Use of an ultrasound cell retention system for the size fractionation of somatic embryos of woody species

Abstract The potential of ultrasonic standing waves for trapping suspended particles was utilized successfully for differential size fractionation of plant somatic embryos. In a flow-through resonator equipped with a 200-kHz piezoceramic transducer, embryos of different sizes corresponding to different developmental stages could be retained by varying the electric power input and flow speed. The system was initially established for carrot (*Daucus carota*) somatic embryos and subsequently adapted for the larger-sized embryos of the woody species cork oak (*Quercus suber*), grapevine (*Vitis Berlandieri × rupestris*) and cherry (*Prunus incisa × serrula*). Separation performance was confirmed by analysing the different fractions for the expression of homeobox genes which are differentially expressed during embryogenesis. No inhibitory effects of embryos on short- and long-term development could be observed.

Key words Ultrasonic filter · Somatic embryogenesis · Woody plants

Abbreviations MS medium: Murashige and Skoog (1962) medium · 2,4-D: 2,4-Dichlorophenoxyacetic acid · NAA α-Naphthalene acetic acid · BA: N\(^6\)-benzyl adenine · BAP: 6-Benzylaminopurine · RT-PCR: Reverse transcriptase polymerase chain reaction

Introduction

Somatic embryogenesis is an efficient tool for plant tissue culture, genetic engineering and plant regeneration. Carrot (*Daucus carota*) has often been used as a model plant for somatic embryogenesis since its discovery in 1958 (Steward et al. 1958; Reinert 1958; Zimmermann 1993). As cultures grow very fast, different embryo stages can be obtained rapidly in gram amounts. Except for large differences in embryo size between different species, embryo development is very similar throughout the plant kingdom. Although somatic embryogenesis can be synchronised for carrot (Fujimura and Komamine 1979), soon after culture initiation, embryos of most species can be found at different stages of development in a suspension. In these cultures synchronisation can be very difficult and achievable only for a short time. In order for embryos to be isolated at different stages, cultures have to be sieved or hand-selected, the first bringing a high risk of contamination, the second being very laborious.

Ultrasound cell filters have been developed over the last few years especially for the fermentation of mammalian cells (Doblhoff-Dier et al. 1994; Trampler et al. 1994; Gröschl et al. 1998). An ultrasonic standing wave can trap cells, if they are much smaller than the acoustic wavelength used, in planes of half-wavelength distance (Gröschl 1998a, 1998b). The cells stay at the antinodes of the sound velocity field, where they are not exposed to significant acoustic forces and thus are not harmed (Pui et al. 1995). An ultrasound standing wave can trap cells, if they are much smaller than the acoustic wavelength used, in planes of half-wavelength distance (Gröschl 1998a, 1998b). The cells stay at the antinodes of the sound velocity field, where they are not exposed to significant acoustic forces and thus are not harmed (Pui et al. 1995). A flow-through sonication system set on the top of a fermenter permits the continuous exchange of the nutrition medium while the cells remain in the sonication chamber. The aggregated cells fall back into the fermenter due to gravity. The system has been successfully applied for the fermentation of hybridoma (Bierau et al. 1998) and insect cells (Zhang et al. 1998).

A selective cell retention system for plant somatic embryos is described below which was initially estab-
lished for carrot and then applied to relatively large embryos of woody species. As the embryos are in the millimeter size range, an acoustic wavelength of about 7 mm was chosen, which is ten times larger than the wavelength typically used for animal cells. Embryos of different sizes were trapped in a flow-through cuvette by varying the acoustic power and the flow velocity, thus making use of the strong dependence of ultrasonic forces on particle size. This is the first time that the ultrasound cell retention system has been successfully used for plant cells and large particles. It provides a good alternative to sieving, since it is easier to fractionate the embryos and wash them whilst maintaining their sterile environment. Batches of 10 ml of culture can be separated in less then 10 min. The system can be used for synchronization of embryogenic cultures and for isolation of developmental-specific genes. Possible effects on cell culture behaviour and embryogenesis are examined and discussed. In particular, cell death caused by cavitation, i.e. formation and collapse of gas bubbles, is analysed.

Materials and methods

Embryogenic cultures

Proembryonic masses of carrot were grown on solid MS medium, supplemented with 2% sucrose and 1 mg/l 2,4-D. Embryogenic callus of Prunus incisa x serrula was grown on solid MS medium containing 2% sucrose supplemented with 0.04 mg/l BAP, 1 mg/l NAA and 0.06 mg/l 3-indolbutyric acid (R6E medium; da Câmara Machado et al. 1995). Embryogenic callus of Quercus suber was grown on solid modified Sommer medium (Sommer et al. 1975) containing 3% sucrose, 100 mg/l myo inositol and 500 mg/l glutamine (M.A. Bueno, personal communication). Embryogenic callus of Vitis Berlandieri x rupestris (grapevine rootstock 110R) was grown on solid half-strength MS medium, supplemented with 2% sucrose, 5 μM naphthoxyacetic acid and 1 μM BA (Le Gali et al. 1994). Except for 110R, which was incubated at 25°C, the cultures were kept at 22°C in the dark and subcultured at monthly intervals. Suspension cultures of cherry, grapevine and carrot were grown in R6E with a doubled calcium concentration. Suspension cultures of cork oak were grown in the same medium as the solid cultures. All suspension cultures were grown in 20–30 ml of medium in 100-ml Erlenmeyer flasks, at 22°C in the dark in a gyratory shaker (Swip, Bühler) at maintained 100 rpm. Nutrition medium was exchanged once every 3 weeks.

Acoustic size fractionation experiments

The separation system was developed initially for carrot somatic embryos using a small flow-through cuvette (8 ml) made of 4.6-mm-thick Pyrex glass with a height of 45 mm and a cross section of 13.5 x 13.5 mm² (PSI Systems, Vancouver, Canada). The cuvette terminated in two conical formed adapters with attached silicon tubes of 3-mm internal diameter. A lead-circonate-titanate piezoceramic (PZT) of 10-mm thickness with a vibration area of 20 x 25 mm² (PSI Systems) with silver electrodes was bonded to the glass with the heat-curing and thermally stable epoxide adhesive Araldite AV 118 (Novaris). The system was powered by the frequency generator BioSep ADI 1015 (Applikon) at the resonance frequency of 190 kHz. A high-frequency ferrite transformer of maximal 5 MHz/5 W with a winding ratio of 1:4 was connected in between the generator and the PZT due to the electric impedance of the ceramic. The acoustic quality factor of the system was estimated according to the measured admittance spectrum (Schmidt et al. 1990) to be about 100. The acoustic energy in the medium was estimated to be about 10% of the total electric energy input (Gröschl 1998b).

The cell suspension was pumped against gravity through the sonication chamber by a peristaltic pump (Fig. 1). The resonator, including the adapters and tubes but without electric connections, was autoclaved at 120°C, 1.3 bar for 20 min.

The separation of carrot somatic embryos was performed in two steps. About 5 ml of concentrated embryogenic culture was diluted to 30 ml. This was slowly pumped into the resonator, followed by 20–30 ml of fresh medium. In the first separation step, the largest embryos were retained in the acoustic chamber at a power input of 0.5–0.7 W which corresponds to an estimated pressure of 1–1.2 bar (Gröschl 1998a). The sound field was pulsed in intervals of 3-s sonication (on) and 1.8-s sonication (off) in order to dissolve agglomerated particles of different sizes. The flow speed was set to approximately 35 ml/min. The smaller embryos were not held in the sound field but left the system with the flow of media. The retained fraction was recovered via the input tube by opening an air inlet between the pump and the resonator (Fig. 1). The unretained fraction could be further separated in a second separation step with a power input of 1.5 W. This corresponds to a pressure amplitude of approximately 1.7 bar, at pulses of 4.5 s (on) and 3–4.5 s (off). The flow-through had to be reduced to approximately 25 ml/min.

For separation of relatively large tree embryos, two different systems were tested. First, adapters with larger connecting pieces were constructed and tubes of 6-mm internal diameter were connected to the 8-ml cuvette. For the second system, a larger cuvette (70 x 30 x 30 mm) than in the original set up (6-mm tubes) was tested. Only one separation step was performed. The small cuvette was operated at 0.7 W, 3 s (on), 1.8 s (off) at a flow-through of 65 ml/min. The large cuvette was operated at 175 kHz, 0.7 W, 9 s (on), 3 s (off) with a flow of 140 ml/min. Washing was repeated at least five times with 60 ml of medium each, which could be recycled, since the cells sedimented rapidly. A further approach to scaling-up the system was investigated by assembling two 8-ml cuvettes. The two ceramics were electrically connected in series and run at 190 kHz at 0.5 – 0.7 W in the first separation step and at 1.2 W in the second step. The flow was increased to 85 ml/min and 65 ml/min, respectively.

Cavitation and viability measurement

Cavitation is the rapid formation and collapse of gas bubbles occurring as a result of pressure differences during sonication. It can affect the vitality of cells and tissues in culture by disrupting cell membranes. As a secondary effect, it involves the formation of H2O2 and free radicals such as OH, which can be chemically detected. The cuvette was filled with a solution of 17 g/l KI, 0.2 g/l potato starch and 2 ml/l CCl4. If cavitation occurs, KI is oxidised to iodine. This interacts with starch and forms a blue colour, which can be quantified spectrophotometrically after sonication by measuring the absorption at a wavelength of 570 nm for 2 min each at different electric power inputs (Weissler et al. 1950).

Free protein in the medium was measured as an indication of cell disruption during sonication. Protein concentration was determined using Bradford reagent (Bio-Rad) and spectrophotometrical determination of the absorbance at 595 nm every 10 min during a 40-min-long sonication. A wetty protein extract from all cells present in the resonator was set as 100% disruption.

In situ cell death was detected by the use of the Cell proliferation kit 1 (MTT) (Roche Molecular Biochemicals) without solubilization of the formazan dye. The embryos were incubated overnight in the MTT solution immediately and 3 weeks after separation. A blue colour indicated viable cells.