Chapter 7

Membranes to reduce adherence of somatic embryos to the cell lift impeller of a bioreactor

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Abstract: Membranes less attractive to embryos were tested as a replacement for nylon screens to prevent adherence of somatic embryos, cell clusters and cells to different sites of the bioreactor, a feature considered undesirable in plant cell suspension cultures. The results showed that the loss of embryogenic cell-mass could be halved by using silicone or track membranes. For aeration purposes, these membranes are as satisfactory as nylon screens conventionally used in cell lift impellers.

Key words: artificial seed, dialysis membrane, microseed, nylon screen, silicone membrane, somatic embryogenesis, somatic seed, track membrane

1. Introduction

Somatic embryogenesis is a highly effective cloning method whereby large numbers of embryos can be produced in a minimum space. The idea of somatic embryogenesis dates back to the 1950's when Reinert (1958, 1959) and Steward and co-workers (1958) reported the differentiation of bipolar structures resembling sexually-produced embryos in carrot cell cultures. One of Reinert's primary observations was that withdrawal of auxin from the nutrient medium triggered the formation of somatic embryos. This observation frequently has been made since that time (Kamada and Harada, 1979; Sung and Okimoto, 1981, 1976; Nomura and Komamine, 1985; Sorvari et al., 1997), and the technique is still successfully applied.

To compete with micropropagation, the production of somatic embryos should be fast and capable of producing millions of new plants daily. However, before it can be as effective as is true seed propagation, and

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economically viable, numerous basic problems need still to be resolved. Above all, both the differentiation of somatic embryos must be a reliable process that can be widely applied to most commercial important plant species, and the genetic stability should be comparable to that of micropropagated plants. The quality of the embryos and the storage techniques need to be satisfactory to ensure that the germination rate will be about the same as for true seeds.

Use of somatic embryos as a competitive cloning method requires that the whole process, from the initiation of suspension culture to the sowing-ready microseeds, should be automated. Bioreactors are of key importance in the development of automated processes for the production of somatic embryos. A key part of the bioreactor vessel is its mixing system. Mixing is needed to oxygenate the suspension culture; also, some physical movement is necessary to keep the developing somatic embryos apart and separate, which would otherwise adhere together. Several types of bioreactors are available commercially, from those with simple marine blade mixing systems, to those with bubble column, air lift loop, liquid impelled loop and cell lift impelled systems (Scragg, 1993). The cell lift impelled bioreactor (CLIB), with or without a double screen, was primarily developed for highly fragile animal cell cultures, nevertheless it seems to be reasonably well suited for plant cell cultures. The CLIB is a satisfactory alternative to the conventional stirred tank reactor (CSTR), which is not well suited for shear-sensitive animal and plant cells and somatic embryos.

CLIB takes advantage of what is known as the cell-lift principle. In the CLIB with double screen, oxygenation of the nutrient medium is carried out by sparging sterile oxygen or air into the cell-free cavity. Even a slow rotation of the impeller discharge ports located in the upper part of the cell lift impeller causes a centrifugal force sufficient to create a weak differential pressure between the lower and upper parts of the impeller. Through this action, cells or embryos are lifted into the central draft tube and then expelled *via* the three discharge ports into a continuous recirculation loop. Shear forces are low in the CLIB and even relatively large somatic embryos can be cultured without major damage. However, even though the double screen is made either of very fine stainless steel or nylon mesh, plant cells and embryos tend to adhere to the finest meshes causing problems with aeration of the medium and the blocking of tubes and probes within the bioreactor. Even the smallest fault in mesh structure allows the cells to pass into the aeration cavity between the double screens causing further clogging of the mesh from inside the cavity.

The aim of this research was to study the possibility of replacing the mesh by membranes to which somatic cells and embryos do not easily adhere, whilst allowing adequate aeration of the growing cell mass.

2. Material and methods

2.1 Initiation of bioreactor culture

The basic protocol for carrot somatic embryo production and maintenance was as follows. The primary explants consisted of hypocotyl sections of seedlings of the domestic carrot (*Daucus carota* L.) var. Duke. Suspension cultures were initiated by placing hypocotyl sections of sterile germinated seedlings, each about 0.5-1 cm long into 100 ml Erlenmeyer flasks. Each flask contained 20 ml of modified MS (Murashige and Skoog, 1962) liquid nutrient medium supplemented with 1 mg l^{-1} 2,4-D: 15 hypocotyl sections were added per flask. The initial cultures were incubated on a gyratory shaker (100 rpm) for 4 weeks with a 16-h day (40-50 µmol $s^{-1}m^{-2}$) at 24/22±1°C day/night temperature. The first subcultures were prepared by sieving the suspension through a stainless steel mesh of pore size 355 µm. The cells passing through the mesh were collected by 5 min centrifugation (100 g), and about 0.5-0.7 ml of the packed cell volume (PCV) was resuspended in 20 ml of fresh MS medium supplemented with 1.0 mg l^{-1} 2,4-D. For maintenance of subsequent subcultures, the same procedure was repeated every 2 weeks.

For the initiation of somatic embryogenesis, the 8-day-old subcultured suspensions were successively sieved through a series of nets of 355, 200, 100, 45 µm mesh, and finally through a net of 27 µm mesh. The fractions remaining on the net of 27 µm mesh were washed 4 times with plant growth regulator (PGR)-free MS solution. The cells were then suspended in PGR-free MS medium to the density of $3x10^4$ cells ml^{-1}, and 20 ml were transferred to a new 100-ml Erlenmeyer flask. For the differentiation of cells to embryos the cultures were kept in the dark on a gyratory shaker (100 rpm) for 2 weeks at 25/23±1°C in a 16h daily photoperiod.

The inoculation volume of packed cells in the bioreactor cultures was 3.0 ml or 2.6 ml of the 45-100 µm fraction per 3.5 l or 3 l of growth medium, respectively. The bioreactor used in this study was the New Brunswick Celligen Plus™ with the CellLift® mixing and aeration system. The revolution speed of CellLift® was, at the beginning of the process 25 rpm, and 35-45 rpm at the end.

2.2 Membranes used in the bioreactor

The membranes used in place of the nylon screen as a cover for the CellLift® were a dialysis membrane, a silicone membrane and a nuclear track membrane. The nylon screen was the original screen from the New Brunswick, developed especially for the CellLift® impeller. The dialysis

membrane was a Spectra/Por® 1 membrane with molecular weight cut-off (MWCO) of 6000-8000Da from Spectrum Medical Industries, Inc. The silicone membrane was acquired from a local industrial plastics reseller; it was 400 µm thick, and the thinnest one available. Nuclear track membrane was obtained from Nerox Filter Oy (Tampere, Finland). Nuclear track membranes are produced by the physico-chemical treatment of autoclavable poly(ethylene terephthalate) films exposed to heavy ion beams. Film thickness is 10 µm, pore size from 0.05 to 2.0 µm and pore density from 10^5 to $3x10^9$ pores per cm^2 (Apel et al., 1992).

2.3 Experimental design

The cell lift impeller has two sites for the aeration screens (Figure 1). One site is on the outer rim and the other is located around the central draft tube. Because factory-made membrane tubes were not available, they were made by hand in the laboratory. The seams were sealed with silicone glue and the self-made sleeves were tightened to the cell lift impeller with O-rings. Depending on the space between the two membranes of the cell lift impeller, the initial volume of the bioreactor was either 3 l or 3.5 l. If the inter-membrane space was kept air filled, the initiation volume was 3 l, otherwise it was 3.5 l. The bioreactor was autoclaved in the Boxer 200/110 autoclave. The total autoclaving time including cooling was 5.5 hours, of which the effective autoclaving at 121°C was 15 min. Because the autoclaving of the bioreactor was a long process, sucrose, meso-inositol, casein hydrolysate and the oxygen probe were autoclaved separately at 121°C for twenty minutes. After the autoclaving process the nutrient components were added in a laminar flow hood and the oxygen probe was assembled into place. After cooling to room temperature in the laminar flow hood, the cell suspension was added to the bioreactor vessel in the volumes as described above. The changes in D.O. (Dissolved Oxygen %) were recorded daily and the changes in pH every second day. No attempts were made to regulate the pH were made, but from about 7^{th} to 10^{th} day additional pure oxygen was given to prevent too low D.O. concentrations.

The cell lift impeller was equipped with membranes and nylon screen in 5 different combinations, as follows. In ‘D/N’, the outer rim was dialysis membrane and the inner nylon screen. In ‘N/N’, both the outer and inner rims were nylon screens. In ‘S/N’, the outer rim was silicone membrane and inner nylon screen. In ‘S/S’, both the outer and inner rims were silicone screens. In ‘T/T’, both the outer and inner rims were track membranes. In S/S and T/T the cavity between the membranes was filled with air, whereas in other cases the cavity was filled with nutrient medium.

Each experiment was run twice, each for 2 weeks. At the end of the experiment, non-adhering embryogenic cell mass was collected separately from the material adhering to the different sites of the bioreactor vessel and the cell lift impeller. For the assay of the dry weight, the collected samples were dried at 60°C overnight and weighed.

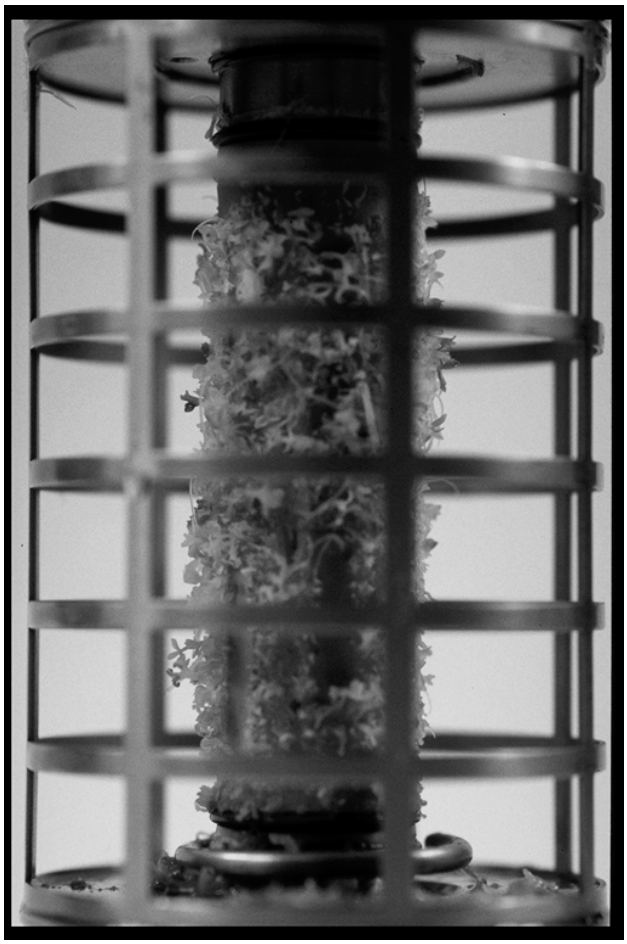

Figure 1: A cell lift impeller with the screen of the outer rim removed to allow a view into the double screen cavity. Growing carrot somatic embryos completely cover the nylon screen of the cell lift draft tube.

3. Results and discussion

Growth of plant cell and somatic embryo cultures in a bioreactor is not without its problems, because the proembryogenic cells tend to form relatively large clusters and somatic embryos have a tendency to adhere for surfaces, and to the tubes and probes that extend into the growing medium. The nylon screen that is commonly used on the cell lift impeller is highly susceptible to the blocking by carrot somatic embryos (Figure 1). One solution could be to use direct air bubbling of the nutrient medium as a mixing system. However, there are indications that direct bubbling disturbs the growth of cells in a suspension culture (Hegarty et al., 1986). Alternatively, it has been shown that a bubble-free silicone tubing system has sufficient capacity to supply oxygen adequate for the growth of *Euphorbia pulcherrima* cell suspension (Luttman et al., 1994). We tested different membranes analogous to the silicone tubing, as replacement for the nylon screen. The most significant result was, that in the experiments S/S and T/T (Figure 2), the loss of embryogenic material was reduced significantly. Whereas the loss of embryogenic material with double nylon screen was 25%, with S/S it was only 10% and with T/T it was 11%. Although the adherence of embryos was reduced markedly, many sites still remained for accumulation of cells and embryos. Experiments D/N and S/N showed that the screen in the central draft tube was one of the main locations for somatic embryo attachment. Evidently, because of centrifugal force, adherence is more pronounced in the central draft tube than on the outer rim of the cell lift impeller, and the central draft tube should therefore have a very smooth surface.

In general, the oxygen (Figure 3) and pH (Figure 4) values followed the same pattern in all experiments. If the pH is not regulated artificially, it reduces for the first 6-8 days and then increases back to the approximate initial value. When air is used, none of the aeration systems can provide sufficient enough oxygen in the second half of the culture period, therefore pure oxygen must be added to the aeration system. For this, simultaneously the circulation speed of the cell lift impeller must be gradually increased up to 35-40 rpm.

Dialysis, silicone and track membranes are materials to which plant cells and embryos do not easily adhere. Theoretically the large mesh size of nylon screen allows greater aeration of the nutrient medium, but in practice the cells, cell clusters and developing embryos quickly block the screen and prevent effective aeration (Figure 3).

For the preparation of different membranes, the dialysis membrane, nylon screen and track membrane are inelastic and especially dialysis membrane and track membrane in particular are difficult to glue into the

form of a sleeve. Therefore new membranes had to be prepared for every experiment. Dialysis membranes are available in sleeve form, but not in the cell lift impeller-fitting diameters required. Silicone membrane is the easiest to use because it is elastic. After the experiments described above, a method was developed in the laboratory to make seamless silicone tubes that fit exactly to the draft tube and the outer rim of the cell lift impeller.

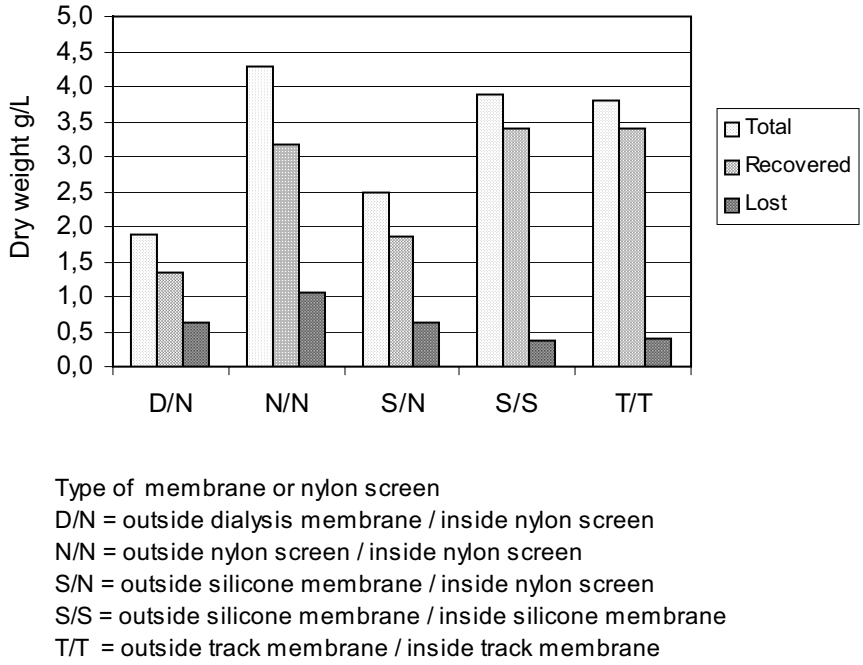

Figure 2: Influence of dialysis membrane, nylon screen, silicone membrane and track membrane on the recovery of carrot somatic cells after culturing of two weeks in a bioreactor equipped with cell lift impeller.

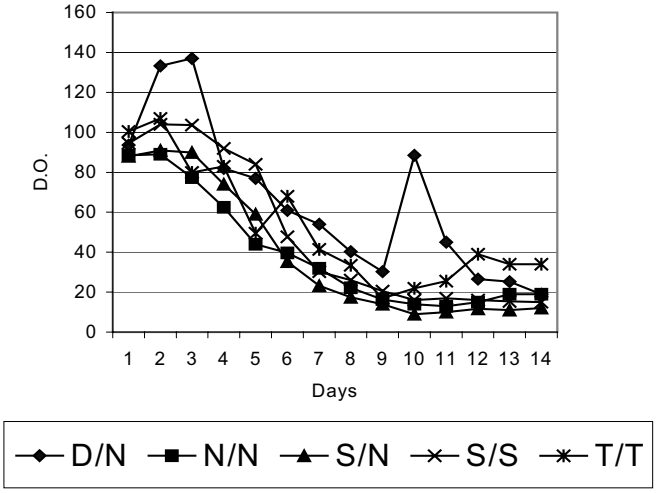

Figure 3: Changes of D.O. (Dissolved Oxygen %) in the bioreactor vessel during the culturing of carrot somatic embryos for two weeks. Abbreviations: see figure 2.

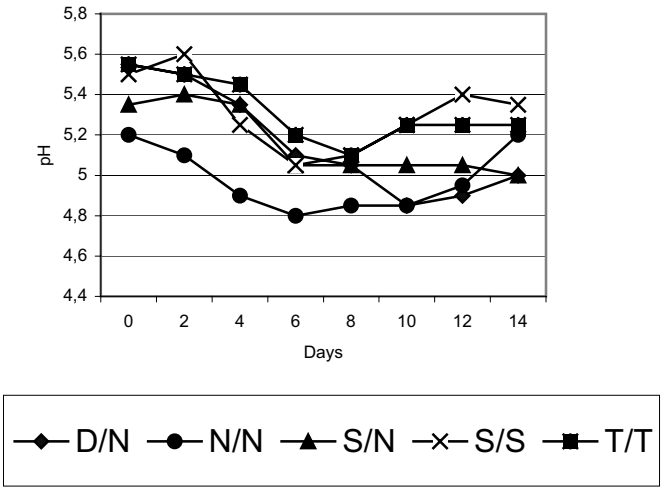

Figure 4: Changes of pH in the bioreactor vessel during the culturing of carrot somatic embryos for two weeks. Abbreviations: see figure 2.

4. Conclusion

Bioreactors are of key importance in the development of automated technology for plant cloning either *via* exposed somatic embryos or *via* microseeds. Plant cells require different conditions from animal or microbial cells and are particularly prone to adhere to bioreactor sites. In this study we have shown that, through choice of suitable material - for example, for the aeration system - the loss of embryogenic material can be reduced substantially.

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