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The effect of explant material on somatic embryogenesis of *Cyclamen persicum* Mill

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Abstract

Somatic embryos of *Cyclamen persicum* Mill. could be produced through a callus phase from juvenile explant material including anthers, ovaries and zygotic embryos. The auxin 2,4-D (1.0–1.5 mg l^{-1}) and coconut milk (10% v/v) in MS medium were important factors for the induction of somatic embryogenesis. Somatic embryos germinated into plantlets in MS medium without growth regulators. The plants grew well in the greenhouse and flowered normally. The plants were phenotypically identical to the mother plants with a few exceptions.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid, NAA – 1-naphthylacetic acid, IAA – 3-indoleacetic acid, BA – 6-benzyladenine, ABA – abscisic acid, CM – coconut milk

Introduction

Cyclamen is one of the most popular pot plants. It is commercially propagated through seeds. The seed is relatively expensive and there is some variation in the seed-derived cultivars. Vegetative propagation (cloning) could result in more uniform cultivars and enable the utilization of unique genetic combinations. Conventional vegetative propagation and micropropagation through axillary or adventitious systems are inefficient and not economical (Geier et al. 1990).

Somatic embryogenesis offers a system where ultimately fermentors could potentially be a rapid and efficient mode of propagation. The concept has been demonstrated on a pilot scale in poinsettia by Preil et al. (1988).

The aim of this study was to evaluate the general feasibility of somatic embryogenesis in cyclamen and assess the impact of explant material on the process of embryogenesis. Only a few reports on somatic embryogenesis on cyclamen are available (Fersing et al. 1982; Wicart et al. 1984).

Materials and methods

Source of explants

Embryogenesis of cyclamen cv. Rosamunde was studied using petioles, peduncles, zygotic embryos, ovaries and anthers as explant materials.

Petioles and peduncles were cut just beneath the leaf or the flower. Flower buds were collected when still closed. Explants were dipped once in 70% ethanol, rinsed 3 times in sterile distilled water, surface disinfested for 3 min (flower buds for 15 min) in 5% sodium hypochlorite solution containing a few drops of detergent (Tween 20), and finally rinsed five times in sterile distilled water. Petioles and peduncles were aseptically cut into 2-mm thick slices. An-

thers and ovaries were removed from flower buds aseptically.

Seeds were surface disinfested immersing them for 15 min in 1% sodium hypochlorite solution containing a few drops of detergent and then rinsed five times in sterile distilled water. After that, zygotic embryos were aseptically prepared (peeling by knife) under microscope.

Culture media

MS (Murashige & Skoog 1962) was used as the basal medium, with half-strength macronutrients. In the germination media all MS salts (macro and micro) were of half-strength. All media were supplemented with 3% sucrose and solidified with 0.8% agar (Bacto-agar, Difco). After growth regulators had been added, the pH was adjusted to 5.7. All growth regulators were added before autoclaving.

Based on the results of the preliminary tests the media for further experiments were chosen. Embryo induction medium contained 1.0 mg l^{-1} 2,4-D with or without 10% CM. The same medium was also used varying the ratio of the nitrogen sources: 10 mM nitrate and 20 mM ammonium ion (in MS with half-strength macrosalts: 10 mM ammonium and 20 mM nitrate ion). Ammonium chloride and sodium dihydrogenphosphate were used in the media in order to keep phosphate and nitrogen ion concentration constant. Embryo differentiation medium contained 0.2 mg l^{-1} NAA and 0.2 mg l^{-1} kinetin. Somatic embryos obtained on induction or on differentiation medium were transferred to the germination medium, which contained 0.2 mg l^{-1} NAA and 0.5 mg l^{-1} ABA (G1) or no growth regulators (G2).

Culture conditions

Cultivation temperature was 23 ± 2°C. Cultures were kept in the dark. The effect of light (daylight, 34 μmol m^{-2} s^{-2}, 16/8 h regime) in the germination phase was also tested. In the induction phase the cultivation time was eight weeks (without subculturing). Cultures were kept for two or three cycles on induction medium before transferring them on to differentiation media. The culture time on differentiation media was also eight weeks (without subculturing).

After two cycles on the induction medium the individual embryos that developed were transferred either onto the germination media G1 or G2 for 8 weeks (no subcultures) in the dark. The germinated embryos were then transferred into the greenhouse.

Embryos that developed after three cycles in the induction media were transferred to the germination medium G2 for 7 weeks either in dark or in light conditions.

Planting into the greenhouse

Germinated embryos were transferred into peat/perlite mixture either:
- by planting them directly into the greenhouse (temperature day/night 22/20°C, light 150–200 μmol $m^{-2}m^{-1}$, humidity 70%). Transparent plastic caps were kept on the cultivation boxes for about two weeks in order to keep humidity high, or
- first planting them into an acclimatization room (temperature 23 ± 2°C, light 50–55 μmol m^{-2} s^{-1}, 20/4 h light regime, humidity 80–85%). After two weeks the plants were transferred to the greenhouse.

Histology

Somatic embryos were fixed in FAA-solution (formalin:acetic acid:50% alcohol, 1:1:19). After dehydration in ethanol, the samples were embedded in paraffin, cut at 10 μm thick, and the sections were stained with fast green and hematoxylin/safranin.

Results and discussion

Preliminary tests

In the preliminary tests all types of explant materials used produced slow growing callus. The growth was better in the dark than in the light. Callus cultures were pale or brownish and hard regardless of the growth regulators applied. Loewenberg (1969) obtained similar results with cyclamen callus (cultivar not known). Usually

both auxin and cytokinin were required for good callus growth, but callus also grew when the cytokinin was replaced by CM. With auxin alone, the callus growth was poor and the explants soon died. Petioles and peduncles produced very homogenous (in terms of colour and morphology) callus, whereas callus induced from more juvenile material was heterogenous and seemed to be more capable of regeneration.

Somatic embryos developed from callus initiated from zygotic embryos and anthers during 24 weeks cultivation. Somatic embryogenesis was induced on basal medium containing 1.0 or 1.5 mg l^{-1} 2.4-D and 10% CM. With zygotic embryos a higher ammonium ion concentration was used (10 mM nitrate and 20 mM ammonium ion). Somatic embryos developed (zygotic embryos: 45% $n = 20$, anthers: 5% $n = 20$) when growth regulators were changed to 0.2 mg l^{-1} NAA + 0.2 mg l^{-1} zeatin with or without ABA. Five per cent of calluses from zygotic embryos produced somatic embryos already on the induction medium during 16 weeks cultivation. Somatic embryos germinated after three months. Callus clumps were inoculated into the liquid medium, but due to the hardness of the callus, suspension cultures could not be made.

Embryo induction and differentiation

Somatic embryos were obtained from ovary and anther cultures (Table 1). They developed in a medium containing 10% CM. A few embryos occasionally also developed in induction medium without CM, when the ammonium ion level was increased (10 mM nitrate and 20 mM ammonium ion). In the early tests somatic embryos were obtained also using zygotic embryos as explants, but in these experiments only a few of the zygotic embryos produced callus in induction media.

Wicart et al. (1984) obtained somatic embryos from cyclamen callus cultures by using different auxin and cytokinin or adenine combinations. Nevertheless, the occurrence of embryo-like structures was inconsistent. In our experiments CM clearly promoted the formation of somatic embryos. This has been observed also in some other species, e.g. in *Antirrhinum majus* (Sangwan & Harada 1975) and *Begonia fimbristipula* (Zhang et al. 1988). Some somatic embryos were also obtained without CM when an increased ammonium ion concentration was used (Table 1). In media containing CM, somatic embryo production was generally poorer with increased ammonium ion level. The best rate of somatic embryogenesis was obtained after two cycles in induction medium. After three cycles the capability of the calluses to produce somatic embryos decreased remarkably, but some embryos still developed (Table 1).

Morphologically mature embryos developed already on the induction medium. Additional somatic embryos were not produced after transfer of the tissue to the differentiation medium with a few exceptions. Thus, 2,4-D did not inhibit embryo development and a separate differentiation medium was not necessary for somatic embryo development. Wicart et al. (1984) also did not report that a particular differentiation

Table 1. Percentage of explants producing somatic embryos after two or three cycles in different induction media all containing 1.0 mg l^{-1} 2,4-D.

Material	Coconut milk (10%)	After two cycles NO_3^-/NH_4^+ (mM/mM)		After three cycles NO_3^-/NH_4^+ (mM/mM)	
		20/10 (%)	10/20 (%)	20/10 (%)	10/20 (%)
Zygotic embryos	+	0	0	0	0
	−	0	0	0	0
Ovaries	+	63	75	32	25
	−	0	67	0	0
Anthers	+	90	69	49	31
	−	0	7	0	7
Peduncles & Petioles	+	0	0	0	0
	−	0	0	0	0

Table 2. Frequency (%) of germination of somatic embryos from anther material (two cycles in induction medium) after eight weeks cultivation in the dark.

	Germination medium			
	G1		G2	
Induction medium[a]	n	(%)	n	(%)
2,4-D + CM normal ammonium level	17	12	22	91
2,4-D + CM increased ammonium level	7	29	6	100

[a] 1.0 mg l^{-1} 2,4-D and 10% CM in both induction media.

Table 3. Frequency (%) of germination of somatic embryos from anther material (three cycles in induction medium) after 6 weeks cultivation in the dark or in light conditions in G2 medium.

Induction medium[a]		n	(%)
2,4-D + CM normal ammonium level	light	38	24
	dark	58	67
2,4-D + CM increased ammonium level	light	81	21
	dark	56	50

[a] 1.0 mg l^{-1} 2,4-D and 10% CM in both induction media.

medium was used. This is not unusual. For example, in *Gasteria verrucosa* and *Haworthia fasciata*, somatic embryos developed on medium containing auxin (Beyl & Sharma 1983). Obviously 2,4-D is needed for the induction of embryogenic cell groups, but after they have developed, 2,4-D can be removed. Yet in every case the germination of embryos required the removal of auxin.

Somatic embryos were white and quite similar to zygotic embryos (Fig. 1A). They arose usually from darkened brownish callus. Once growth began, development was rapid and different developing stages were difficult to recognize. In one piece of callus there were approximately 5–15 somatic embryos. Embryos occurred either individually or in clusters. Histological examination showed root and shoot meristems, which were connected to each other by vascular strands. There were no such vascular connections to the callus (Fig. 1B).

In the absence of 2,4-D, somatic embryos germinated and produced plantlets (Fig. 2). The highest frequency of germination was achieved in a medium containing no growth regulators (Table 2). Germination was significantly better in the dark than in the light (Table 3, Fig. 2B). As with somatic embryo formation, the embryo germination frequencies were better from cultures that had been only two cycles in the induction medium (Tables 2 and 3).

Greenhouse establishment

Plantlets derived from somatic embryos (two cycles in induction medium) grew well when established in the greenhouse. The viability of the plantlets after four weeks and after eight weeks in greenhouse conditions is given in Table 4. First, it seemed that planting directly into the greenhouse might give better results than using the acclimatization room, but after eight weeks in the greenhouse no real difference in the survival of plantlets could be observed.

Plants and flowers formed were morphologically indistinguishable with mother plants (Figs 3A and 3B). After six months of cultivation only a few abnormalities were noticed (Table 5, Fig. 3C). Abnormalities could be found in the shape and size of the leaves. One plant produced flowers with altered shape. Usually the abnormal plants were smaller. After six and a half months cultivation (two cycles on different induction

Table 4. The survival of plantlets germinated from somatic embryos derived from anther material (two cycles in induction medium) after 4 and 8 weeks in greenhouse.

Induction medium[a]	Germination medium	Direct planting			Acclimatization		
		n	4 wk (%)	8 wk (%)	n	4 wk (%)	8 wk (%)
2,4-D + CM normal ammonium level	G1	9	89	33	9	33	22
	G2	54	61	46	63	54	40
2,4-D + CM increased ammonium level	G1	7	71	43	6	100	50
	G2	20	75	50	22	77	55

[a] 1.0 ml l^{-1} 2,4-D and 10% CM in both induction media.

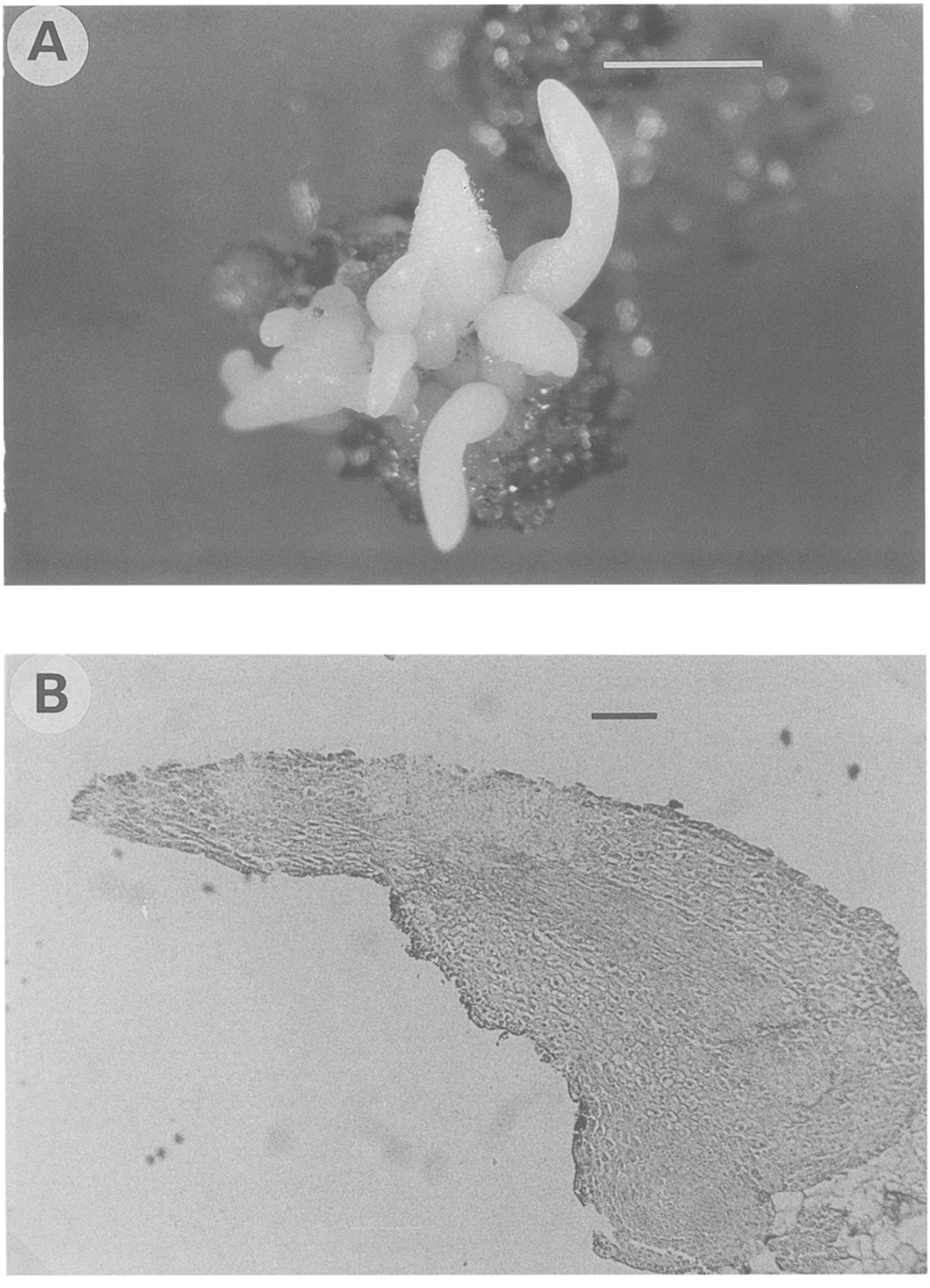


Fig. 1. (*A*) Somatic embryos developed from anther callus in basal medium containing 1,0 mg l^{-1} 2,4-D and 10% CM. (*B*) 10 μm thick section of a somatic embryo. Bar = 0,05 mm.

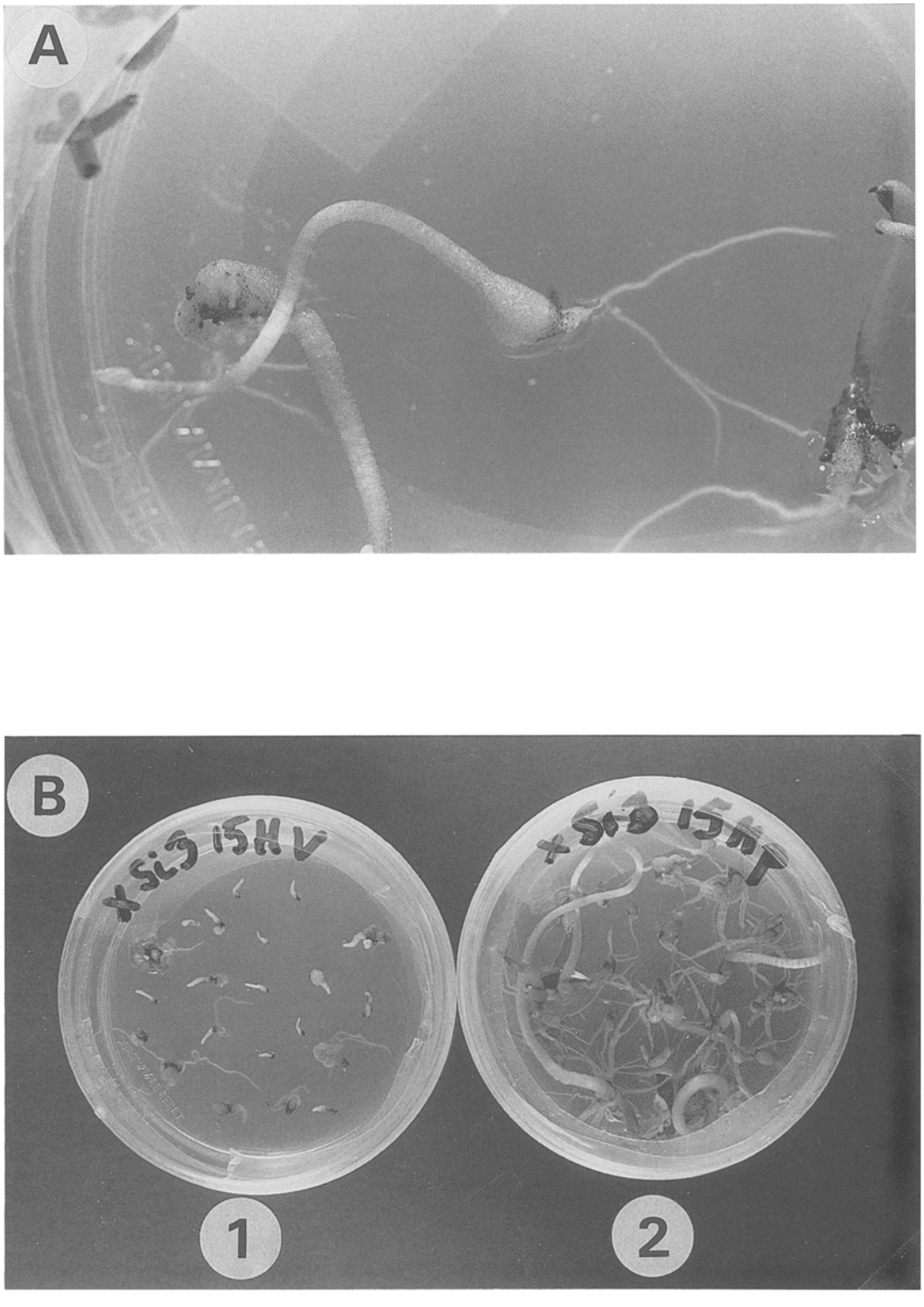
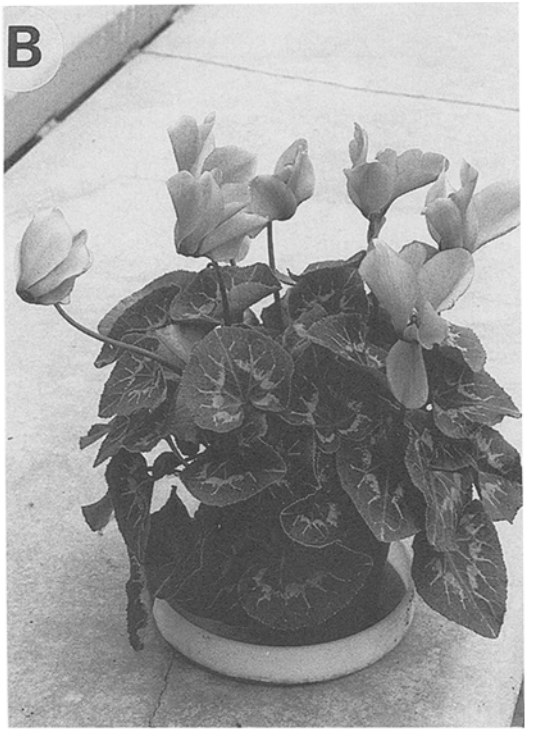


Fig. 2. (*A*) Germinating somatic embryo. (*B*) Somatic embryos germinated five weeks in light conditions (1) or in the dark (2).



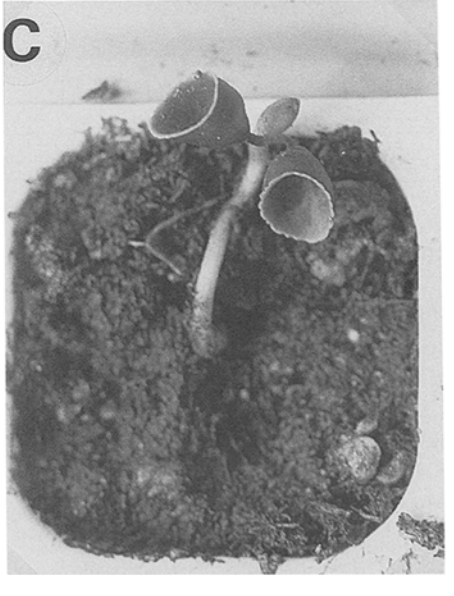


Fig. 3. Cyclamen plantlets originated from somatic embryos: (*A*) plants after 15 weeks growing in the greenhouse. (*B*) Flowering plant after 6 month growing in the greenhouse. (*C*) Abnormal cyclamen plantlet with tube like leaves after eight weeks in the greenhouse.

media) 19 flowering plants, 83 plants having flower buds and 8 abnormal plants were found in a total of 117 plants (based on combined data of all experiments started from somatic embryos derived from different explant materials).

The survival of plantlets that had been three cycles on induction medium was poor and the number of plants having abnormal leaves was quite high (Table 6). Plants germinated in the light had clearly more abnormalities than plants germinated in the dark. These plants have not flowered yet.

Table 5. Percentage of flowering plants and abnormal plants (started from somatic embryos developed from anther material after two cycles in induction medium) in the greenhouse after six and a half months cultivation time.

Induction medium[a]	Germination medium	n	Flowering plants (%)	Plants having flower buds (%)	Abnormal plants (%)
Direct planting					
2,4-D + CM	G1	3	33	67	0
normal ammonium level	G2	24	21	53	17
2,4-D + CM	G1	2	0	100	0
increased ammonium level	G2	10	10	80	10
Acclimatization					
2,4-D + CM	G1	2	0	100	0
normal ammonium level	G2	27	7	89	4
2,4-D + CM	G1	3	33	67	0
increased ammonium level	G2	12	25	67	8

[a] 1.0 ml l^{-1} 2,4-D and 10% CM in both induction media.

Table 6. The survival of plantlets from anther material (3 cycles in induction medium) germinated in medium G2 in light or in dark after 8 weeks in greenhouse and percentage of survived plants having abnormal leaves after 5 months cultivation in the greenhouse.

Induction medium[a]		n	Surviving plants (%)	Abnormal plants (%)
2,4-D + CM normal ammonium level	light	4	0	0
	dark	42	12	20
2,4-D + CM increased ammonium level	light	17	41	86
	dark	25	40	20

[a] 1.0 ml l^{-1} 2,4-D and 10% CM in both induction media.

Conclusions

The aim of our study was to examine the feasibility of somatic embryogenesis in cyclamen. Somatic embryos could be produced repeatedly through a callus phase from juvenile explant material in the presence of coconut milk in the medium. They germinated and developed in greenhouse conditions into normal plants. We did not optimize any of the process steps and currently the method is not effective enough for commercial micropropagation due to long culture times and low frequencies in embryo production, germination and plant establishment. The most critical step that requires further optimization is the induction phase. Currently it is far too inconsistent. Since we experimented with only one cultivar, the effect of genotype requires further studies. Also the genotypic and phenotypic fidelity of the process has to be ascertained. It is likely that the phenotypic aberrations observed were caused by tissue culture environment. Since no genetic analyses were made, genetic changes can not be positively excluded.

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