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Invited paper

MICROPROPAGATION THROUGH SOMATIC EMBRYOGENESIS OF *CYCLAMEN PERSICUM* MILL. GENOTYPES FOR CUT FLOWER PRODUCTION – FEASIBILITY STUDY

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Abstract

The regeneration of *Cyclamen persicum* through somatic embryogenesis (SE) has been described by several groups for a wide spectrum of genotypes. Here, we studied this regeneration system for the first time with 30 genotypes which are valued as cut flowers. The aim of this study was to analyse the efficiency of the micropropagation through SE, to investigate the genetic fidelity and performance of the regenerants and to estimate production costs of young plants. The genotype significantly influenced the frequency of callus induction that ranged from 8 to 80% and also the frequency of calluses with differentiation of somatic embryos that ranged from 0 to 92%. Only three of the 30 genotypes were not able to regenerate somatic embryos, while plants were obtained from the remaining 27 genotypes. In total, 2,783 regenerants were transferred to the greenhouse, 2,003 (72%) of which could be acclimatized. Some genotypes were extremely uniform in terms of flower colour and flower shapes, whereas in others somaclonal variation was detected. Estimation of micropropagation cost was 2-3 € per acclimatized young plant at our research facility. If genotypes with high regeneration efficiencies are selected, plant production costs could be reduced to 0.56-0.80 € or even more.

Key words: *Cyclamen*, genetic fidelity, performance test, production costs, *in vitro* propagation, somaclonal variation

INTRODUCTION

Cyclamen cultivars are known as potted ornamental plants with significant economic importance in many European countries and Japan. However, the production of *Cyclamen* for the use as cut flowers has been restricted to some areas in Germany and it would be of interest to expand their production, since *Cyclamen* flowers are rewarding for florists due to their attractive flower morphology and generally long vase life of more than 12 days and even up to three weeks (Neumaier et al. 2009). According to the currently limited plant numbers produced for cut flowers *Cyclamen* breeding is focused mainly on pot plants. Only a few open pollinated older cultivars were bred for cut flowers (mainly belonging to the series Luckenwalder Rasse or Luwa cultivars). Moreover, in special flower shape types, like 'Victoria' (white with fringed red margin), 'Barbarossa' (so-called bearded flowers) or 'Striata' (petals with stripes of different colours), selections with long peduncles exist. All these selections are propagated by seed resulting from open pollination within a group of plants. Therefore, compared with modern F_1 hybrid cultivars, which dominate the market for potted plants in *Cyclamen*, these selections are characterized by high heterogeneity regarding growth, yield and flower quality. Since single plants in these seed propagated groups combine high yield with excellent flower quality and longevity, their selection and vegetative propagation would be desirable. Vegetative propagation in *Cyclamen* is only possible if *in vitro* techniques are applied, and somatic embryogenesis (SE) was already achieved for *C. persicum* cultivars used as potted plants (Wicart et al. 1984, Fukui et al. 1988, Otani and Shimada 1991, Kiviharju et al. 1992, Kreuger et al. 1995, Takamura et

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al. 1995, Takamura and Tanaka, 1996, Bach et al. 1998, Schwenkel and Winkelmann 1998) as well as other *Cyclamen* wild species (Seyring et al. 2009, Prange et al. 2010a,b).

Objectives of this study were (i) to investigate the applicability of the SE protocol to 30 *Cyclamen persicum* genotypes that had been selected for use as cut flowers, (ii) to describe their growth in the greenhouse, their genetic fidelity and performance, and (iii) to calculate the price of a young plant regenerated through SE in order to evaluate the economic feasibility of this regeneration system.

MATERIALS AND METHODS

Plant material

For this study, 20 *C. persicum* genotypes were selected based on previous investigations on yield and flower quality (S11-S30, Table 1). In addition, two F_1 hybrid cultivars bred for being used as potted plants (S6, S7), three rare plants originating from a Swiss breeding programme (S8-S10) with scent and mini-type flowers, three plants with special flower shapes or colour (S3, S4, S5) and two F_1 hybrid plants selected for cut flower use (S1, S2) were also included.

Callus induction

Flower buds were harvested one to three days before anthesis and subjected to surface disinfection as described previously (Schwenkel and Winkelmann 1998, Winkelmann 2010). Ovules were excised from these flower buds, cultured on Murashige and Skoog (1962) based medium containing 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.8 mg l^{-1} 6-(γ,γ-dimethylallylamino)purine (2iP) for eight weeks at 24°C in the dark, and thereafter evaluated for callus formation (a detailed technical description of the protocol can be found in Winkelmann 2010). Calluses of a minimal diameter of 2 mm were transferred to fresh

Table 1. Origin and description of the plant material used.

Internal No.	Single plant out of cultivar/experimental line*	Origin (Propagator)	Cultivar type
S1	'Decora Lila'	Schott	F_1 hybrid
S2	'Decora Rosa'	Schott	F_1 hybrid
S3	'Luwa Lila'	Schott	Open pollinated
S4	Nebelung 'Barbarossa'	Schott	Open pollinated
S5	Heinecke 'Striata'	Schott	Open pollinated
S6	'Halios Gefranste Mischung' exp.	Morel	F_1 hybrid
S7	'Halios Fantasia Dunkel Violett' exp.	Morel	F_1 hybrid
S8	'Wirz Weiss'	Depot	Open pollinated
S9	'Wirz Rot'	Depot	Open pollinated
S10	'Wirz Lachs'	Gartenbau Ruelcker	Open pollinated
S11	'Luwa Lulu'	Schott	Open pollinated
S12	'Luckenwalder Schnitt Lulu'	Chrestensen	Open pollinated
S13	'Luckenwalder Schnitt Lucie'	Chrestensen	Open pollinated
S14	'Luckenwalder Schnitt Lucie'	Hofmann	Open pollinated
S15	'Luckenwalder Schnitt Lunav	Hofmann	Open pollinated
S16	'Luckenwalder Schnitt Luna'	Chrestensen	Open pollinated
S17	'Franz List' (Off type colour: Victoria)	Spruenken	Open pollinated
S18	'Victoria'	Spruenken	Open pollinated
S19	'Luwa Lulu'	Schott	Open pollinated
S20	'Beethoven Gefranst'	Spruenken	Open pollinated
S21	'Luwa Luise'	Schott	Open pollinated
S22	LW18/03	Gartenbau Luckenwalde	Open pollinated
S23	'Cut Flower Mix'	S&G Syngenta	Open pollinated
S24	'Luckenwalder Schnitt Luna'	Chrestensen	Open pollinated
S25	LW17/03	Gartenbau Luckenwalde	Open pollinated
S26	'Luwa Lulu'	Schott	Open pollinated
S27	'Beethoven Gefranst'	Spruenken	Open pollinated
S28	'Luwa Luna'	Schott	Open pollinated
S29	'Decora F1-Mix-Schnitt'	Schott	F_1hybrid
S30	'Luwa Luna'	Schott	Open pollinated

* Cultivar names are indicated by quotations, experimental lines by numbers.

medium of the same composition and cultured for another eight weeks under identical conditions.

For each genotype, there were three replicates with two flower buds each and with a time lag between two replicates of at least 10 days. One replicate consisted of 100 ovules (50 per flower bud) distributed to 4 Petri dishes.

Regeneration of somatic embryos

For realisation of SE the calluses were transferred to plant growth regulator (PGR) free medium (Winkelmann 2010). After an 8-weeks-culture at 24°C in darkness the percentage of calluses with regenerated somatic embryos was recorded.

Thereafter, somatic embryos were singled out and cultured further on the PGR-free medium at 18°C in the dark until the cotyledons had elongated to a length of about 2 cm. Subsequently, plantlets were again subcultured and transferred to light (16 h photoperiod, 30 µmol m^{-2} s^{-1}).

Acclimatization

Plantlets with two to three leaves were acclimatized in a greenhouse: After a thorough removal of the culture medium they were planted into peat substrate mixed with perlite (2 : 1) in multi-trays (diameter of growing cell: 4 cm) and preventively sprayed once with a fungicide. For 14 days the plantlets were kept at high humidity under a tent of transparent plastic foil, before gradual reduction of relative humidity and cultivation in a greenhouse at 18/16°C day and night temperature. After about six-week-acclimatization the survived plants were counted and potted into 16 cm pots. Further cultivation took place at 16/14°C day/night temperature and liquid fertilizer was applied at a concentration of 0.03-0.05% (Ferty 3 green: 15% N, 10% P_2O_5, 15% K_2O). Flowers were harvested weekly and all plants were observed for visible abnormalities representing somaclonal variation.

Calculation of propagation costs

In order to study the economic feasibility of *in vitro* propagation through SE we calculated the production costs by recording the labour time needed for the different production stages as they were performed in this particular set of genotypes. Moreover, the prices for media components and culture vessels were collected in 2010.

Statistics

Data collected for callus formation and regeneration of somatic embryos were subjected to one-way analysis of variance (ANOVA) for the factor genotype. Means were compared based on Fisher's least significant difference (LSD) procedure at 95% confidence interval.

RESULTS AND DISCUSSION

Callus induction

Most ovules did not show any signs of contaminations, except for three genotypes (S10, S20 and S21) for which a higher number of up to 100 ovules had to be discarded due to bacterial infections. Therefore, the suitability of ovules as starting material was confirmed, which was also recommended for a high number of diploid cyclamen genotypes used as potted plants (Winkelmann and Serek 2005).

Calluses were induced in all 30 genotypes, however of different sizes and consistencies (Fig. 1) and at different frequencies (Fig. 2). Genotypes with more than 60% ovules forming callus were S8, S9, S10 (all diploid mini-types) as well as S12, S16 and S24 (all presumably tetraploid genotypes of the Luckenwalder series). In contrast, poor callus induction (less than 16%) was recorded for S13, S14, S15, S28 and S30 (genotypes of the Luckenwalder series as well). Differences between single genotypes belonging to one cultivar were observed, for instance in 'Luna' (S15, S16, S24, S28,

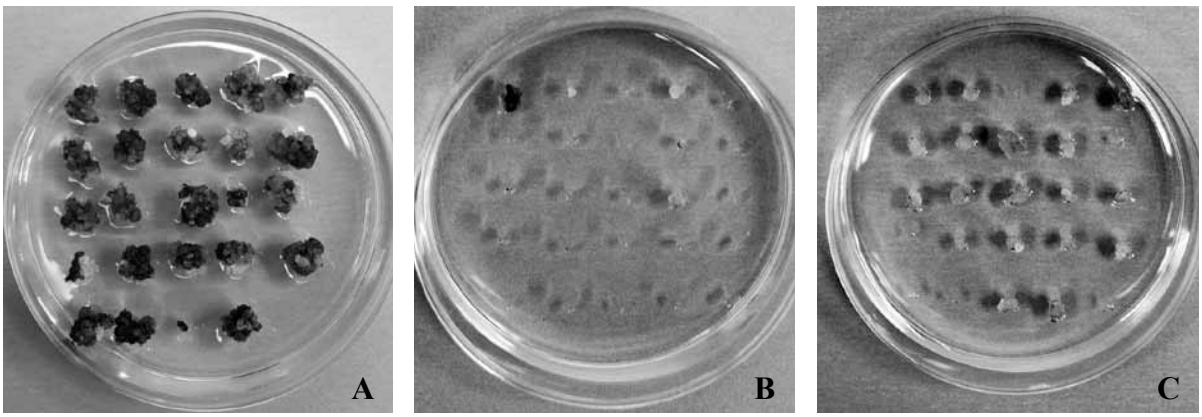

Fig. 1. Callus induction from ovules after eight weeks of culture (6 cm Petri dishes). A) Genotype S8, B) Genotype S15, C) Genotype S20.

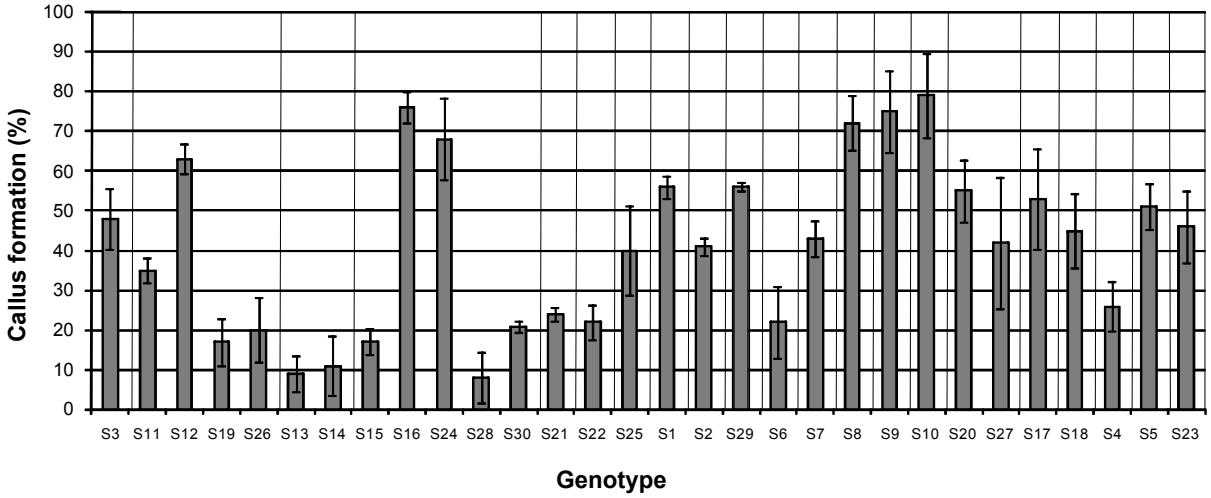

Fig. 2. Callus formation after eight weeks of 30 genotypes. Bars are means and standard deviations of three replicates with 100 ovules each, differences bigger than 25.04% were significant after Fisher's least significant difference (LSD) procedure at 95 % confidence interval. Vertical lines represent borders between cultivars/races according to Table 1.

S30; Fig. 2). Interestingly, genotypes of one origin of the plants, where the propagation via open pollination was conducted over many generations, were found to react more uniformly (e.g. S16 and S24 were provided by the same company).

Standard deviations in Fig. 2 representing the variation among the three replicates clearly indicated the influence of the bud and/or time of preparation on callus induction.

Of all genotypes investigated in this study an average callus formation frequency of 41.4% was observed. In comparison with this, a higher frequency of 61.4% was obtained in a study of 32 F_1 hybrid *Cyclamen persicum* cultivars for potted plant production (Winkelmann and Serek 2005). Those F_1 hybrid cultivars were more uniform in callus formation when single plants of one cultivar were compared than the single plants of the open pollinated cultivars derived from different propagators in the present study.

Regeneration of somatic embryos

Normally the trigger for regeneration of somatic embryos in our system is the transfer of calluses to the PGR-free medium. Nevertheless, at the end of the second culture passage on medium containing 2,4-D first somatic embryos may become visible, probably due to depletion of 2,4-D in some areas of the callus culture. In 11 out of the 30 genotypes investigated, regeneration of somatic embryos after 16 weeks was detected with an outstanding high percentage of 82% of the calluses of genotype S6.

After another eight weeks of culture on PGR free medium the differentiation of somatic embryos was evaluated and revealed strong genotypic differences (Fig. 3, Fig. 4). For 27 out of the 30 genotypes somatic embryos were obtained, but with a variation in frequency of 5.6% (S17) up to 100% (S6) (Fig. 4). Moreover, not only the percentage of calluses with differentiation of somatic embryos varied, but also the

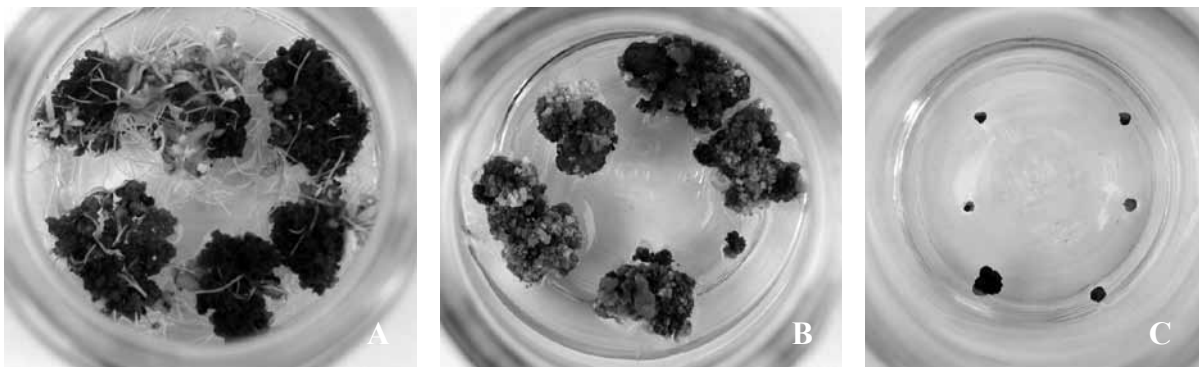

Fig. 3. Regeneration of somatic embryos from callus eight weeks after transfer to plant growth regulator-free medium (diameter of vessel: 5 cm). A) Genotype S6, B) Genotype S17, C) Genotype S15.

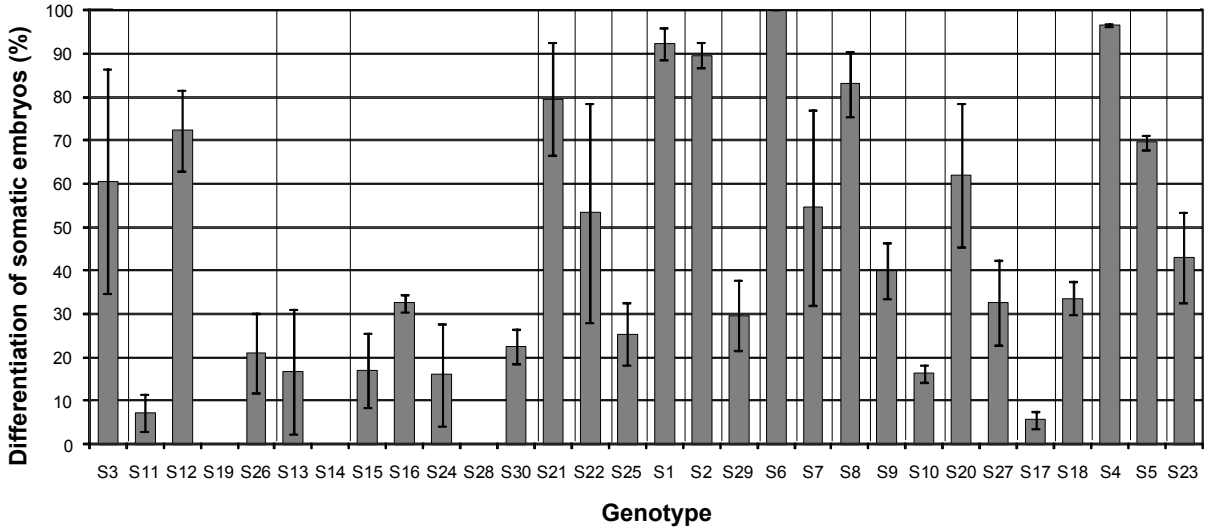

Fig. 4. Regeneration of somatic embryos eight weeks after transferring the calluses of 30 genotypes to plant growth regulator free medium. Bars are means and standard deviations of three replicates each, differences bigger than 33.72% were significant after Fisher's least significant difference (LSD) procedure at 95% confidence interval. Vertical lines represent borders between cultivars/races according to Table 1.

number of somatic embryos formed on a single callus (Fig. 3). A closer look at the different genotypes within one cultivar demonstrated high genetic variability, for example for 'Lulu' in which one genotype regenerated somatic embryos from more than 70% of the calluses (S12), but another one (S19) did not produce any somatic embryos.

These data are in agreement with previous reports demonstrating strong genotypic influences on SE in *Cyclamen persicum* showing that only a minority of genotypes was not able to regenerate through SE (Schwenkel and Winkelmann 1998, Winkelmann and Serek 2005), while Takamura and Tanaka (1995) observed SE regeneration only in five out of 13 genotypes. Püschel et al. (2003) postulated two major complementary genes to be responsible for regeneration ability through SE in *C. persicum*.

Acclimatization, further growth and stability

As a result of genotypic influences on callus formation as well as on regeneration efficiency between 0 and 431 *in vitro* plantlets were obtained per genotype in this given period of time (Table 2). Over all genotypes a relatively low acclimatization rate of 72% was achieved (Table 2), whereas in previous experiments an acclimatization success of 80-90% was observed. Probable explanation for the lower rate could be very high temperatures in the greenhouse due to intensive solar irradiation in the first days of acclimatization.

One of the problems in SE is the lack of synchronization which causes heterogeneity in development of somatic embryos. Therefore, in some genotypes pronounced variation in plant sizes that had already been observed in tissue culture continued to give rise to variations in young plants (Fig. 5). Selection of big and strong plants would be one solution in cases where high plant numbers are available. A better understanding of the whole regeneration system on the molecular level that has recently been initiated by studies of transcriptome (Rensing et al. 2005, Hoenemann et al. 2010) and proteome (Winkelmann et al. 2006, Lyngved et al. 2008, Bian et al. 2010, Rode et al., unpublished) might be another strategy to improve synchronization and plant quality.

Due to the extreme variation in plant numbers and differences in plant size, the performance test and yield determination were difficult to realize. Regarding flower size and flower shape the *in vitro* propagated plants were more uniform than the seed propagated controls (Fig. 6). Especially in salmon coloured varieties, which normally show variation in pigmentation when propagated from seeds, flowers of vegetatively propagated plants were found more homogenous. In contrast, the variation of colour patterns in 'Victoria' could not be reduced by *in vitro* propagation (Fig. 7A). While the typical bearded shape of 'Barbarossa' flowers was maintained after *in vitro* propagation, colour intensity was found to be variable (Fig. 7B).

One interesting observation was the segregation of flowers regarding colour in the two 'Striata' cultivars (S5 and S23, Fig. 7C). It appeared that these striped flowers were genetically instable. However, the reason for this flower pattern remains unclear: chimeric composition of the plant seems not likely, because somatic embryos are considered to originate from single or a few cells. Possibly transposon activity could have caused

Fig. 5. Acclimatized young plants. A) Homogenous regenerants of genotype S7, B) Inhomogeneity in terms of plant size in genotype S27.

Fig. 6. Flowering plants regenerated through somatic embryogenesis in the performance test.

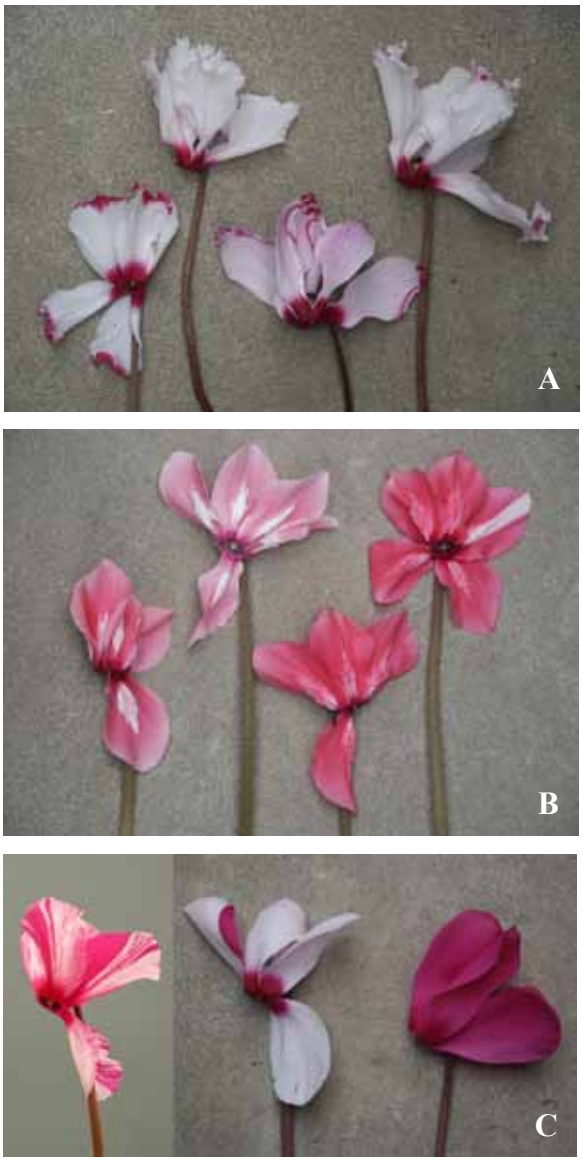

Fig. 7. Variation in flower morphology. A) Variation in colour pattern which is also found in the same extent in seed propagated plants of 'Victoria', B) Variation in colour intensity, but stability in the bearded flower shape of 'Barbarossa', C) Segregation of flowers showing the original 'Striata' phenotype and more or less uniformly coloured flowers.

this particular flower patterning.

Various types of aberrations were found in plants regenerated via SE (Table 3). It is clear that in some cultivars somaclonal variations occurred more frequently than in others, a fact that has also been described by Schwenkel and Winkelmann (1998). However, since in many cases very few plants were investigated (Table 2) no conclusion could be drawn. In contrast, after regeneration of *Cyclamen persicum* from protoplasts, much higher levels of somaclonal variation have been observed (Winkelmann et al. 2008).

The exact estimation of yield was impeded by the variation in plant sizes and time limit. For well developed young plants the following numbers of cut flowers could be harvested: 40-60 for open pollinated cultivars, 130-150 for the mini-types (S8, S9, S10) and 70-130 for the F_1 hybrids. These numbers are comparable with yields of seed propagated plants of the respective cultivars.

Calculation of propagation costs

Costs of *in vitro* propagated plants were mainly associated with manual labour in the laboratory (Table 4). Obviously the biggest part of the total cost was

Table 2. Plants regenerated through somatic embryogenesis and acclimatized.

Internal No.	No. of plants suitable for acclimatization	Acclimatization success (%)
S1	131	95
S2	20	70
S3	5	60
S4	104	93
S5	3	100
S6	431	64
S7	329	75
S8	139	83
S9	4	75
S10	22	68
S11	21	81
S12	200	56
S13	8	88
S14	0	--
S15	60	75
S16	55	100
S17	11	100
S18	270	63
S19	0	--
S20	241	72
S21	2	50
S22	35	57
S23	74	84
S24	132	79
S25	56	100
S26	13	100
S27	386	60
S28	0	--
S29	33	85
S30	0	--
Sum/Average	2,783	72

caused by singularization and subculturing the somatic embryos (Table 4A). Since ratings should not be taken into account for the economic feasibility, the time needed for propagation of one genotype was estimated at 12.6 h. The salaries of employees in the lab vary from company to institutes and from one country to another. If we assume a wage per hour of 10-15 €, 12.6 h cost 126-190 €.

The amount of material that is used for the propagation of one genotype giving a sum of 5.07 € (Table 4B). Furthermore the capital costs of electricity and maintenance of the building and other infrastructure could only be roughly estimated with 10 €/ genotype ending up with a sum of 15 € in total. These data point to the well-known fact that labour accounts for the majority of costs in micropropagation.

If the total number of acclimatized plants obtained is 2,003 (Table 2: 72% of 2.783) and is divided by the number of genotypes (30), the result is an average of 67 plants for one genotype. The costs per genotype were estimated at 141-205 € depending on the wages resulting in average cost per plant of 2-3 €.

These cost calculations are based on the experimental design in this study. However, if genotypes are selected with high propagation efficiencies of 200-400 plants, the costs would be greatly reduced. One should assume slightly higher work load of 15 h per genotype due to more manual labour for separation of somatic embryos resulting in costs of 150-225 €. If more material would be needed (7 €) and further capital costs remain the same (10 €), this would give a sum of 167-242 € for 300 plants, corresponding to costs of 0.56-0.81 € per plant and approximate prices of 1-1.5 €/plant. However, we are convinced that in the situation of a commercial laboratory much lower costs could be achieved than under the research institute

Table 3. Type of deviations from the original morphology found in plants regenerated through SE.

Genotype	Somaclonal variation
S1	Different leaf colour and pattern
S4	Variation of flower colour (Fig. 7A)
S5	Segregation into totally red flowers next to flowers with stripes (‘Striata’ type, Fig. 7C)
S6	Segregation in flower colour, more bluish
S7	Variation flower colour: distinct type of dark red, variation in width of the white margin
S8	Pink sectors in flowers and plants
S10	Doubled flowers, anthers transformed into petals
S11	One plant with variegated leaves (out of 12)
S12	One plant with variegated leaves (out of 104)
S18	Variation in flower colour pattern (same extent as in seed propagated plants, Fig. 7B)
S23	Segregation into uniformly coloured flowers and flowers of ‘Striata‘ type, about 20-30 % of plants with original phenotype
S24	One plant with variegated leaves (out of 104)
S27	About 30% with greyish leaves, different leaf pattern, flowers with blueish and necrotic margins, less vigorous

Table 4. Calculation of costs for propagation via SE.

A) Working time calculation			
Production stage	Working time (summed for all 30 genotypes)	Working time without evaluations (summed for all 30 genotypes)	Working time without evaluations (for one genotype)
Medium preparation	4 h	4 h	0.1 h
Explant preparation and callus induction	55 h	55 h	1.8 h
Evaluation of callus formation and transfer to fresh medium	60 h	25 h	0.8 h
Evaluation of callus growth and transfer to fresh medium (PGR free)	60 h	25 h	0.8 h
Evaluation of somatic embryo formation	80 h	0 h	0 h
Separation of somatic embryos and one further subculture	250 h	250 h	8.3 h
Acclimatization	60 h	20 h	0.7 h
Sum	569 h	379 h	12.6 h
B) Materials calculated for one genotype			
Type of material	Amount needed for one genotype		Costs
Medium	1.6 l		1.60 €
Petri dishes	12		0.92 €
Culture vessels	18		1.05 €
Other lab materials	(NaOCl, Parafilm, scalpel blades…		0.50 €
Materials for acclimatization	(substrate, multi-trays…)		1.00 €
Sum			5.07 €

conditions of this study.

Our calculated price would compare with actual prices of young plants from seed of 0.33-0.40 €. Thus, *in vitro* propagated plants must achieve higher flower yields of 0.62-1.17 € corresponding to only about 8 flowers.

In conclusion, *in vitro* propagation of *Cyclamen persicum* through SE could become an attractive alternative, if better homogeneity and quality of flowers would result in higher gains. However, genotypes with high regeneration efficiencies have to be selected and the quality and synchrony of development need to be improved further.

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