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CO_2 accumulation in bioreactor suspension cultures of *Cyclamen persicum* Mill. and its effect on cell growth and regeneration of somatic embryos

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Abstract CO_2 accumulation in different culture systems containing embryogenic cell suspension cultures of cyclamen (*Cyclamen persicum* Mill.) was analyzed. In bioreactors equipped with a bubble-free or a bubble aeration system, CO_2 mole fractions in the gas phase of more than 10% were determined whereas in Erlenmeyer flasks, CO_2 mole fractions were below 2%. CO_2 accumulation in bioreactors was severely growth inhibiting in comparison to the flasks. By removing CO_2 in the aeration gas of a bubble-free aerated bioreactor, cell growth comparable to that in flasks was achieved. The regeneration ability of cell suspensions after being cultured in bioreactors with CO_2 accumulation was better than those after culture in bioreactors without CO_2 accumulation or in flasks.

Key words Carbon dioxide · Cell suspension culture · Bioreactor · *Cyclamen persicum* Mill. · Somatic embryogenesis

Abbreviations *FDA* Fluorescein diacetate · *pcv* Packed cell volume · *PEM* Proembryogenic mass · pO_2 Relative O_2 partial pressure · *TCD* Thermal conductivity detector

Introduction

Micropropagation of cyclamen (*Cyclamen persicum* Mill.) via somatic embryogenesis offers a means of vegetative propagation of this species, which is of interest for clonal multiplication of seed parents and for mass propagation of selected single plants. As micropropagation on solid media is very labor intensive, liquid cultures have been established (Kreuger et al. 1995; Winkelmann et al. 1998), which enable more rapid and uniform cell growth and are therefore a prerequisite for automation. Our aim of bioreactor culture was to study the effects of different growth factors in order to be able to control cell growth and differentiation.

One important growth factor is the gas composition in the culture vessels. Whereas O_2 is usually controlled in bioreactor culture, less attention is paid to CO_2 in heterotrophic cultures. For cultures of *Catharanthus roseus*, amounts of up to 2% CO_2 have been found to be beneficial for cell growth (Maurel and Pareilleux 1985; 1986, Ducos and Pareilleux 1986; Hegarty et al.1986). On the other hand, CO_2 accumulation has also been found to have a negative effect on cell growth in suspension cultures of *Picea glauca* (Kumar et al. 1989) and *Prunus persica*× *amygdalus* GF 677 (Marino et al. 1995). Little information is available about a possible negative effect of CO_2 accumulation in plant cell suspension cultures in bioreactors. Preil (1991) reported that this might occur especially in bioreactors equipped with bubble-free or microsparging aeration systems.

The aim of this study was to determine the amount of CO_2 accumulation in embryogenic suspension cultures of cyclamen in flasks and bioreactors with different aeration systems and its impact on cell growth and subsequent differentiation of somatic embryos.

Materials and methods

Suspension cultures in Erlenmeyer flasks and bioreactors

The experiments were performed with two cell lines: 3738-14, derived from an I_1-plant of the cultivar 'Purple Flamed' (diploid) and 3736-12, derived from an I_1-plant of the cultivar 'Leuchtfeuer' (tetraploid). Suspension cultures of 3738-14 were established as described by Winkelmann et al. (1998), suspension cultures of 3736-12 as described by Kreuger et al. (1995) but starting from somatic embryos instead of seedlings. Maintenance of long-term suspension cultures of both cell lines was performed as described by Winkelmann et al. (1998).

All experiments were started with an inoculum density of 2% pcv (packed cell volume). Cultures in 3 Erlenmeyer flasks sealed with

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caps of aluminium foil on a rotary shaker (100 rpm) served as a control in all experiments. The cultures in flasks and bioreactors were incubated at 24 °C under low light conditions.

The bioreactor equipment (Applikon, Schiedam, The Netherlands) was consisting of 4 identical 2 l-vessels. These were equipped either with a bubble aeration system *via* a sintered metal, run with pure oxygen, or with a bubble-free aeration system *via* silicone tubes (length: 1.7 m, ∅: 4 mm, thickness: 0.4 mm), run with oxygen enriched air. The latter system was closed, so that the aeration gas was circulating. By oxygen addition to this aeration gas pressure was built up inside the tubes, which was limited by a pressure regulator to a maximum of 0.5 bar. The relative O_2 partial pressure (pO_2) in the liquid phase was adjusted to 10% pO_2 (calibration of the pO_2 electrodes with pure oxygen), since 10% pO_2 had proved to be not growth limiting in former experiments (data not shown). Stirring was performed by large blade stirrers (25 rpm).

Sampling

Cell growth

The packed cell volume (pcv) was determined by taking samples of a defined volume, which were centrifuged (700 *g*, 5 min) in graduated tubes. The data given for cell growth in flasks are the mean of three samples, each from one flask. For determining cell growth in the bioreactors, three samples were taken from each.

Cell viability

Cell viability was determined by FDA staining according to Widholm (1972) using the scheme for cell clusters described by Winkelmann et al. (1998) with a slightly changed method of calculation: cell clusters were grouped into class I (fully viable), II (2/3 viable), III (1/3 viable), and IV (dead). About 100 clusters were analyzed and the viability calculated as follows: viability (%) = 100 ($n_I \cdot 1 + n_{II} \cdot 2/3 + n_{III} \cdot 1/3 + n_{IV} \cdot 0)/n_{total}$.

CO_2 mole fraction (gas phase)

Gas samples of 4 ml were taken from the headspace of the flasks and the headspace and the aeration tubes of the bioreactors. The samples were analyzed using a gas chromatograph (Autosystem XL, Perkin Elmer, Überlingen, Germany) equipped with a 10-m-long Porapak Q column and a thermal conductivity detector (TCD). The carrier gas was helium (25 ml/min). The temperatures were 150 °C and 60 °C for the TCD and the oven, respectively. The data given for the CO_2 mole fractions are the mean of two samples each. In experiment 1, samples were taken at the end of the culture period, in experiment 2, twice a week.

Regeneration of somatic embryos

Seventeen days after inoculation in experiment 2, cell suspensions were sieved to obtain the fraction 200–500 µm, which was adjusted to a density of 1% pcv (cell line 3738-14) and 0.5% pcv (cell line 3736-12), with liquid hormone-free medium and plated (1 ml/jar, 20 replicates) on solid hormone-free medium according to Winkelmann et al. (1998). The cultures were incubated at 18 °C in the dark. The number of normally developed somatic embryos per jar was counted 10 (cell line 3736-12) and 13 (cell line 3738-14) weeks after plating.

Experimental design

Experiment 1: CO_2 accumulation in the different aeration systems

One bioreactor was equipped with each aeration system (sintered metal and silicone tubes, respectively). CO_2 accumulation and cell growth in the bioreactors were compared to the control in Erlenmeyer flasks.

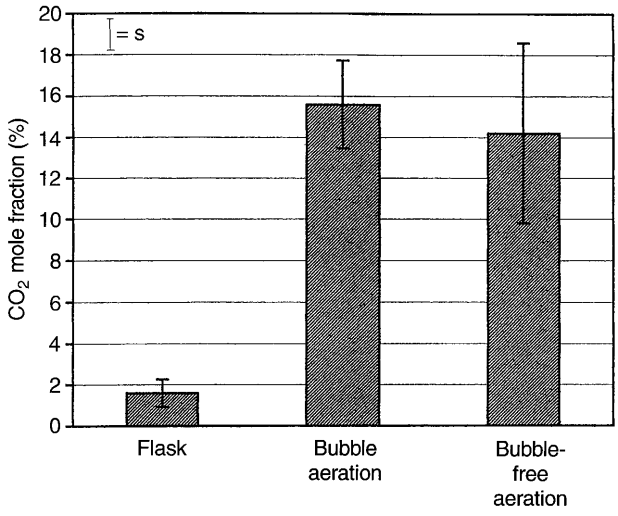

Fig. 1 Experiment 1: CO_2 mole fractions in the headspace of flasks and bioreactors equipped with different aeration systems

Experiment 2: effect of CO_2 on cell growth and differentiation

The bioreactors were equipped with the bubble-free aeration system. In this system, a bottle was installed either filled with water or with 3 N NaOH to remove CO_2 from the aeration gas. CO_2 accumulation, cell growth, and regeneration ability were compared to the control in Erlenmeyer flasks.

Experiment 1 was repeated twice with cell line 3738-14. Experiment 2 was repeated twice with cell line 3738-14 and once with cell line 3736-12. As all results were highly reproducible for both experiments, only the data of one replication are presented.

Results and discussion

Experiment 1: CO_2 accumulation in the different aeration systems

The CO_2 mole fraction in the headspace gas of the different culture vessels is shown in Fig. 1. The CO_2 accumulation in flasks was limited to about 2%, indicating that there was considerable gas exchange through the aluminum caps. Similar results with aluminum-capped flasks were obtained by Marino et al. (1995) with *P. persica×amygdalus* GF 677 cell suspension cultures. These authors reported a slight CO_2 accumulation with aluminum caps in comparison to cotton plugs, where CO_2 accumulation was negligible.

CO_2 accumulation in the bioreactors was about ten times higher than in flasks (Fig. 1), although neither aeration system was completely closed, as the headspace was in contact with the room air through an air filter and considerable amounts of fresh air were pumped into the headspace during sample taking. There was no difference regarding CO_2 accumulation between the bioreactors equipped with different aeration systems. Although the bubble aeration is an open system, where waste air is stripped out, CO_2 accumulation was similar to that in the

bioreactor with the closed bubble-free aeration system, where aeration gas is only released when the pressure inside the aeration tubing exceeds 0.5 bar. This was probably due to the low gas volume which was necessary to run the bubble aeration (oxygen supply summed up over the culture period of 26 days: 0.82 l), as this system was run with pure oxygen and a sintered metal sparger to avoid excessive bubbling, which can cause foaming problems and callus growth above the liquid surface. These results correspond with information given by Preil (1991) and Ozturk (1996). Preil (1991) mentioned that the use of silicone tubing and microsparging aeration systems might cause problems with CO_2 accumulation. Ozturk (1996) reported that the efficiency of CO_2 removal in sparging systems diminished with decreasing bubble size and that the problem of CO_2 accumulation was even more pronounced in silicone tubing aeration systems because there the mass transfer coefficient of CO_2 was about four times lower than that of O_2.

When considering the absolute values of the measured data of the described experiments, one has to take into account that the CO_2 mole fraction was measured in the gas phase, which is in contact with the suspension, not in the suspension itself. As CO_2 is continuously produced by the cells in the culture and as the headspace mass transfer rate is far too low to remove the endogenously produced CO_2 (Payne et al. 1990), the CO_2 mole fraction in the headspace gas is always lower than the dissolved CO_2 tension. Thus CO_2 accumulation in the bioreactors is probably underestimated by the given data.

The growth curves of the suspension cultures corresponding to the data on the CO_2 accumulation are given in Fig. 2. In the flasks, the cells grew to a density of 30% pcv within 4 weeks, indicating that the slight CO_2 accumulation in the flasks was not growth limiting. This corresponds to the results of Marino et al. (1995), where cultures of *P. persica×amygdalus* GF 677 cell suspensions grown in flasks closed with aluminim caps or cotton plugs grew equally well. The cultures in the bioreactors showed clearly reduced growth in comparison to that in flasks starting with day 8 after inoculation. The maximum cell density in the bioreactors was only 8% pcv and there was no difference between the bioreactors equipped with the different aeration systems. This corresponded with the data on CO_2 accumulation, so that it was concluded that the growth-limiting factor in the bioreactors was the high CO_2 mole fraction, which was checked with experiment 2.

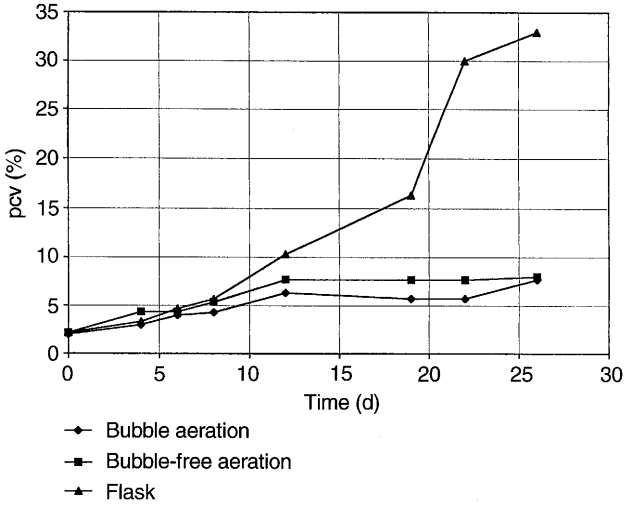

Fig. 2 Experiment 1: cell growth in flasks and bioreactors equipped with different aeration systems

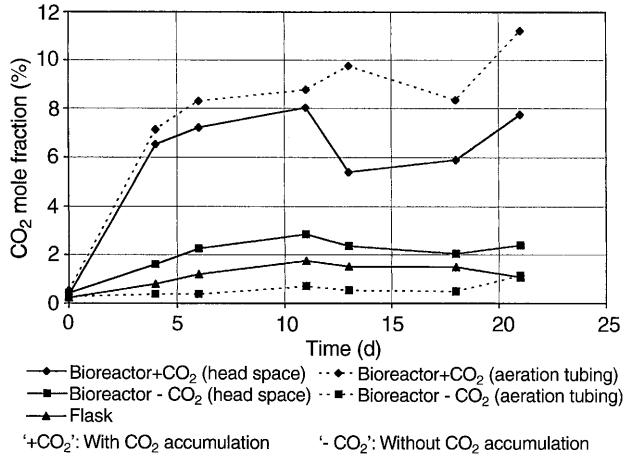

Fig. 3 Experiment 2: CO_2 mole fractions in flasks and bioreactors with and without CO_2 accumulation (cell line 3738-14)

Experiment 2: effect of CO_2 on cell growth and differentiation

The course of CO_2 accumulation in the headspace of the culture vessels and the circulating aeration gas of the bioreactors is given in Fig. 3 for cell line 3738-14. The CO_2 mole fractions in the flask and in the headspace of the bioreactor without CO_2 removal in the aeration gas were similar to those found in experiment 1. The time course showed that the CO_2 mole fraction increased sharply during the first 6 days of the culture period. After that only minor changes were observed. If the aeration gas was conducted through NaOH, the CO_2 mole fraction was reduced markedly both in the aeration gas and in the headspace gas (Fig. 3), indicating that mass transfer between these two compartments was given by the contact with the suspension.

The growth curves of the suspension cultures in this experiment are shown in Fig. 4. The cells grew equally well in the flasks and in the bioreactor with CO_2 removal, whereas cell growth was nearly halted after day 6 in the bioreactor with CO_2 accumulation. This corresponded with the data on CO_2 accumulation, which increased up to the same time (Fig. 3). Cell viability also clearly decreased in the treatment with CO_2 accumulation (Fig. 5). However, a marked effect on cell viability could not be seen before day 12. This delayed effect on cell viability is probably a consequence of the method of FDA staining, which also proved

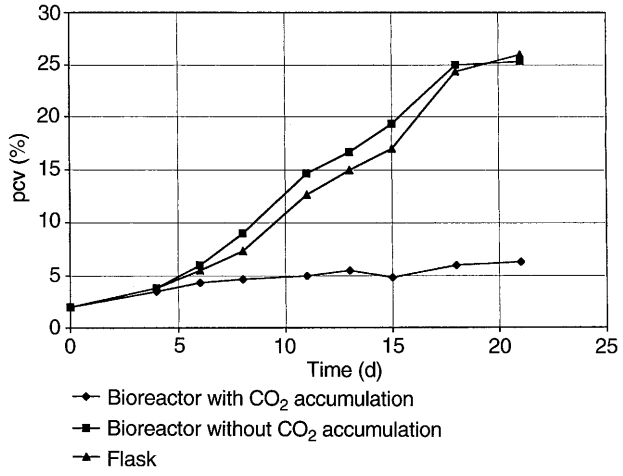

Fig. 4 Experiment 2: cell growth in flasks and bioreactors with and without CO_2 accumulation (cell line 3738-14)

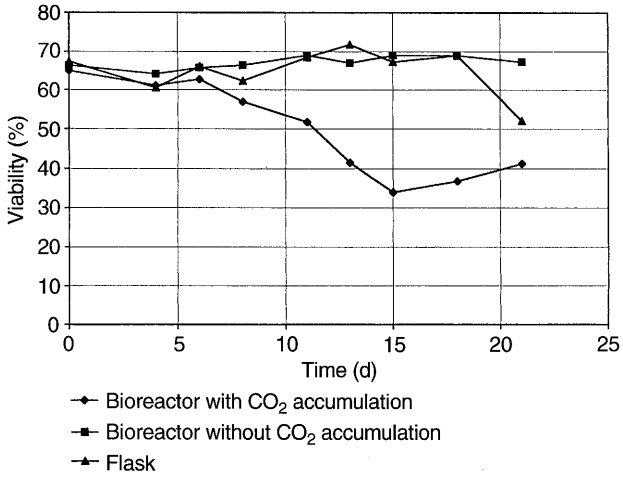

Fig. 5 Experiment 2: viability of cell clusters in flasks and bioreactors with and without CO_2 accumulation (cell line 3738-14)

to be less sensitive than other methods in experiments on shear stress (Dunlop et al. 1994).

These results confirm the supposition of experiment 1 that CO_2 accumulation was the growth-limiting factor in the bioreactors. Hydrodynamic forces created by aeration and stirring, which are often mentioned as growth-limiting factors in plant cell bioreactor culture (Namdev and Dunlop 1995) were apparently of no importance in this experiment. High CO_2 mole fractions affecting plant cell growth were also reported by Kumar et al. (1989) for cell suspension cultures of *P. glauca* and by Marino et al. (1995) for *P. persica*×*amygdalus* GF 677 cell supension cultures. Their experiments were performed in flasks comparing closures with different gas exchange rates. In these publications, the range of the amount and the time course of CO_2 accumulation were similar to the data presented here, showing the strongest increase in the CO_2 mole fraction within the first quarter of the culture

Table 1 Number of normally developed somatic embryos regenerating from cell suspensions grown in flasks and bioreactors with and without CO_2 accumulation (mean ± standard deviation)

	Cell line	
	3738-14	3736-12
Bioreactor with CO_2 accumulation	10.1±4.5	58.1±16.3
Bioreactor without CO_2 accumulation	0.4±0.7	42.2±8.5
Flask	4.9±5.2	33.2±14.9

period. However, in the experiments described by Marino et al. (1995), the suspension culture was performed in gastight closed flasks, so that the observed effect on cell growth could be due at least partly to lack of O_2. This possibility was excluded in the experiments of Kumar et al. (1989) by incubating suspension cultures under a continuous flow of gas mixtures of known composition, where a gas mixture containing 15% CO_2, 7 ppm ethylene, and 20% O_2 was clearly growth limiting in comparison to air. This is in accordance with the data presented here, as a constant and not growth limiting (unpublished data) pO_2 of 10% was maintained in the bioreactors with and without CO_2 accumulation, so that an O_2 effect could be excluded. An effect of the ratio of pO_2 to pCO_2 could not be determined, since the pO_2 was kept constant in all experiments. Marino et al. (1995) and Kumar et al. (1989) reported an accumulation of ethylene corresponding to the CO_2 accumulation. Ethylene accumulation has not been determined in the experiments described here, but accumulation of this gas might also be partly responsible for the growth-inhibiting effect.

The results presented here could be obtained with two cell lines (3738-14 and 3736-12) which differed in their origin, growth characteristics, and cell morphology. Thus the observed effects were not restricted to a single cell line.

The effect of CO_2 accumulation during suspension culture on the regeneration of somatic embryos after transfer to hormone-free medium is given in Table 1. For cell line 3736-12, this value is within the same range for the suspensions grown in the control flasks and in the bioreactor without CO_2 accumulation, whereas it was increased for the suspension which was only slowly growing due to CO_2 accumulation. For cell line 3738-14, the results were slightly different: the most embryos regenerated from the suspension grown in the bioreactor with CO_2 accumulation, less from the control flasks and only very few after culture in the bioreactor without CO_2 accumulation.

The results for 3736-12 might be an effect of stress, here in the form of CO_2 accumulation. Different forms of stress are known to enhance the induction of somatic embryogenesis (Dudits et al. 1995), although this suspension consisted (microscopically assessed) only of proembryogenic masses (PEMs), aggregates of cells which are already determined for differentiation and arrested in this state by high auxin levels (Merkle et al. 1995).

The different results in the case of cell line 3738-14 might be due to the typical composition of cell types of this

suspension: besides PEMs, large, vacuolated cells were present. The growth of the latter cell type was very much enhanced in the bioreactor without CO_2 accumulation whereas the suspension in the bioreactor with CO_2 accumulation consisted almost exclusively of PEMs (microscopic assessment, data not shown). Thus – apart from a stress effect, which might exist here too - these results were probably an effect of an altered cell type composition.

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