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Plant cell proliferation inside an inorganic host

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Abstract

In recent years, much attention has been paid to plant cell culture as a tool for the production of secondary metabolites and the expression of recombinant proteins. Plant cell immobilization offers many advantages for biotechnological processes. However, the most extended matrices employed, such as calcium-alginate, cannot fully protect entrapped cells. Sol–gel chemistry of silicates has emerged as an outstanding strategy to obtain biomaterials in which living cells are truly protected. This field of research is rapidly developing and a large number of bacteria and yeast-entrapping ceramics have already been designed for different applications. But even mild thermal and chemical conditions employed in sol–gel synthesis may result harmful to cells of higher organisms. Here we present a method for the immobilization of plant cells that allows cell growth at cavities created inside a silica matrix. Plant cell proliferation was monitored for a 6-month period, at the end of which plant calli of more than 1 mm in diameter were observed inside the inorganic host. The resulting hybrid device had good mechanical stability and proved to be an effective barrier against biological contamination, suggesting that it could be employed for long-term plant cell entrapment applications.

Keywords: Plant cell immobilization; Silica matrix; Sol–gel

1. Introduction

The use of whole plants for the biosynthesis of recombinant proteins is a subject of great biotechnological interest. Plant-based production systems have also an established track record for the production of valuable therapeutic secondary metabolites. Compared to other more conventional platforms such as

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microbial and mammalian cultures, plants offer many advantages in terms of economy, scaling up and safety (Abranches et al., 2005). As a counterpart, the lack of precise control on growth conditions and the variations in product yield and quality make difficult the production of added-value proteins in compliance with good manufacturing practices. Plant cell cultures emerge as a promising alternative, combining advantages from whole plants with those from conventional cell cultures (Hellwig et al., 2004). However, mass transfer limitation of oxygen and nutrients, inhomogeneous culture conditions and plant cell sensitiveness to shear stress are responsible for extensive cell death in bioreactors. Moreover, as most secondary metabolites are produced during the plateau phase (Bourgaud et al., 2001), slow growth of plant cells becomes a problem in the design of large-scale systems where contamination by microorganisms implies huge economic losses, both as production delays and decontamination costs. These constraints have triggered our research in plant cell immobilization for bioreactor applications.

Some immobilization strategies were identified to increase plant cell bioproduction of secondary metabolites (Dörnenburg, 2004). Among them, the most successful are, organic polymer gelation (carrageen, pectate, alginate) or direct growing of cells onto polyuretane foams (Vaněk et al., 1999). However, all these methods are biosensitive. Though being the most extended method for plant cell encapsulation (Onishi et al., 1994), alginate gelation does not provide a good protection to entrapped cells (Ripp et al., 2000). Biologically modified ceramics (biocers) which combine biocomponents with ceramic-like matrices surge as an interesting alternative. The sol–gel chemistry of silicates provides a biocompatible route for the synthesis of the inorganic host that can be achieved at moderate temperatures and physiological conditions. It constitutes a versatile means for the encapsulation of microorganisms (Davis, 2002) or cell derived subunits (Vidinha et al., 2006). In recent years, a large number of bacteria and yeast-entrapping ceramics (‘living ceramics’) have been developed for different applications (Böttcher et al., 2004; Nassif et al., 2002). But even mild thermal and chemical conditions of sol–gel synthesis may prove harmful to cells of higher organisms, greatly affecting cell viability (Kuncova et al., 2004). In standard procedures cells are not only exposed to an unnatural environment during the inorganic host synthesis, but tightly encapsulated cells are kept in permanent contact with gel’s silanol groups throughout the immobilization process. This could seriously damage cell integrity and limit their survival (Ferrer et al., 2006), which is unsuitable for plant cell culture, where biomass is supposed to be continuously reused in long-term culture systems.

Another constraint of the space limitation suffered by confined cells is the impossibility to perform cellular division (Nassif et al., 2002). As a consequence, initial cell load cannot further proliferate and may result insufficient for high cell density-demanding processes. In order to create space inside the matrix for cells to complete cellular division, some strategies of encapsulation use macroporous scaffolds (Kataoka et al., 2005). This approach fails to prevent the entrance of contaminating agents, which is one of the benefits pursued with the immobilization. Some other procedures in which groups of animal or vegetal cells are encapsulated inside a siliceous layer have been described (Boninsegna et al., 2003). The inorganic synthesis occurs in the periphery only affecting closely-in-contact cells, but this system does not confer the mechanical stability required for bioreactor applications.

Thus, the challenge for encapsulation of plant cells is to produce a biocompatible matrix that allows free diffusion of nutrients and metabolic products, acts as a barrier against microbial contamination, provides mechanical support but permits cell proliferation inside. This is of particular importance for plant cell bioreactor applications that require high cell density for the biosynthesis of value-added proteins (Wen Su et al., 1996).

Here we present a simple strategy, based on the concept of multiple-cell immobilization (Boninsegna et al., 2003). In a two-step procedure, cells are previously immobilized in Ca-alginate beads that are subsequently trapped in the inorganic matrix, avoiding any harmful contact of cells with silica precursors. Once silica consolidation is completed, alginate is liquefied and cells remain confined into macrocavities containing a liquid medium inside a solid silica monolith. This procedure based on inexpensive silica precursors has already been employed for yeast (*Sacharomyces cerevisiae*) and bacteria (*Escherichia coli* and *Bacillus subtilis*) (Perullini et al., 2005), and is now extended to more sensitive

cells, such as carrot phloematic tissue and tobacco BY2 line.

2. Materials and methods

2.1. Biological materials and culture conditions

Plant cells used in these experiments were initiated from BY2 tobacco (Nagata et al., 1992) calli and from carrot phloematic tissue. Cell suspensions were obtained from calli cultured under controlled conditions on semisolid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Stock cell suspensions were grown in the dark at $(24 \pm 1)\,^{\circ}C$ under orbital agitation (120 rpm) and were subcultured every 7 days for a period of 1 month in MS medium supplemented with 40 μM nicotinic acid, 2.4 μM pyridoxine·HCl, 0.175 M sucrose and 0.5 μM 2,4-dichlorophenoxiacetic. Previous to immobilization procedures, cell suspensions were filtered through sterilized meshes (50–100 μm pore-size) in order to obtain isolated cell cultures.

Contamination experiments were carried out using *E. coli* (strain DH5α), *S. cerevisiae* (strain ∑1278b) and *Trametes trogii* (strain BAFC 463). Triptein-Soya, Yeast Nitrogen Base and Malt Extract Agar were used as semi-solid culture media, respectively.

2.2. Plant cell immobilization

Fifty microlitres of cell culture, containing about 1.5×10^4 cells, were mixed with 5 ml of 1.5% sodium-alginate and dropped into a 0.1 M $CaCl_2$ solution with the aid of a syringe provided with a needle 0.8 mm in diameter. After 10 min of stirring, Ca-alginate beads of 3.0–3.2 mm in diameter were collected by filtration. Beads were dispensed into glass tubes, placing 15 beads/tube. Silica encapsulation procedure was performed as previously described (Perullini et al., 2005). Briefly, 200 μl of 1.25 M sodium silicate, 100 μl of commercial colloidal silica (LUDOX HS40, Aldrich) and 100 μl of succinic acid (5%, w/w) were added to each tube containing alginate-cells beads and vortexed for 30 s. Once the sol–gel polymerization reaction was completed, tubes were exposed for 2 h to 0.05% potassium citrate to solubilize the Ca-alginate matrix. Average diameter of the macrocavities obtained by this procedure was 3.1 mm. After solubilization of Ca-alginate, tubes were rinsed once with distilled water and introduced into a flask containing liquid MS medium. The flask was maintained under orbital agitation (120 rpm) at $(24 \pm 1)\,^{\circ}C$, and the medium was replaced periodically every 7 days.

2.3. Evaluation of viability at initial time and periodic evaluation of plant cell proliferation

Individual tubes were withdrawn at different times and sampled for microscopic observations (10 individual macrocavities were sampled at every specified time). Samples of individual macrocavities were obtained by introducing a syringe into the semi-solid matrix and carefully applying suction to avoid gel disruptions. Microscopic observations were performed in a Zeiss fluorescent microscope (Axioplan). The total volume of a single macrocavity (about 15 μl) was withdrawn and diluted when necessary with distilled water. Ten microlitres of this dilution were dispersed on a glass slide and a drop of a solution containing 100 μg/ml fluorescein diacetate and 10 mg/ml propidium iodide was added to stain the cells. The total number of cells was calculated from the microscopic images by direct counting all the viable isolated cells and estimating the number of cells forming clusters in each slide (hexagonal packing was assumed). Percentage of viable cells was assessed as already described (Kato et al., 1972).

3. Results

As described in Section 2, plant cells are immobilized in Ca-alginate beads that are further immersed in tubes where a mixture of sodium silicate, colloidal silica and succinic acid is vigorously mixed. This colloidal solution undergoes a rapid sol–gel transition, and alginate beads are quickly covered with a nanoporous silica gel. Throughout the inorganic synthesis, Ca-alginate matrix preserves plant cells from harmful silica precursors. However, once silica synthesis is completed, the cavity content is turned to liquid by complexation of Ca^{2+} with citrate. During the encapsulation process cells are starved for more than 2 h (because after 2 h potassium citrate is rinsed with distilled water and tubes are placed into flasks containing liquid MS

culture medium, but diffusion of nutrients through the silica matrix is not immediate). It becomes indispensable to evaluate this procedure's effect on plant cell viability. Assessment of cell viability conducted at the beginning of the assay by averaging the cell contents of 10 macrocavities revealed that $(97 \pm 1)\%$ of carrot cells and $(95 \pm 2)\%$ of tobacco BY2 cells remained metabolically active after the encapsulation procedure. Hereinafter, immobilized cells are kept in near-standard culture conditions, as each macrocavity containing liquid MS culture medium can be regarded as a mm-scale bioreactor (see Scheme 1 in Supplementary data).

Plant cell proliferation and monolith physical stability were monitored along a period of 6 months. Macrocavities content was evaluated after 10, 20, 30, 45 and 60 days by fluorescent microscope images of encapsulated cells at different post-immobilization times. Representative fluorescent microscope images of BY2 cells are shown in Fig. 1; similar results were obtained from carrot cells (data not shown). The total number of cells per cavity was estimated as described in methods by averaging over 10 cavities. Growth curve in Fig. 1 shows that the total number of tobacco BY2 cells per cavity increases more than 20 times after 45 days of

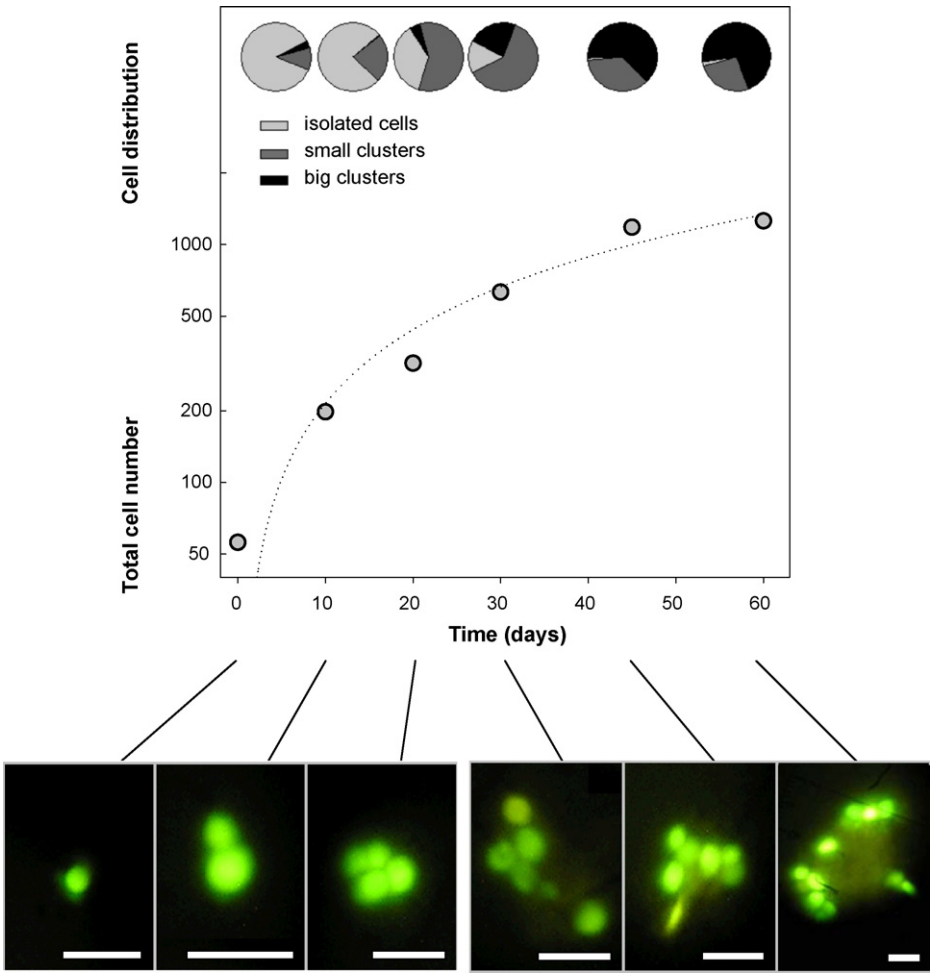

Fig. 1. Plant cell proliferation inside the inorganic host. (Top) Total cell content of a single cavity (15 μl) sampled after 0, 10, 20, 30, 45 and 60 days of culture inside the silica matrix (logarithmic scale). Pie charts show the distribution of cells: isolated cells (light grey), cells forming small clusters of 2–8 cells (dark grey) and cells forming big clusters of more than 8 cells (black). (Down) Representative fluorescence microscope images corresponding to each sampling time. Bars represent 50 μm.

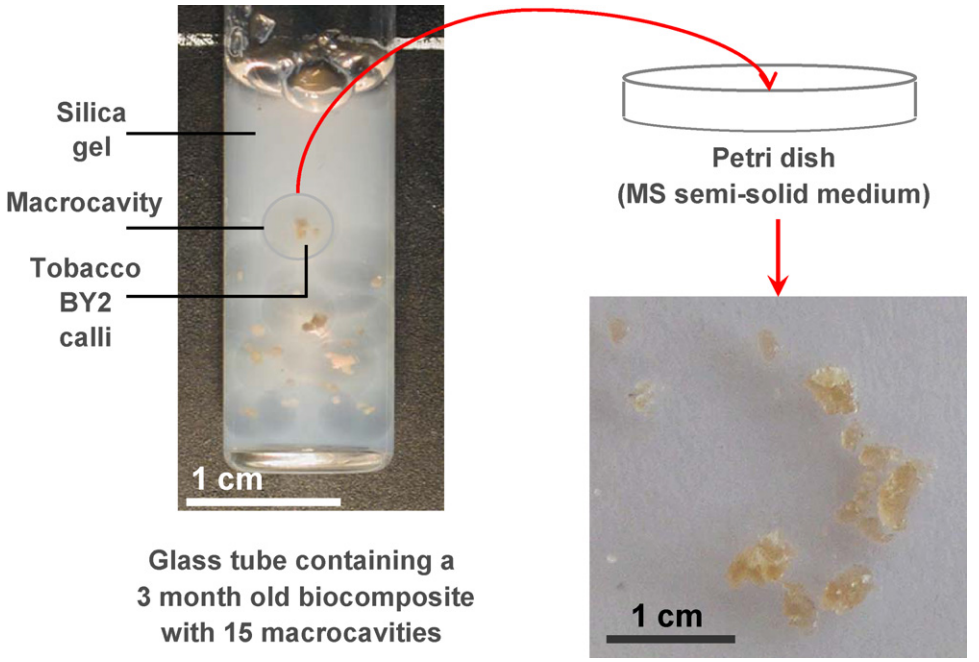

Fig. 2. Evaluation of calli formation inside the monoliths' cavities. (Left) Photograph of a glass tube containing a 3-month-old biocomposite with 15 cavities. Tobacco BY2 calli can be seen with naked-eye inside each cavity. (Right) Photograph of calli formed 2 months after plating on MS semi-solid medium one single cavity's content rescued from the 3-month-old biomaterial.

assay (doubling time = 250 h). After 45 days of growth inside the cavities most of the cells were organized in big clusters making it increasingly difficult to accurately count the total number of cells. Besides, at later immobilization times, each macrocavity was occupied by plant calli growth of which could be assessed with naked-eye. After 3 months of culture, two to four small calli (0.1–1 mm) were observed inside each cavity as can be seen in the photograph of Fig. 2. As time elapsed, calli size progressively increased and the number of calli per cavity showed a tendency to decrease. Inside each 6-month-old biocomposites cavity, single calli of more than 1 mm in diameter were seen. In order to study the possible effect of encapsulation procedure on the potential for cell division, the content of cavities sampled after 3 months of immobilization culture was plated on MS semi-solid medium. For both cell types assayed, large number of calli developed normally on Petri dishes (Fig. 2, right).

To demonstrate that the silica matrix effectively acts as a barrier against biological contamination, a contaminating agent was inoculated into the external culture medium of a 6-month-old biocomposite. After 48 h samples were withdrawn from external medium and from the interior of cavities. Previous to cavities' content sampling, the contaminated external medium in contact with the biocomposite was eliminated. The monolith was rinsed twice with sterilized distilled water, and it was submitted to a surface sterilization procedure by 10 min immersion in a flask containing alcohol 70%. Aliquots of both samples were sown onto MS semi-solid medium and onto appropriate semi-solid culture medium (see Section 2 for details). Experiments were carried out using different polluting cells or microorganisms as contaminating agents (bacteria, yeasts and filamentous fungi). Regardless of the biological agent assayed, contamination was only detected in the external medium. A schematic design of the experiment can be seen in Scheme 2 of Supplementary data.

4. Discussion

Employing a two-step immobilization strategy, we accomplished plant cell proliferation, from the single-cell stage to calli formation, inside a glass-like matrix.

The protection offered by Ca-alginate ensures isolation of plant cells from cytotoxic silica gel precursors during sol–gel synthesis. Porosity of the silica matrix is large enough to allow fast diffusion of citrate and low molecular weight nutrients, allowing the whole encapsulation process to take place in less than 3 h. Both facts are determinant in the high plant cell initial viability obtained with this procedure (≥95%). Intents of direct immobilization of plant cells in silica matrices reported by other authors (Kuncova et al., 2004) showed a much lower viability.

Plant cells are significantly slower growing than most microbial organisms, with doubling times ranging from 20 to 100 h (Kieran et al., 1997). In the present study we reach a doubling time of 250 h. Though being slow, cell growth within the inorganic material is established.

Fast diffusion of nutrients and metabolic products is of particular interest in the design of devices for bioreactor applications. Due to the protection provided by the Ca-alginate encapsulation, silica synthesis could be attempted in even more cytotoxic conditions (i.e., through condensation of silicon alcoxide precursors). Thus, nanoporous size distribution can be exquisitely regulated in order to adapt to specific requirements of transport properties for bioreactor systems, in particular the diffusion of biosynthesis products.

The silica monoliths reported here were synthesized using an inexpensive silica source. They maintained their mechanical properties along a period of several months. Absence of cracking, shrinkage or sineresis above the nanoscale was demonstrated by the effectiveness of the silica gel as a barrier against contamination.

Immobilized plant cells offer an alternative to batch culture. One of the clear advantages of this technology is the easier separation between the biosynthesis products and the producing plant cells. But additional benefits arise from the use of plant cell encapsulating devices for bioreactor applications, since plant cells are generally weak against hydrodynamic stress generated by agitation or aeration in conventional bioreactor designs (Moon et al., 1999). Furthermore, the encapsulation procedure reported here permits a good cell-to-cell contact, known to favor secondary metabolite production and hinders the contamination by biological pollutants. Biological contamination to a culture, regardless in the culture medium or within the cell clusters, renders the same kind of detrimental effect. However, in silica modular bioreactors, surface sterilization processes can be easily implemented to eliminate the biological pollutant.

Since plant cells are particularly susceptible to culture conditions, high initial viability obtained suggests that this technique could be advantageously extended to other higher eukaryotic cells. Thus, this synthesis strategy could provide a general approach to the production of different biological ceramic composites (biocers), regarding both the biocomponent and the silica matrix.

Although promissory the results shown here must be complemented with further research in order to optimize matrix performance (transport properties, rate of cell growth) and to adapt the shape of silica gel monoliths to a modular bioreactor design. Work in both directions is currently in advance in our laboratories.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.jbiotec.2006.07.024.

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