Somatic Embryogenesis of *Cyclamen persicum* Mill. in Bioreactors

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Propagation experiments were carried out in a series of six self-constructed and self-built bioreactors controlling temperature, oxygen level, stirring speed/ direction, and light quality. The bioreactors are all fully automated and computer controlled. They provide excellent experimental conditions for controlled environments of the liquid cultures. Light quality, the factor investigated in these experiments, showed that blue light had a positive effect on production of proembryogenic masses (PEM) and somatic embryos in hormone-free medium. The experiments also showed that somatic embryogenesis responded to CO_2 level. Higher CO_2 levels were correlated with increased PEM, but the CO_2 level was not controlled, only measured. Ethylene concentration is believed to be another important factor, but was not measured in these experiments. Comparisons between cultures grown in bioreactors and suspension cultures in flasks on shakers clearly demonstrated that bioreactors have an enormous effect on cell proliferation and embryo formation. However, bioreactors require higher technical skills of the operator, as well as extreme care to prevent microbial contamination of the cultures, since the loss of 2 liters of suspension is a substantial loss in a commercial setting, compared to a flask of 100 ml.

INTRODUCTION

Today the commercial propagation of *Cyclamen persicum* Mill. is through seeds, preferably by F_1-hybrid seeds which are relatively expensive. *Cyclamen* is used as a pot plant and as a bedding plant (southern Europe in winter) and is one of Europe's largest flower crops, especially in Germany, Italy, and France. Hybrid seed production requires pure parent lines produced by six to seven generations of selfing. Even F1-hybrid seeds are sometimes not uniform enough in production. *Cyclamen* suffers from inbreeding depression which can show up as a sudden breakdown of viability during inbreeding. Vegetative propagation of *C. persicum* has so far been performed only on a small scale due to a number of obstacles. Tuber division yields low numbers as a meristem on each part is required to produce shoots. In vitro techniques have only recently become efficient enough, even though the first report on in vitro propagation was published more than 40 years ago (Mayer, 1956). However, the work published since Mayer (1956), such as Geier et al. (1983), Hoffman and Preil (1987), or Schwenkel and Grünewaldt (1991), has not solved the fundamental problems for an in vitro mass propagation scheme for *Cyclamen*.

Somatic embryogenesis (SE) would be an ideal way to produce masses of uniform "clonal seeds". One of the advantages of SE compared to axillary or adventitious bud propagation techniques, is that embryos are bipolar structures with functional

shoot and root meristems (Preil, 1994). Also, propagation through SE seems to give less problems with somaclonal variation than other in vitro techniques, especially techniques based on regeneration from callus formation (Geier et al., 1992). There seems to be a selection towards normal plants with diploid nucleus through SE in poinsettia, even though suspension cultures contain high variation in ploidy level. Recent publications have reported SE on solid medium (Kiviharju et al.,1992; Takamura et al., 1995; Schwenkel and Winkelmann, 1995). Kreuger et al. (1995) successfully produced masses of SE from liquid cultures with no variation in ploidy level.

The process of SE production can be divided into several steps: initiation of embryogenic cultures, proliferation of pro-embryogenic masses (PEMs), development of SE, maturation, and germination of SE into plants. The first three stages take place in liquid medium, as described by Kreuger et al. (1995). Large-scale production of *Cyclamen* embryos in liquid cultures would be greatly enhanced by the use of bioreactors. Bioreactors are controlled growth vessels for liquid in vitro cultures under sterile conditions.

Schwenkel and Winkelman (1995; 1998) in Germany have developed a protocol for SE in *Cyclamen* using ovaries for initiation of embryogenic callus. Through COST 822, working group 2, our laboratory has been given two of the German genotypes and their protocol for production of SE on solid medium. Our contribution to the COST 822 cooperation in the Cell and Tissue Culture Group of the Agricultural University of Norway is the development of methods for SE in bioreactors, investigation of light quality effects, and the protein changes occurring during development from single cells/clusters to SE in *Cyclamen*. This research is possible because of our six identical bioreactors built by our Department of Agricultural Sciences (Heyerdahl et al., 1995) which enabled factorial experiments in bioreactors and our previous work on embryo specific protein changes in birch suspension cultures (Hvoslef-Eide and Corke, 1997).

MATERIALS AND METHODS

The six identical bioreactors in the Cell and Tissue Culture Group enables us to accurately monitor and control of growth conditions in liquid cultures. Our computer-controlled bioreactors are equipped for on-line measurement and control of temperature, agitator speed and direction, pH, and pO_2. Furthermore, we can vary light conditions (daylength, quantity, and quality). Bioreactors may be compared with a phytotron, a greenhouse compartment where the physical environment is under full control. The bioreactors in our laboratory have been especially designed for plant cell growth with low shear forces and gentle agitation to produce as little stress as possible and support proliferating cultures with high viability. Using bioreactors for plant propagation is a great challenge with many pitfalls along the road (Heyerdahl et al., 1995). Worst case scenario is to believe fully in the measurements the computer gives you, without cross checking. All the parameters measured may be wrong and give a false answer far from reality. Having six identical bioreactors enables us to run factorial experiments, as well as interchanging the bioreactors when repeating experiments. The reactors are based on batch cultures, which means that we dilute the cultures every 7th or 8th day, after sampling and cell counts. Oxygen is supplied through thin-walled (0.2 mm) silicone tubes which the gas diffuses through into the suspension dependent upon concen-

trations. This can be done with nonsterile air enriched with oxygen because the pore sizes through the tubes are small enough to filter the microbes, but allow gas exchange. Temperature is regulated in a waterbath, where cold water is added, or heat is provided. The pH is regulated by acid/alkali supplement through sterile filters. Each bioreactor is separately controlled by a computer, and each parameter may be varied with 1-h intervals over 24 h.

The advantages of using bioreactors compared to Erlenmeyer flasks on rotary shakers are: more accurate control of temperature, much improved control of oxygen, the possibility of controlling or monitoring pH, and the ability to grow larger volumes. The disadvantages may be the loss of larger volumes in case of contaminations, and it is more difficult to exchange all medium when removing auxin to induce SE. In spite of this, bioreactors provide an opportunity to produce large volumes with less labor, although they require greater accuracy and higher skills (computer and electronics).

The cultures and the protocol originated from Germany through the COST 822 cooperation. Laboratories in Germany, Italy, Switzerland, The Netherlands, France, and Norway all work on the same genotypes, and the same protocols for maintaining callus cultures and initiating suspensions (liquid cultures). The callus cultures are maintained in jars, in the dark, at 25C and are subcultured every 4 weeks.

Our liquid cultures are initiated by inoculation of 7 g embryogenic callus into a 250 ml Erlenmeyer flask containing 100 ml medium with auxin, placing on a rotary shaker (100 rpm) at 25C for 7 days, thereafter sieving through a mesh of 500 µm, and inoculating into one bioreactor. The bioreactor temperature set point is 25C and growth occurs in darkness for 7 days. When proliferating nicely, this bioreactor culture is used as inoculum for the other bioreactors in the experiments and provides identical starting material. To enhance SE production, the suspensions are sieved through sieves, collected on a 75-µm mesh, and flushed with auxin-free medium prior to inoculating the bioreactors. The bioreactors are autoclaved with 200 ml medium with/without hormones, cooled to setpoint temperature and calibrated for oxygen and pH in advance.

The light quality experiments were performed with four light qualities; darkness, daylight (OFT bioLIGHTSYSTEMS), blue filter (Strand filter No 419, primary dark blue), and red filter (Strand filter No 406, primary red). All light treatments were given the same light quantity; 5 µmol $m^{-2}s^{-1}$ for 18 h. The bioreactors were kept constantly at 25C, 100% saturation of oxygen (calibrated with 100% air through medium at 25C, without plant cells for 24 h), 30-rpm stirring speed, and change of stirring direction every 10 sec.

Plant cell growth was monitored through cell counts, packed cell volume (PCV) and proliferation of PEM. The latter two were done by sampling 10 ml aliquot of suspension, letting it sink in a pipette for 10 min, and reading the volume of PCV/PEM directly in milliliters.

RESULTS AND DISCUSSION

Factorial experiments revealed differences between bioreactors with regard to cell growth and embryo production. We, therefore, had to develop as uniform conditions in the bioreactors as possible and investigated the possible causes for the variation. By providing darkness in all bioreactors, and keeping the temperature, oxygen content, and stirrer speed constant, we discovered that the gas exchange varied from

bioreactor to bioreactor. There was a high degree of correlation between gas exchange and embryo production (Table 1). Bioreactor 1 had a high gas exchange, with depletion of CO_2, and poor growth/PEM over the period from Day 9 till Day 16. The other three bioreactors had more CO_2 trapped and had better embryo production over the same period. These results could be due to high CO_2, or possibly ethylene accumulation. We did not measure ethylene and this provides an uncertainty. Kvaalen and von Arnold (1991) have published results for Norway spruce clearly showing the importance of CO_2 and oxygen, but have also provided data on *the importance of ethylene for embryo formation.*

Table 1. Measured CO_2 level in head space of bioreactors compared to packed cell volume (PCV) of *Cyclamen persicum* Mill. under somatic embryogenesis inductive conditions (hormone free medium).

Bioreactor no	Gas flow (ml min^{-1})	CO_2 level (ppm) 9 days	CO_2 level (ppm) 16 days	PCV % 9 days	PCV % 16 days
1	206 + 20.0	0.10	0.08	2.0	2.0
3	186 + 7.1	0.70	0.90	2.7	3.7
5	265 + 24.0	0.65	0.90	2.4	3.7
6	205 + 23.3	0.50	0.71	2.4	3.2

Table 2. The effect of light quality on *Cyclamen* suspension cultures in bioreactors and Erlenmeyer flasks on PCV in percent in two different experiments (I and II), number of embryos per 5 ml and number of globular embryos per 15 ml.

Light quality	PCV % I	PCV % II	Embryos per 5 ml (no.)	Globular embryos per 15 ml (no.)
Dark	2.2	3.0	100 + 34.5	2
Daylight	6.3	1.6	55 + 8	0
Red	2.3	2.9	69 + 25	1
Blue	8.2	1.4	71 + 16	5
Dark (flask)	-	0.1	0	0
Light (flask)	-	0.1	0	0

A plausible explanation for the difference in gas exchange between bioreactors, despite the fact that the gas flow was equivalent, could be the age and thereby number of autoclave treatments of the silicon tubes. They were only changed if a leak was observed. We tried new sets of tubing, autoclaved them several times, measured the gas exchange between each autoclave sterilization and found that the gas exchange deteriorated slowly. This is yet another factor to take into consideration when performing experiments in bioreactors, besides all the other pitfalls described by Heyerdahl et al. (1995).

In medium with hormones, light quality effects on *Cyclamen* cultures were difficult to interpret because the internal variation was too large. Earlier results in birch and *Begonia* (Hvoslef-Eide and Sæbø, 1991) have shown that red light was beneficial compared to darkness or blue light. However, all the cultures grew better in bioreactors compared to Erlenmeyer flasks; the doubling time of *Cyclamen* in bioreactors was 2 days, while Erlenmeyer flask cultures doubled in 1 week. This was similar to results for poinsettia suspension cultures (Preil, 1991; Preil and Beck, 1991).

When the medium lacked hormones and the aim was production of SE, Erlenmeyer flasks hardly gave any growth at all and no embryos were produced in 4 weeks (Table 2). Two experiments were contradictory regarding PCV results after 4 weeks. However, when counting embryos beyond the globular stage after 4 weeks, blue light clearly gave better and more advanced embryos. When taking previous experiments with birch (unpublished results) into consideration, as well as other literature (Chee, 1986), we would recommend blue light to enhance SE.

Our theory on the effect of light quality is that blue and red light resemble hormone effects, either through direct influence on production and/or degradation or hormone sensitivity (Kendrick and Kronenberg, 1994). In birch we could use red light to replace auxin in the medium, and obtain similar growth, while blue light triggered embryo formation (unpublished results). Chee (1986) states that blue light can enhance IAA destruction and shoot growth through IAA oxidation. Red light is reported to stimulate the formation of the IAA oxidation inhibitor quercetin (Mumford et al., 1961) and will therefore favor cell growth, but inhibit embryogenesis. It is well established that a strong auxin is a prerequisite for embryogenesis, but it must be removed to allow embryo development (Fujimura and Komamine, 1980). Blue light would, therefore, be beneficial to stimulate embryo development after red light has enhanced auxin-promoted cell growth. This would explain why we obtained the best quality embryos under the blue light regime. It is plausible to believe that removal of auxin/blue light treatment may well inactivate genes producing proteins that are inhibitory for embryogenesis, since removal of auxin results in proteins disappearing both in carrot (Kiyosue et al., 1991) and birch (Hvoslef-Eide and Corke, 1997).

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