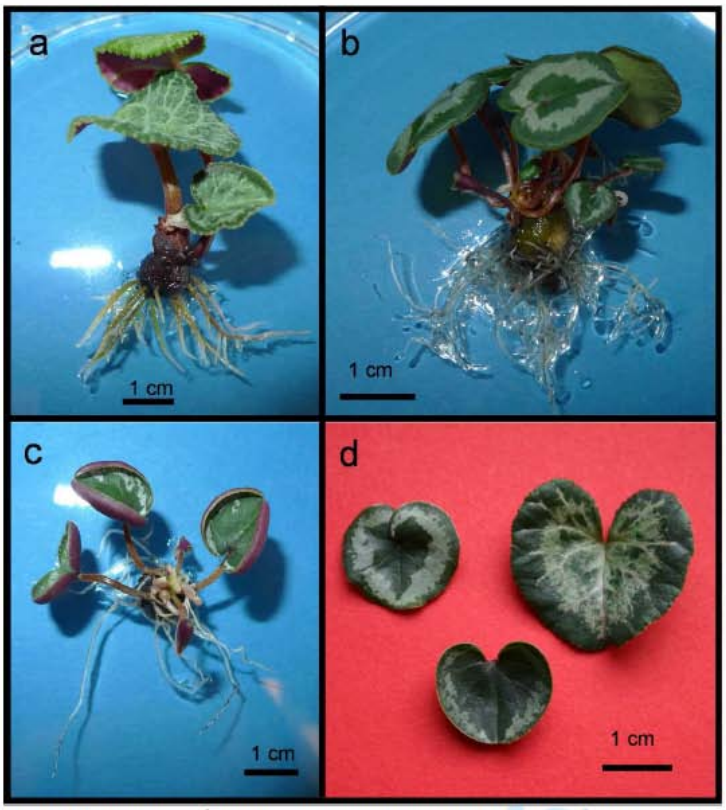



Fig 6: Phenotypes of in vitro grown (regenerated) plants of a) *C. persicum* parent b) *C. coum* parent c) Hybrid plant No 31 d) Comparison of the leaves of the *C. coum* parent (top, left), the *C. persicum* parent (top, right) and the hybrid plant No 31 (bottom)

## Annexe I: Staining of Protoplasts

### Protoplast staining with Rhodamine B isothiocyanate (RBITC)

In addition to scopoletin and FDA, RBITC was successfully used for staining of protoplasts. As described for FDA (**Prange *et al*., 2010c**), the RBITC-staining [the recommended staining procedure after Durieu & Ochatt (2000)] had to be modified because of the high destructiveness of the dye or its solvent (acetone) on *Cyclamen* protoplasts. The modified procedure was performed as described for FDA in **Prange *et al*. (2010c)**. After these modifications were made, the viability of the protoplasts was retained after the staining treatments, as confirmed by the viability screenings with *C. persicum* and *C. graecum* protoplasts described in **Prange *et al*. (2010c)** (data not shown), and by the regeneration via somatic embryogenesis, yielding similar results to FDA for both species (cf. **Prange *et al*., 2010c**) (data not shown). The RBITC fluorescence was visualised as described in **Prange *et al*., (2010c)** using the (RBITC) TRITC Bright Line HC filter set F36-502 (593/40 nm excitation, 562 nm beam splitter, 543/22 nm emission; AHF Analysentechnik AG, Germany). RBITC staining was visible for more than five or seven days in the protoplasts and did not bleach when visualised under a fluorescence microscope. After fusion experiments with FDA and RBITC, the stained protoplasts of *C. persicum* (FDA stained) and the *C. graecum* (RBITC stained) heterofusion products were monitored as described by Durieu & Ochatt (2000) (Fig. 4). Repeated fusion experiments, especially with low quality protoplasts or harsher fusion methods, resulted in protoplast cultures with up to 100% RBITC-stained protoplasts. When FDA and RBITC stained protoplast were incubated in liquid protoplast isolation buffer or culture medium without PEG treatment, the occurrence of double stained protoplasts was observed. The amount of RBITC stained protoplasts was always increasing, while the amount of FDA stained protoplast remained unchanged (data not shown). It was assumed that the high amount of RBITC stained and double stained protoplasts (Fig. 4) were not only attributed to successful fusion, but mainly to a leaching of active dye (RBITC) from the protoplasts.

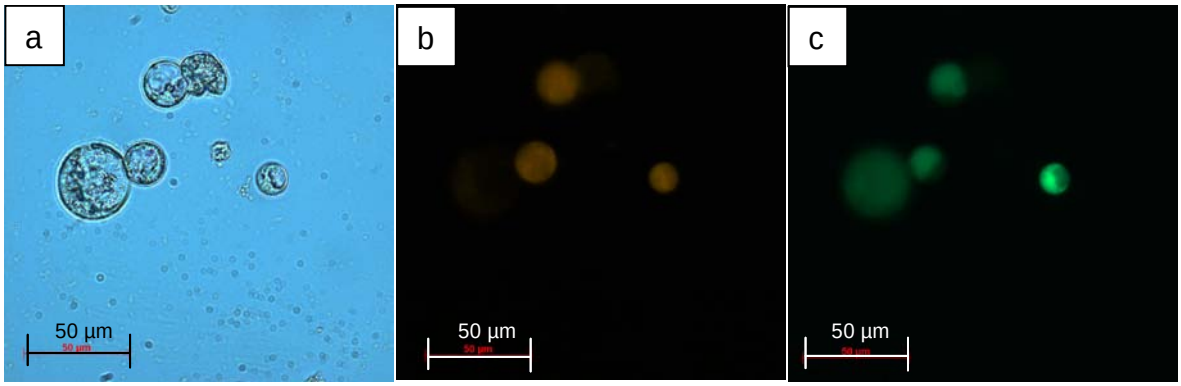



**Figure 4:** Protoplasts of *C. graecum* (FDA-stained, green fluorescence) and *C. persicum* (RBITC-stained, red fluorescence) after protoplast fusion under the microscope. The high number of double stained protoplasts indicates either a high heterofusion rate or a leaching of dye from the cells a) transmitted light: all protoplasts are visible b) RBITC Bright Line HC filter set: RBITC stained protoplasts are visible by their red fluorescence c) GFP Bright Line HC filter set: FDA stained protoplasts are visible by their green fluorescence.

### Protoplast staining with Fluorescein diacetate (FDA) and Scopoletin

FDA and scopoletin were well tolerated by the plant protoplasts, which was confirmed in our viability screening in *C. persicum* (**Prange *et al.*, 2010c**) and *C. graecum* (data not shown), yielding similar results. Enhanced concentrations and incubation times of FDA resulted in the death of the protoplasts, but with the appropriate staining procedure no toxicity of the dye was observed (**Prange *et al.*, 2010c**). FDA bleached only slightly during the monitoring under the fluorescence microscope. Scopoletin did not harm the protoplasts, but during monitoring under the fluorescence microscope the dye bleached rapidly after light exposure of about 5 seconds (Fig. 5). This problem was partly solved by changing the excitation filter of the standard DAPI filter to a narrower excitation filter, which excluded the harsh UV-light. This modification prolonged the potential screening time under the microscope in which the intensive fluorescence of the cells was retained and additionally possible mutagenic effects of the UV radiations were minimised.

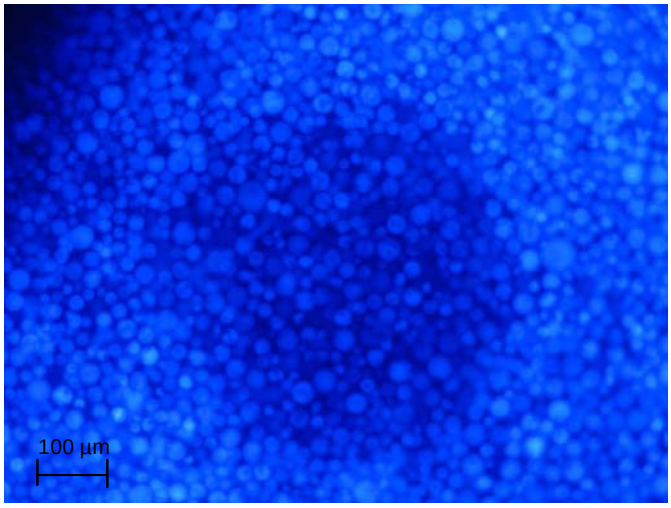



**Figure 5:** Protoplasts of *C. graecum* (scopoletin-stained) under the fluorescence microscope (DAPI Bright Line HC filter set). The protoplasts were exposed to light in a 20x enlargement objective for about 5 seconds. Then, the enlargement was switched to a 10x magnification and a picture was immediately taken after light exposure. The bleached protoplasts from the previous light exposure are visible as a dark circle in the middle of the picture.

In contrast to RBITC, scopoletin and FDA were successfully used for the monitoring of the fusion process and the determination of heterofusion frequencies in hybridisation experiments of *C. persicum* with *C. coum* (**Prange *et al.*, 2010c**) and *C. graecum*. The results produced with scopoletin and FDA were also reliable.

The tested staining agents revealed different advantages and disadvantages that were partly, but not fully, described in the literature and will be discussed in chapter 4.2.1.

## Annexe II: Protoplast Fusion

Analogous to the protoplast fusion experiments in **Prange *et al.* (2010c)** with *C. persicum* and *C. coum*, protoplast fusion was also achieved in the species combination *C. persicum* x *C. graecum* (Fig. 6). In protoplast fusions with *C. persicum* x *C. graecum*, heterofusion frequencies averaging between 1.6 and 12.1% were achieved. As in *C. coum,* the statistical analysis (ANOVA) revealed significantly lower heterofusion frequencies with a density of 4 x $10^6$ protoplasts per mL than with the lower cell density (**Prange *et al.*, 2010c**), but there was no significant effect from the different fusion treatments.

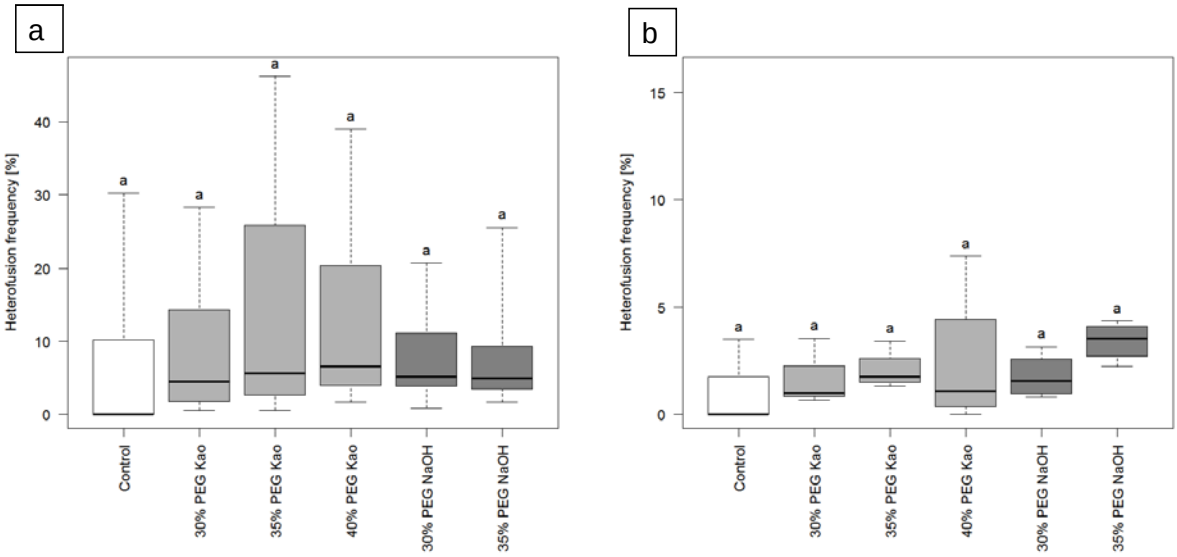



**Figure 6:** Heterofusion frequencies (*C. graecum* x *C. persicum*) after different fusion treatments. a) Cell density 1 x $10^6$ protoplasts per mL, n= 9 to 14; b) Cell density 4 x $10^6$ protoplasts per mL, n=3 to 4; box whiskers extend to the maximum (minimum) values within +(-) 1.5x box width from the box (more extreme values are marked as dots); (compare to **Prange *et al.*, 2010c**). Two factorial ANOVA revealed significant differences between the two densities ($p<0.0001$), but no significant treatment effects. Each mean value (± standard deviation) followed by the same lower-case letter did not differ significantly at $p>0.05$.

In Table 3, the different experiments are displayed with their minimal and maximal heterofusion frequency of the PEG treated cells and their corresponding control. The variability of the heterofusion rates between the different experiments was very high. In four fusion experiments the heterofusion frequency of the untreated control was 0%, whereas in four other experiments (No. 3, 4, 5, 7) the heterofusion frequency was enhanced, yielding up to 30.3% heterofusion products. In two experiments it was even higher than the lowest yielded heterofusion frequency in the PEG treated protoplasts (Table 3). Comparable high fusion rates have also been reported in the literature for

PEG-mediated fusion of *Vicia hajastana* Grossh.-soybean (*Glycine max* L.) (23% heterokaryocytes) and barley (*Hordeum vulgare* L.)-soybean (35% heterokaryocytes) (Kao *et al.*, 1974). Possible reasons for these results will be discussed in chapter 4.2.3.

**Table 3:** Heterofusion frequencies in the experiments with *C. persicum* and *C. graecum*. The lowest (Min) and the highest (Max) heterofusion frequency per fusion experiment is displayed. The experiments were accomplished at different days with all 5 tested fusion variants and 1 to 4 repetitions per variant.

| Experiment No. | Heterofusion frequency control [%] | Heterofusion frequency with fusion treatment [%] | | Protoplast density during fusion [cells per mL] |
|---|---|---|---|---|
| | | min | max | |
| 1 | 0.0 | 2.8 | 6.0 | $1 \times 10^6$ |
| 2 | 0.0 | 1.0 | 6.0 | $1 \times 10^6$ |
| 3 | 2.9 | 5.0 | 16.2 | $1 \times 10^6$ |
| 4 | 30.3 | 11.1 | 46.3 | $1 \times 10^6$ |
| 5 | 17.5 | 20.3 | 28.8 | $1 \times 10^6$ |
| 6 | 0.0 | 0.0 | 4.8 | $1 \times 10^6$ |
| 7 | 3.5 | 1.0 | 7.4 | $4 \times 10^6$ |
| 8 | 0.0 | 0.0 | 3.9 | $4 \times 10^6$ |

Unfortunately, the high heterofusion rates were not reflected in the amount of hybrid plants regenerated from those experiments. About 50 to 80 plants could be regenerated from fusion experiments No. 4, 5 and 7. The phenotype of the plants resembled *C. graecum*, which was confirmed by genetic and molecular methods (species-specific-molecular marker, flow cytometry). Until now, no hybrid between these two species has been detected (Annexe IV).

## Annexe III: Selection of the Heterofusion Products

### Cell Finder Slides

Cell Finder Slides (POG Präzisionsoptik Gera GmbH, Germany) were tested for the selection of stained *Cyclamen* protoplasts after fusion (**Prange *et al.*, 2010c**). These microscopic slides are marked with a coordinate system and enable the localisation of labelled heterofusion products. After fusion, FDA- or scopoletin-stained protoplasts were embedded in agarose directly on the slides or by fixation of the solidified alginate films with agarose (**Prange *et al.*, 2010a,b,c**). Both protoplast films were additionally fixed on the slides with agarose, screened for heterofusion products (=dual labelled protoplasts) under an epifluorescence microscope and cultivated as described earlier (**Prange *et al.*, 2010a,b,c**), with some modifications: Cell division and microcallus formation in the protoplasts was only achieved when the Cell Finder Slides were embedded in a cavity of solid 8pmC.1/8pmC.2 medium in 9-cm diameter Petri dishes and floated with 3.5 mL liquid 8pmC.1/8pmC.2 culture medium (Fig. 7). In contrast, protoplasts did not develop to microcalluses when the films were cultivated in liquid medium in 9-cm diameter Petri dishes as described by Meyer (2006).

However, two major problems prevented a successful selection of heterofusion products using this technique. Firstly, the developing *Cyclamen* calluses were very friable. Thus, the calluses could not be kept intact when separated and generally could not be cultivated further. Secondly, a high cell density (**Prange *et al.*, 2010c**) was obligatory for cell division and further development after protoplasts fusion. This made the selection of the identified heterofusion products extremely difficult.

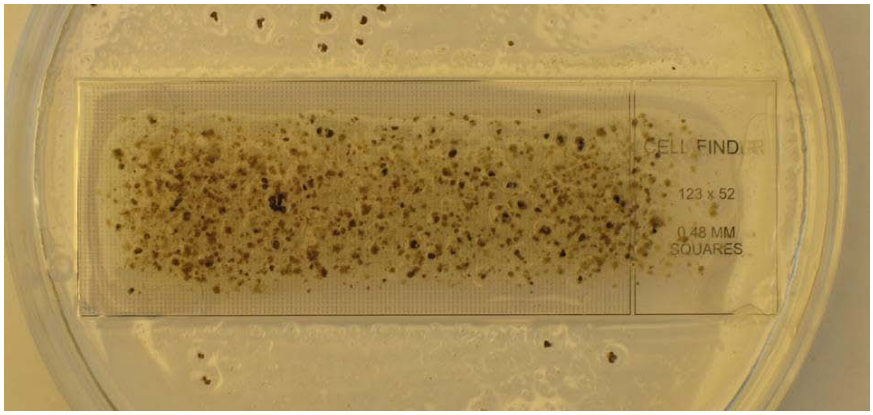



**Figure 7:** Microcalluses derived from a *C. coum* and *C. persicum* protoplast fusion, embedded in agarose cultivated on a Cell Finder Slide.

**Fluorescence Activated Cell Sorting (FACS)**

In cooperation with Dr. Jörg Fuchs, at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, the fluorescence activated cell sorting (FACS) of FDA-stained, scopoletin-stained and unstained protoplasts of *C. persicum* and C*. graecum* was tested. The protoplasts were analysed and sorted using a FACSAria flow cytometer (BD, Bioscience, USA) equipped with three lasers (407 nm, 488 nm, 633 nm). Protoplasts were isolated as described earlier (**Prange *et al.*, 2010a,b**) and sorted under semi-sterile conditions using W5 solution (125 mM $CaCl_2$ x 2 $H_2O$, 155 mM NaCl, 5 mM KCl, pH 5.6) as a sheath fluid (for methodical details, see Galbraith, 2007).

Figure 8 and 9 display the FDA-stained and unstained protoplast during the sorting process. A separation of the protoplasts by potential morphological or structural differences employing scatter light parameters was not possible (compare Fig. 8a, d and 9 a, d). While the FDA-stained protoplasts could be successfully discriminated from unstained protoplasts using the 488 nm laser and a 530/30 filter (compare Fig. 8b, c, e, f and Fig. 9b, c, e, f), the successful detection of the scopoletin-stained protoplasts was not possible with the available equipment (407 nm laser and a 450/40 filter) (data not shown). According to the excitation profile of scopoletin (e.g., Funke, 2007) the optimal excitation would be in the range of 230 – 380 nm. The 407 nm laser that was used was not suitable for the sufficient excitation of scopoletin. Figure 10 shows cultures of FDA-stained protoplasts of *C. persicum* after flow cytometric analyses and sorting according to the fluorescence intensities. Population P7 contained mainly damaged, non-viable protoplasts without FDA fluorescence (Fig. 10a) - c)). Population P6 was mainly composed of non-damaged, viable protoplasts with green fluorescence (Fig. 10d) – f)). The protoplasts might have been damaged by the sorting process itself, but the overall quality was also lowered by transport over a long distance by car from Hannover to Gatersleben (170 km) in liquid medium.

To test the propagation potential of sorted protoplasts (unstained and FDA-stained), they were sorted into different fractions (Fig. 8, 9) and embedded in alginate or agarose. After successful cultivation for several days the cultures became overgrown by microorganisms that prevented the regeneration to microcalluses. To minimise the risk of contamination, the flow cytometer has to be better prepared for aseptic sorting in future experiments.

***Cyclamen persicum*** (56/2)

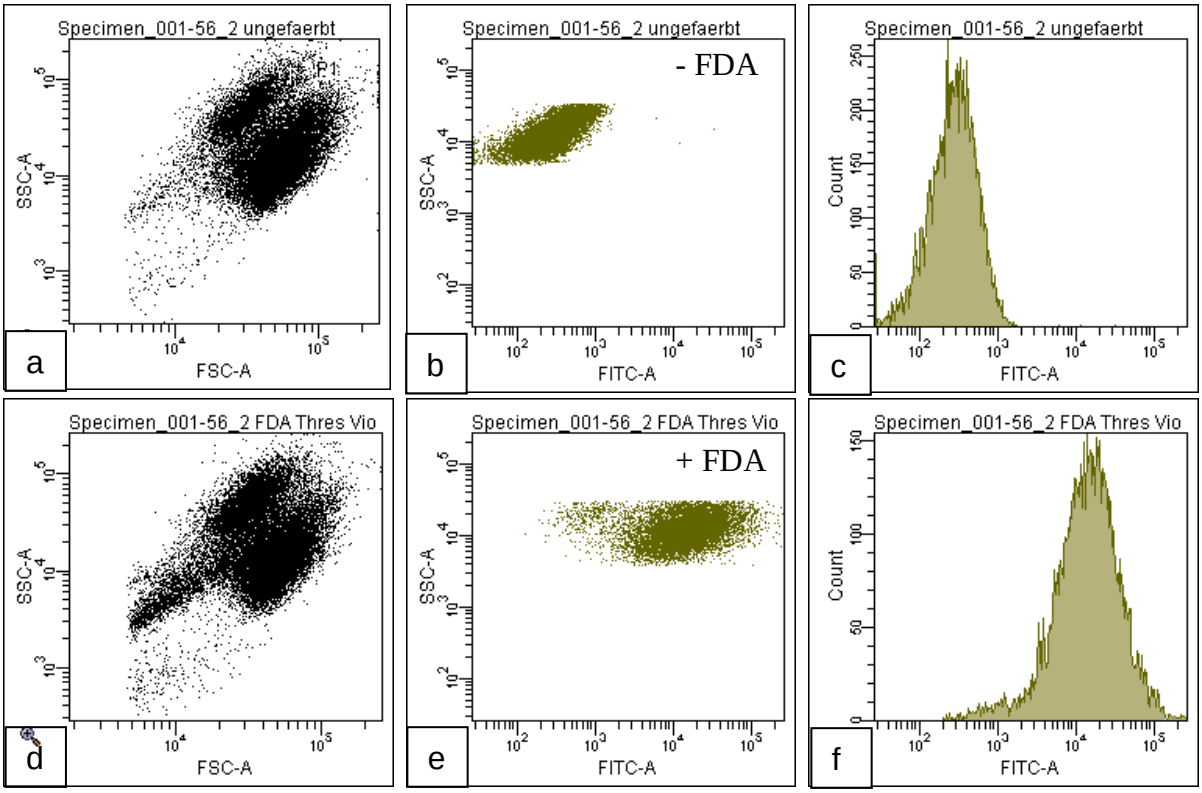


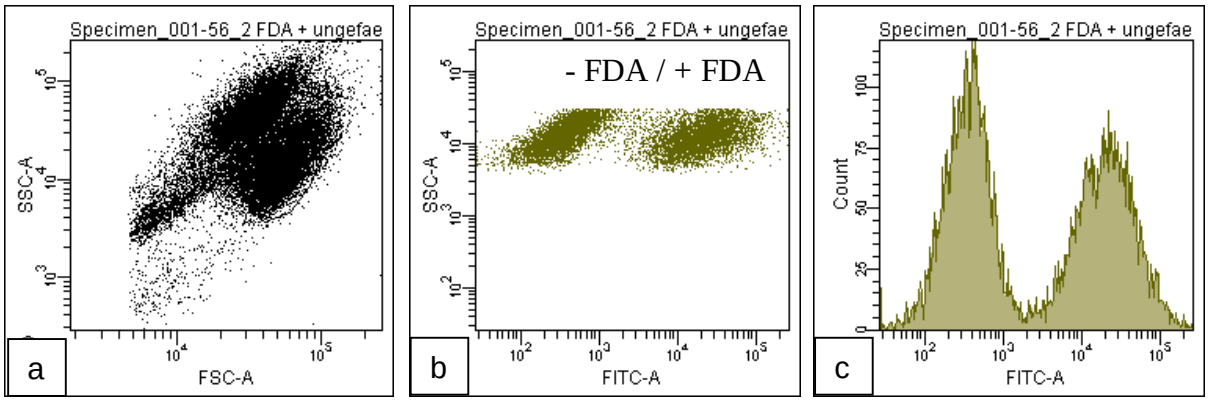

**Figure 8:** Flow cytometric analyses of unstained (a-c) and FDA-stained (d-f) protoplasts of *C. persicum.*

a) + d) SSC (Side Scatter, representing the granularity of the measured particles) plotted against the FSC (Forward Scatter, correlating with the size of the measured particles). Compare to Fig. 9.

b) + e) SSC plotted against the intensity of the FDA staining. The stained and the unstained protoplasts have the same SSC, but differ in their green fluorescence.

c) + f) Histogram of the fluorescence intensity (counts/number of particles plotted against the fluorescence intensity)

***Cyclamen persicum*** (56/2)




***C. persicum*** (56/2) FDA stained + ***C. graecum*** unstained

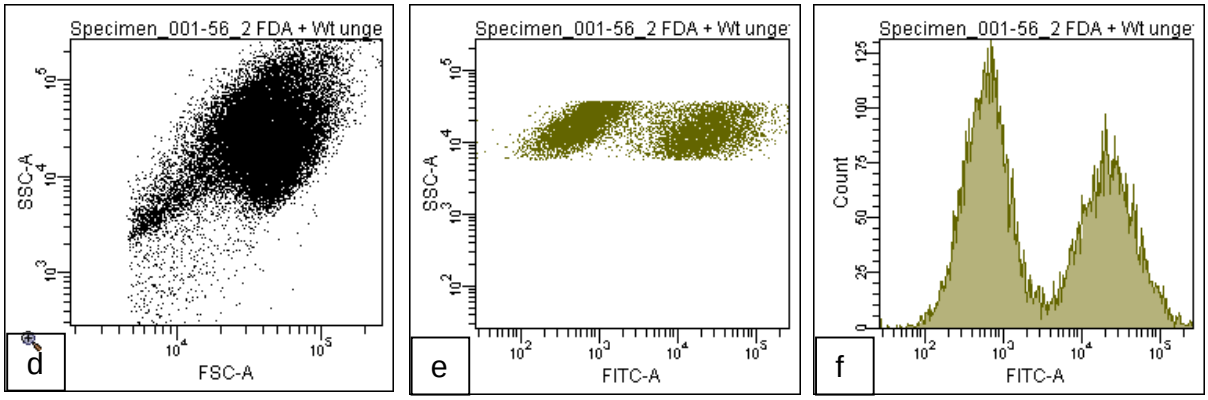



**Figure 9:** Flow cytometric analyses of mixtures of FDA-stained and unstained *C. persicum* protoplasts (a-c) and of FDA-stained *C. persicum* and unstained *C. graecum* protoplasts (d-f).

a) + d) SSC (Side Scatter, representing the granularity of the measured particles) plotted against the FSC (Forward Scatter, correlating with the size of the measured particles).

b) + e) SSC plotted against the intensity of the FDA staining. The stained and the unstained protoplasts have the same SSC, but differ in their green fluorescence. The two populations (FDA-stained and unstained protoplast) can be distinguished by their fluorescence and, therefore, can be separated by sorting.

c) + f) Histogram of the fluorescence intensity (counts, number of particles plotted against the fluorescence intensity). Also in this visualisation, two clearly distinct populations can be differentiated.

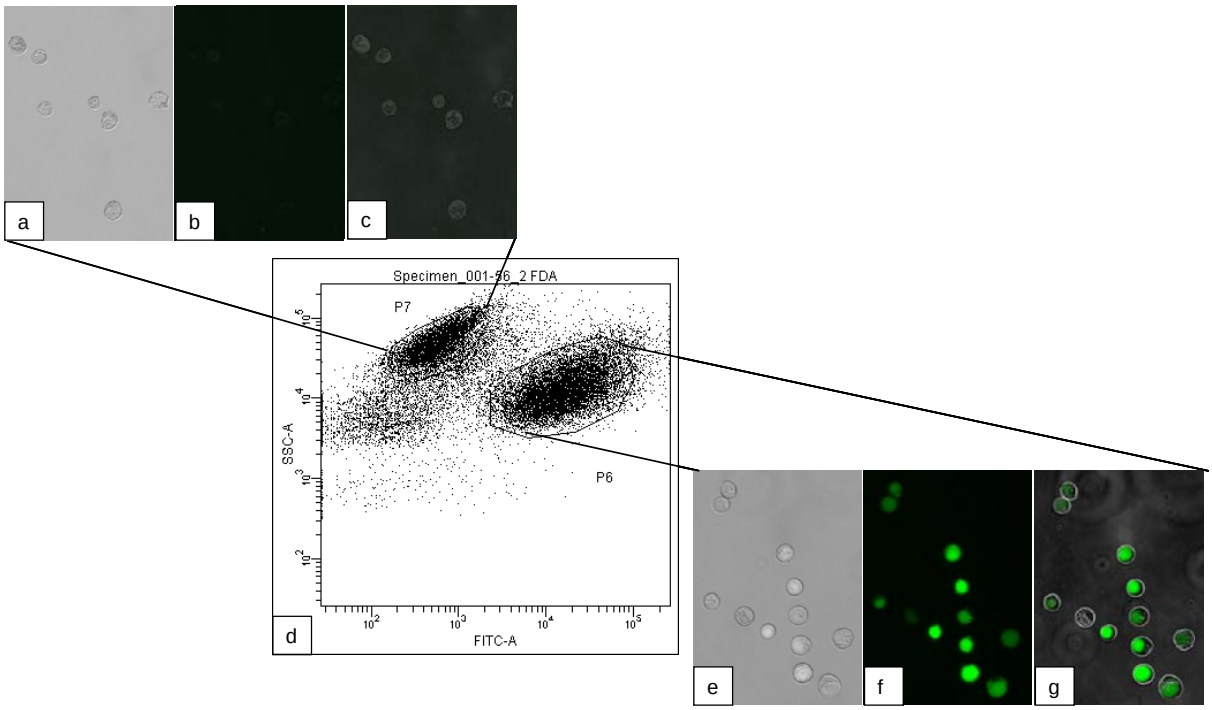



**Figure 10:** Flow cytometric analyses and microscopic evaluation of sorted *C. persicum* protoplasts (FDA-stained), from the two separate populations (P7 and P6), encircled in d).

a) + e) transmitted light, b) + f) FDA Filter (**Prange *et al.*, 2010c**; Annexe I), c) + g) merge. Population P6 contains mainly viable protoplasts, indicated by their green fluorescence and their spherical shape. In contrast to this population, P7 mainly contains dead and/or damaged protoplasts. This is indicated by the lack of fluorescence and their irregular shape.

### Microfluidic Chips

To investigate the unstained *Cyclamen* protoplasts, a “modular fluidic chip assembly” (microfluidic chip) was designed and optimised for protoplast sorting by Anika Prange, Proyag Datta and Jost Goettert. Following engineering and fabrication (micromilling, hot embossing), the microfluidic chip was successfully tested: The system facilitates easily handling, identification, counting, sorting and isolation of cells (protoplasts) for cellular level experiments. The microfluidic chips (Fig. 11) provide a flexible, adaptable, user-friendly platform that is compatible with standard laboratory equipment like a light microscope (Fig. 12) (Datta *et al.*, 2008).

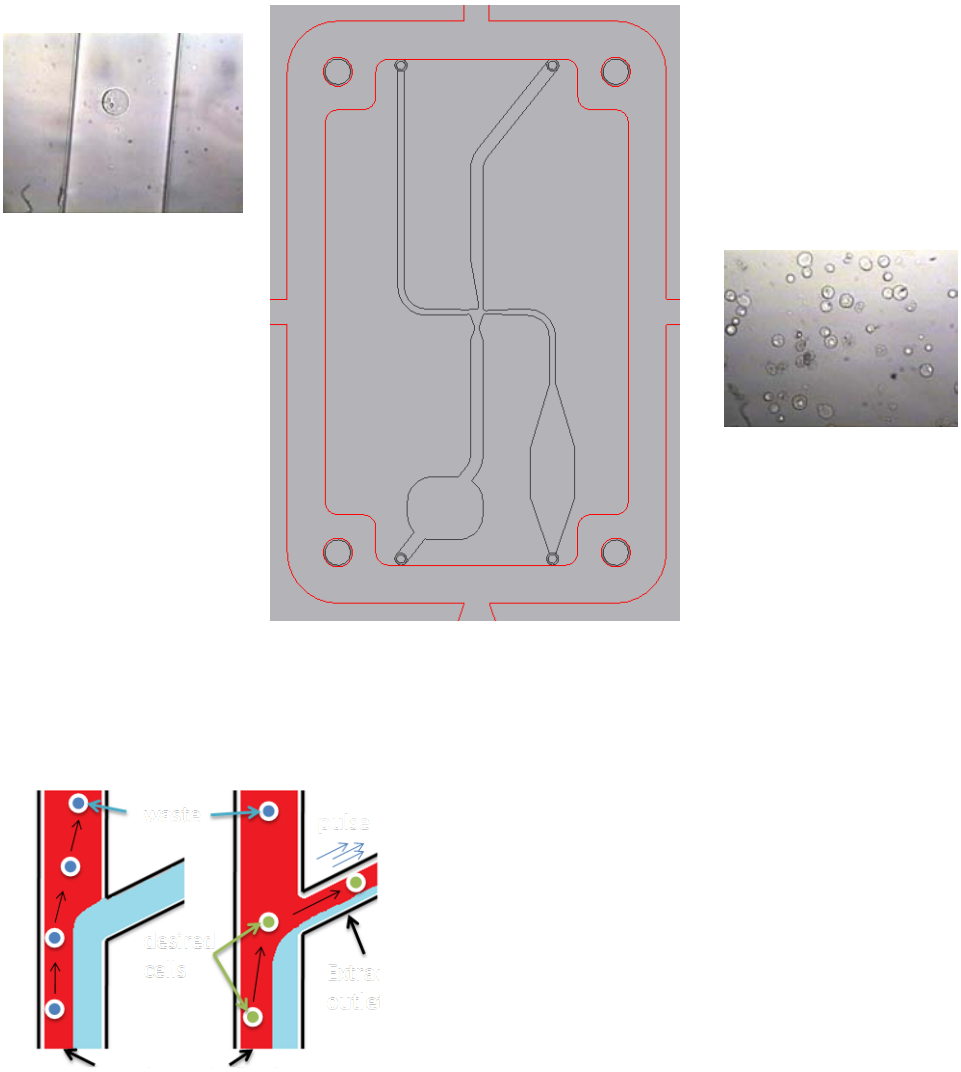



**Figure 11:** Design of the Microchip used for visual separation of protoplasts (Figure adopted from Datta *et al.*, 2008); a *Cyclamen graecum* protoplast during its passage through a microfluidic channel (diameter of the channel: approx. 100µm) (top). Schematic sketch of the microfluidic flow during sample observation (left) and separation of the desired cells (right) (bottom).

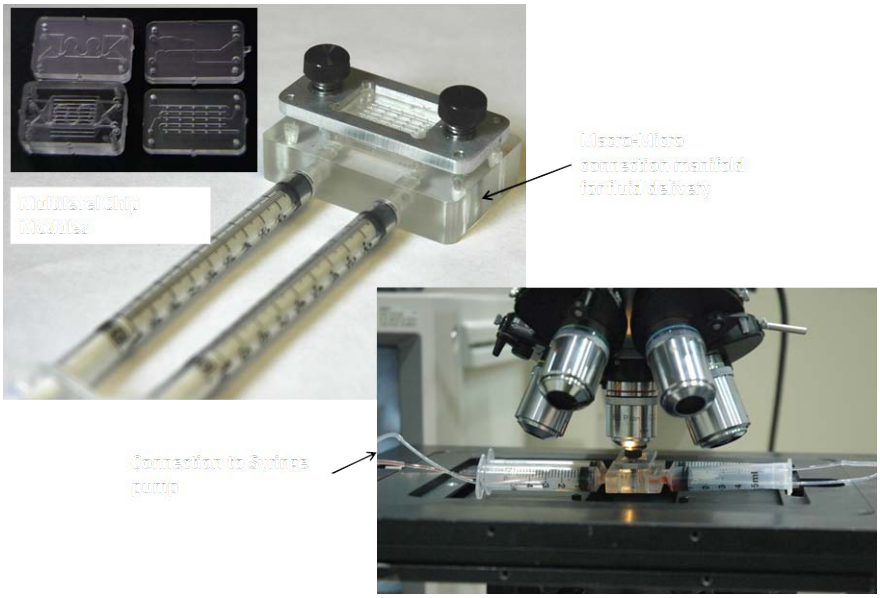



**Figure 12:** Standard equipment for using microfluidic chips (assembled system): Chip modules, syringes (connected to syringe pump) and a standard light microscope (Figure adopted from Datta *et al*., 2008).

The details of the chip design, material (polymer), fabrication and its functions are described in Datta (2007) and Datta *et al.* (2008). The microfluidic chip used in this thesis was additionally equipped with a micropump (or syringe) and a button that regulates the direction of the flow and enables the manual sorting process. The protoplasts are gently pressed through the channel into the observation and sorting zone. The desired protoplast can be manually sorted out by the use of a button that changes the flow of the surrounding liquid and leads the protoplast of interest (e.g., the heterofusion products) into a special channel and a reservoir where the cells are collected.

## Annexe IV: Species-Specific Molecular Markers

For species combinations *C. persicum* x *C. mirabile* and *C. persicum* x *C. graecum,* a specific molecular marker was developed according to the one described for *C. persicum* x *C. coum* (**Prange *et al*., 2010c**) (Table 4; Fig. 13, 14). The *C. mirabile* specific marker was highly specific, whereas the *C. graecum* primer combination always amplified a weak fragment in the negative control with *C. persicum* DNA as a template (Fig. 13, encircled in red).

**Table 4:** Primers for the detection of the 5.8S gene in the ribosomal nuclear DNA and its flanking internal transcribed spacers ITS1 and ITS2 (Anderberg *et al.,* 2000) in DNA of the two parental species *C. mirabile* and *C. graecum*.

| **Primer name** | **Primer sequence** | **Expected fragment length [bp]** | **Annealing temp. [°C]** |
|---|---|---|---|
| C_mirabile_forward | 5′- AAT ACC CCG GGC GCG TCG GAC -3′ | 440 | 67 |
| C_mirabile_reverse | 5′- GAC CGC GCA CAT CTG CGC AC -3′ | | |
| C_graecum_forward | 5′- ATA TCA CCT CGG ACG TGT CGG A -3′ | 439 | 67 |
| C_graecum_reverse | 5′- GCC GCG TGC AGC TGC ACA -3′ | | |

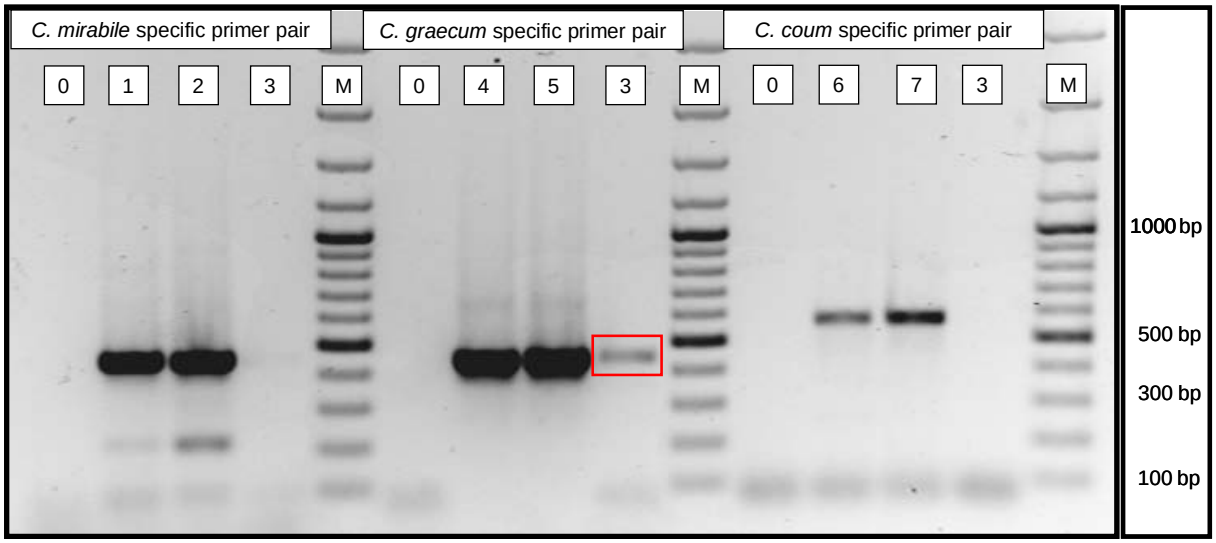



**Figure 13:** Results of three independent PCR reactions with the primer pair specific for the respective wild *Cyclamen* species (*C. mirabile*, *C. graecum*, *C. coum*) with different DNA templates: M = 100 bp DNA ladder; 0 = negative control ($H_2O$); 1 = DNA of *C. mirabile* and *C. persicum*; 2 = DNA of *C. mirabile*; 3 = DNA of *C. persicum*; 4 = DNA of *C. graecum* and *C. persicum*; 5 = DNA of *C. graecum*, 6 = DNA of *C. coum* and *C. persicum*; 7 = DNA of *C. coum.*

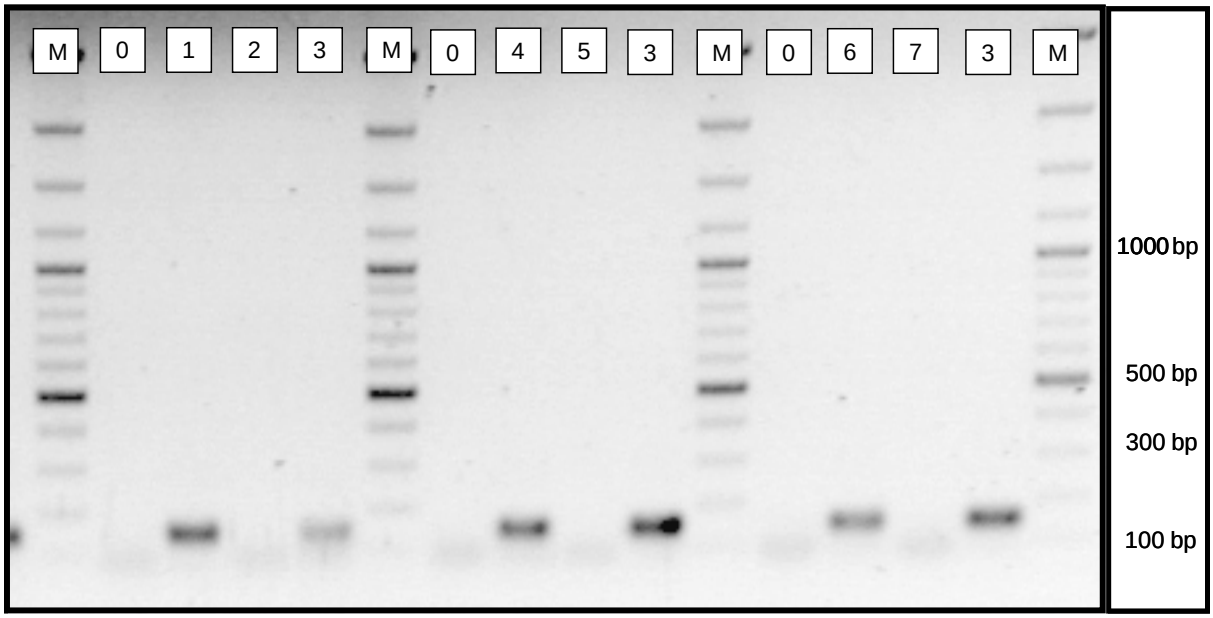



**Figure 14:** Results of three PCR reactions with the primer pair specific for *C. persicum* with DNA templates of the different wild *Cyclamen* species (*C. mirabile*, *C. graecum*, *C. coum*): M = 100 bp DNA ladder; 0 = negative control ($H_2O$); 1 = DNA of *C. mirabile* and *C. persicum*; 2 = DNA of *C. mirabile*; 3 = DNA of *C. persicum*; 4 = DNA of *C graecum* and *C. persicum*; 5 = DNA of *C. graecum*, 6 = DNA of *C. coum* and *C. persicum*; 7 = DNA of *C. coum.*

For all of the designed primer pairs (*C. coum*, *C. graecum*, *C. mirabile* and *C. persicum*) the species-specific DNA fragment was not amplified when the DNA of the other four species (one genotype per species) was used as DNA template. The transferability of the species-specific primer was confirmed with four to five additional genotypes per species.

# 4 Discussion

The two main parts of this thesis are the establishment of wild *Cyclamen in vitro* cultures (shoot cultures and embryogenic cultures) and the somatic hybridisation of these wild *Cyclamen* species with *C. persicum* cultivars. In this chapter, aspects of these two main topics that have not been discussed or were only partially discussed in chapter 3 and its annexes will be discussed[1].

## 4.1 *In Vitro* Culture of Wild *Cyclamen* Species

For the establishment of *in vitro* cultures, seven different species of the genus *Cyclamen* were chosen due to their relationship to *C. persicum*, their availability and their different morphological and physiological characteristics. Each is distinguished from *C. persicum* cultivars by specific traits, which will be valuable for *Cyclamen* breeding, if combined with established *C. persicum* cultivars by hybridisation and, later on, introduced into breeding programs.

*C. hederifolium and C. coum* were chosen for their frost hardiness (Grey-Wilson, 2003; Ishizaka, 2008). *C. hederifolium* is characterised by ivy-shaped leaves and *C. coum* by small, round to kidney-shaped leaves with interesting leaf patterns and flowers with rounded petals. *C. graecum* is resistant to some important *Cyclamen* diseases (Grey-Wilson, 2003; Ishizaka, 2008) and is characterised by high variance in leaf shape, size and pattern. *C. mirabile* is the only species with a pink leaf pattern (Grey-Wilson, 2003), whereas *C. alpinum* has a unique flower shape and a sweet primrose scent (Grey-Wilson, 2003).

Except for *C. coum*, in which the tissue cultures were established from mature tissue, sterile seedlings served as initial material for the *in vitro* cultures (**Prange *et al.*, 2008**). Due to the low germination rates and slow growth of *C. repandum* (*) and *C. libanoticum* (**Prange *et al.*, 2008**) no *in vitro* cultures were established.

The established *in vitro* cultures (**Prange *et al.*, 2008; 2010a,b**) served as the initial material for protoplast isolation and fusion experiments (**Prange *et al.*, 2010c**) and were

[1] Additional results that are not part of the publications, manuscripts and annexes of chapter 3 are partly included in the discussion (chapter 4) and presented in a combined “results and discussion” style. Additional results are marked with an asterisk.

evaluated for their appropriateness for the preservation of the germplasm of the putatively endangered genus *Cyclamen*.

In the next four sub-chapters (4.1.1, 4.1.2, 4.1.3, 4.1.4), the influence of the explant, the genotype, technical factors, stress, as well as genetic, epigenetic and other factors that might be of importance for the induction of shoots and embryogenic callus and the regeneration of somatic embryos will be discussed. Future prospects of the established *Cyclamen* tissue cultures will be briefly summarised in sub-chapter 4.1.5.

### 4.1.1 Shoot Induction

Vegetative propagation without the usage of *in vitro* culture is very inefficient in *C. persicum* and, therefore, has no commercial relevance. Many attempts have been made to improve the shoot induction in tissue culture (cf. chapter 1.2.2.1), but the quality and uniformity necessary for horticultural plant production have never been achieved via this propagation pathway. Vegetative propagation via shoots is valuable for the preservation of the germplasm, e.g., for the maintenance of important breeding varieties of *C. persicum* or for the preservation of the germplasm of wild and endangered species of the genus *Cyclamen* (Karam & Al-Majathoub, 2000a,b; **Prange *et al.*, 2008**). Another application is the production of high amounts of sterile plant material for protoplast isolation, which is one focus of the present study.

Adventitious shoot cultures were induced on root segments, tuber discs and cotyledon explants from seedling tissue of *C. coum*, *C. graecum*, *C. hederifolium* and *C. mirabile* on MS- (Murashige & Skoog, 1962) based media. Two culture media with different concentrations of plant growth regulators (PGRs) were compared: 1) Shoot induction medium I containing α-naphthylacetic acid (NAA) and 6-benzylaminopurine (BA) and 2) Shoot induction medium II containing indole-3-acetic acid (IAA), BA, 6-(γ,γ-dimethylallylamino)purine (2iP) and kinetin. Adventitious shoots were induced in all four species with various successes depending on the explant type, the species, the genotype and the culture medium (**Prange *et al.*, 2008**). Karam & Al-Majathoub (2000a,b) induced shoots from seedling and mature tissue of wild *C. persicum* collected in Jordan on MS-based culture media containing thidiazuron (TDZ) or TDZ and NAA. The authors failed to induce shoots from mature tissue on BA-containing media (Karam & Al-Majathoub, 2000a). BA resulted in regeneration success in other studies dealing with *C. persicum* cultivars (Loewenberg, 1969; Ando & Murasaki, 1983; Wainwright & Harwood, 1985; Hawkes & Wainwright, 1987; Dillen *et al.*, 1996) as well as in this study

dealing with different wild species (**Prange *et al.*, 2008**). As described by **Prange *et al.* (2008)**, one exception was *C. coum*, in which the lowest amount of adventitious shoot formation was observed in the combination of *C. coum* and shoot induction medium I (containing NAA and BA). Moreover, due to their location at the top of the tuber, these shoots most likely originated from the apical meristem and, therefore, were not adventitious shoots. Interestingly, Seyring *et al.*, 2009 also did not obtain shoots from *C. coum* on modified N69-medium (Nitsch & Nitsch, 1969) containing BA and IAA, indicating that BA, IAA and NAA at their respective concentrations, are not suitable for shoot induction in *C. coum*. In contrast, shoot induction medium II (**Prange *et al.*, 2008**), containing IAA, BA, 2iP and kinetin, resulted in shoot development from cotyledon and tuber explants from one out of three *C. coum* genotypes, demonstrating that this species is able to form adventitious shoots. In summary of the results of Karam & Al-Majathoub (2000a,b), TDZ-containing media are worth testing in future experiments for shoot induction in this species.

The lack of shoot development in *C. persicum* wild species on BA-containing culture media observed by Karam & Al-Majathoub (2000a; b) might also have resulted from genotypic differences, but unfortunately the exact number of genotypes tested was not stated by the authors (Karam & Al-Majathoub, 2000a; b). In the shoot induction experiments presented in this thesis, a very high variation in the tissue culture response of different genotypes was found in all species (e.g., 0% shoot induction compared to 82% shoot induction in *C. graecum* on the same medium at the same point in time). Due to the fact that, for technical reasons, only one genotype (=one seedling) could be evaluated on one medium, the shoot induction results on the two shoot induction media, I and II, are difficult to compare (**Prange *et al.*, 2008**). As an indicator of the high genotypic variation, the shoot formation frequency in *C. graecum* and *C. hederifolium*, of which 9 to 12 genotypes were evaluated, is quite similar on the two media, between the two species and between the different explants. These genotypic effects were very pronounced in all of our tissue culture observations with the different *Cyclamen* species. The high genotypic variation in the reactions to *in vitro* culture conditions has frequently been described for other species (e.g., Jain *et al.*, 1988; Ochoa-Alecho & Ireta-Moreno, 1990; Gulati *et al*., 1992; Schween & Schwenkel, 2003), for *C. persicum* cultivars (e.g., Schwenkel & Winkelmann, 1998; Winkelmann & Serek, 2005; Takamura, 2007) and for other *Cyclamen* species, such as *C. hederifolium* and *C. coum* (Seyring *et al.*, 2009). In this thesis (**Prange *et al.*, 2008**), often particular genotypes (= seedlings) did not show any reaction to the tissue culture media and turned brown or black without enlargement of the explants, whereas others showed proliferation, such as growth of the explants or formation of

callus, shoots or roots. These losses might have obscured some effects of the different PGRs, which, thus, could only be mentioned as statistically insignificant “tendencies” or not be evaluated at all. For some genotypes, the culture medium used might have not been suitable, resulting in the death of all explants of the particular genotypes. The disruption of the explants during preparation or the age/developmental stage of the seedling used as the explant source might also be causal factors. In some species, like *C. coum*, damage to the seedlings during preparation due to their small size might also be a possible explanation. In future experiments, the use of later developmental stages and different preparation methods (e.g., thicker tuber slices as explants) should be tested in species with low frequencies of organogenesis. Environmental effects of the times of preparation (Winkelmann & Serek, 2005) and seasonal differences (Püschel, 2000) also influenced the *in vitro* callus and somatic embryo formation in *C. persicum* cultivars.

Using sterile seedling explants from the petiole and lamina (wounded blades, blades with midrib, blades without midrib, central lamina, petiolated lamina), Karam & Al-Majathoub (2000b) observed a positive effect of the midrib and the petiole on shoot regeneration. According to Kumar *et al.* (1998), this effect could be attributed to the presence of vascular tissue. Kumar *et al.* (1998) induced shoots in lamina and petiole explants of *Paulownia* and found a positive effect of the petiole on shoot induction and an additional positive effect of attached parts of the lamina. In accordance with these results, Karam & Al-Majathoub (2000a) observed no regenerative potential in leaf discs of mature tissue, whereas petiole, petal and peduncle explants from mature tissue showed 7 to 54% shoot formation on TDZ containing culture media.

Further experiments with the shoot cultures of *C. graecum*, *C. hederifolium*, *C. mirabile* and *C. coum* obtained within this thesis (**Prange *et al.*, 2008**) were performed. The suitability of leaf blades as explants was tested on various media but did not result in shoot formation (Metzenauer, 2007). However, shoot regeneration from dark-grown petioles was successful in *C. hederifolium* and *C. graecum* (**Prange *et al.*, 2008**) and in *C. coum* (Metzenauer, 2007). In *C. graecum*, 33.4% (shoot induction medium I) and 38.7% (shoot induction medium II) of explants formed shoots after 8 weeks, compared to 2.4% (Shoot induction medium I) and 16.9% (shoot induction medium II) of *C. hederifolium* explants forming shoots after 16 weeks. Additionally, genotypic variations were very high in these randomised experiments (0 to 70.5% explants with shoot formation on the same medium at the same point of time) (**Prange *et al.*, 2008**). In *C. graecum,* the number of explants with shoot formation decreased after 16 weeks because the shoots started to become wilted and necrotic after 8 weeks of culture. In

contrast, in *C. hederifolium* shoot induction rose after 16 weeks of culture, and after prolonged culture in the dark shoot formation increased immensely*. Therefore, the timeframe for future shoot induction experiments has to be widened for this species. Shoot cultures of *C. hederifolium* were vigorous and firm, and they rooted partly on the shoot induction medium or on medium without additional PGRs. In these rooted cultures, the medium often liquefied. In *C. graecum*, spontaneous rooting was rarely observed; for efficient rooting the shoot cultures often had to be transferred to a medium without PGRs or a special rooting medium with lowered $NH_4NO_3$ content and NAA as PGR (after Schwenkel, 1991). The base of the non-rooted shoot cultures of *C. graecum* often turned black at the surface in contact with the medium and the shoots became wilted and necrotic*. These reactions were observed throughout all *C. graecum* genotypes (**Prange *et al.*, 2008**)*.

In all media, the solidification agent used was Gelrite, which has also been successfully used to cultivate *C. persicum in vitro*. Medium solidified with Gelrite (= gellan gum, phytagel) has better water availability for the cultured plants than agar-solidified medium. The greater water availability benefits some cultures but can also lead to vitrification/hyperhydricity in susceptible cultures. The gel strength in Gelrite-solidified media is not only determined by the amount of solidification agent added to the culture medium but also by the pH, basal salt composition, sucrose concentration, mannitol concentration, presence of activated charcoal, autoclaving methods, other additives and the storage time before use (Huang *et al.*, 1995; Cameron, 2001). As a consequence, the availability of various medium components, such as magnesium, calcium, zinc and manganese, is reduced in media solidified with Gelrite, whereas the availability of other components (e.g., potassium) is increased (Van Winkle *et al.*, 2003). As a result, the use of Gelrite-solidified media can exacerbate problems with the supply of some nutrients, such as calcium and magnesium. In many studies, insufficient calcium supply to the upper parts of the plant was probably a factor in the development of malformations *in vitro*. In field-grown mango (*Mangifera indica* L.) and its seedlings, the calcium content in malformed tissue was significantly lower in necrotic shoots than in healthy tissue (Singh *et al.*, 1991). Martin *et al.* (2007) effectively reduced shoot necrosis in banana and plantain (*Musa* ssp.) by the addition of extra calcium chloride to the *in vitro* culture medium. Bangerth (1979) proposed that the insufficient distribution of calcium to the apical parts was more likely caused by absence of transpiration in *in vitro* cultures due to high humidity than to insufficient uptake alone. This hypothesis was proven by Cassels & Walsh (1994) who correlated the calcium accumulation in the leaves of *in vitro* grown plants with the gas permeability of the vessel lid and the stomatal function *in vitro*. In this study, a combination

of the above-mentioned factors may have led to the wilting and necrosis of shoots in *C. graecum*.

It can be assumed, that the well-developed root system of *C. hederifolium* was able to extract calcium and magnesium from the gel and, thereby, dissolve the Gelrite-solidified culture media. In contrast, *C. graecum* shoot cultures, mainly without root systems, were not able to extract sufficient amounts of nutrients from the culture media, which resulted in calcium-deficient necrotic shoots. The developmental stages of the vascular system in *C. graecum* shoot cultures might also be rudimentary, and nutrient transport under the described culturing conditions would only be possible by diffusion. Therefore, we tested and used a modified culture medium with double the amounts of $CaCl_2$, $MgSO_4$ and microelements and a reduced amount of Gelrite (3 g $L^{-1}$) (medium 2.25) for the culture of wild *Cyclamen* species*. *C. graecum* shoot cultures were more vigorous and could be stabilised *in vitro* as well as regenerated into plants, when this modified medium was used*.

In the study of Karam & Al-Majathoub (2000a), etiolated petioles exhibited a higher shoot regenerative potential than non-etiolated petioles. The influence of the light conditions during preculture on the explant material was not investigated in detail in this thesis. Explant material (seedlings and shoot cultures) was exclusively grown in the dark, which might have improved the results.

Studies with petiole explants of *C. graecum* and *C. hederifolium* (two genotypes of each) showed a clear positional effect, with the upper part of the petiole being more responsive to the shoots induction medium than the lower part (Metzenauer, 2007). However, Kumar *et al.* (1998) also found a correlation between regenerative ability and the length of the petiole parts of the petiole-lamina explants of *Paulownia* that suggested that the cells located at the base of the petiole (closest to the stem axis) are most competent to differentiate shoots.

In the shoot induction experiments presented in this thesis (**Prange *et al.*, 2008**), generally cotyledon and tuber explants had the highest shoot regeneration yields. On average, the most responsive species were *C. graecum* and *C. hederifolium* with shoot induction up to 59% and 18%, respectively. In general, in all species except *C. graecum*, shoot induction medium II with a combination of cytokinins led to better shoot formation than shoot induction medium I. Root explants often mainly developed roots. In *C. mirabile*, only roots were induced on root explants while in *C. hederifolium* and *C. graecum* mainly roots were induced on root explants, but low numbers of shoots were induced as well.

*C. coum* root explants did not shown any differentiation. In all species, except for *C. coum*, vigorous formation of roots was detected, presumably due to a very high endogenous auxin content. In future experiments, other cytokinin combinations must be tested, especially in *C. mirabile* and *C. coum*, for which the shoot induction has yet to be optimised. The suppression of the vigorous root formation by antiauxins, such as p-chlorophenoxybutyric acid (PCIB), as described by Bellamine *et al.* (1998), merits consideration.

### 4.1.2 Induction of Embryogenic Cultures

Embryogenic cultures (callus and suspension cultures) of wild *Cyclamen* species served as the initial material for the regeneration of plants via somatic embryogenesis (**Prange *et al.*, 2010a,b**) and the isolation and regeneration of protoplasts (**Prange *et al.*, 2010a,b**). The success of the experiments was influenced by many factors, such as the choice of the explant, the genotype and the application of stress as well as physiological and epigenetic reasons that will be discussed in this chapter. The morphological characteristics of embryogenic and non-embryogenic cultures of the different *Cyclamen* species will be compared. Additionally, recent proteomic and transcriptomic studies in *Cyclamen* and perspectives for the application of these results to *Cyclamen in vitro* culture will be discussed.

Explants of mature leaves from greenhouse-grown *C. coum* and explants of tissue derived from sterile *C. alpinum*, *C. graecum* and *C. mirabile* seedlings were successfully used for the induction of embryogenic callus (**Prange *et al.*, 2010a,b**).

The choice of an appropriate explant is often described as being of paramount importance for somatic embryo induction (Lo Schiavo, 1995; Winkelmann, 2004; von Arnold, 2008). Often, juvenile/ontogenetically young tissue, such as microspores, ovules, embryos and seedlings (von Arnold, 2008), are recommended as explant source and have been successfully used in the past (e.g., Tulecke & McGranahan, 1985; Gray & Mortensen, 1987; Rai *et al.*, 2007). However, in many species, there have been numerous reports of somatic embryogenesis from mature tissue (e.g., Poirier-Hamon *et al.*, 1974; Trolinder & Goodin, 1988; Kintzios *et al.*, 1999; Wilhelm, 2000).

Somatic embryogenesis has been induced on various explant sources, such as seedling tissue (Fukui *et al.*, 1988; Kreuger *et al.*, 1995; Takamura *et al.*, 1995; Bach *et al.*, 1998), anthers, ovaries, zygotic embryos (Kiviharju *et al.*, 1992), young leaf blades (Otani & Shimada, 1991) and ovules (Schwenkel & Winkelmann, 1998; Winkelmann, 2010), in

*C. persicum* cultivars. However, reports of somatic embryogenesis in wild species are rare (Seyring *et al.*, 2009, Furukawa *et al.*, 2002) and based on seedling and mature tissue from different sources. Furukawa *et al.* (2002) report the induction of somatic embryos from petiole and leaf explants of sterile seedlings of *C. africanum*, *C. cilicium*, *C. persicum*, *C. rohlfsianum* and *C. trochopteranthum* (=*C. alpinum*). Unfortunately, due to the unavailability of an English translation of the full article, we lack detailed information about the methodology. Seyring *et al.* (2009) described the formation of somatic embryo-like structures on various explants of mature plants of *C. africanum* (peduncle explants), *C. cilicium* (petiole explants), *C. coum* (placenta, peduncle, leaf and petiole explants), *C. hederifolium* (peduncle, leaf and petiole explants), *C. persicum* (placenta and peduncle explants), and *C. purpurascens* (placenta explants). Summarising these observations, juvenile tissue might be advantageous for the induction of somatic embryos in *Cyclamen*, but somatic embryogenesis can also be achieved on mature tissue. In this study, embryogenic callus and somatic embryos were induced on mature leaf tissue of *C. coum* (**Prange *et al.*, 2010a**) in one genotype out of ten. Additionally, in this species, Seyring *et al.* (2009) were able to induce somatic embryo-like structures on all tested explants (placenta, peduncle, leaf and petiole explants) in only one genotype. In other genotypes of this species, the *in vitro* response was limited to some explant sources (Seyring *et al.*, 2009).

In this thesis, somatic embryos were also induced on seedling explants of *C. alpinum*, *C. graecum*, and *C. mirabile* (**Prange *et al.*, 2010b**). Of the ten genotypes examined per species, one (*C. graecum*, *C. mirabile*) or two (*C. alpinum*) formed embryogenic callus and somatic embryos. Cotyledons, tubers and roots were used as explants. In *C. graecum* and *C. alpinum*, all explants formed embryogenic callus and somatic embryos, whereas in *C. mirabile* only cotyledon explants did (**Prange *et al.*, 2010b**). No morphological or physiological differences in the callus cultures derived from different explants of the same genotype were observed.

Due to the low proportion of responsive genotypes in both studies (10 to 20%) (**Prange *et al.*, 2010a,b**) and the successful induction of embryogenic callus and somatic embryos from mature and juvenile tissue, the role of the genotype seems to be more important than the source of the explants in the case of wild *Cyclamen* species. Kiviharju *et al.* (1992) observed that highly embryogenic callus was frequently induced on juvenile tissue of *C. persicum*, whereas the callus obtained from mature tissue was rarely embryogenic. Karam & Al-Majathoub (2000b) observed microtuberisation on seedling explants but not on mature explants of wild *C. persicum* (Karam & Al-Majathoub, 2000a). This

microtuberisation was also observed in the embryogenic cultures obtained from mature tissue of *C. coum* (**Prange *et al.*, 2010b**). This microtuberisation can be regarded as a kind of somatic embryogenesis in which the organ formation does not follow the classical succession of embryonal stages. The resulting small embryos/plants were not distinguishable from plants developed from classic somatic embryos, unlike plantlets obtained by adventitious shoot formation, few of which developed proper tubers (**Prange *et al.*, 2008**). Both forms (microtubers, and "classical" somatic embryos) occurred in parallel and in all intergradations and, therefore, were treated in this thesis as somatic embryos. In wild *Cyclamen* species more detailed studies are needed to reveal the influence of the age of the explant material on the ability to form embryogenic callus and somatic embryos. There is evidence that juvenile tissue is advantageous for the induction of somatic embryogenesis (Kiviharju *et al.*, 1992; Karam & Al-Majathoub, 2000a; b), but it is not obligatory (Seyring *et al.*, 2009; **Prange *et al.*, 2010a**). For *Arabidopsis thaliana* (L.) Heynh, only 5 to 6-day old seedling were responsive *in vitro* and produced somatic embryos (Ikeda-Iwai *et al.*, 2003).

Moreover, this study revealed different sensitivities of the different *Cyclamen* species to plant growth regulators during shoot induction and protoplast culture experiments (**Prange *et al*. 2008; 2010a,b**). Therefore, different PGR combinations and concentrations might also be tested in the induction of somatic embryogenesis. These changes to the protocol might increase the proportion of genotypes developing embryogenic callus and somatic embryos, as demonstrated by Bailey *et al.* (1993) in soybean or Close & Ludeman (1987) in maize.

The induction of embryogenic callus succeeded in only 10% to 20% of the genotypes in *C. alpinum*, *C. coum*, *C. graecum* and *C. mirabile* (n=10) under our experimental conditions (**Prange *et al.*, 2010a,b**). The capacity to form somatic embryos and their growth *in vitro* also differed between genotypes of the same cultivars in *C. persicum* *, which were grown from seeds and, therefore, closely related but not identical clones.

High genotypic influence on the induction of somatic embryogenesis was also observed in many other species [e.g., soybean (Parrott *et al.*, 1989) and cotton (Trolinder & Xhixian, 1989); for a general overview, see Henry *et al.* (1994)] and in *C. persicum* (Takamura & Miyajima, 1996; Takamura & Tanaka, 1996; Schwenkel & Winkelmann, 1998; Winkelmann & Serek, 2005). Takamura & Miyajima (1997) induced embryogenic callus from aseptic seedlings of different *C. persicum* cultivars from various breeders and origins with a positive response in 31% genotypes (n=13), which is comparable to our results obtained in wild *Cyclamen* species. Schwenkel & Winkelmann (1998) were able to induce

embryogenic callus from 97% of *C. persicum* genotypes (n=30), breeding lines and $F_1$ hybrid cultivars, when ovules were used as the explant source. With the same protocol, Winkelmann & Serek (2005) obtained the same percentage (97%) of embryogenic genotypes (n=32) from $F_1$ hybrids of various breeders. These differences in the *in vitro* response might have their origin in the explant source (aseptic seedlings versus ovules), the genotype or the culture medium. Comparing the results of this thesis with the studies of Schwenkel & Winkelmann (1998) and Winkelmann & Serek (2005) the last reason can be excluded because the same medium composition was used. Therefore, the genetic predisposition of the genotype might be of importance. Püschel *et al.* (2003) postulated that for *C. persicum* the ability to regenerate via somatic embryogenesis is controlled by two dominant major genes. Additionally, F1 hybrids that are heterozygous for many traits might be more likely to have a positive *in vitro* response than the inbred breeding lines that are homozygous at many gene loci. In accordance with these results, the impact of the culture media and the explant tissue might be less important than the genetic background of the genotype under investigation. The high percentage of plants with the ability to undergo somatic embryogenesis found among the *C. persicum* cultivars observed by Schwenkel & Winkelmann (1998) and Winkelmann & Serek (2005) might be attributable to a constricted genetic variability in the *C. persicum* cultivars. Over the centuries of breeding history, some particular cultivars/plants became the basis for the many existing cultivars (Bongartz, 1999). This selection process has narrowed the gene pool of *C. persicum* cultivars and, by chance, might have favoured the ability for somatic embryogenesis.

The genotype seems to be of paramount importance for the ability to form somatic embryos. One scientist stated, “somatic embryogenesis can probably be achieved for all plant species provided that the appropriate explant, culture media and environmental conditions are employed” (von Arnold, 2008). Regarding this statement, additional factors that can influence the ability to form somatic embryos, such as technical reasons, stress, epigenetic factors and genetic factors will be discussed.

Technical causes might be responsible for the failure to obtain somatic embryos in some genotypes. Gawel *et al.* (1986), for example, stated that in their study only three out of twenty explants formed somatic embryos (out of one embryogenic genotype and the same explant source). This finding implies that in this thesis (**Prange *et al.*, 2010a,b**) the amount of explants might have been insufficient to reveal somatic embryogenesis in each genotype. When seedling tissue in seed-propagated plants (one seed = one genotype) is used, as in this study, the available explant material is naturally limited. It was

demonstrated that the juvenility of the explant material is not obligatory in *C. coum* (**Prange *et al.*, 2010a**) as well as in other *Cyclamen* species (Seyring *et al.*, 2009). Therefore, the use of older and bigger seedlings and, thereby, of enhanced amounts of explants should be tested in future experiments. Still, there may be a positive effect of juvenility (see above).

The protocol for the induction of somatic embryos from seedling tissue in *C. alpinum*, *C. graecum* and *C. mirabile* was modified from the protocol of Schwenkel & Winkelmann (1998). After about eight weeks, the authors obtained embryogenic cultures from ovules of explants from *C. persicum* cultivars. This protocol was adapted to seedling tissue of different wild *Cyclamen* species (**Prange *et al.*, 2010b**), but the development of embryogenic cultures took four to six months. The explants were cultivated for 16 weeks and transferred to fresh medium after 8 and 16 weeks. After this period, the explants and developing cultures were kept on the same medium. Embryogenic cultures (undifferentiated, embryogenic callus and somatic embryos) were only obtained in these aged cultures. In contrast to the protocol of Schwenkel & Winkelmann (1998), stress seemed to be a crucial factor for the induction of somatic embryos in this thesis. In the ageing cultures, the availability of water and nutrients was probably reduced and the osmolarity was enhanced due to the beginning desiccation of the medium. In contrast to these results, Schwenkel & Winkelmann (1998) obtained embryogenic callus during the culturing period, when the cultures were frequently subcultured. The induction of somatic embryogenesis by stress has often been demonstrated by high macronutrient stress in ginseng (*Panax ginseng* C. A. Meyer) (Choi *et al.*, 1998), osmotic stress in carrot (*Daucus carota* L.) (Kamada *et al.*, 1993), explant wounding in soybean [*Glycine max* (L.) Merr.] (Santarem *et al.*, 1997), osmotic-, heavy-metal-ion- and desiccation stresses in *Arabidopsis thaliana* (L.) Heynh. (Ikeda-Iwai *et al.*, 2003) and other stresses (reviewed by Dudits *et al.*, 1995; Karami & Saidi, 2009). 2,4-D is applied in more than 65% of the protocols for the induction of somatic embryos (Gaj, 2004). The application of this PGR *in vitro* has been demonstrated to change the physiology and gene expression of cells (Dudits *et al.*, 1995; Karami & Saidi, 2009).

In *Chenopodium rubrum* suspension cells, osmotic changes and 2,4-D-application can induce the generation of reactive oxygen species (ROS), known as an oxidative burst (Pfeiffer & Höftberger, 2001). Therefore, 2,4-D itself is regarded as a putative stress factor and a causal agent for the induction of an oxidative burst and/or other physiological changes that trigger the formation of embryogenic patterns in plant cell cultures (Dudits *et al.*, 1995; Feher *et al.*, 2003; Gaj, 2004; Karami & Saidi, 2009).

The up-regulation of many genes or the abundance of the corresponding gene products involved in the detoxification of ROS have been described in the early stages of somatic embryogenesis [e.g., in soybean (*Glycine max* L. Merr.) (Thibaud-Nissen *et al.*, 2003) and in *C. persicum* (Rensing *et al.*, 2005; Hoenemann *et al.*, 2010)]. Additionally, the definition of stress has been widened by many authors (Karami & Saidi, 2009 and references therein) to include the "normal" treatments applied during *in vitro* culture, such as cutting, sterilisation, artificial culture media and non-physiological PGR concentrations. All of those stresses induce similar or identical physiological and genetic changes (Karami & Saidi, 2009) resulting in genetic reprogramming for somatic embryogenesis (Dudits *et al.*, 1995; Karami & Saidi, 2009 and references therein). Due to their observations, many authors hypothesised that somatic embryogenesis itself is an extreme stress response or an extreme case of adaption of cultured plant cells (Dudits *et al.*, 1995; Pasternak *et al.*, 2002; Karami & Saidi, 2009). The molecular basis for these observations was presumed by Dudits *et al.* (1995) to be hormonal- or stress- induced signal transduction cascades. These cascades lead to the re-programming of gene expression and induction of cell division, which results in non-differentiated cell proliferation or somatic embryogenesis. More recent results have partly confirmed this concept and the putative molecular connections of auxin, other PGRs and stress to somatic embryogenesis Karami & Saidi (2009).

Besides the induction of an oxidative burst (see above), shortly after the application of 2,4 D and various stresses, the expression of some histone H3 gene variants increased during somatic embryogenesis in *Medicago* (Kapros *et al.*, 1992). The application of 2,4-D and other auxins for the induction of somatic embryos also results in enhanced DNA methylation [e.g., in pumpkin (*Cucurbita pepo*) (Leljak-Levanic *et al.*, 2004) and carrot (*Daucus carota* L.) (LoSchiavo *et al.*, 1989)]. Additionally, both DNA hypermethylation and somatic embryogenesis were blocked by the DNA hypomethylating drugs, 5-azacytidine, ethionine and 2-amino5-ethoxy-carbonyl-pyrimidine-4(3H), in carrot (LoSchiavo *et al.*, 1989, Yamamoto *et al.*, 2005). DNA methylation and histone modification, in addition to other epigenetic changes, lead to a modulation of the chromatin structure and, thereby, of the gene expression pattern (Bender, 2004, Feher *et al.*, 2003). This genetic reprogramming takes place in the presence of 2,4-D and other auxins and results in genetic reprogramming and somatic embryogenesis (Karami & Saidi, 2009).

Abscisic acid (ABA) and ethylene are suggested to be two regulators of the response to abiotic as well as *in vitro* stress, and an up-regulation of their biosynthesis has been demonstrated in response to stress as well as during somatic embryogenesis (Karami &

Saidi, 2009). Molecular evidence from genetic studies in *Arabidopsis* (Sheen, 1996) suggests that the biological function of ABA in the induction of somatic embryogenesis is connected with calcium-dependent protein kinases that activate a stress- and ABA-inducible promoter (Karami & Saidi, 2009). Additionally, ethylene is putatively connected to stress by mitogen-activated protein kinase (MAPK) cascades. In *Medicago truncatula*, somatic embryogenesis is induced by the application of auxin and cytokinin as well as the biosynthesis of ethylene; the latter is most likely induced by wounding (Mantiri *et al.*, 2008a; b). The expression of the transcription factor *Mt SOMATIC EMBRYO RELATED FACTOR 1* (*MtSERF1*) that belongs to the *ERF* family of ethylene responsive elements (Nakano *et al.*, 2006, Mantiri *et al.*, 2008a; b), is essential for somatic embryogenesis. As observed by Mantiri *et al.* (2008a, b), *MtSERF* from *M. truncatula* has binding sites for ethylene and additional putative binding sites for auxin and cytokinin. Therefore, this gene likely links stress, auxin, cytokinin and somatic embryogenesis. Other examples of such connections have been reviewed by Karami & Saidi (2009).

In this thesis (**Prange *et al.*, 2010b**), the development of undifferentiated cell proliferation/callus growth and the formation of somatic embryos was observed in ageing, and thereby, additionally stressed cultures of *C. alpinum*, *C. graecum* and *C. mirabile*. In future experiments, an optimisation of the protocol for somatic embryogenesis in wild *Cyclamen* species, utilising stress as important factor for the induction of somatic embryogenesis, should be considered. Short-term exposures to directed desiccation, high macronutrient and/ or osmotic stresses or higher 2,4-D concentrations can be evaluated for their impact on embryogenity and the time needed for somatic embryo induction. In parallel, the expression patterns of embryo- and stress-related genes, their methylation patterns and their gene products should be investigated in more detail during these protocol optimisations (e.g., Feher *et al.*, 2003; Karami & Saidi, 2009).

Some general morphological aspects of embryogenic and non-embryogenic tissue will be discussed with a special emphasis on *Cyclamen* embryogenic cultures, and some examples of the detection of somatic embryo-specific genes and proteins in *C. persicum* will be given.

Nabors *et al.* (1983) were able to visually distinguish embryogenic from non-embryogenic callus and cells in various cereal species in a mixed culture. Embryogenic cells are often characterised as smaller and less vacuolated than non-embryogenic cells, and they are composed mainly of cytoplasm (e.g., Nabors *et al.*, 1983; Van Engelen & De Vries, 1992). Embryogenic cultures are mostly a mixture of embryogenic and non-embryogenic cells, which has been demonstrated e.g., for sweet potato (*Ipomoea batatas* Poir.) (Chee &

Cantliffe, 1988) and carrot (*Daucus carota* L.) (Van Engelen & De Vries, 1992). In *C. persicum*, embryogenic and non-embryogenic callus lines of the same genotype exist as individual, separate cultures, as in many other species. The two callus types can easily be distinguished by their macroscopic appearance and consistency (Winkelmann *et al.*, 1998). In general, non-embryogenic callus is yellow and friable, whereas embryogenic callus is brownish, soft or friable and often contains bigger aggregates. Embryogenic callus in wild *Cyclamen* species was comparable to that obtained from some *C. persicum* cultivars (**Prange *et al.*, 2010a,b**). Most of the freshly induced non-embryogenic calli were very hard and could not be subcultivated. The embryogenic callus obtained from *C. graecum* closely resembled the non-embryogenic callus of *C. persicum* described by Winkelmann et al. (1998), both were friable to smooth, white yellowish in colour, but the one derived from *C. graecum* (**Prange *et al.*, 2010b**) had a high embryogenic capacity, whereas the one from *C. persicum* (Winkelmann *et al.*, 1998), never gave rise to somatic embryos. Recent comparative microarray expression profiling in *C. persicum* cells lines (Hoenemann *et al.*, 2010) identified three homologous of pectin modifying enzymes involved in pectin degradation and methylesterification that were upregulated in embryogenic cultures in comparison to non-embryogenic cultures. The authors hypothesised that pectin degradation and modification is necessary for active pre-embryogenic callus growth in *C. persicum* and correlated this with the reduced friability observed in the embryogenic cell line compared to the non-embryogenic cell line (Hoenemann *et al.*, 2010). In this thesis, the opposite observation was made with the *C. graecum* embryogenic culture: this callus exhibited a high resemblance to the (identical) non-embryogenic cell line from *C. persicum* described by Hoenemann *et al.* (2010) in terms of colour and friability, but it had a high capacity to form somatic embryos (**Prange *et al*., 2010b**). This cell line would be an interesting addition for the further investigations intended by the authors (Hoenemann *et al.*, 2010) regarding the connection of pectin metabolism, friability and the ability to form somatic embryos. Due to these observations, a visual classification of embryogenic and non-embryogenic callus cannot be generalised for the genus *Cyclamen*. *In vitro* screening for somatic embryogenesis or other markers for somatic embryogenesis must always be used. The research in progress on the proteomic and transcriptomic levels has revealed many putative targets for the development of such markers in *C. persicum*, but their transferability to other *Cyclamen* species remains unproven.

Since the establishment of a very efficient system for the induction of embryogenic callus in 1998 by Schwenkel & Winkelmann, *C. persicum* was established as a model system for somatic embryogenesis, similar to that in carrot (*Daucus carota* L.) (Zimmerman, 1993;

Rensing *et al.*, 2005). In the last five years, the poor genetic and proteomic knowledge of *C. persicum* (and other species in this genus) has been rapidly enhanced by the establishment of an expressed sequence tag (EST) library from different stages of somatic embryos and embryogenic cultures of *C. persicum* (Rensing *et al.*, 2005) and the use of this data in cDNA microarray and real-time PCR expression profiling (Hoenemann *et al.*, 2010). These genetic studies were complemented by the proteomic studies comparing zygotic *versus* somatic embryos (Winkelmann *et al.*, 2006c; Rode *et al.*, 2010), embryogenic *versus* non-embryogenic cultures (Lyngved *et al.*, 2008) and somatic and zygotic embryos versus non-embryogenic cultures (Bian *et al.*, 2010) performed in the same species. These studies gave new insights into the biochemical changes during *Cyclamen* somatic embryogenesis that revealed many molecular similarities to other somatic embryo systems, such as the one established in carrot (Zimmerman, 1993; Rensing *et al.*, 2005). All of these basic research studies were additionally aimed at improving the *in vitro* culture methods for mass propagation of *C. persicum*, proper somatic embryo development, the detection of seed storage compounds for the improvement of the artificial seed technique (Winkelmann *et al.*, 2004b, Winkelmann *et al.*, 2006c; Winkelmann, 2010; Hoenemann *et al.*, 2010) and the detection of embryo-specific genes/ proteins for the development of molecular markers for the detection of embryogenic cell lines (Lyngved *et al.*, 2008; Hoenemann *et al.*, 2010). Some examples of putative somatic embryo-related genes and proteins will be given, with special emphasis on *C. persicum.*

Typical genes (or specific isoforms of those genes), such as specific genes involved in signal transduction (somatic embryo receptor-like kinases (SERKs), GTP-binding proteins), heat shock proteins (HSPs), peroxidases, late embryogenesis abundant (LEA) proteins, arabinogalactan proteins (AGPs) and chitinases, have often been observed to play essential roles during somatic embryogenesis in various species (Lyngved *et al.*, 2008 and references therein, Hoenemann *et al.*, 2010). Their occurrence in *C. persicum* and their putative functions and usability as markers will be discussed.

Genes for signal transduction are often found in high abundance in embryogenic tissue. One example is the somatic embryogenesis receptor-like kinases (SERKs), first described by Schmidt *et al.* (1997) in carrot. Due to their expression in somatic embryos from the competent cell stage up to the globular stage of somatic embryos and the undetectability of transcripts in non-embryogenic stages of embryogenic cultures, they were designated as embryo-specific genes (Schmidt *et al.*, 1997; Chugh & Khurana, 2002). Hoenemann *et al.* (2010) found only one out of five SERK homologues to be over-expressed in

embryogenic tissue of *C. persicum* in all of their experiments. This finding is in accordance with observations summarised by Chugh & Khurana (2002) that specific SERK genes are connected to somatic embryogenesis, but not all genes that belong to the SERK family. G proteins, calreticulin, protein phosphatase 2A (PP2A) and GF14-D are proteins involved in signal transduction that were found to be more abundant in *C. persicum* embryogenic callus than in non-embryogenic callus (Lyngved *et al.*, 2008). Three corresponding gene products were found to be correlated with somatic embryogenesis in *Cyclamen* by Rensing *et al.* (2005).

In comparative studies of different stages in somatic embryos of *C. persicum*, Hoenemann *et al.* (2010) observed a high expression of a chitinase gene and a peroxidase gene three days after somatic embryo induction. Mutant cell cultures of *D. carota* that were arrested in a pre-globular stage due to disturbed protoderm development could be rescued by the addition of a glycosylated acidic endochitinase that was excreted from non-mutated cell cultures of *D. carota* (De Jong *et al.*, 1992). Additionally, the investigated *C. persicum* embryos resembled the arrested *D. carota* embryos morphologically (Hoenemann *et al.*, 2010). Therefore, the authors (Hoenemann *et al.*, 2010; De Jong *et al.*, 1992) deduced that the endochitinases in both species play an essential role in protoderm formation, which is necessary for embryo development (Hoenemann *et al.*, 2010; De Jong *et al.*, 1992). In *D. carota*, the putative substrates of the chitinase genes are arabinogalactan proteins (Van Hengel *et al.*, 2001), active compounds in conditioned medium with the ability to stimulate somatic embryo formation in non-embryogenic or recalcitrant tissue (Egertsdotter & von Arnold, 1995; von Arnold *et al.*, 2005).

A positive correlation between enhanced specific peroxidase activity and somatic embryogenesis has often been observed [e.g., date palm (*Phoenix dactylifera* L.) (El Hadrami & Baaziz, 1995) and *C. persicum* (Hoenemann *et al.*, 2010)]. The addition of the antibiotic tunicamycin inhibited somatic embryogenesis, but not unorganised proliferation, in carrot cell suspensions (Lo Schiavo *et al.*, 1986; Cordewener *et al.*, 1991; Van Engelen & de Vries, 1992). Embryo development was retained by the addition of conditioned medium from embryogenic cultures, and a cationic peroxide isoenzyme was found to be the responsible agent (Lo Schiavo *et al.*, 1986; Cordewener *et al.*, 1991; Van Engelen & de Vries, 1992). Peroxidases can crosslink structural cell wall glycoproteins. Therefore, they are candidates for the restriction of cell expansion (Van Engelen & de Vries, 1992), resulting in the development of small, cytoplasm-rich cells, characteristics of cells that will undergo somatic embryogenesis. The substrates of peroxidases can be hydrogen peroxide or organic hydroperoxides, and, thus, one can assume that these

enzymes are also involved in the response to oxidative stress (Thibaud-Nissen *et al.*, 2003). In comparisons of the expression profiles of somatic and zygotic embryos of *C. persicum*, different specific glutathione S-transferases (GST), a superoxide dismutase (SOD), catalase and two cytochrome P450 homologues were found to be up-regulated in early-stage somatic embryos (after auxin removal) (Hoenemann *et al.*, 2010). Winkelmann *et al.*, (2006c) identified a highly abundant SOD in somatic and zygotic embryos of *C. persicum*. These enzymes are involved in oxidative stress response (see above) and are active in other cellular detoxification processes. A connection between oxidative stress and somatic embryogenesis has been suggested by many authors.

Genes of the argonaut (AGO) family have proven to be essential for or involved in somatic embryogenesis in *Picea glauca* (Tahir *et al.*, 2006) and *D. carota* (Takahata, 2008). These genes are known to be part of the RNA-induced silencing complex, which is involved in the gene silencing via RNAi (Mello & Conte, 2004; Tolia & Joshua-Tor, 2007). Hoenemann *et al.* (2010) identified the transcript of a significantly up-regulated putative AGO homologue in non-embryogenic cell line that they could not detect in embryogenic cells and somatic embryos, and indicated that RNAi might be involved in the loss of embryogenic competence, a common tissue culture problem that will be discussed later in this thesis.

Also, some of those genes are known to be expressed in the whole plant, but transcripts of specific isoforms have often been identified in embryogenic tissue or somatic embryos. The genes, transcripts and gene products of those specific genes/ isoforms might be suitable candidates for the development of markers for the detection of genotypes, cells or cell lines with the ability to form somatic embryos or of cells that are undergoing somatic embryogenesis. Püschel *et al.* (2003) proposed the control of somatic embryogenesis in *C. persicum* by two dominant major genes. Based on this proposal, it should be possible to develop a simple, PCR-based marker to screen for genotypes with the ability to form somatic embryos. Due to the putative involvement of epigenetic silencing in non-embryogenic tissue (Hoenemann *et al.,* 2010) and other epigenetic mechanisms involved in the control of somatic embryogenesis (see above), one might have to distinguish between callus with or without the genetic predisposition to form somatic embryos and callus that is arrested in its non-embryogenic state for various epigenetic or physiological reasons. Therefore, depending on the experimental goal, the marker developed should target the genes, the transcripts or the gene products.

### 4.1.3 Regeneration of Somatic Embryos from Embryogenic Cultures

Somatic embryos were regenerated from embryogenic cultures (callus cultures and suspension cultures) of four wild species (*C. alpinum*, *C. coum*, *C. graecum* and *C. mirabile*) with various frequencies of somatic embryo formation, embryo quality, regeneration, maturation and germination success (**Prange *et al.*, 2010a,b**)*. Again, the differences in the embryogenic capacities between the species/genotypes were very pronounced. The regeneration via somatic embryogenesis proved to be a very efficient propagation method in wild *Cyclamen* species, especially in *C. coum* and *C. mirabile*. The effect of the media composition on the formation of somatic embryos will be briefly summarised (for a detailed discussion, see **Prange *et al.*, 2010a,b**). The main focus is set on the problems or side effects during somatic embryo regeneration, such as hyperhydricity, secondary embryogenesis and decreasing embryogenic capacity, which were observed with different intensities in each species.

Different media compositions were tested to improve the embryo formation, maturation and germination from embryogenic cultures in wild *Cyclamen* species (**Prange *et al.*, 2010a,b**). The content/concentrations of PGRs (kinetin), calcium, magnesium, microelements, activated charcoal and ammonium nitrate were modified. Positive effects on the embryo formation were observed in media that contained activated charcoal (*C. mirabile*) and additional $CaCl_2$ (*C. persicum*). The reduction of ammonium nitrate reduced the number of somatic embryos (*C. mirabile*), and the addition of kinetin resulted in improved embryo formation in *C. mirabile*, but reduced embryo formation in *C. persicum*. In *C. graecum*, *C. alpinum* and *C. coum*, no effect of the tested media was observed.

The effects of activated charcoal in tissue culture media have been reviewed by Pan & van Staden (1998). In embryogenic cultures of *C. mirabile*, the positive effect on the embryo formation could have been caused by the binding of PGRs. Activated charcoal can absorb endogenous and incorporated (natural or artificial) PGRs that are released by the cultured plant material into the culture medium (Pan & van Staden, 1998 and references therein) and, thereby, lower the absolute concentration of PGRs in the culture. This reduction in the PGR concentration can promote somatic embryo formation, which normally occurs in *Cyclamen* on PGR-free medium (**Prange *et al.*, 2010b**). The positive effect of calcium and magnesium has already been discussed in sub-chapter 4.1.1. The impact of these media changes on embryo formation was tested to prevent problems such as the putatively insufficient nutrient supply observed in shoot cultures and to improve somatic embryogenesis (**Prange *et al.*, 2010b;** cf. sub-chapter 4.1.1).

Symptoms of hyperhydricity (vitrification) and browning during somatic embryogenesis were frequently observed in somatic embryos of *C. graecum* and caused the loss of most of the embryos, which were previously obtained in high quantities from the freshly induced embryogenic culture*. Medium modifications (**Prange *et al.*, 2010b**) did not improve the regeneration results in this species. Given the previously discussed properties of Gelrite as a solidification agent (cf. sub-chapter 4.1.1) and the fact that many studies report enhanced hyperhydricity in Gelrite-solidified culture media (e.g., Nairn *et al.*, 1995; Ivanova *et al.*, 2006), a change to agar as solidification agent should be considered to improve the embryo development in this species. However, agar bears many disadvantages, such as mineral (e.g., copper and sodium) and organic impurities (e.g., Debergh, 1983), which can be toxic to sensitive tissues, reduced water availability, which can cause water stress (e.g., Bonga & von Aderkas, 1992), as well as a cytokinin-binding capacity (e.g., Debergh, 1983; Bonga & von Aderkas, 1992). Agar-solidified culture media have less pH buffering capacity than Gelrite-solidified media, which can cause pH drops in aged cultures (Bonga & von Aderkas, 1992). Due to these problems, in many cultures the use of Gelrite as the gelling agent improved growth and development in comparison to agar [e.g., *Gossypium hirsutum* (Zimmerman & Robacker, 1988); *Poa pratensis* (Van Ark *et al.*, 1991)]. The use of a mixture of Gelrite and agar is worth testing, as this mixture has proven to combine the advantages of both gelling agents and was superior to the use of either gelling agent alone in conifers [e.g., *Pinus sylvestris* (Häggman *et al.*, 1996); *Larix decidua* (McLaughlin & Karnosky, 1989)] and other species.

Besides the gelling agent, many other factors, such as insufficient ventilation in the culture vessels, can lead to hyperhydricity (Thorpe *et al.*, 2008). An improvement might be achieved by using bigger vessels or lids that enable a better gas exchange.

The embryogenic capacity of the *in vitro*-grown cultures was often retarded, decreased or lost after subsequent subculture (**Prange *et al.*, 2010a,b**)*. The time after which this effect appeared varied between the different genotypes/species and culturing methods. In general, suspension cultures were more susceptible to this effect than callus cultures. The embryogenic cultures of *C. graecum* were most strongly affected (**Prange *et al.*, 2010b**), but the effect was also observed in *C. coum* embryogenic cultures after about one year subsequent subculture *in vitro**, although it was less pronounced.

Negrutiu *et al.* (1979) observed a decrease in the embryogenic and organogenic capacities in *Arabidopsis thaliana* after subculturing on media containing PGRs, especially 2,4-D. The authors presumed that this effect was caused by an accumulation of PGRs. The high receptivity of suspension cultures can be easily explained following this concept.

Suspension cultures grow faster than callus cultures on solid medium, giving generally higher mutation rates and making them more assailable. Additionally, each cell/cell-cluster is in full contact with culture medium and is thereby continuously exposed to PGRs, enhancing the accumulation. During cultivation on solid medium, only the lowermost cell layer has contact with the culture medium and, thereby, the PGRs.

As a consequence, one approach in this thesis was to minimise the amounts of PGRs in all tissue culture media to minimise this effect (**Prange *et al.*, 2010a,b**). Especially in the protoplast culture experiments, the optimal concentration of PGRs was evaluated to speed up and optimise the embryo development for regeneration to plants. The reduction of PGRs to a quarter of the original concentration used by Winkelmann *et al.* (2006b) slightly reduced the speed of growth and improved the formation and germination of somatic embryos in C*. persicum* cultivars*. In wild *Cyclamen* species, the PGR concentrations had to be evaluated separately for each species/ genotype because of pronounced differences in their *in vitro* responses (**Prange *et al.*, 2010a,b**). The reduction of PGRs often did not affect the growth rate of the cells (callus cultures, suspension cultures, protoplasts) and, therefore, was adopted as the standard method in wild *Cyclamen* species (**Prange *et al.*, 2010a,b**). Also for *C. persicum*, the concentration of PGRs during protoplast culture was reduced, which ought to minimise the effect of PGR accumulation, reduce the mutation rate and, additionally, save money.

The application of PGRs, especially 2,4-D, is also known to induce genetic reprogramming, changes in the DNA methylation pattern and other epigenetic modifications (Karami & Saidi, 2009 and references therein) (see above). The mechanisms and importance of methylation and histone modification as well as the impact of both epigenetic changes on the chromatin structure, gene expression and genetic content of the underlying information have been reviewed by Bender (2004). These epigenetic changes might be the reason for the decrease or loss of embryogenic capacity in embryogenic cultures. Hoenemann *et al.* (2010) additionally suggested the involvement of RNA interference (RNAi) in the loss of embryogenic capacity in *C. persicum* (see above).

Secondary embryogenesis is a “special case of direct somatic embryogenesis” (von Arnold, 2008), in which “secondary embryos develop directly from epidermal or sub-epidermal cells of the cotyledons or hypocotyls” of somatic embryos (Thomas *et al.*, 1976; in von Arnold, 2008). This process can positively influence the propagation rate of the regenerated plants. However, if the formation of additional somatic embryos cannot be

stopped, this process is termed “continuous, recurrent or accessory” and the regeneration of plants is problematic (von Arnold, 2008).

The formation of secondary embryos was observed in all four wild *Cyclamen* species (*C. alpinum*, *C. coum*, *C. graecum* and *C. mirabile*). Continuous somatic embryogenesis was observed especially in *C. alpinum* and *C. graecum*. Successive cycles of separation onto fresh medium without PGRs had to be performed until a few plants could be regenerated (**Prange *et al.*, 2010b**). In *C. coum* and *C. mirabile*, secondary embryogenesis was less pronounced and stopped in medium without PGRs after one or two cycles of separation and increased the number of regenerated plants (**Prange *et al.*, 2010a,b**)*. Winkelmann *et al.* (2006b) observed more frequent secondary embryogenesis after prolonged culture on 2,4-D-containing medium. The authors suggested that a reduction of PGRs would be beneficial. Additionally, there may be an accumulation of PGRs in this situation, but it remains to be proven. Some authors suggest the application of ABA to the culture medium during a maturation step to reduce secondary embryogenesis (e.g., von Arnold, 2008).

In general, five steps of plant regeneration via somatic embryogenesis can be differentiated (von Arnold, 2008): 1) the initiation of embryogenic cultures, 2) their proliferation, 3) the pre-maturation of somatic embryos, 4) their maturation and 5) their germination and regeneration to plants. In the protocol for the regeneration via somatic embryogenesis established by Schwenkel & Winkelmann (1998) for *C. persicum*, which was modified and applied to wild *Cyclamen* species in this thesis, the five steps proceeded on two culture media. The initiation and proliferation of the embryogenic cultures was performed on medium containing 2,4-D and 2iP, and the last three steps were performed on medium without PGRs. Variations in the micro- and macronutrient composition of the PGR-free differentiation medium as well as the use of additives, such as activated charcoal and kinetin, were tested, as discussed above (**Prange *et al.*, 2010a, b**). For many species, it is necessary or beneficial to include a maturation step on a different culture medium that is not identical to the medium used for initiation or germination/ regeneration of plants [e.g., in soybean (*Glycine* ssp.) (Komatsuda *et al.*, 1992 and references therein)]. A maturation phase may improve the proper development and reduce the occurrence of secondary embryogenesis in wild *Cyclamen* species.

During the maturation phase of somatic embryos, many physiological similarities to the maturation phase in zygotic embryos can be observed. Consequently, maturation protocols for somatic embryos often try to mimic the processes known from zygotic embryo maturation. The embryo maturation process in zygotic embryos includes the

synthesis of storage products, the induction of water loss, the prevention of precocious embryo germination and the establishment of a dormancy period (Goldberg *et al.*, 1994; von Arnold, 2008). Understanding the mechanisms that control zygotic embryogenesis and seed maturation will help to improve the development of somatic embryos *in vitro*. In zygotic embryos, the accumulation of storage compounds is presumably regulated by ABA or water stress (Dodeman *et al.*, 1997). In somatic embryos, the regulation often seems to be incomplete, and it is often necessary to apply these triggers exogenously during the maturation phase to enable or optimise their development. The application of ABA to the culture medium has been demonstrated to induce the expression of maturation genes in wheat embryos (Morris *et al.*, 1990; Williamson *et al.*, 1985), to suppress some proteins related to germination in barley (Bartels *et al.*, 1988) and to inhibit precocious germination in interior spruce somatic embryos (Roberts *et al.*, 1990). The supplementation of the maturation medium with ABA often results in the accumulation of storage products in somatic embryos similar those in zygotic embryos (e.g., Roberts *et al.*, 1990; Etienne *et al.*, 1993) and has often improved embryo quality, as in *Hevea brasiliensis* Müll. Arg. (Etienne *et al.*, 1993), *Larix* x *leptoeuropaea* (Lelu *et al.*, 1994), *Picea glauca* (Moench (Voss) (Attree *et al.*, 1991) and *Aesculum hippocastanum* L. (Capuana & Debergh, 1997).

The application of ABA is in most cases combined with the application of osmotic stress, achieved by supplementing the media with sucrose (e.g., Komatsuda *et al.*, 1992; Etienne *et al.*, 1993, Lelu *et al.*, 1994), PEG (e.g., Attree *et al.*, 1991; Capuana & Debergh, 1997), mannitol (Roberts, 2006) or maltose (Li *et al.*, 1998) or by a dehydration procedure (Roberts, 2006; von Arnold, 2008), resulting in improved embryo quality. The osmoticum has to be evaluated for suitability in each species because negative effects of PEG on somatic embryo germination in *Picea abies* (Bozhkov & von Arnold, 1998) and detrimental effects of sucrose on the somatic embryos of *Picea glauca* (Attree *et al.*, 1991) have been reported.

The application of PGRs other than ABA, such as $GA_3$ (e.g., Komatsuda *et al.*, 1992; Choi *et al.*, 2002; Park & Facchini, 1999), has been successfully applied to improve maturation and has also been used in later steps to support the conversion of somatic embryos into plants (Gaj, 2004). In the *D. carota* and other somatic embryo systems, $GA_3$ is an essential regulator for somatic embryogenesis, but it can inhibit proper development when it is applied at the wrong point in time (Tokuji & Kuriyama, 2003). Therefore, the application of $GA_3$ during *Cyclamen* somatic embryogenesis might be worth testing due to

the putative physiological similarities of the two systems (Rensing *et al.*, 2005; Hoenemann *et al.*, 2010), but the correct timing of the application must be evaluated.

The improvement of somatic embryo maturation after the application of $AgNO_3$ (Roustan *et al.*, 1990; Kong & Yeung, 1994; Park & Facchini, 2001) has been attributed to its action as putative ethylene inhibitor, but the simple application of metal ion stress might also increase endogenous ABA levels (Kong & Yeung, 1994), positively effecting somatic embryo development.

In *C. persicum*, 7S globulins, 11S globulins and xyloglucans have been identified as storage compounds (Braccini *et al.*, 1995; Winkelmann *et al.*, 2006c). Winkelmann *et al.* (2006c) observed different quantities of 7S and 11S globulins in zygotic embryos than in somatic embryos and a high abundance of xyloglucan endotransglycosyltransferase (XTH, an enzyme capable of xyloglucan biosynthesis and hydrolysis) in endosperm tissue of *C. persicum*. Hoenemann *et al.* (2010) found a high expression of xyloglucan endotransglycosylase (XET, an enzyme capable of xyloglucan biosynthesis and hydrolysis) in *C. persicum* somatic embryos. Therefore, somatic embryos in *C. persicum* might not accumulate sufficient or the correct storage compounds, as reported in other somatic embryo systems [e.g., carrot (*D. carota*) (Dodemann *et al.*, 1998); oil palm (*Elais guineensis*) (Morcillo *et al.*, 1998)], or somatic embryos might accumulate storage compounds that are confined to the endosperm, as also suggested by Hoenemann *et al.* (2010).

According to these observations, some approaches can be suggested to improve embryo maturation and germination in wild *Cyclamen* species. On the basis of the experiments presented in **Prange *et al.* (2010a,b),** first, the application of the maturation treatment after a pre-maturation step on medium without PGRs should be considered. Second, ABA and PEG or other osmotica merit testing during a maturation step. Therefore, PEG with a $M_r$>4000 should be used because the larger PEG molecules cannot penetrate the cell wall and enter the symplast while water is withdrawn (von Arnold, 2008). Additionally, the application of other PGRs, such as $GA_3$ or kinetin, as well as the application of $AgNO_3$ should be considered. Additionally, supplementation of the culture medium with the natural *Cyclamen* storage compounds might mimic the endosperm and support proper somatic embryo development. An analysis of the accumulation of storage compounds (globulins and xyloglucans) in *Cyclamen* somatic embryos should be considered. The accumulation of storage compounds was often used as indicator for the maturation stage of somatic embryos [e.g., in interior spruce (*Picea glauca engelmanni*) (Roberts *et al.*, 1990) and in *Medicago sativa* L. (Fujii *et al.*, 1990)]. The storage compound compositions

in somatic embryos should preferably be similar to those in zygotic embryos as already described by (Winkelmann *et al.*, 2006c). In the case of wild *Cyclamen* species, the availability of high amounts of seeds for such comparative analysis might be problematic. The storage compounds should be analysed in *C. persicum* zygotic embryos in more detail as already performed by Winkelmann *et al.* (2006c), and the applicability of these results to other *Cyclamen* species should be determined. Alternatively, comparative studies of the gene expression pattern of somatic and zygotic embryos can be performed, but for this approach more detailed information about the involved genes and transcripts is necessary.

### 4.1.4 Protoplast Isolation and Regeneration from Protoplasts to Plants

In this thesis, shoot cultures (petioles, leaves and adventitious shoots)* and embryogenic suspension cultures were tested as initial material for protoplast isolation and the regeneration from protoplasts to plants (**Prange *et al.*, 2010a,b**). So far, the occurrence of somaclonal variation has been evaluated in plants regenerated from protoplasts of *C. coum* (**Prange *et al.*, 2010a**). A putative mechanism of somaclonal variation and different strategies for its reduction will be discussed in sub-chapter 4.1.5

The induction of adventitious shoots in wild *Cyclamen* species was done with the aim of establishing *in vitro* cultures of these species (**Prange *et al.*, 2008**), which served *inter alia* as initial material for protoplast isolations.

The isolation of mesophyll protoplasts from sterile *in vitro* leaves and petioles (grown in the light) of *C. graecum*, *C. persicum* and *C. hederifolium* did not yield viable and intact protoplasts*. Viable protoplasts were isolated from *in vitro*-grown young adventitious shoot cultures of *C. graecum* (grown in the dark) before the unfolding of leaves. Suitable amounts of protoplasts (about $1 \times 10^5$ protoplasts per gram fresh mass) were obtained, which were cultivated for the regeneration to plants as described for the other wild *Cyclamen* species (**Prange *et al.*, 2010a,b**)*. The protoplasts divided, but the development of multi-cellular colonies (microcalluses) was only very rarely observed*. Cell proliferation stopped before the cells reached a size suitable for transfer to solid medium without PGRs for regeneration of somatic embryos*. Additionally, with other combinations of PGRs in the protoplast culture medium, the regeneration of plants via somatic embryos or shoots was not achieved*. The development of microcalluses is described by different authors as a critical step in the regeneration from protoplasts to plant. In *C. persicum*, microcalluses were formed in only half of the protoplast isolations (Winkelmann *et al.*,

2006b). Beyond this critical step, the regeneration of high frequencies of somatic embryos was observed (Winkelmann *et al.*, 2006b). Protoplasts of sour cherry (*Prunus cerasus* L.), pear (*Pyrus communis* L.) and honeysuckle (*Lonicera nitida*) were arrested at the ten-cell stage when cultivated under suboptimal macronutrient conditions or in media rich in organic compounds (e.g., Km8P, MS) (Ochatt & Power, 1988a,b,c; Ochatt, 1991). In this thesis, Km8P-based protoplast culture media that were modified in their PGR content but not in their nutrient composition were used. For optimisation of protoplast growth and the development of microcalluses, the nutrient composition (e.g., ammonium) and PGRs in the protoplast culture media may be varied.

The success in regeneration of somatic embryos from protoplasts depends on the embryogenic capacity of the starting material (e.g., Wilhelm, 2000; von Arnold, 2008). Embryogenic suspension cultures, embryos, ovules, seedlings and juvenile tissue are classical examples of starting materials. The use of mesophyll protoplasts or other somatic tissue from mature plants has also often been used for isolation of protoplasts. Plant regeneration from these protoplasts was achieved in many plant species by somatic embryogenesis [e.g., wild tomato (*Lycopersicon peruvianum*) (Zapata & Sink, 1981); alfalfa (*Medicago sativa* L.) (Dijak & Simmonds, 1988)] or adventitious shoot formation [e.g., tobacco (*Nicotiana tabacum* L.) (Thomas & Rose, 1983); *Petunia* and *Calibrachoa* ssp. (Meyer *et al.*, 2009)]. In this thesis, the isolation of mesophyll protoplasts from *in vitro*-grown *Cyclamen* plant material was not successful without major modifications of the protoplast isolation protocol*. Adventitious shoot-derived protoplasts could be isolated, but not regenerated to plants with the tested PGR combinations*.

Due to technical reasons, the genotypes used for shoot induction experiments could not be evaluated for their ability to form somatic embryos. Therefore, no information about their regenerative ability via somatic embryogenesis is available, and genotypic reasons for the lack of regeneration from protoplasts to plants cannot be excluded.

Regarding these results, we concentrated on the induction of embryogenic suspension cultures as initial material for protoplast isolation.

Until now, plant regeneration from protoplasts using (embryogenic) suspension cultures as the initial material for protoplast isolation has been successful and often very efficient for various plant species, such as roses (*Rosa* ssp.) (Matthews *et al.*, 1991), iris (*Iris germanica* L.) (Shimizu *et al.*, 1996), coffee (*Coffea arabica* L.) (Acuna & de Pena, 1991), litchi (*Litchi chinensis* Sonn.) (Yu *et al.*, 2000), pearl millet (*Pennisetum americanum*) (Vasil & Vasil, 1980), rice (*Oryza sativa* L.) (Abdullah *et al.*, 1986) and others. Embryos

developed from all embryogenic suspension cultures established from wild *Cyclamen* species (*C. alpinum*, *C. coum*, *C. graecum* and *C. mirabile*) and *C. persicum* cultivars and were successfully regenerated to plants. The regenerative capacity and the success of regeneration after protoplast isolation in all *Cyclamen* species under investigation were always comparable to the regenerative capacity of the originating embryogenic culture (callus and suspension culture)*. This transferability of established propagation pathways to regeneration protocols from protoplasts to plants has already been proposed by Davey *et al.* (2010). General aspects and problems during embryo regeneration in the different *Cyclamen* species have already been discussed in sub-chapter 4.1.3.

### 4.1.5 Somaclonal Variation

Somaclonal variation summarises the phenotypic or DNA variations in plant clones propagated by tissue culture (Larkin & Scowcroft, 1981; Kaeppler *et al.*, 2000). It can be either beneficial if used as a "simple form of genetic engineering" (Davey *et al.*, 2010). Or it can be undesirable if a uniform, genetically true-to-type population is needed for mass propagation of valuable genotypes and/or if the kind of somaclonal variation is more a malformation than a desirable new trait, as reported by Winkelmann *et al.* (2008) for some *C. persicum* genotypes. In this thesis, the somaclonal variation of plants regenerated from wild *Cyclamen* protoplasts was investigated.

The plants regenerated from callus (control) and from protoplasts of *C. alpinum*, *C. graecum* and *C. mirabile* were either acclimatised in insufficient numbers or have yet to reach a suitable size for phenotypic evaluation (**Prange et *al.*, 2010b**), and, therefore, the assessment of somaclonal variation of greenhouse-grown plants is in progress. In *C. coum*, the regenerated plants grown in the greenhouse were very uniform, with some phenotypically aberrant plants expressing different leaf sizes or patterns and slightly different flower colours (**Prange *et al.*, 2010a**). Flow cytometric analyses revealed tetraploidy in plants regenerated from protoplast and from callus cultures (**Prange *et al.*, 2010a**); these plants were characterised by gently upturned leaf edges (forming a concave leaf surface). About 20% of the regenerated calluses gave rise to either diploid and tetraploid or tetraploid plants. Similar kinds of somaclonal variation have been reported for *C. persicum* plants regenerated from protoplasts (Winkelmann *et al.*, 2008). In *C. persicum*, about 28% of the plants (= calluses) regenerated from protoplasts were found to produce putatively tetraploid plants (mean frequency over eight regenerated genotypes) (Winkelmann *et al.*, 2008). The occurrence of polyploidy in plants regenerated from protoplasts can be explained by the spontaneous fusion of protoplasts during protoplast isolation or by the doubling of the chromosome set during the callus phase

(endopolyploidisation). Endopolyploidisation may occur by endomitoses, endoreduplication or the formation of restitution nuclei (Geier, 1991). In contrast to the study in *C. persicum* (Winkelmann *et al.*, 2008), in *C. coum* more than one plant per callus (= per protoplast) was analysed (**Prange *et al.*, 2010a**). Only one callus generated exclusively tetraploid plants, whereas the other calluses regenerated either only diploid or diploid and tetraploid plants. In *Saintpaulia ionantha*, diploid and tetraploid regenerants from single protoplast-derived calluses were also detected (Winkelmann & Grunewaldt, 1995). Therefore, in *C. coum* the doubling of the chromosome set occurred more often during callus culture or the regeneration phase, most likely by endopolyploidisation and only rarely by spontaneous fusion of protoplasts. However, mistakes during callus separation and merging of calluses cannot be fully excluded. Endopolyploidisation during the callus or regeneration phase explains not only the occurrence of mixoploid calluses, but also the tetraploid plants in the regenerated somatic embryos originating directly from embryogenic cultures (callus), which served as a control (**Prange *et al.*, 2010a**).

Winkelmann *et al.* (2008) observed additional kinds of somaclonal variation in some genotypes of *C. persicum* protoplast regenerants, such as serious flower malformations (flowers with multiple petals, central sepals, uncoated ovaries, additional flowers within the flower and partly missing anthers), very late flowering and the formation of multiple vegetative meristems, which were absent in *C. coum*. The types of somaclonal variation and their extents in *C. persicum* were found to be genotype specific (Winkelmann *et al.*, 2008). In comparison, the *C. coum* genotype under investigation is quite stable. Major malformations, which would limit its horticultural cultivation and value, were not observed.

Despite different genotypic- and species-specific susceptibility, one plausible cause of the decrease/loss of the embryogenic capacity and of somaclonal variation might be the prolonged culture on PGR-containing culture media, especially that containing 2,4-D, and prolonged callus phases (von Arnold, 2008). These factors are probable inducers of genetic and epigenetic variation (von Arnold, 2008). In *C. persicum*, prolonged culture on 2,4-D-containing culture medium also enhanced the frequency of secondary embryogenesis (Winkelmann *et al.*, 2006b). It is recommended that embryogenic cultures (and other tissue cultures) should regularly be established anew from greenhouse-grown stock plants (Borchert *et al.*, 2007 and references therein) or should be frozen directly after establishment and freshly thawed to minimise the cultivation time (von Arnold, 2008). The cryopreservation of embryogenic suspension cultures of C. *persicum* has previously been established (Winkelmann *et al.*, 2004c) and can be applied in future experiments for the preservation of embryogenic cultures of wild *Cyclamen* species.

Cassels & Curry (2001) hypothesised that many common tissue culture problems, such as the loss of organogenic potential (recalcitrance), hyperhydricity (vitrification) and somaclonal variation, are a consequence of or at least related to oxidative stress damage caused by explant preparation (wounding), media components and other environmental factors. The induction of an oxidative burst and the generation of reactive oxygen species (ROS, including superoxide, hydrogen peroxide hydroxyl, peroxyl radicals and alkoxyl radicals) can be induced by 2,4-D (see above), osmotic stress, wounding and other factors (Henry *et al.*, 1998; Cassels & Curry, 2001 and references therein; Pfeiffer & Höftberger, 2001). Oxidative stress can induce changes in the DNA methylation pattern (including hypo- and hypermethylation) as well as chromosomal changes (including polyploidy, aneuploidy, strand breakage and DNA base deletions) (Cassels & Curry, 2001 and references therein). Besides the induction of genetic variation by stress, Kaeppler *et al.*, (2000) suggested that *in vitro* culture bypasses the “normal” epigenetic patterning during plant development, which results in an error-prone genetic patterning, contributing to epigenetic changes and, thereby, somaclonal variation.

Preece (2008) observed that adventitious shoot formation, especially from callus, is more prone to somaclonal variation than proliferation from axillary shoots (*de novo* meristem generation *versus* preformed meristems) but did not attribute this to the use of PGRs. Additionally, in many other studies, prolonged callus culture probably contributes to ploidy instability (Borchert *et al.*, 2007, and references therein).

The stem cell concept of Laux (2003) provides another possible explanation for the discussed problems in tissue culture. The occurrence of so-called founder cells in a slowly dividing central zone of the shoot meristem is discussed by the author in analogy to the stem cell concept in animals. These founder cells evolve (postembryologically) all organs (Stewart & Dermen, 1970; Laux, 2003) and are characterised by an exclusive gene expression pattern, including the meristem-specific expression of the *CLAVATA3* (*CLV3*) gene. The maintenance of this stem cell state requires signals from surrounding cells and depends on the position of these cells in the meristem (Laux, 2003). In plants, meristems can be constituted *de novo* from almost any cells when tissue culture techniques are applied. The evolutionary context for the development of such a stem cell concept has to be considered. Rapid cell proliferation, as in callus cultures, results in the accumulation of mutations. This problem is avoided by the stem cell concept, because of the slow growth of the founder cells. The position-dependent information is the crucial factor for the maintenance of these founder cells. Tissue culture techniques circumvent this security

concept and thus enhance the mutation rate in the propagated tissue, according to this theory.

Recommendations such as the avoidance of adventitious shoot proliferation (Preece, 2008) and the regular establishment of tissue cultures from stock plants (Borchert *et al.*, 2007) are taking advantage of the stem cell concept.

### 4.1.6 Future Prospects for Wild *Cyclamen in vitro* Cultures

The established methods of vegetative propagation, adventitious shoot formation and regeneration via somatic embryogenesis, have proven to be very efficient ways of vegetative propagation especially in some species and genotypes. The complete capture of the gene-pool for the preservation of the germplasm cannot be assured by the usage of tissue culture techniques at the present state of the art. However, if the observed genotypic differences can be overcome, both adventitious shoot formation and regeneration via somatic embryogenesis might be utilised for the propagation and preservation of endangered wild *Cyclamen* species.

Besides the discussed problems with somatic embryogenesis, the established wild *Cyclamen in vitro* cultures proved to be very efficient for mass propagation of particular genotypes with a high quality and uniformity of the regenerated plants, as described for many *C. persicum* cultivars by Winkelmann (2010). The established *in vitro* cultures are the basis for the transfer of other approaches that have already been established in *C. persicum* to wild *Cyclamen* species, such as the preparation of artificial seeds and their conservation by desiccation (Winkelmann *et al.*, 2004a; Winkelmann, 2010) and the cryopreservation of embryogenic suspension cultures (Winkelmann *et al.*, 2004c; Winkelmann, 2010).

The established *in vitro* cultures of wild *Cyclamen* species, especially the embryogenic suspension cultures, served as good sources for the isolation of protoplasts and their regeneration to plants. These cultures were easy to maintain and could be scaled up to yield high amounts of initial material for the protoplast isolation and fusion experiments discussed in the following chapter.

## 4.2 Somatic Hybridisation in the Genus *Cyclamen*: Protoplast Fusion and the Development of Different Selection Systems for Heterofusion Products

In this chapter, somatic hybridisation via PEG-mediated protoplast fusion, including optimisation of the protoplast isolation and the fusion conditions, selection of heterofusion products and the characterisation of the somatic hybrids, is discussed. Finally, a short perspective for future research and breeding will be given.

### 4.2.1 Protoplast Isolation and Culture

For successful protoplast isolation in wild *Cyclamen* species, some modifications were introduced into the isolation protocol of Winkelmann *et al.* (2006b). Pre-plasmolysis was omitted due to the often very fine suspension cultures of the species. The enzymatic digestion of wild *Cyclamen* species was not performed on a rotary shaker, due to the observed disruption of the wild *Cyclamen* protoplasts and reduced protoplast yields after this treatment. The centrifugation steps were slowed down, which enhanced the protoplast yields, and some other modifications were made, including the use of double the amount of sucrose solution (10 mL instead of 5 mL), the use of a modified enzyme solution and a prolonged enzymatic digestion (for details, see **Prange *et al.*, 2010a,b**).

High variations in protoplast yields between the different isolation experiments were observed. Similar variations were also reported in other studies dealing with plant protoplast isolation (e.g., Meyer *et al.*, 2009). The yields ranged, on average, between 2.6 ± 1.9 x $10^5$ protoplasts per gram fresh mass (*C. coum*) and 13.7 ± 8.0 x $10^5$ protoplasts per gram of fresh mass (*C. graecum*) (**Prange *et al.*, 2010a,b**), which was comparable to the yields obtained from *C. persicum* cultivars (Winkelmann *et al.*, 2006b). Yields of 1 - 6 x $10^6$ protoplasts per gram of fresh mass in single isolation experiments were also obtained from various *Cyclamen* suspension cultures. Comparable protoplasts yields are described in the literature [e.g., coffee (*Coffea arabica* L.) (Acuna & de Pena, 1991)], but in some species higher yields (above 1 x $10^6$ protoplasts per gram fresh mass) are typically achieved [e.g., *Petunia*, *Calibrachoa* ssp. (Meyer *et al.*, 2009); sunflower (*Helanthus* ssp.) (Chanabe *et al.*, 1991)]. It would have been advantageous to achieve yields above 1 x $10^6$ protoplasts per gram of fresh mass as the standard yield for the application in the protoplast isolation experiments. The obtained yields were sufficient and the isolated protoplasts were used in protoplast fusion experiments (**Prange *et al.*, 2010a,b,c**).

The protoplasts of *Cyclamen* wild species showed different reactions to PGRs and the two embedding agents that were tested. As expected, the minimisation of the amount of PGRs during protoplast culture was one of the experimental approaches needed to prevent negative influences of enhanced levels of PGRs. For most of the species (*C. coum*, *C. graecum*, *C. mirabile*), a reduction of plant growth regulators to a level of 0.5 mg $L^{-1}$ 2,4-D and 0.2 mg $L^{-1}$ 2iP (medium 8pmC.2) affected their growth (evaluated by the division frequency) positively or insignificantly. For *C. alpinum*, PGR concentrations of 1 mg $L^{-1}$ 2,4-D and 0.4 mg $L^{-1}$ 2iP (medium 8pmC.1) were necessary to achieve cell division rates above 1% after one or two weeks. The reduced PGR concentrations were also tested with the *C. persicum* cultivars in 8pmC.1 (Winkelmann *et al.*, 2006b) and 8pmC.1 and 8pmC.2 media (*), and yielded division frequencies that were comparable, or only slightly reduced, to the original protoplast culture medium. These division frequencies were similar to the ones determined in the wild species. With regard to the intended protoplast fusion experiments, a synchronous development of the wild *Cyclamen* protoplasts with the potential fusion partner *C. persicum* would be beneficial. In protoplast fusion experiments, the 8pmC.1 medium should be used for *C. mirabile* x *C. persicum* combinations, and 8pmC.2 should be used for the other species combinations.

Another important characteristic of the various *Cyclamen* species was their development in the different embedding agents: alginate and agarose. The division frequency and the morphology of the protoplasts of the two species differed in both embedding agents (**Prange *et al.*, 2010b**). Malformed and enlarged cells were observed in protoplast cultures of *C. persicum*, *C. mirabile* and *C. alpinum* during culture in agarose, which was partly paired with reduced division frequencies and the failure of plant regeneration. According to similar observation by other authors, this disturbed development was attributed to irregular cell wall development. For a detailed discussion see **Prange *et al.* (2010c)**.

### 4.2.2 Staining of Protoplasts

One common set of problems in protoplast fusion experiments is monitoring the protoplast fusion for protocol optimisation, the detection of heterofusion products and their selection. Embryogenic suspension cultures of different *Cyclamen* species, as used in this thesis, release colourless and undistinguishable protoplasts when observed by light microscopy. Therefore, different staining protocols were established using fluorescent dyes (**Prange *et al.*, 2010**; Annexe I). The dyes were selected and tested for their compatibility with the

protoplasts, their detectability and distinguishable colour, their stability in the cell and their tendency to leach from the protoplasts.

Scopoletin (blue fluorescence) (Kanchanapoom *et al.*, 1985), fluorescein diacetate (FDA) (green fluorescence) (Durieu & Ochatt, 2000) and rhodamine B isothiocyanate (RBITC) (red fluorescence) (Durieu & Ochatt, 2000) were successfully used to stain protoplasts isolated from suspension cultures of different *Cyclamen* species (**Prange *et al.*, 2010c;** Annexe I). The tested staining agents revealed different advantages and disadvantages that were partly, but not fully, described in the literature and will be discussed in the following narrative.

Rhodamine B isothiocyanate has been described as a promising staining agent for use in monitoring protoplast fusion and selecting heterofusion products (Durieu & Ochatt, 2000). However, in some fusion experiments in this study, the dye was presumably leaching from the cells, as nearly all of the protoplasts were RBITC stained after fusion (cf. Annexe I). Due to the increased occurrence of the problem with low qualities of protoplasts and harsh fusion methods, it can be assumed that the disruption of protoplasts leads to the release of RBITC in high amounts from the cell. This resulted in the staining of the protoplasts of the respective fusion partner without fusion. Kanchanapoom & Boss (1986) localised RBITC labelling in the vacuole and the cytoplasm of RBITC stained protoplasts. During staining, RBITC reacts with the free amino groups of proteins and phosphatidylethanolamine, forming complexes with these molecules (Kanchanapoom & Boss, 1986). Due to the intensive staining property of this dye, one promising approach for the improvement of the RBITC staining could be a further reduction of dye concentration. The amount of unconverted dye in the protoplasts can therefore be minimised to prevent the leaching of residual dye from the cell during cell disruption or protoplast fusion, or by diffusion. The observed problems might be specific to the protoplast system used here because RBITC has been successfully used for protoplast staining and heterokaryon selection in pea (*Pisum sativum* L.) (Durieu & Ochatt, 2000), *Phaseolus coccineus* L., *Phaseolus polyanthus* Greenm. (Geerts *et al.*, 2008) and strawberry (*Fragaria* × *ananassa*) (Geerts *et al.*, 2009) with FDA as corresponding staining partner.

FDA and scopoletin have also been frequently used for the selection of heterofusion products after protoplast fusion (e.g., Yarrow *et al.*, 1986; Rasmussen *et al.*, 1997; Waara *et al.*, 1998; Durieu & Ochatt, 2000). The non-toxic effects of the staining solutions on the protoplasts of *C. persicum* (**Prange *et al.*, 2010c**) and *C. graecum* (cf. Annexe I) and their

regenerative ability were observed. Both dyes were actively taken up and converted by the protoplasts. Scopoletin was mainly localised in the vacuole.

FDA, a non-fluorescing, non-polar compound, is freely permeable through plasma membranes (Rotman & Papermaster, 1966; Larkin, 1976). It is hydrolysed by esterase activity into the polar, fluorescing compound fluorescein, which is accumulated in the cytoplasma when the plasmalemma is intact (Rotman & Papermaster, 1966; Larkin, 1976). Therefore, FDA stains only viable cells. Fluorescein lacks the properties of FDA as a stain and is not portable through membranes, which was tested by Rotman & Papermaster (1966) in mammalian cells. Therefore, the leaching of the active stain from the cells should not occur. FDA showed good compatibility with the cells when the appropriate concentrations were used (**Prange *et al.*, 2010c**; Annexe I) and only bleached slightly during monitoring under a fluorescence microscope. However, the stain did not last as long as RBITC and, therefore, screening for heterofusion products had to be performed within 24 to 48 h.

Scopoletin is actively taken up into the plant cells and converted into scopolin, a 7-O-glucoside of scopoletin, and is then actively translocated into the vacuole (Kanchanapoom *et al.*, 1985; Taguchi *et al.*, 2000). The leaching of dye from the protoplasts seems to be prevented by active uptake and enclosure into two membranes (the plasma membrane and the tonoplast). In tobacco cell cultures, the active uptake into the cell was found to be stimulated by the addition of 2,4-D to the culture medium (Taguchi *et al.*, 2000). The 2,4-D was a standard PGR in the culture medium for the suspension cultures used in this thesis, which might have supported the uptake of the dye in *Cyclamen* suspension cells.

Scopoletin is a cumarine derivative that is known to be involved in hypersensitive reactions in plants (e.g., Massala *et al.*, 1980; Dorey *et al.*, 1999; Gachon *et al.*, 2004). As expected, due to its common occurrence (Andreae, 1952), staining procedures with enhanced concentrations did not harm the *Cyclamen* cell suspensions and protoplasts*. Scopoletin is suspected to be an inhibitor of IAA oxidase (Schaeffer *et al.*, 1967), which may increase callus growth under certain circumstances (Machakova *et al.*, 2008). Callus growth, embryo development or regeneration in *Cyclamen* protoplast cultures was not notably affected after scopoletin treatment. The *Cyclamen* protoplasts showed a blue basic fluorescence in unstained protoplasts that varied depending on the protoplast isolation experiment. Waara *et al.* (1998) also observed this phenomenon in protoplasts of potato and attributed it to secondary metabolites such as phenolic compounds. Due to the involvement of scopoletin and other coumarin compounds in the hypersensitive reaction of

plants (Dorey *et al.*, 1999), and the observed fluctuation in the blue fluorescence between the different experiments, it stands to reason that the formation of the compounds causing this fluorescence might be correlated with factors inducing the expression of genes for secondary metabolism. These factors might be stress factors in the pre-culture of the *Cyclamen* suspension cultures or more general factors, inducing genes connected to the synthesis of secondary metabolites or more specific to the hypersensitive reaction.

One disadvantage of scopoletin as a stain was the bleaching of the blue fluorescence during monitoring under the fluorescence microscope (cf. Annexe I). An exchange of the excitation filter prolonged the potential screening time under the microscope in which the intensive fluorescence of the cells was retained (cf. Annexe I).

In contrast to RBITC, scopoletin and FDA were used successfully and with reliable results to monitor the fusion process and to determine heterofusion frequencies in fusion experiments with *C. persicum* with *C. coum* (**Prange *et al.*, 2010c**) and *C. graecum* (Annexe I), respectively. Scopoletin and RBITC have been reported to affect the membrane proteins or lipids, which impeded protoplast fusion in fusogenic carrot protoplasts (Kanchanapoom & Boss, 1986; Pattanavibol *et al.*, 1998). After long incubation times in RBITC, Kanchanapoom & Boss (1986) observed an inhibition of the fusion process by the stain. However, incubation times equal to the critical length were not used. The inhibiting effect of the stains was not observed in detail and, therefore, an inhibition of the fusion process by RBITC or scopoletin cannot be excluded. Successful protoplast fusion and heterokaryon selection using RBITC (Durieu & Ochatt, 2000; Geerts *et al.*, 2008; Geerts *et al.*, 2009) or scopoletin (Rasmussen *et al.*, 1997; Waara *et al.*, 1998) as a protoplast dye have been reported; as a result, both staining agents were tested for their applicability in fusion experiments with different *Cyclamen* protoplasts.

The approaches developed for the mechanical separation of these fusion products will be discussed in sub-chapter 4.2.4.

### 4.2.3 Protoplast Fusion

Protoplast fusion was achieved with all of the tested protocols in species combinations *C. persicum* x *C. coum* (**Prange *et al.*, 2010c**) and *C. persicum* x *C. graecum* (Annexe II). Other species combinations were only tested once or not at all. *C. persicum* x *C. coum* fusion has already been discussed in detail in **Prange *et al.* (2010c)**. In the following section, the main focus of the discussion is put on the *C. persicum* x *C. graecum* fusion.

In *C. coum,* all PEG treated fusion variants resulted in significantly enhanced heterofusion frequencies in comparison to the untreated control (**Prange *et al.*, 2010c**). The heterofusion frequencies by spontaneous fusion in the controls were below 1%, and in the PEG, treated variants were between 2.8% ± 2.3 (Kao protocol, 30% PEG) and 4.5% ± 4.3 (NaOH-glycine buffer protocol, 35% PEG). In *C. graecum,* the heterofusion rate could not be significantly enhanced by treatments with PEG (Annexe II), even though the determined heterofusion frequencies were 1.6% to 12.1% (average per protocol) higher than in *C. coum* (Annexe II). In two experiments, the untreated controls yielded heterofusion frequencies of 17.5% and 30.3%. These high heterofusion frequencies are desirable and have been reported by other authors (e.g., Kao *et al.*, 1974), but in the fusion experiments carried out in this thesis, they were not reproducibly obtained (cf. Annexe II).

One feasible explanation for the extremely high heterofusion rates in some experiments using the *C. persicum* x *C. graecum* combination might be the lab temperature at that time. These experiments were conducted in very hot summer months. On some days, the average lab temperature ranged from 35 to 40°C. Unfortunately, the exact lab temperature was not recorded for each fusion experiment. Elevated temperature (up to 40°C) is known to enhance PEG-mediated protoplast fusion (e.g., Senda *et al.*, 1980) and temperatures of 34°C have been used for successful fusion in tobacco (*Nicotiana tabacum* L.) (Patnaik *et al.*, 1982). This might explain the outliers in this study, especially in experiment No 4 with 30.3% heterofusion products in the controls without the PEG treatment. Such high temperatures are not necessarily detrimental to the cells. The protoplasts in this thesis recovered from these experiments and were regenerated to plants.

Due to the high fusion rates, nearly every third plant would have been expected to be a hybrid. No hybrid plant has been detected visually or through the examination of 31 randomly chosen plants using flow cytometry and 12 plants with developed species-specific markers.

One possible explanation for the lack of regenerated hybrid plants would be the genetic incompatibility of the two species resulting in the death of the hybrid cells. However, the genetic compatibility of *C. persicum* (2n=48 chromosomes) and *C. graecum* (2n=84 chromosomes) has been proven by successful hybridisation using the embryo rescue technique (Ishizaka, 1996; Ishizaka, 2003; Ishizaka, 2008). Both fertile (with tetraploid *C. persicum* plants as parental plants with 2n=96 chromosomes) and infertile hybrids were obtained containing half chromosome sets of each parental species (2n=66 or 2n=90)

(Ishizaka, 2008). Both species belong to the subgenus Eucosme (Compton *et al.*, 2004) and, based to this and other cladistic studies, *C. graecum* is probably one of the closest relatives to *C. persicum*. Ishizaka (2008) determined the genomic affinity between *C. persicum* and *C. graecum* by examining the frequency of chromosome pairing during diakinesis or metaphase-I in the pollen mother cells of haploid plants (2n=45 chromosomes) obtained from the anther culture of *C. persicum* and *C. graecum* hybrids (2n=90 chromosomes). The author observed univalents and loosely-paired bivalents resulting in sterile pollen. Thus, the author suggested a low degree of genetic affinity and a distant relationship in the genus *Cyclamen* (Ishizaka, 2008). The conclusion drawn by Ishizaka (2008) that states there is a very distant relationship between *C. graecum* and *C. persicum* contradicts many of the cladistic observations in this genus *Cyclamen* (Compton *et al.*, 2004) and has to be considered with caution. However, symmetric somatic hybrids would contain two chromosome sets of both parents and, therefore, behave like fertile amphidiploids.

Considering the high fusion rates and the absence of regenerated hybrid plants, the reliability of the developed system for the determination of heterofusion products has to be reviewed. The observed high fusion rates in this thesis imply a high stringency in the fusion treatment and/or the low quality of protoplasts (**Prange *et al.*, 2010c**). Good results (extraordinary high heterofusion rates) might entail a deterioration of the protoplasts that result in the death of many cells. One can assume that low or medium heterofusion frequencies enhance the probability of obtaining hybrid plants because the cells are less affected and stressed by the protoplast isolation and fusion treatment. As a consequence, extraordinary high heterofusion rates should be considered with caution. The failure rate of the method used for the determination of the heterofusion frequencies might be less reliable with very high fusion rates due to the death of many cells during the ongoing culture. This hypothesis has to be observed in future studies with more quantitative results aiming at this subject.

For future fusion experiments, it would be desirable to enhance the heterofusion frequencies and to obtain more stable fusion results. Protoplast fusion with enhanced density was one approach to stabilise and enhance the heterofusion frequencies, which resulted in more stable, but overall reduced heterofusion frequencies in comparison to a cell density of $1 \times 10^6$ protoplasts per mL (**Prange *et al.*, 2010c**; Annexe II). Therefore, intermediate cell densities of $2 \times 10^6$ and $3 \times 10^6$ protoplasts per mL are worth testing in future fusion experiments. According to the observation made in *C. graecum* (see above), fusion under high temperature conditions (about 30 to 35°C) should be tested for its effect

on improving the heterofusion frequency. Protocol modifications such as longer incubation times and an increase in the pH of the respective high-pH-fusion-solutions can also be tested. Additives to the fusion solution such as dimethylsulfoxide improved the heterofusion frequency in other species [e.g., carrot (*D. carota*) - barley (*Hordeum vulgare* L.) fusion (Haydu *et al.*, 1977); *Brassica napus* - *B. campestris* or *B. napus* - *Nicotiana plumbaginifolia* fusion (Menczel & Wolfe, 1984)], presumably by making the cells more susceptible to PEG. Concanavalin A, a lectin isolated from jack bean (*Canavalia ensiformis*), has been described to improve the attachment of the protoplasts during PEG-mediated fusion, resulting in improved heterofusion rates (Glimelius *et al.*, 1978). Electrofusion has often been reported to have advantages over PEG-mediated chemical fusion because fusion conditions can be more easily controlled, the fusion of single protoplast pairs is higher and it can be applied to protoplasts that do not survive under chemical fusion conditions (Waara & Glimelius, 1995). PEG has also been shown to be advantageous in increasing the amount of binary fusion products in *Musa* ssp. (Assani *et al.*, 2005). According to these diverse observations, electrofusion should be compared to PEG-mediated fusion in *Cyclamen* and tested for its putative advantages in *Cyclamen* protoplast fusion.

### 4.2.4 Selection of Heterofusion Products

Although many strategies for the selection of heterofusion products have been developed for a variety of species, the transfer of these methods to other systems is limited by specific physiological or morphological characteristics of the parental cell cultures (e.g., Waara & Glimelius, 1995; Kanchanapoom *et al.*, 1985) such as the use of petal and mesophyll protoplasts that can be selected visually (e.g., in *Petunia* ssp., Phillips & Darrell, 1992; Meyer, 2006). However, often the respective plant parts are not a suitable tissue for protoplast isolation and/or the protoplasts of these tissues cannot be regenerated into plants, as in *Cyclamen*. Even in theoretically transferable systems applications to other plant systems are often cumbersome (e.g., Waara & Glimelius, 1995).

The time consuming and cost-intensive genetic modification of plant material, such as the use of transgenic reporter lines (e.g., Olivares-Fuster *et al.*, 2002; Guo & Grosser, 2005), is another possible selection method with limited application.

The application of theoretically transferable systems is often problematic. As confirmed in this thesis, the reaction of the plant material to applied treatments such as staining

(**Prange *et al.*, 2010c**; Annexe I), physiological inhibition (cf. chapter 1.5.2) or others is dependent on the species, the genotype, the tissue and, presumably, many other factors. The adjustment of these protocols might not only be time consuming, it might also be impossible. It may not yield the desired reproducibility that is needed for use as a reliable selection marker in protoplast fusion experiments. As reported by Meyer (2006), the extent of physiological inhibition using iodoacetamide and rhodamine 6G on protoplasts of *Petunia* and *Calibrachoa* ssp. varied from one experiment to another and did not provide reproducible results (Meyer, 2006). The author attributed this to the changing quality of the isolated protoplasts in *Petunia* and *Calibrachoa* ssp. (Meyer, 2006).

Due to these problems and the limitations of the existing selection systems, hybrid plants in many studies, such as the ones in this thesis, are obtained without the selection of heterofusion products by cultivating all protoplasts after fusion (e.g., Melchers *et al.*, 1978; Chaput *et al.*, 1990; **Prange *et al.*, 2010c**). At the abandonment of selection after fusion, morphological differences or hybrid vigour (e.g., Handley *et al.*, 1986) are beneficial for the detection of hybrid plants but are not always present (e.g., Gleddie *et al.*, 1986). The selection of heterofusion products bears many advantages: The often laborious cultivation of vast amounts of developing calluses can be minimised. Very fragile hybrids or hybrids with less vigour than the parental plants might be overgrown in mixed cultures and, therefore, will be hard, or even impossible, to detect due to their putatively smaller size. The selection of these hybrids directly after fusion might capture these hybrids and facilitate their development (compare to sub-chapter 4.2.4.1 and 4.2.4.2). In *C. persicum* the hybrid vigour at the plant level is not always observed. Because of this, different systems for the selection of heterofusion products were evaluated that enable the capture of potentially fragile heterofusion products.

In this thesis, three different selection methods based on the established staining methods (**Prange *et al.*, 2010c**; Annexe III) have been evaluated.

#### 4.2.4.1 Cell Finder Slides

The use of Cell Finder Slides caused problems, and some of them could not be fixed with the use of this system for hybrid callus selection (**Prange *et al.*, 2010c**; Annexe III).

The successful cultivation of the protoplasts in the agarose or alginate films on the slides was only achieved in high cell densities after protoplast fusion (**Prange *et al.*, 2010c**;

Annexe III) and by replacing parts of the liquid medium with solid culture medium (Annexe III).

Problems are mainly correlated with the cell density of the cultivated protoplasts. The protoplast density after fusion, in general, had to be higher than 1.5 x $10^5$ protoplasts per mL - the density that was used for protoplast culture without fusion. The cultivation with this density after protoplast fusion resulted in the death of the entire culture in most cases*. The cells were likely damaged by the fusion treatment, causing the death of protoplasts after fusion and a subsequent lowering of the cell density after embedding. This low density in turn led to the death of the whole culture when the initial density became too low. The death or the cessation of further development of entire cultures is often described as a phenomenon in protoplast culture experiments (e.g., Nagata & Takebe, 1971; Ochatt, 1991; Vardi *et al.*, 1975; Winkelmann *et al.*, 2006b). The protoplast density has often been described to be crucial for proper protoplast development, with decreasing cell densities resulting in lowered plating efficiencies and a failure of colony (microcallus) formation [e.g., in tobacco (*Nicotiana tabacum* L.) (Nagata & Takebe, 1971), *Citrus* (*Citrus sinensis* (L.) Osbeck) (Vardi *et al.*, 1975)]. Unfortunately, the extent of the deterioration of the *Cyclamen* protoplasts varied between the experiments and, therefore, the protoplast density could not be adjusted depending on the grade of damage. As standard procedure, a relatively high cell density was used in all further experiments (**Prange *et al.*, 2010c**).

The same factors that caused the cell death mentioned above might also be correlated with problems during the cultivation of the protoplast films in high amounts of liquid medium. The inability of the protoplasts to grow at low population densities is attributed by some authors to the diffusion of metabolic intermediates into the surrounding culture medium, which can stimulate growth at high cell densities, but leads to a deficiency of these substances in low population densities (Kao & Michayluk, 1975). Medium developed with the addition of nucleic-acid bases, amino acids, other sugars, sugar alcohols, and organic acids, enabled the growth of *Vicia hajastana* Grossh cells at low population densities (25-50 down to 1-2 protoplasts per mL) (Kao & Michayluk, 1975). A modified variant of this medium was used as standard medium in *Cyclamen* protoplast cultures (Winkelmann *et al.* 2006b, **Prange *et al.*, 2010a,b,c**). Nevertheless, for the successful cultivation of *Cyclamen* protoplasts in this medium, a cell density of about 1.5 x $10^5$ cells per mL is required (Winkelmann *et al.*, 2006b; **Prange *et al.*, 2010a,b,c**). Another possible explanation might be the conditioning of the medium by the protoplasts based on the pH or by the excretion of specific substances or other modifications that are required for

development and cannot be performed by low amounts of cells (Davey *et al.*, 2010). The use of a preconditioned medium in which protoplasts have already been cultured, or the use of a nurse culture system, are two possible ways to solve the problem of low densities after protoplast fusion (Davey *et al.*, 2010). The establishment of a nurse culture system is worth testing in *Cyclamen*. This method uses actively growing nurse-/feeder-cells (protoplasts) that are cultivated in the same medium as the desired low density protoplasts, and are often separated from them by enclosure in alginate or agarose, or other methods (e.g., Kyozuka *et al.*, 1987; Larkin *et al.*, 1988). The nurse-/feeder-cells enable the growth of the low density protoplast by conditioning the medium (e.g., Kyozuka *et al.*, 1987; Larkin *et al.*, 1988; Davey *et al.*, 2010).

The high cell density, which was necessary for protoplast culture on Cell Finder Slides, often complicates the selection of single cells from the coordinate system. To overcome this problem Meyer (2006) selected additional surrounding calluses for further analyses of the hybrid status. In contrast to the developing *Petunia* or *Calibrachoa* calluses (Meyer, 2006) the developing *Cyclamen* calluses were very friable. During separation, the calluses broke apart. The cultivation of small callus parts and of small calluses selected in earlier developmental phases on solid medium was in most cases impossible. They died, presumably due to their small size or due to a damage of the few cells during separation. Few putative hybrid calluses were separated. Further investigations verified the hybrid status of one plant out of five using flow cytometry.

Thus, selection with Cell Finder Slides using a fluorescence microscope equipped with two filter sets for the respective stains was possible. The necessary manual filter change during the determination of heterofusion products was time-consuming, and, therefore, a dual filter would have been advantageous.

#### 4.2.4.2 Fluorescence Activated Cell Sorting (FACS) and the Development of a Microfluidic Chip

Fluorescence activated cell sorting (FACS) has been successfully used for the separation of FDA-stained and unstained protoplasts of *C. persicum* and *C. graecum*. Flow sorting has been used many times for the determination and recovery of heterokaryons after protoplast fusion (e g., Galbraith *et al.*, 1984; Alexander *et al.*, 1985; Glimelius *et al.*, 1986; Sundberg & Glimelius, 1991, Waara *et al.*, 1998). For the stains used in this thesis, the cell sorter was not fully optimal or suitable (improper laser equipment for the detection of scopoletin). The laser equipment (375 nm laser) is now available and the detection of

scopoletin stained protoplasts should be possible and may be tested (Dr. Jörg Fuchs, pers. communication). In future experiments, sorting should be performed under aseptic conditions. A test is necessary to determine if sterilisation of the cell sorter by pre-flushing with 70% ethanol will be sufficient to keep the sorted protoplasts sterile, or if a decontamination of the whole system with hypochloride is necessary. Waara *et al.* (1998) combined the sterilisation of the cell sorter by pre-flushing the internal system with 70% ethanol with addition of an antibiotic (Claforan = Cefotaxim-Natrium) to the culture medium of the protoplasts after sorting. The compatibility of the antibiotic with *Cyclamen* cell cultures was proven in genetic transformation experiments, and cell cultures were not negatively affected by 500 mg $L^{-1}$ Cefotaxim (Prof. Dr. Traud Winkelmann, pers. communication). With suitable laser equipment and an aseptic cell sorting system, the sorting of FDA-, scopoletin-stained protoplasts and their heterofusion products and their subsequent regeneration to plants should be possible, according to other authors (Rasmussen *et al.*, 1997; Waara *et al.*, 1998).

In cooperation with Dr. Proyag Datta and Prof. Dr. Jost Goettert (Center for Advanced Microstructures and Devices (CAMD), Louisiana State University, Baton Rouge, LA, USA), a completely new approach for protoplast sorting was designed. This approach was part of a broader project on the observation and study on individual cells based on microfluidic technologies (”Microfluidic Solutions for Living Cell Research”, Board of Regent Project at CAMD; PI Dr. Datta), developing a better understanding of how cells interact under “controlled environmental conditions”. Controlled environmental conditions (e.g., pH, concentrations of certain gases or chemicals within the solution) might yield valuable information with far-reaching impacts in areas including stem cells, tissue engineering, genetics, drug discovery and agriculture. In order for life scientists to conduct systematic cell based studies similar to those conducted in protoplast research, it is crucial to isolate and interact with individual cells in well-controlled, predefined conditions. In addition to protoplast research, microfluidic solutions also offer unique opportunities in numerous biological fields, for example, cell counting in the study of microbial activity levels or pharmacological studies at the cellular level for drug reactivity. Though microfluidic solutions offer unique opportunities in cell studies and are easily adaptable to specific questions and topics, they are currently not widely used in the life science community for cutting edge cell research.

The microfluidic chip was successfully used for the separation of unstained protoplasts for the first time. As a tool for protoplast sorting, the microchip has not been tested with stained protoplasts and heterofusion products yet, but it should work in the same way as

tests with unstained protoplasts in combination with a fluorescence microscope. This promising and new approach bears many advantages in comparison to the use of a fluorescence activated cell sorter. The latter approach has a much higher throughput than a microchip, in which each single protoplast has to be sorted manually. In contrast to this, the microchip is a much cheaper (one microchip values approximately to 2-4 €). Furthermore, the chip design can be adjusted directly to the experimental demands. The conditions of the protoplasts during sorting can be directly observed and adjusted if necessary on the microchip. Moreover, the establishment of FACS as a method requires a lot of time, experience and money. The microchip can be used as a microscopic slide, with some added pumping equipment. The installation of a sterile work area under a clean bench can be realised with a microscope and the microchip.

After the successful sorting with a microchip, or a FACS, the amount of selected heterofusion products might be very low. Therefore, the application of these separation methods will require modifications in the protoplast culture of heterofusion products (cf. sub-chapter 4.2.4.1). Small culture vessels with a reduced volume adjusted to the amount of cells such as 24-well multidishes (Waara *et al*., 1998), 48-well multidishes (Rasmussen *et al.*, 1997) or 96-microwell plates (e.g., Chuong *et al.*, 1988) have often been used after separation of heterofusion products. Due to the sensitivity of *Cyclamen* protoplasts to culture in liquid medium, the protoplast should be additionally embedded in alginate or agarose (Winkelmann *et al.*, 2006b; **Prange *et al.*, 2010b**). Such a downscaled culture system should be tested and optimised first, because modifications regarding aeration and other factors might be necessary. Another way of cultivating protoplast at low population density is the use of a nurse culture system (cf. sub-chapter 4.2.4.1).

### 4.2.5 Identification of Somatic Hybrids and Characterisation of Hybrid Plants

Plants were regenerated from three independent *C. persicum* x *C. coum* (**Prange *et al.*, 2010c**) and *C. persicum* x *C. graecum* protoplast fusion experiments (*) without the selection of heterofusion products, respectively. Most of the regenerated plants resembled the two parental wild species (*C. coum* or *C. graecum*) (**Prange *et al.*, 2010c**; Annexe II). In the *C. persicum* x *C. coum*, putative hybrid plants with malformed and deviating leaves were visually detected, and their genetic background was preferable investigated (**Prange *et al.*, 2010c**). In the *C. persicum* x *C. graecum* fusion combination no deviating plants were detected and plant were chosen at random for hybrid analysis. Few *C. persicum* plants were regenerated in the combination *C. persicum* x *C. coum*. In the second

combination (*C. persicum* x *C. graecum*), only the *C. graecum* parent could be regenerated to mature plants.

The choice of agarose as the main embedding agent in the protoplast fusion experiments was presumably the selection factor that favoured the development of the two wild species *C. coum* and *C. persicum*. Agarose is only poorly tolerated by the *C. persicum* protoplasts, if at all (Winkelmann *et al*. 2006b; **Prange *et al.*, 2010a,b,c**). The regeneration of one parental species (*C. persicum*) was therefore reduced or prevented by the choice of agarose as main embedding agent. Interestingly, the obtained hybrid plants were regenerated from the fusion experiments where both hybridisation partners in general survived the stressful fusion and regeneration process, and some *C. persicum* plants were also regenerated. The degree of the detrimental effect of agarose on *C. persicum* protoplasts - as demonstrated by preliminary tests and the development of the parental protoplast cultivated in parallel to the fusion experiments (*) - differed from one experiment to another and was also dependent on the quality of the protoplasts in the respective isolation experiment. Varying protoplast quality has also been described by other authors (e.g., Durieu & Ochatt, 2000; Meyer, 2006; Meyer *et al.*, 2009).

The temperatures in the lab were relatively high (35 to 40°C), especially in the *C. graecum* fusion experiments, which might have had an additional negative effect on the *C. persicum* protoplasts, resulting in the death of the cells in combination with the suboptimal embedding agent. Selection methods that are based on the reduced or absent development of at least one hybridisation partner during the regeneration process have been successfully applied to obtain somatic hybrid plants. The use of albino cell lines that are unable to regenerate to plants (Power *et al.*, 1979), the combination of non-regenerable cell lines and cell lines that are unable to divide (Kisaka *et al.*, 1998) and the irradiation and/or the use of iodoacetamide (e.g., Böttcher *et al.*, 1989; Ge *et al.*, 2006) and other physiological inhibitors were effective tools for the selection of hybrid plants. Böttcher *et al.*, (1989) described the occurrence of “escapes” (parental species that survived the putative lethal selection pressure) in addition to the detected hybrid plants. In synopsis, with the results in this thesis, the advantage of the complete elimination of one hybrid partner in early stages of the development to the development and regeneration of the somatic hybrids must be determined. It might be more favourable to sue moderate selection pressure on the development of hybrid cells and their regeneration to plants.

In addition to morphological screenings, hybrid status was examined in this thesis using flow cytometry, RAPD-PCR and a specific molecular marker (Annexe IV; **Prange *et al.*, 2010c**).

In the regenerated plants from *C. persicum* x *C. coum* fusion experiments, morphological screening in the early stages of development with one cotyledon or two to three secondary leaves revealed many plants with unusual leaf shapes and malformations (**Prange *et al.*, 2010c**). These plants were preferably chosen for hybrid investigations. These morphological abnormalities in most cases vanished with progressive development. In the regenerated plants from *C. persicum* x *C. graecum* fusion experiments, no abnormalities were visible, and they were chosen at random for hybrid identification. Hybrids were detected among the regenerated plants from the *C. persicum* x *C. coum* fusion with the different genetic screening methods but not among the plants regenerated from the *C. persicum* x *C. graecum* fusions (cf. sub-chapter 4.2.3). The lack of hybrids in the *C. persicum* x *C. graecum* fusions has already been discussed in sub-chapter 4.2.3, and the following chapter is mainly focused on the *C. persicum* x *C. coum* regenerants.

Seven putative hybrid plants (out of 46) were detected in the plants regenerated from the *C. persicum* x *C. coum* fusions using flow cytometry (**Prange *et al.*, 2010c**). Due to the high fluctuations of the peak positions in measurements of the parental *C. coum* plants (**Prange *et al.*, 2010a,c**), internal standardisation as recommended by Dolezel (1991), Dolezel & Bartos (2005) and Bennett *et al.* (2008) with the *C. coum* parent or both parents was performed when the relative DNA content of the putative hybrids was determined. Using internal standardisation the putative hybrids were recognized by the appearance of two peaks or by very broad peaks with two pinnacles in repeated measurements on different dates (**Prange *et al.*, 2010c**). Flow cytometry has proven to be a fast screening method for the detection of somatic hybrid plants.

Variations in the flow cytometric measurements have been reported by other authors during the determination of absolute DNA contents with propidium iodide (PI) staining or Feulgen microspectrophotometry, often leading to the misinterpretation of intraspecific genome size variation (Bennett *et al.*, 2008 and references therein). The authors reviewed numerous studies that reported on intraspecific genome size variation. In most cases, these were artefacts, mainly due to the occurrence and variable content of DNA staining inhibitors in the used tissue. Various substances or classes of substances have been clearly identified, or are suspected to act as DNA staining inhibitors such as phenolic compounds, furanocumarins, anthocyanins and other compounds with potential DNA binding capacities (e.g., tannin, caffeine, chlorogenic acid, cyanidin-3-rutinoside, quercetin). *Cyclamen* leaves visibly contain anthocyanins in variable amounts depending on the growth conditions, the species and the genotype. Phenolic substances, derived

from the phenylpropanoid pathways are ubiquitous and prevalent in the plant kingdom. Bennett *et al.* (2008) demonstrated the inhibiting influence in flow cytometric measurements of poinsettia (*Euphorbia pulcherrim*a Willd. ex Klotzsch) by using components of red bracts and by sample preparation with added anthocyanin (cyanidin-3-rutinoside). In mixed sample preparation with one of the former additives containing anthocyanin, the inhibiting influence affected the sample tissue and the internal standard in the same way (Bennett *et al.*, 2008). Noirot *et al.* (2002) found similarities in the inhibiting effect of the cytosolic components of *Coffea liberica* var. *dewevrei* in flow cytometric sample preparations using PI or DAPI as dye. According to these results, it can be assumed for the experiments in this thesis that: 1) the influence of putative and identified DNA staining inhibitors is probably similar in flow cytometric measurements using PI or DAPI; 2) that the inhibitors (most likely phenolic substances and/ or anthocyanin) influence the internal standard in the same manner as the sample tissue; 3) the fluorescence intensities (peak positions) in the measurements are shifting, but the spacing between the peaks is probably not affected (**Prange *et al.*, 2010c**). The determination of the absolute DNA content in poinsettia was still significantly reduced (2.8%; $P < 0.001$) when determined using red bracts in comparison to green leaves of the pea (*Pisum sativum* L.) as the internal standard (Bennett *et al.*, 2008). A failure induced by DNA staining inhibitors remaining in flow cytometric measurements must be considered if they are present. Bennett *et al.* (2008) revealed a dose-dependent inhibition in case of anthocyanin. According to this result, it can be presumed that there is an inhibiting effect with lower amounts of anthocyanin, and *C. coum* is probably lower in these than poinsettia red bracts.

Future experiments should take into account that the content of phenolic compounds and anthocyanins is also dependant on the growth conditions (Bennett *et al.*, 2008). To enhance the stability of the peak positions in flow cytometric measurements and, thereby, the comparability between different measurements the sample and standard, material should be cultivated under optimal growth conditions. If the same genotypes are used, plants should be grown at least under identical conditions to induce the production of probably similar contents of the putative DNA staining inhibitors.

The relative DNA content of 31 randomly chosen plants regenerated from *C. persicum* x *C. graecum* fusions was determined using flow cytometry and DAPI staining according to the measurements in *C. coum*, but no hybrid plant was detected*. During the measurements at least one parental plant was measured as the internal standard (see above) together with the putative hybrid plants. The DNA content of *C. graecum* (2n=84

chromosomes) has not been published so far and the absolute DNA content of diploid *C. persicum* cultivars (2n=48 chromosomes) was described by Borchert *et al.*, 2007 with 3.21 ± 0.003 pg/ 2C. The relative DNA content of *C. graecum* was determined in comparison to *C. persicum* in four different measurements, yielding on average an about 1.46 times higher DNA content than *C. persicum* and a calculated absolute DNA content of about 4.69 pg/ 2C*. Putative hybrid plants were expected to have an additive DNA content of both species. Therefore, the peaks of hybrid plants would be easy to distinguish from the peaks of the parental plants. Due to the variation in the peak positions, which were also observed in these measurements and are comparable to the observations in *C. coum* (**Prange *et al.*, 2010c**), a differentiation between tetraploid parental species and somatic hybrids might be problematic. The measurements revealed no putative hybrid plants with an additive DNA content of both parental species. But the peaks of the putative somatic hybrids and *C. graecum*, which usually appeared at the same relative fluorescence intensity, were often very broad, and in some plants broad peaks with two pinnacles were detected. This might indicate an aneuploid hybrid status of the measured plants. Due to the phenotypic resemblance of the putative hybrid plants to *C. graecum* the developed species-specific marker for *C. persicum* has been applied to twelve plants for the detection of *C. persicum* DNA, but up to now no amplification product was obtained. As suggested in the *C. persicum* x *C. coum* fusion combination, the target sequence might have been lost during genomic reorganisation. The observation of these plants is still in progress and also RAPD-markers still have to be applied, for hybrid identification. Other possibilities of hybrid identification, especially the detection of *C. persicum* DNA will be discussed in the following in context with *C. persicum* x *C. coum* hybrid identification.

In *C. persicum* x *C. coum* hybrids, the hybrid identification using the specific molecular marker confirmed the hybrid status of two plants (No 31 and 32*) (**Prange *et al.*, 2010c**), but not for the other six designated hybrids from the flow cytometric measurements. Using the specific marker for *C. persicum,* the amplification of the marker fragment using hybrid DNA was absent in these plants, except for the hybrid plant No 31 and 32*. For the latter two, the amplification of the *C. persicum* specific fragment was always very weak in comparison to the control with *C. persicum* DNA or a mixture of *C. persicum* and *C. coum* DNA (1:1 and 1:5), respectively.

RAPD PCR has been described as clear marker for the detection of somatic hybrid plants and as a screening method in *Solanum*, *Citrus*, *Actinidia* (Xu *et al.*, 1993; Takemori *et al.*, 1994; Grosser & Chandler, 2000; Xiao *et al.*, 2004) and other species. At least two replications and several primers were necessary to obtain reliable results in putative

hybrids of *C. persicum*/*C. coum* (**Prange *et al.*, 2010c**). Using this marker technique for the characterisation of plant No 31, the *C. coum* genome was predominantly detected (23 reproducible amplification products in the hybrid DNA), whereas evidence was scarcely seen for the *C. persicum* genome in the hybrid plants (one reproducible amplification product in the hybrid DNA). The absences of parental bands and the emergence of new bands corroborate the hybrid status of the designated hybrid plant (No 31) and reflect the results obtained with the specific marker. According to the results in this thesis, RAPD markers alone did often not give interpretable results, had to be replicated very often and many primers were necessary to obtain comprehensive results.

Other RAPD primer or RAPD primer combinations might be worth testing to improve the detection of the *C. persicum* genome parts in the somatic hybrid plants. It has to be considered to sequence the amplified *C. persicum* specific bands from the hybrid DNA and compare their sequence to the corresponding sequence obtained from the *C. persicum* DNA. Similar band pattern obtained by RAPD PCR are not necessarily amplified from homologous sequences as revealed by other studies (e.g., Quiros *et al*., 1995; Degani *et al.*, 1998). The sequencing of the rare putative *C. persicum* specific amplification products might therefore be a good proof for the substantiation and characterisation of the somatic hybrid plants. By this method, a specific marker can also be established by designing long specific primer for the respective fragments which might than serve as additional specific marker for *C. persicum* [Sequenced Characterized Amplified Region Marker (SCAR) (e.g., Naqvi & Chattoo, 1996; Castrillo *et al*., 2003)].

The results of both, the specific marker technique and the RAPD PCR most likely indicate an elimination of alleles or chromosomes of the target sequence, as reported for other species combinations after somatic hybridisation (Kao, 1977; Binding & Nehls, 1978; Wetter & Kao, 1980; Karp *et al.*, 1982) (for a detailed discussion see **Prange *et al.*, 2010c**). The putative loss of chromosomes was not reflected in the estimated relative DNA content of these hybrids using flow cytometry and DAPI staining. This method revealed the addition of both the *C. persicum* and the *C. coum* genome. As discussed above, these measurements include an internal variation and might additionally cause a chance of failure for the determination of the DNA content. The determination of the absolute DNA content with PI staining of the designated hybrid plants and their parents will give more precise results. Due to the large differences in chromosome size between the two parental species [(Glasau, 1939) *C. coum* with 2n = 30 chromosomes and a DNA content of 14.71 ± 0.13 pg/ 2C (**Prange *et al.*, 2010c**) and *C. persicum* with 2n = 48 chromosomes and a DNA content of 3.21 ± 0.003 pg/ 2C (Borchert *et al.*, 2007)], the

determination of the absolute DNA content might not be precise enough to detect the loss of single *C. persicum* chromosomes. In this case, the exact counting of chromosomes has to be performed in order to detect these losses. To document chromosome recombination or loss, a chronological series of analyses would be advantageous. One basic problem with this kind of analysis is the limited amount of plant material. Both the putative hybrid plants and the control plants developed slowly, especially in the very early stages of development. Subsequent analysis was limited by the available plant material and analysis was not possible without the destruction of the whole plant. Some putative hybrid plants have not been examined for their hybrid status until recently because their fragile and malformed phenotype did not provide suitable plant material for analysis. The existence of these malformed plants and the slow development of the other putative hybrid plants provide evidence that chromosome reorganisation/loss might be a prerequisite for normal plant development is these hybrids. Similar observations have been made in *Citrus* by Fu *et al.* (2002). *Citrus* somatic hybridization is capable of circumventing sexual and graft incompatibility, but somatic hybrids with the double chromosome sets (amphidiploids) were often characterised by a low vigour, which indicated that the incompatibility phenomenon still exists in these plants (Guo & Deng, 1998; Fu *et al.*, 2002). In contrast to this, hybrids with asymmetric chromosome sets (after chromosome doubling or elimination), often exhibited high vigour and good development, and the authors assumed that these reorganisations were necessary for overcoming hybrid incompatibility (Fu *et al.*, 2002). Compared to symmetric hybrids, asymmetric hybrids often exhibited a higher regeneration and rooting capacity [e.g., *Petroselinum hortense* - *Daucus carota* somatic hybrids (Dudits *et al.*, 1980); *Nicotiana plumbaginifolia* - *Atropa belladonna* somatic hybrids (Gleba *et al.*, 1988)] or increased fertility (*Nicotiana* somatic hybrids Bates *et al.*, 1987). In this study, no regenerated calluses gave rise to plants of either parental species. Therefore no chimeric calluses (i.e., two calluses grown together) were regenerated. Due to these results, future experiments should include a verification of the hybrid status at the callus level. In this manner a very early stage of development will be analysed, and if a hybrid callus has been detected, it can easily be propagated at the callus level to obtain more plant material for further subsequent analyses. If chromosome reorganisation or loss in *Cyclamen* hybrids takes place at the callus or embryo/plant level still remains unproven and must be analysed.

In addition to chromosome elimination, the weak amplification of the *C. persicum* specific marker can also originate from genetic recombination inside the target sequence of the 5.8S RNA gene as reported by Cluster *et al.* (1996) in *Medicago* somatic hybrids for the 18S RNA gene (for a detailed discussion **Prange *et al.*, 2010c**). *C. persicum* alleles might

have been sorted out completely (putative hybrids plants, without detection of *C. persicum* specific marker) or partially (hybrid plant No 31) during the reorganisation of the two genomes and recombination of alleles, which can explain the weak amplification of the marker in the PCR reaction. Other methods such as AFLPs or microsatellite based molecular markers have to be applied for the detection of these and other putative recombinations. The establishment and application of FISH or GISH techniques to the somatic hybrids can give detailed information about the localisation of the parental genome parts in the genome of the hybrid plant.

Other authors successfully established an ITS-based specific marker for the detection of somatic hybrids [e.g., in *Cucumis* ssp. (Dabautza *et al*., 1998)] and obtained reliable results for the characterisation of their hybrid plants. In this thesis, the specific marker was technically a good screening method, but the C*. persicum* marker should be improved because apparently it did not exhibit the reliability that was expected (see above). Additionally, the primer should be tested for their amplification results in a multiplex PCR, which would facilitate the applicability of the marker. For all of the established marker combinations, except for the combination *C. persicum*/*C. coum*, multiplex PCR could be used without major modifications because the annealing temperatures of the primers are identical. It must be assumed that this marker cannot be utilised as a single screening method when comparing the results obtained by the species-specific marker with the flow cytometric results. At least in the fusion combination under investigation (*C. persicum* x *C. coum*), the danger of losing the target sequence of the marker seems to be very high. For the screening of somatic hybrid plants, it would be beneficial to have a set of specific markers that enable the detection of different parts of the genome. Completely new specific molecular markers can be established that will provide information about other target genes in the hybrid plants, and also about the organellar composition by establishing markers for mitochondria or chloroplasts. Very few other genes are available in the NCBI database for *Cyclamen* wild species, and only a few chloroplast genes have been published for *C. coum* (http://www.ncbi.nlm.nih.gov) by Yesson *et al.* (2008). Based on this sequence, a specific marker can be established, providing information about the organellar composition of the *C. persicum* x *C. coum* hybrid plants. For *C. persicum* more nucleotide sequences are available (about 30-40), and since 2005 over 2000 expressed sequence tags (EST) representing 1980 assembled transcripts have been published by Rensing *et al.* (2005) (www.cyclamen-est.de). This sequence information can serve as the basis for the detection of unknown genes in the other *Cyclamen* species and for the development of other specific markers. The establishment of EST-based markers does not aim at the amplification of highly conserved regions. For the establishment of other

classical marker techniques based on RNA, ITS, microsatellite and/or other repetitive, highly conserved or non-coding DNA regions for a subset of general information are available today (Weising *et al.*, 2005); it would be worthwhile to establish and optimise them for the genus *Cyclamen* to enable a further characterisation of the hybrid plants.

### 4.2.6 Perspectives for *Cyclamen* breeding

Many hybrids in *Cyclamen* species were obtained using the embryo rescue technique, resulting in both fertile and sterile vigorous plants (Ewald, 1996; Ishizaka, 2008; for details cf. chapter 1.4). The cross combination *C. persicum* x *C. coum* has not been achieved using this method and is likely due to incompatibility reactions. These incompatibility reactions might have an impact in somatic hybrids and, therefore, might have led to the reorganisation of the genome by recombination or chromosome elimination resulting in putatively aneuploid, but vigorously growing, plants as reported in *Citrus* (Fu *et al.*, 2002).

Further investigations on fully grown plants will reveal if the hybrids are fertile. The strong morphological and genetic resemblance to the *C. coum* parent indicates that incorporation into breeding programs with *C. persicum* might not be possible. The hybrid itself might have retained parts of *C. persicum* genome, which might improve its horticultural value, such as enhanced flower size.

Future somatic hybridisation experiments with other diploid or tetraploid *C. persicum* cultivars should be conducted, and the resulting hybrids should be tested for their chromosome balance. If the reorganisation of the genome is not always directed against the *C. persicum* parent, the introducing parts of the *C. coum* genome into the *C. persicum* genome might provide a solution. The might be achieved spontaneously or by asymmetric hybridisation using irradiated *C. coum* protoplasts by which only parts of the *C. coum* genome will be combined with the *C. persicum* genome (e.g., Dudits *et al.*, 1980; Parokonny *et al.*, 1992; Forsberg *et al.*, 1998). Both methods might results in asymmetric hybrids, or even cybrids that offer the incorporation of genetic material from *C. coum* into the *C. persicum* gene pool, such as frost tolerance, which would be a very desirable trait to establish *C. persicum* as an outdoor perennial in the temperate zone.

The protoplast fusion combination *C. persicum* x *C. graecum* was expected to result in viable and fertile plants, because of the existence of hybrids obtained by embryo rescue. Most likely due to technical reasons, no hybrids of this fusion combination were obtained in this thesis. These hybrids would be of scientific interest for their comparison to gametic

hybrids obtained via embryo rescue to study characteristics of maternal inheritance. Based on the experience made in this thesis, the protoplast fusion technique can be improved to obtain somatic hybrids in future experiments. In general, the genomic reorganisation in somatic hybrids can often not be foreseen and the suitability of somatic hybrids for comparative studies has to be proven. Therefore, the emphasis should be put on the establishment of molecular marker techniques and FISH and GISH techniques that will reveal more detailed information about the genomic composition of somatic hybrid plants in the genus *Cyclamen*.

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# PUBLICATIONS

## Journal research papers (peer-reviewed)

1. **Kurz, M., Brünig, A.N.S. & Galinski, E.A. (2006).** NhaD type sodium/proton-antiporter of *Halomonas elongata*: a salt stress response mechanism in marine habitats? *Saline Systems* **2**: 10.

2. **Winkelmann, T., Prange, A.N.S., Specht, J. & Serek, M. (2008).** Morphological characterization of plants regenerated from protoplasts of *Cyclamen persicum* Mill. *Propagation of Ornamental Plants* **8**: 9-12.

3. **Prange, A.N.S., Serek, M. & Winkelmann, T. (2008).** Vegetative propagation of different *Cyclamen* species via adventitous shoot formation from seedling tissue. *Propagation of Ornamental Plants* **8**: 204-209.

4. **Prange, A.N.S., Serek, M., Bartsch, M. & Winkelmann, T. (2010).** Somatic embryogenesis as suitable basis for efficient and stable regeneration from protoplast to plant in *Cyclamen coum* Miller. *Plant Cell, Tissue and Organ Culture* **101**: 171-182.

5. **Prange, A.N.S., Bartsch, M., Serek, M. & Winkelmann, T. (2010).** Regeneration of different *Cyclamen* species via somatic embryogenesis from callus, suspension cultures and protoplasts. *Scientia Horticulturae* **125**: 442-450.

6. **Prange, A.N.S., Bartsch, M., Serek, M. & Winkelmann, T. (2010).** Interspecific somatic hybrids between *Cyclamen persicum* and *C. coum*, two sexually incompatible species. *Plant Cell Reports* (manuscript in review).

## Miscellaneous Research Papers

1. **Brünig, A.N.S., Hormes, J. & Prange, A. (2006/2009).** Are microbes involved in the reducing of arsenate and chromate by *Nasturtium officinale*: An *in vitro* cultivation and X-ray absorption spectroscopy study. In: The ROSE Committee (Ed.) *CAMD Annual Report 2006* and *The ROSE Report: CAMD, Past, Present and Future*, pp. 287-289. Center for Advanced Microstructures and Devices, Baton Rouge, LA, USA. (internal review)

2. **Datta, P., Goettert, J., Frische, N., Prange, A. & Prange, A.N.S. (2008).** Microfluidic platform for education and research. COMS Conference Puerto Vallarta, Mexico*, Commercialization of Micro and Nano Systems Proceedings* (peer-reviewed).

## Conference Contributions

### (a) Lectures

1. **Kurz, M., Brünig, A., Gräf, C. & Galinski, E.A. (2004).** Heterologous protein expression in *Halomonas elongata* – why halophilic organisms offer a viable alternative to *E. coli*. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM) 28.-31.03.2004, Braunschweig, Germany, *Biospektrum-Sonderausgabe* 2004, p. 58.

2. **Brünig, A., Serek, M. & Winkelmann, T. (2007).** Vegetative propagation of different *Cyclamen* species via adventitous shoot formation from seedling tissue. Fifth International Conference "Propagation of Ornamental Plants", 05.-08.09.2007, Sofia, Bulgaria, *Book of Abstracts*, p. 39.

**(b) Poster**

3. **Winkelmann, T., Specht, J., Brünig, A. & Serek, M. (2007).** Effiziente Pflanzenregeneration aus Protoplasten von *Cyclamen persicum* Mill.. Jahrestagung der Deutschen Gartenbaulichen Gesellschaft (DGG) 21.-24.02.2007, Erfurt, Germany, *DGG Conference Transcript*, p. 116.

4. **Winkelmann, T., Brünig, A., & Serek, M. (2007).** Plant regeneration from protoplasts isolated from embryogenic suspension cultures of *Cyclamen persicum* Mill.. Fifth International Conference "Propagation of Ornamental Plants", 05.-08.09.2007, Sofia, Bulgaria, *Book of Abstracts*, p. 81.

5. **Prange, A., Metzenauer, A., Serek, M. & Winkelmann, T. (2008).** Vegetative propagation of different *Cyclamen* species via adventitous shoot formation from seedling tissue. First Symposium on Horticulture in Europe (SHE), 17.-20.02.2008. Vienna, Austria, *Book of Abstracts*, p. 275.

6. **Bartsch, M., Vázquez Antequera, J., Borrego Padilla, A., Prange, A. & Winkelmann, T. (2010).** Vermehrung von vier *Cyclamen* Wildarten über somatische Embryogenese. Jahrestagung der Deutschen Gartenbaulichen Gesellschaft (DGG) 24.-27.02.2010, Hohenheim, Germany, *DGG Conference Transcript*, p. 125.

**Affirmation/Erklärung**

Hiermit erkläre ich, Anika N. S. Prange, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen Hilfsmittel als die angegeben verwendet habe. Jene Stellen, die ich anderen Unterlagen und Arbeiten dem Wortlaut oder dem Sinn entsprechend entnommen habe, sind durch Quellenangaben gekennzeichnet. Weiterhin versichere ich, dass die vorliegende Arbeit nicht in einem anderen Prüfungsverfahren verwendet wurde.

Brilon, den 15. Juni 2010

Anika Nadja Sabine Prange

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