A novel method for the immobilisation and culture of plant cells

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A novel technique is described for the immobilisation of plant cells. The method is simple and does not require the use of potentially toxic gels for the entrapment process. Liquid-suspended cells of two species, *Capsicum frutescens* Mill. and *Daucus carota* L. were found to invade, and were strongly retained in, polyurethane foam particles over a 21 day culture period. The viability of the immobilised cells was high (70–80% were alive after 21 days). On agitation of the loaded foam particles in fresh liquid medium, at least 95% of the cells remained immobilised after 3 or 4 days.

Cell immobilisation *Secondary metabolite production* Capsicum frutescens *Mill.* *Cell differentiation* Daucus carota *L.* *Polyurethane foam*

1. INTRODUCTION

The employment of culture systems in which plant cells are immobilized on an inert support is now recognised as a means by which the environment of the cells can be simply manipulated, and the yields of specific secondary metabolites increased over those of liquid-suspended cells [1–4]. The first techniques reported for the immobilization of plant cells [5,6] have been derived from those used for enzyme and microbial cell entrapment, and have made use principally of a variety of gels. The cells are in direct contact with the gel matrix and so are inevitably subjected to high concentrations of a variety of ions and organic compounds which, especially during the immobilization procedure, may have undesirable effects on cell metabolism. Furthermore, for both research-scale and industrial-scale applications it is essential that, in order to minimise the incidence of microbial contamination, the number of steps in the immobilization procedure be kept to the minimum. Cell entrapment in gels usually involves at least two steps, but immobilization can be simplified further still. Atkinson et al. [7] have described the use of different types of metal biomass support particles for the entrapment of yeasts and fungi in a purely 'passive' way. Here, we similarly describe the invasion of polyurethane reticulate foam particles (described in [8]) by cells of two plant species, *Capsicum frutescens* Mill cv. annuum (Chilli pepper), and *Daucus carota* L. (carrot) in a single step process.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

Suspension cultures of *Capsicum frutescens* were grown in 250 ml Ehrlenmeyer flasks containing 60 ml liquid medium comprising 4.71 g/l Murashige and Skoog's medium (Flow Labs., Irvine) supplemented with 2 μg/l (10^{-8} M) indolyl-3-acetic acid (IAA), 20 μg/l (10^{-7} M) kinetin, 20 g/l sucrose, and 50 ml/l coconut milk, at pH 6.0 (pepper medium).

Carrot (*Daucus carota*) suspended cells were similarly cultured in liquid medium containing 4.71 g/l Murashige and Skoog's medium (Flow Labs.) supplemented with 0.2 mg/l (10^{-6} M) 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/l

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(10^{-6} M) kinetin, and 30 g/l sucrose (pH 5.8) (carrot medium). The cultures were agitated on a rotary shaker of orbital diameter 1.5 cm at 96 rev./min.

Cells (1 g fresh wt) were transferred to fresh medium at intervals of 2 weeks, and maintained at 24 ± 1°C in continuous fluorescent illumination (Warmwhite) of an illuminance of 20 μmol.m^{-2}.s^{-1}.

2.2. *Immobilisation of cells*

Three (for carrot) or six (for pepper) foam particles, each of dimensions 10 mm × 10 mm × 10 mm and of 18 pores/cm, were added to newly sub-cultured flask cultures. At intervals flasks were harvested and determinations were made of the fresh and dry weight and, for the pepper cultures, the number of immobilized cells/culture and the fresh and dry weight of suspended cells/culture. Loaded foam particles (i.e., containing cells) from each flask were resuspended in 100 ml Murashige minimal organics medium in the absence of sucrose or growth substances, to limit cell growth; after 3 or 4 days' agitation on a rotary shaker the % cell retention was determined.

2.3. *Determination of cell weight, number and viability*

The fresh weight of suspended and immobilized cells was determined after the removal of excess medium on a Buchner funnel. The dry weight of suspended and immobilized cells was determined after drying at 90°C for 24 h in a hot air oven. Cell numbers were determined by a method based on that in [9]. Immobilized or suspended cells of known fresh weight were macerated in 10 or 15 ml of 15% (w/v) aqueous chromium trioxide solution for 30 min at 70°C. The solution was then allowed to cool, shaken for 1 min, and the cells were counted on a haemocytometer (Hawksley Cristalite). Cell viability was determined as in [10].

3. RESULTS

3.1. *Cell immobilisation*

The data in fig.1 and 2 describe, respectively, the changes in the fresh weight and the dry weight of immobilised, suspended and total carrot cells; in fig.5 and 6 are described, respectively, the changes in fresh weight and dry weight and cell number of

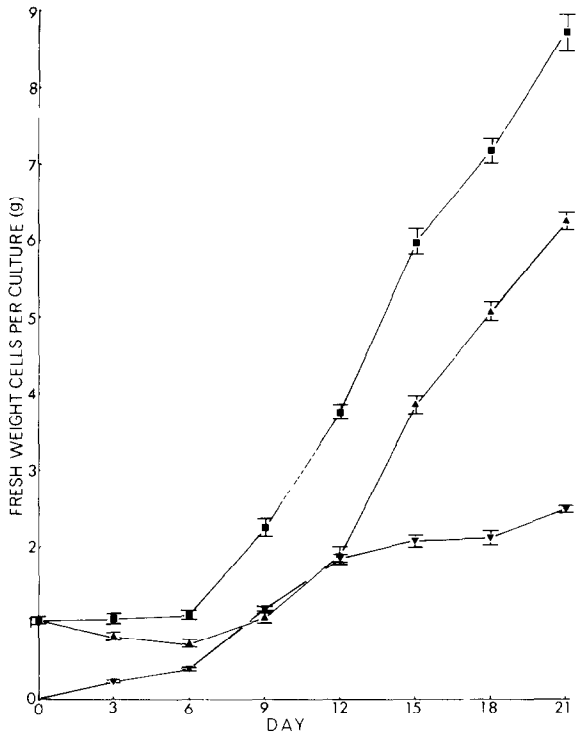

Fig.1. Changes in the fresh weight of suspended (▲), immobilized (▼) and total carrot cells (■) during a 21 day culture period. Each point represents the mean for 3 replicate flask cultures with standard errors. The fresh weight of immobilized cells represents the total weight of cells immobilized on 3 foam particles/culture.

immobilized and suspended pepper cells.

During the first 6–9 days of the carrot cell culture period, there was a lag phase in which the *total* cell fresh and dry weight increased only very slowly. However, by day 3, cells became entrapped in the foam particles, followed by a continued immobilisation of cells until about day 15. After this time, there was a sharp reduction in the rate of increase of the dry weight of the suspended cells, and no further increase in the dry weight of immobilized cells (fig.2). Nevertheless, the fresh weight of the immobilized and suspended cells continued to increase to day 21, presumably due to cell expansion (fig.1). By day 21, cells had grown out through the surface of the foam particles, were extremely compact, and displayed a variety of shapes and sizes, usually 20–100 μm long, and were occasionally organised into globular structures (fig.3,4). By the end of the 21 day culture period,

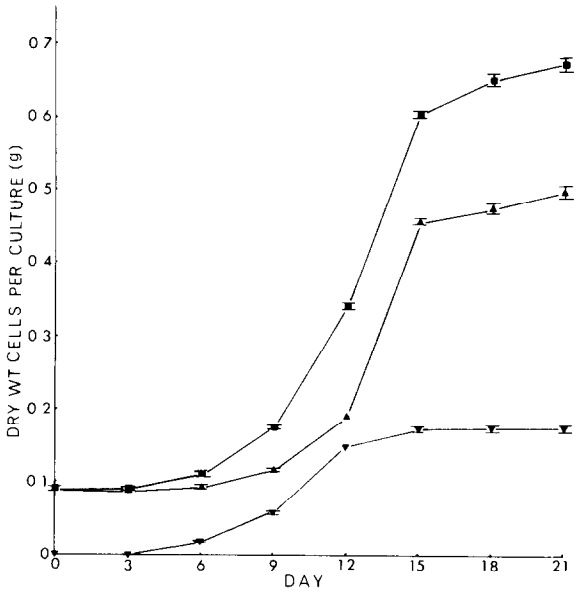

Fig.2. Changes in the dry weight of suspended (▲), immobilized (▼) and total carrot cells (■) during a 21 day culture period. Each point represents the mean for 3 replicate flask cultures with standard errors. The dry weight of immobilized cells represents the total weight of cells immobilized on 3 foam particles/culture.

each foam particle contained ~2.9–3.0 × 10^6 carrot cells.

The results presented in fig.5 and 6 show that, unlike the carrot cells, the growth and immobilization process of the pepper cells had no lag phase. The fresh and dry weights and numbers of immobilized cells increased in an approximately linear manner for the first 12 days of the culture period; after this time the rate of increase became greater, and, unlike the case for the carrot cultures, there was no indication of a stationary phase of growth by day 21. By day 3, the fresh weight of the immobilised pepper cells was greater than that of the suspended cells, and this situation was sustained throughout the period of culture. In this respect, it is also interesting that the final fresh weights of 'control' pepper cultures, containing no foam particles, were significantly higher (11.0 ± 0.6 g fresh wt/culture) than in cultures which did contain foam particles (6.6 ± 0.1 g fresh wt/culture).

The final total fresh weight of the pepper cells was lower than that of the carrot cells, despite the absence of a lag phase. On a basis of fresh and dry weight of cells per foam particle, there was a greater weight of carrot than pepper cells immobilized after 21 days although the total fresh weight of immobilised pepper cells (i.e., in 6 foam support particles) was greater than that of carrot cells (in 3 foam support particles). This suggests that, at least in the latter part of the growth period, the immobilization process is limited by the availability of 'immobilization sites'; i.e., the number of foam pores in a culture, and also, the more immobilization sites there are available, the more slowly will the total fresh weight of the culture increase. Nevertheless, although there was a greater fresh weight of immobilized carrot cells than pepper cells per foam particle, there was a greater number of immobilized pepper cells than carrot cells (~4.8 × 10^6 cells/particle compared with 2.9–3.0 × 10^6 cells/particle), due to the smaller size of the pepper cells at the time of analysis.

3.2. *Cell retention and viability*

The results in table 1 show that the % retention of both carrot and pepper cells on transfer to, and agitation in, fresh medium, was usually ~99%, and never <95%. Throughout the growth cycle the viability of the immobilized cells remained high, at 70–80%. Sectioning of foam support particles demonstrated that, as early as day 1 in the culture period, cells of either species became irreversibly entrapped in the centre of the foam support particles, although in these early stages there was a readily reversible invasion of the peripheral regions of the particles. The irreversible entrapment appeared to begin from the centre and gradually spread through each particle, when the cells became embedded as a result of cell expansion and division. By day 21, the cells had become so densely packed in the foam particles that cells remained immobilized even after prolonged flushing of the particles under a fast-flowing tap of water. Furthermore, dried cells could be retrieved only after the particles had been pulled apart.

3.3. *Capsaicin production by cells of* C. frutescens

The results in table 2 demonstrate that pepper cells immobilized in polyurethane foam produce significantly more capsaicin (μg levels/g dry wt) than do suspended cells (ng levels/g dry wt). The capsaicin is not accumulated intracellularly but is released into the surrounding nutrient medium.

Fig.3.

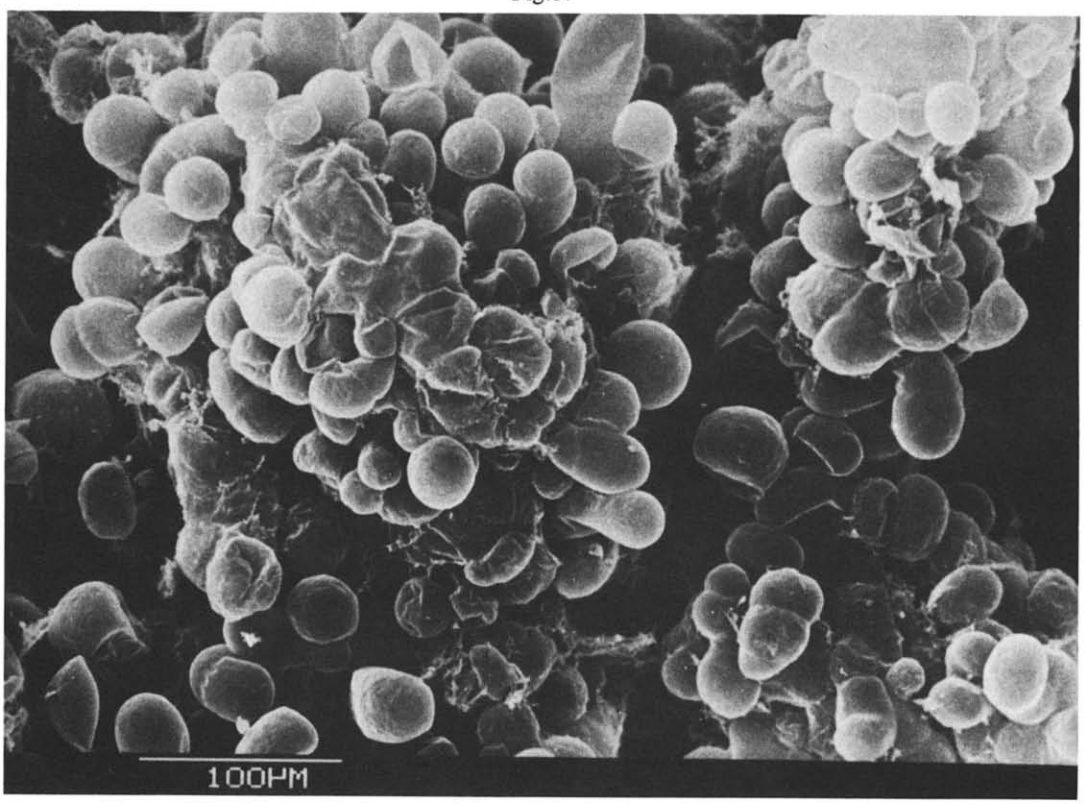

Fig.4.

Fig.3,4. Scanning electron micrographs of immobilised cells of *Daucus carota* after 21 days' immobilization. Loaded foam particles were fixed overnight in 4% glutaraldehyde + 2% accolein (w/v, in pepper medium) at 20°C, washed for 20 min in pepper medium and for 1 h in 0.1 M phosphate buffer (pH 7.0). The cells were then fixed in 1% aq. osmium tetroxide for 2 h, washed for 3 h in distilled water and dehydrated in an acetone series (10% to absolute, overnight, by diffusion dehydration) followed by critical point drying via CO_2. The specimens were coated with 10 mm gold in an EMSCOPE SC500 sputter coater.

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4. DISCUSSION

There is an increasing body of evidence to suggest that the metabolism of a plant cell will be realised fully only if that cell is cultured in a way which resembles, as closely as possible, the physical and chemical environments which obtain within the intact plant [4,11]. More often than not, cells which are rapidly grown and form colourless, highly dispersed suspension cultures, and are consequently subjected to little polarity and few gradients, synthesise and accumulate only low levels of secondary metabolites and may be deficient in at least some enzymes [12,13]. On the other hand, highly differentiated structures within callus cultures and stationary phase suspension cultures accumulate much higher levels of such products. Such evidence points to immobilized cell technology as a means by which slow-growing, differentiated or partially differentiated cells can be obtained and treated chemically in a sequential fashion which would mimic at least some of the biochemical processes which determine the production of specific secondary compounds.

A number of successful attempts have been made to immobilize whole plant cells, with little adverse effect on primary metabolic functions, and the activities of particular enzymes of secondary pathways have been found to be enhanced in immobilized cells compared with freely suspended

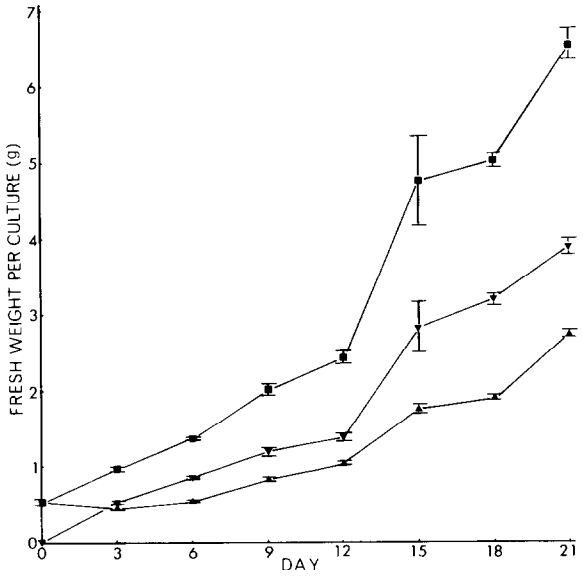

Fig.5. Changes in the fresh weight of suspended (▲), immobilised (▼) and total (■) pepper cells during a 21 day culture period. Each point represents the mean for 3 replicate flasks with standard errors. The fresh weight of immobilised cells represents the total weight of cells immobilised on 6 foam particles/culture.

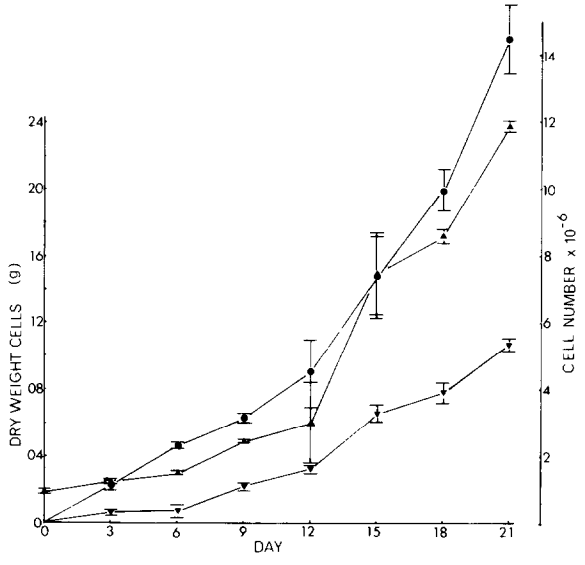

Fig.6. Changes in the number of immobilised pepper cells (●) and dry weight of suspended (▲) and immobilised (▼) pepper cells during a 21 day culture period. Each point represents the mean for 3 replicate flask cultures with standard errors. The dry weight and number of immobilised cells represents the total for cells immobilised on 3 foam particles respectively per culture.

Table 1

Day of culture	Immobilized carrot cells		Immobilized pepper cells	
	Mean % retention	Mean % viability	Mean % retention	Mean % viability
3	95.0 ± 1.2	77.2 ± 1.7	98.9 ± 1.6	74.3 ± 2.1
6	99.8 ± 0.9	78.3 ± 0.9	99.4 ± 1.4	82.6 ± 3.7
9	98.9 ± 0.9	74.7 ± 1.7	99.8 ± 1.5	79.4 ± 2.9
12	99.0 ± 1.1	73.6 ± 1.2	99.6 ± 0.9	78.4 ± 1.8
15	98.4 ± 0.2	80.1 ± 4.0	99.5 ± 0.8	72.5 ± 2.0
18	99.0 ± 0.3	76.6 ± 2.0	98.5 ± 0.5	76.6 ± 1.7
21	99.8 ± 0.5	75.4 ± 1.8	99.8 ± 0.4	78.8 ± 3.1

The % retention and viability were calculated as the mean of 3 replicate foam particles; 300 cells were counted per replicate for the viability data. % retention was calculated as the % fresh weight of cells still immobilized after resuspension of loaded foam particles after 3 days (carrot cells) or 4 days (pepper cells)

Table 2

The capsaicin contents of cells and nutrient medium from cell suspension and immobilized cell cultures of *Capsicum frutescens* without precursors

Replicate	Suspension cultures		Immobilized cell cultures[a]	
	Cells (ng/g dry wt)	Medium (ng/ml)	Cells (ng/g dry wt)	Medium (μg/ml)
1	<0.1	10.4	<0.1	1.5
2	<0.1	8.3	<0.1	0.9
3	<0.1	1.2	<0.1	1.7
4	<0.1	20.4	<0.1	1.0

[a] Yields of capsaicin can be enhanced 5-fold by the addition of precursors (e.g., 5 mM isocapric acid) to the circulating nutrient medium

Suspension cultures (60 ml) were analysed 10 days into a culture period. For immobilised cell cultures, cells were immobilised for 21 days before insertion into a glass column (15 cm × 1.5 cm) through which 60 ml nutrient medium was circulated for a further 10 days, when cells and medium were analysed. Capsaicin was extracted from the cells in chloroform, and the extracts and nutrient medium were analysed using an enzyme-linked immunosorbent assay (ELISA), checked by TLC and GLC. Suspension cultures contained ~1 g dry wt cells, immobilised cell cultures 0.8–1.0 g dry wt cells. Four replicate culture samples were analysed; the data for each replicate represent the mean of 8 optical density ELISA measurements

cells [1,4]. Four categories of immobilization techniques may be listed: immobilization in an inert substratum (usually in one or more gels), the adsorption of cells to an inert substratum (usually for charged animal cells), the absorption of cells to inert substrata via biological macromolecules, and finally the covalent binding of cells to a substratum (such as carboxymethyl cellulose) (review [4]). All these techniques, however successful, require a number of steps to immobilize the cells, and a method which is more simple but effective would have obvious advantages, particularly in an industrial context.

This method fulfils a number of requirements. Not only is it very simple, but it also eliminates some of the specific problems encountered with other immobilization methods. The % cell viability data in table 1 demonstrate that the polyurethane foam support particles do not have detectable cytotoxic properties, as are associated with such immobilization matrices as polyacrylamide, gelatin, alginate plus gelatin and agarose plus gelatin [1,6], or with molten agar which must be used at 35–40°C [1,4,14]. Furthermore, as can be seen from fig.3 and 4, the immobilized cells are in direct contact with the nutrient medium; there is no permeability barrier to nutrients and metabolites which could be created by a gel.

As discussed in [4,11,15], the slow growth and high cell–cell contact of the cells would be expected to be an essential pre-condition to the establishment of a differentiating culture, and

some morphological differentiation is indeed apparent in the immobilized cells. It is believed that, as a result, cells immobilized in the above manner are closer, metabolically, to cells 'immobilized' in the whole plant than to the liquid-suspended cells from which they were derived.

This may explain the apparent increased capacity for capsaicin production by immobilized compared with suspended pepper cells, a result which indicates the potential of this technique for the large-scale production of this and other secondary metabolites.

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