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Development of somatic embryos of *Cyclamen persicum* Mill. in liquid culture

Entwicklung somatischer Embryonen von *Cyclamen persicum* Mill. in Flüssigkultur

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Summary

For differentiation of somatic embryos of *Cyclamen persicum* Mill. in liquid culture a protocol was developed regarding the parameters "fraction of the inoculum", "period of liquid culture", "plating density of globular embryos" and "fraction of the globular embryos plated". Moreover development of somatic embryos of cyclamen was performed in bioreactor culture which improved the yield of globular and germinating embryos in comparison to culture in flasks. Starting from one liter of a suspension culture in growth-regulator-free medium within 38 weeks a yield of 27,000 acclimatized young plants in the greenhouse was calculated.

Zusammenfassung

Es wurde ein Protokoll für die Differenzierung somatischer Embryonen von *Cyclamen persicum* Mill. in Flüssigkultur erarbeitet. Dabei waren die Parameter „Größenfraktion des Inokulums", „Dauer der Flüssigkulturphase", „Dichte globulärer Embryonen beim Plattieren auf Festmedium" sowie „Größenfraktion der plattierten globulären Embryonen" Gegenstand der Untersuchungen.

Ergänzend dazu konnte durch den Einsatz von Bioreaktoren die Ausbeute an globulären, aber auch an keimenden somatischen Embryonen im Vergleich zur Kultur in Erlenmeyerkolben deutlich gesteigert werden.

Ausgehend von einem Liter einer Suspension in hormonfreiem flüssigen Medium wurde die erzielbare Anzahl akklimatisierter Jungpflanzen berechnet. Mit dem vorgestellten System lassen sich innerhalb von ca. 38 Wochen aus einem Liter dieser Suspension 27.000 Jungpflanzen im Gewächshaus etablieren.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid, germ.s.e. – germinating somatic embryos, glob.s.e. – globular somatic embryos, 2iP – 6-(γ,γ-dimethylallylamino)purine, pcv-packed cell volume.

Introduction

Vegetative propagation of *Cyclamen persicum* Mill. in vitro is of interest for clonal propagation of parents for F_1-hybrids as well as for mass propagation of single valuable elite plants. Regarding the latter purpose high regeneration rates are required, which can be achieved in this species by somatic embryogenesis (WICART et al. 1984, OTANI and SHIMADA 1991, KIVIHARJU et al. 1992, TAKAMURA et al. 1995, SCHWENKEL and WINKELMANN 1998). Liquid culture systems offer the advantage of being less labour-intensive and allowing automation in large scales.

Cyclamen can successfully be propagated *via* somatic embryogenesis from suspension cultures (KREUGER et al. 1995, WINKELMANN et al 1998). The protocol published by SCHWENKEL and WINKELMANN (1998) which was also applied in this study is a two-step-system: After cell proliferation of embryogenic suspension cultures in growth-regulator-containing medium the cells were transferred to growth-regulator-free gelled medium for development of somatic embryos.

Application of this method for commercial mass propagation requires automation by use of bioreactors. Bioreactor culture of embryogenic suspensions in growth-regulator-containing medium has already been established (HOHE et al. 1999a, b). Here we describe a protocol for development of cyclamen somatic embryos in liquid culture and the production of the globular somatic embryos in bioreactors.

Materials and Methods

Development of cyclamen somatic embryos in liquid culture (experiments A–D)

Embryogenic cultures of cell line 3738-14 were maintained as long-term suspension cultures by subculturing at two week intervals (WINKELMANN et al. 1998) in a modified MS-medium (MURASHIGE and SKOOG 1962). This medium contained the macro- and micronutrients half concentrated, except Fe-EDTA (full strength), 30 g/l sucrose, 2 g/l glucose, 0.5 mg/l nicotinic acid, 0.1 mg/l thiamine-HCl, 0.5 mg/l pyridoxine-HCl, 250 mg/l casein-hydrolysate, 100 mg/l inositol, 2 mg/l glycine, 2 mg/l (9.05 µM) 2,4-D and 0.8 mg/l (3.94 µM) 2iP as described by WINKELMANN et al. (1998). Development of somatic embryos was induced by transferring cell clusters to the same medium lacking growth regulators (SCHWENKEL and WINKELMANN 1998). Liquid cultures were maintained in Erlenmeyer flasks (culture volume: 1/5th of the total volume) on a rotary shaker (100 rpm) at 24 °C in the dark. In the case of subsequent culture of the embryos on gelled medium 3.7 g/l Gelrite were added and 40 ml of the medium

Table 1. Variation of parameters for improving development of somatic embryos in liquid culture
Variation der Parameter in den Versuchen zur Optimierung der Entwicklung somatischer Embryonen in Flüssigkultur

Experiment	parameter	variation
A	size fraction of the inoculum	[µm]: 0–200; 200–500; 500–1000
B	period of liquid culture	[weeks]: 1; 2; 3; 4; 5; 6
C	plating density of the embryos	[glob.s.e./jar]: 25; 50; 100; 200; 400
D	size fraction of the globular embryos plated	[µm]: 200–500; 500–800; 800–1000, > 1000

were filled in 210 ml-glass jars (diameter: 6.5 cm) with twist-off-caps. These cultures were kept at 18 °C in the dark.

For development of somatic embryos in liquid culture four independent experiments (A–D) were performed. In each of the experiments one of the following culture parameters was varied: size fraction of the inoculum, period of liquid culture, plating density of the embryos and size fraction of the embryos plated. Table 1 shows the variation of the parameters in the four experiments. For the remaining parameters, not under investigation in the respective experiments, the following standard conditions were chosen:

Size fraction of the inoculum:	200–500 µm
Period of liquid culture:	3 weeks
Plating density of glob.s.e.:	not adjusted, except exp. D: 100 glob.s.e./jar
Size fraction of glob.s.e. plated:	no fractionation, except exp. C: 800 –1000 µm

In all experiments cell clusters from two week old suspension cultures in growth-regulator-containing medium were inoculated at a density of 0.5 % packed cell volume (pcv) in liquid medium without growth regulators (experiments A and B: three flasks per treatment, experiment C: one bioreactor, experiment D: two flasks). After a defined period of development in liquid medium the globular embryos were plated (1 ml/jar) on solid medium lacking growth regulators for germination (experiment A: 10 jars/treatment, experiment B: not plated, experiments C and D: 20 jars/treatment).

The experiments were evaluated by determining the number of developing globular somatic embryos (glob.s.e.) in liquid culture or germinating somatic embryos (germ.s.e., defined as embryos with roots and a cotyledon longer than 5 mm) 10–13 weeks after plating, respectively. The differences between the number of germinating somatic embryos in experiments A and D were tested statistically by a Tukey-Test (Proc GLM, SAS). In experiment B size, number and the standard deviation of the number of globular somatic embryos are given. The average diameter of the globular somatic embryos in this experiment has been determined by measuring 50 randomly chosen embryos. In experiment C differences between the number of germ.s.e. could not be statistically tested, because the original data (germ.s.e./jar) were transformed to obtain the "number of germ.s.e./100 glob.s.e.".

Bioreactor culture

A bioreactor with a working volume of 2 l (Applikon, Schiedam, The Netherlands) was equipped with a bubble free aeration system via silicone tubes (total length: 2 m, diameter: 4 mm, wall thickness: 0.4 mm) and a large blade stirrer running with 25 rpm. The setpoint of the O_2 control was 10 % of full O_2 saturation, CO_2 was removed by conducting the aeration gas through 3N NaOH (HOHE et al. 1999a). The cultures in the bioreactor as well as in three control flasks on a rotary shaker (100 rpm) were kept at 24 °C under low light conditions (approximately 100 lux, "Narva neutralweiß 20", 16 h day). The bioreactor and the flasks were inoculated with a two week old suspension culture of cell line 3738-14 (fraction: 200–1000 µm) with a density of 0.5 % pcv (growth-regulator-free medium, see above). Twice a week the number of globular somatic embryos was determined.

Results and Discussion

Development of cyclamen somatic embryos in liquid culture

Experiment A (size fraction of the inoculum): Following three weeks development in liquid culture the numbers of globular somatic embryos per milliliter were 184, 238 and 164 for the inoculum size fractions 0–200 µm, 200–500 µm and 500–1000 µm, respectively. Ten weeks after plating the number of germinating somatic embryos was counted as 58, 44 and 42 per jar, respectively, for each category of inoculum size (fig. 1). These differences were statistically not significant ($\alpha = 0.05$, Tukey-Test). KREUGER et al. (1995) and WINKELMANN et al. (1998) obtained maximum numbers of somatic embryos of cyclamen using the fractions 150–300 µm and 200–500 µm, respectively. The difference in response to the results reported here might be due to either the use of different cell lines or the culture conditions used for somatic embryo development, as these authors plated directly on gelled growth-regulator-free medium without development in liquid culture.

Experiment B (period of liquid culture): Globular embryo production in liquid cultures increased during the first three weeks of culture up to an average of 290 glob.s.e./ml (fig. 2). During the following three weeks this number did not change markedly. The average diameter of the embryos was increasing until two weeks after inoculation, when it reached 508 µm. Embryo growth arrest after two to three weeks was probably caused by exploitation of the medium, as described by HUANG et al. (1993) in somatic embryos of *Daucus carota* using batch culture. In their studies both the number of embryos and the embryo development reached a stationary phase, when the carbohydrate supply of the medium was exploited.

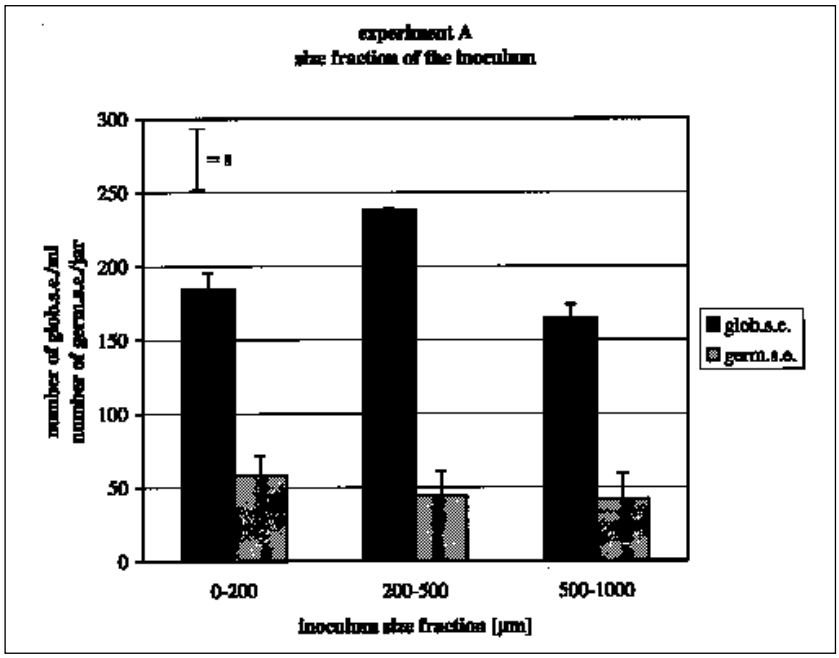
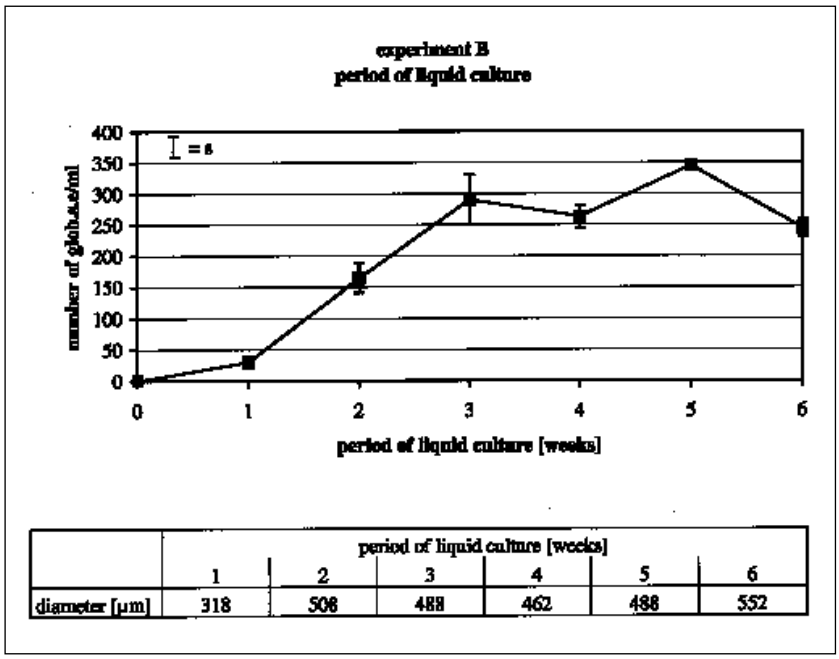


Fig. 1. Number of globular somatic embryos after three weeks development in liquid culture and number of germinating embryos ten weeks after plating in relation to the size fraction of the inoculum
Anzahl globulärer somatischer Embryonen nach dreiwöchiger Entwicklung in Flüssigkultur und Anzahl keimender somatischer Embryonen zehn Wochen nach dem Plattieren in Abhängigkeit von der Größenfraktion des Inokulums



	period of liquid culture [weeks]					
	1	2	3	4	5	6
diameter [µm]	318	506	488	462	486	552

Fig. 2. Number and size of globular somatic embryos in relation to the period in liquid culture
Anzahl und Größe globulärer somatischer Embryonen in Abhängigkeit von der Dauer der Flüssigkulturphase in hormonfreiem Medium

Experiment C (plating density of the embryos): Up to 200 glob.s.e./jar the plating density had no marked effect on the number of germinating somatic embryos 13 weeks after plating, varying between 22 and 27 germ.s.e. per 100 glob.s.e. plated. In contrast only 12 out of 100 globular embryos germinated when plated with a density of 400 glob.s.e./jar (fig. 3). This might be an effect of competition for nutrients or, as reported by OSUGA et al. (1997) and HIGASHI et al. (1998) of an inhibitory conditioning factor which is produced at high embryo densities.

Experiment D (size fraction of the globular embryos plated): Eleven weeks after plating the highest number of germinating embryos per jar was obtained from globular embryos of the fraction 800–1000 µm. This number was significantly lower for smaller and larger fractions of globular embryos (fig. 4). As all variants were evaluated at the same date the lower number of germinating embryos from smaller fractions of globular embryos might be an effect of synchronization of the embryo development by fractionation, thus selecting less developed globular embryos in the smaller fractions. The low yield of germinating embryos from larger fractions might be due to a higher number of malformed embryos in the fraction > 1000 µm which did not germinate.

From these results the following protocol for development of somatic embryos of cyclamen in liquid culture was deduced:

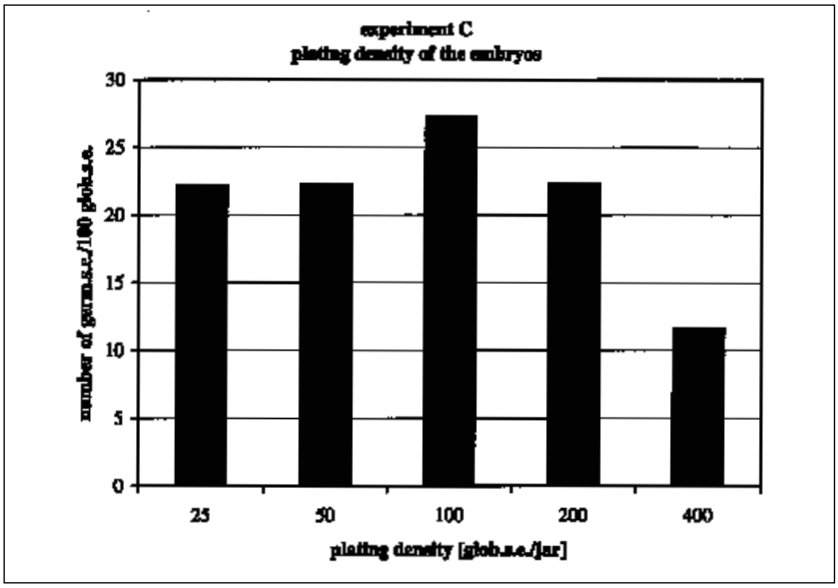


Fig. 3. Effect of the plating density of globular somatic embryos on the number of germinating embryos evaluated 13 weeks after plating (Because of transformation of the data no standard deviation was calculated)
Einfluss der Plattierungsdichte der globulären somatischen Embryonen auf die Anzahl keimender Embryonen (Auswertung erfolgte 13 Wochen nach dem Plattieren. Aufgrund der Transformation der Daten konnte keine Standardabweichung errechnet werden.)

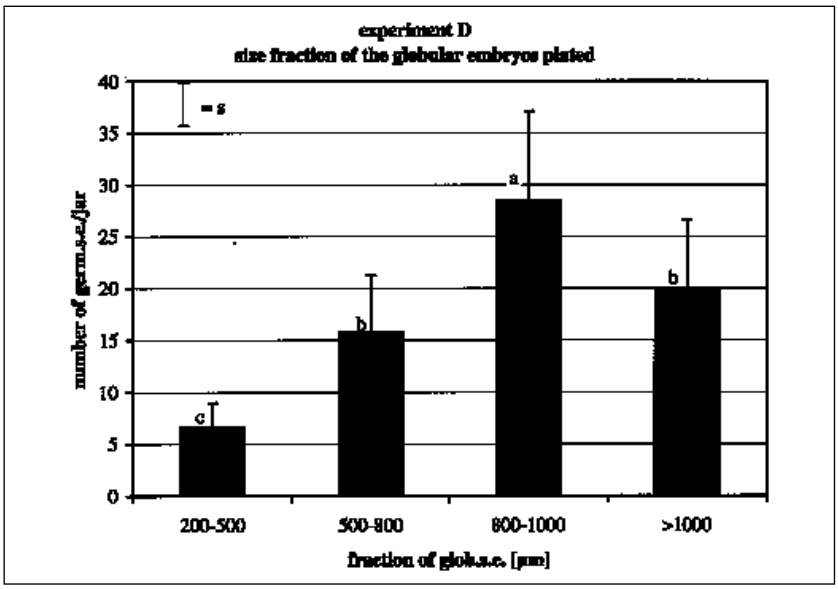


Fig. 4. Number of germinating somatic embryos in relation to the size fraction of the plated globular embryos evaluated eleven weeks after plating (Statistically significant differences are indicated by different letters, $\alpha = 0.05$, Tukey-Test)
Anzahl keimender somatischer Embryonen in Abhängigkeit von der Größenfraktion der plattierten globulären Embryonen (Auswertung erfolgte elf Wochen nach dem Plattieren. Statistisch signifikante Unterschiede sind mit unterschiedlichen Buchstaben gekennzeichnet, $\alpha = 0.05$, Tukey-Test.)

Size fraction of the inoculum: no fractionation
Period of liquid culture: 3 weeks
Fraction of plated embryos: 800–1000 µm (at least > 500 µm)
Plating density: max. 200 glob.s.e./jar

Bioreactor culture

After three weeks development more than 500 glob. s.e./ml were obtained from bioreactor culture. For the culture in flasks this number was only slightly above 300 glob.s.e./ml (fig. 5). The higher yield of globular somatic embryos after bioreactor culture was possibly an effect of better supply of the embryos with nutrients and oxygen due to effective stirring and aeration.

The yield of a propagation system of cyclamen via somatic embryogenesis using liquid culture is shown in fig. 6. By determining the number of globular embryos ($n_{total} = 150$) in the different size fractions after three weeks development in bioreactor culture and using the germination rates which were obtained in experiment D the yield of germinating embryos was calculated. Thus starting with 500,000 glob.s.e./l of a bioreactor culture (fig. 5) 52,000 germinating embryos could be obtained. On average 65 % of these germinating embryos developed into plantlets, of which 80 % (SCHWENKEL and WINKELMANN 1998) survived the acclimatization to greenhouse conditions. Thus a yield of about 27,000 acclimatized young plants in the greenhouse was calculated starting from one liter of a suspension culture in growth-regulator-free medium.

Thus not only the propagation of embryogenic suspension cultures in growth-regulator-containing medium (HOHE et al. 1999a, b), but also the development of somatic embryos of cyclamen can successfully be performed in liquid culture. Moreover the yield of the propagation system can be improved by culture under controlled environmental conditions in bioreactors. The production of high numbers of globular somatic

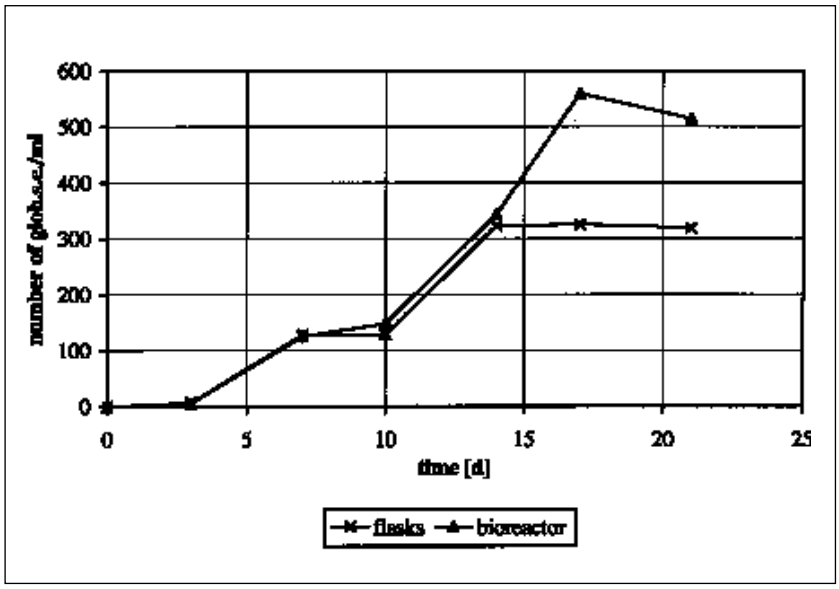


Fig. 5. Number of globular somatic embryos developing in growth-regulator-free medium in flasks and bioreactor culture
Entwicklung globulärer somatischer Embryonen in hormonfreiem Flüssigmedium in Erlenmeyerkolben und im Bioreaktor

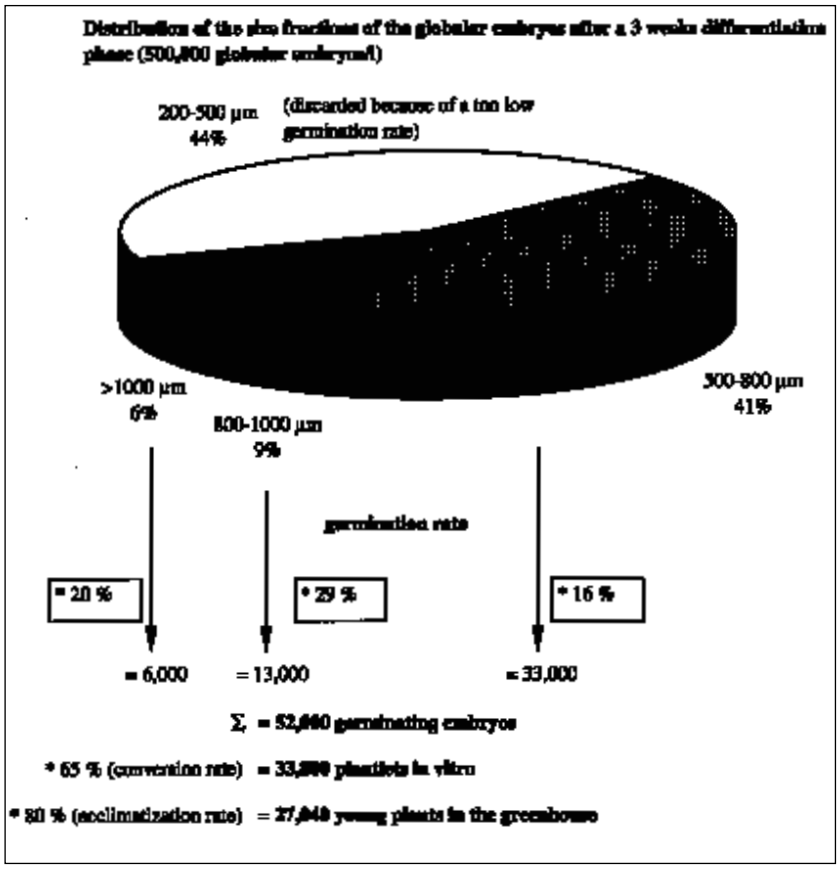


Fig. 6. Yield of young plants in the greenhouse from 1 l of suspension culture in growth-regulator-free medium
Ausbeute an Jungpflanzen im Gewächshaus ausgehend von einem Liter einer Suspension nach dreiwöchiger Kultur in hormonfreiem Medium

embryos is the prerequisite for the production of artificial seeds (REDENBAUGH et al. 1987), which could be of interest for cyclamen as a plant with high priced seed.

These data clearly demonstrate, that mass propagation of cyclamen *via* somatic embryogenesis is possible. However, the high propagation rates reported here have been obtained only with few genotypes so far. The inheritance of the trait "regeneration ability" is under investigation with the aim of introducing this character into commercially important varieties in breeding programmes (PUESCHEL 2000).

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