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Achievement of thousand-fold accumulation of rosmarinic acid in immobilized cells of sweet basil (*Ocimum basilicum* L.) by ten-fold increase of the volume of the immobilization matrix

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A very high rosmarinic acid (RA) production (20 mg g^{-1} dry weight) was achieved by immobilizing sweet basil (*Ocimum basilicum* L.) cells at a high density (approximately 25×10^4 cells ml^{-1}) in a 4-ml gel matrix, a production performance that was 1400 times higher than in basil cells immobilized in spherical beads, with a 14-fold lower volume. Therefore, doubling of the volume of the immobilization matrix was associated with an increase of RA accumulation by a hundred times. In addition, RA was excreted into the culture medium, where it was collected without terminating the culture of immobilized cells. A complex relationship between oxygen transfer, ascorbate and RA biosynthesis was observed, with L-phenylalanine ammonia lyase (PAL) activity appearing to be a critical common link. The results are discussed in consideration of stress under conditions of nutrient and oxygen transfer restriction to immobilized cells.

Key words: calcium alginate, immobilization, *Ocimum basilicum*, rosmarinic acid.

INTRODUCTION

Sweet basil (*Ocimum basilicum* L.) is a source of the caffeic acid ester rosmarinic acid (α-*O*-caffeoyl-3,4-dihydroxyphenyllactic acid) (RA), a natural antioxidant widespread in the families Lamiaceae and Boraginaceae (Petersen *et al.*, 2009). In a previous study, we have used leaf-derived suspension cultures of sweet basil in order to accumulate rosmarinic acid up to 10 mg g^{-1} dry weight (d.w.), a value up to 11 times higher than in callus cultures or in leaves of donor plants (Kintzios *et al.*, 2003). During the same study, we investigated RA production in immobilized cell cultures, with rather disappointing effects. More analytically, sweet basil cells immobilized in 1.5, 2 or 3% w/v calcium alginate beads (each having a diameter of approximately 2 mm) accumulated RA at a much reduced rate (< 15 μg g^{-1}) compared to cell suspensions of tissue cultures.

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Bioreactors can also be used for the culture of cells immobilized in various substrates. Immobilisation helps in stabilising the cultured biomaterial for re-use. Whole cell immobilisation can be defined as the physical confinement or localisation of intact cells to a certain defined region of space with the preservation of some desired activity (Karel et *al.*, 1987; Willaert & Baron, 1996). The successful application of an immobilised cell system as a biocatalyst relies on the proper choice of the main components of the system, namely the matrix material, the cell type and the configuration of the immobilization system. Among the desirable characteristics for immobilized cell systems are a high surface-to-volume ratio, chemical and mechanical stability and optimum diffusion of oxygen and nutrients (Looby & Griffiths, 1990). However, the use of bioreactors specifically for immobilized cells is still in its infancy. Relative reports in the literature commonly refer to the application of basically pneumatically agitated reactors for the scale-up culture of immobilized cells from various plant species, such as *Daucus carota* (Majerus & Pareilleux,

1986), *Capsicum frutescens* (Lindsey & Yeoman, 1984) and, more recently, *Taxus cuspidata* (Han & Yuan, 2004).

In the present study, we report on the immobilization of sweet basil cells at high density in a 4-ml gel matrix, as a method to achieve considerable scale-up of RA production and extracellular release into the culture medium. Doubling of the volume of the immobilization matrix was associated with an increase of RA accumulation by a hundred times.

MATERIALS AND METHODS

Source of plant material and preparation of explants

Leaves from young sweet basil plants (purchased from a local nursery) were surface-sterilized for 12 min in 0.1% (w/v) $HgCl_2$, containing 1% (v/v) Tween 80, and then rinsed 3 times in sterilized distilled water. Leaf segments, 1 cm long, were excised and used as explants for callus induction.

Establishment of cell suspension cultures

For callus induction, leaves from young sweet basil plants were surface-sterilized as above. Leaf pieces, 1 cm long, were cut and grown in MS (Murashige & Skoog, 1962) basal medium supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} meso-inositol, 1 mg l^{-1} kinetin and 2 mg l^{-1} α-naphthaleneacetic acid (NAA) as plant growth regulators. For suspension culture, callus tissues, grown for three weeks on solid medium, were aseptically transferred into Erlenmeyer flasks containing liquid medium of the same composition and shaken at 100 rpm. In order to investigate the effect of ascorbate on RA biosynthesis in sweet basil cells, 10 mg l^{-1} of ascorbate were added to the MS medium. In this way two different nutrient media (one with and one without ascorbate) were used in parallel.

Cell immobilization

For cell immobilization, cell suspensions (derived from the same stock culture and aliquoted in 50-ml culture flasks) growing in liquid MS (± ascorbate) medium for one week were mixed with 2% (w/v) sodium alginate (diluted to MS medium ± ascorbate) and 4 ml of the mixture were transferred to 15 ml Cellstar® tubes (Greiner bio-one), the lower part of which was filled up with 1.5 ml of liquid MS medium (± ascorbate) without phosphate and Fe-EDTA and supplemented with 0.8 M $CaCl_2$. This solution was separated from the cell suspension (in sodium alginate) by means of sterile filter paper. An additional 1.5 ml of liquid MS medium was added on top of the cell suspension, separated from it with filter paper (Fig. 1) and the tube aseptically sealed with Parafilm®. Consequently, immobilization of the cells took place into the cylindrically shaped calcium alginate gel matrix, at a density of 25×10^4 cells ml^{-1} approximately. A total of 50 tubes containing immobilized cells were prepared according to this procedure. In a parallel experiment, oxygen was provided to each tube by an air pump (at a rate of 1 l m^{-1} at 0.2 bar) through an autoclaved silicone tube sealed with a hole at the bottom (air input), while an air vent with a 0.2 μm pore size sterilizing filter was attached to a hole in its screw cap (air output). In this way, we investigated the effect of oxygen transfer on RA accumulation in immobilized cells.

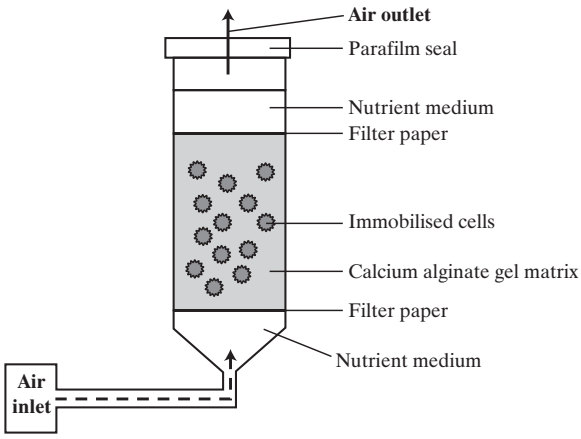

FIG. 1. Schematic representation of the 4-ml immobilization system for sweet basil cells.

In order to compare RA biosynthesis with cells immobilized under smaller scale conditions, cells were also immobilized in alginate beads, each having a diameter of 4 mm (i.e. a volume of 0.27 ml) and cultured in liquid MS medium.

All cultures were continuously shaken on a rotary shaker at 100 rpm and incubated under a photosynthetic photon flux density of 150 μmol m^{-2} s^{-1} (16 hrs of light daily, from cool white fluorescent lamps) at 24°C.

Biochemical analyses

For rosmarinic acid extraction and isolation, immobilized cells were extracted with 10 ml of 70% (v/v) methanol. The whole content (the cell containing gel, having a volume of 4 ml) of each bioreactor was used for the extraction. Extracts were centrifuged (4000 g;

10 min; 20°C) and the supernatant was concentrated under vacuum (40°C; N_2 stream) to 2 ml. Extracts were partitioned using acetonitrile/petroleum ether 40-60°C (1:1, v/v) to remove the non-polar fraction and the resulting samples were concentrated to a small volume using a rotary evaporator. Dry samples were rediluted in methanol (1 ml methanol per 100 mg d.w.) and kept at –20°C until analysis. Rosmarinic acid was analysed by HPLC (Jasco) using a Hypersil BDS C18 column (bead size 5 μm, 25 cm × 4.6 mm) and eluted with an isocratic elution [acetonitrile/formic acid (4:1, v/v) at 1.2 mL min^{-1}; detection at 300 nm]. Rosmarinic acid in the samples was identified using pure standard (Extrasynthese SA). Quantitative determination was made according to a reference curve using the peak areas.

Firstly, in order to see the activity of L-phenylalanine ammonia lyase (PAL) immobilized cells were homogenized in 5 ml protein extraction buffer (50 mM Tris, 700 mM Glycerol, 200 mM NaCl, 2 mM β-mercaptoathanol, 1 mM EDTA, pH 8.5). Afterwards the samples were centrifuged (11000 rpm; 40 min; 4°C). Two ml of the supernatant was mixed with 2 ml Tris/EDTA buffer containing 12 mM L-phenylalanine. After incubation at 40°C for 60 min, the reaction was stopped by adding 200 μl of 6N HCl and PAL activity (mM trans-cinnamic acid) was estimated by spectrophotometrically measuring the concentration of trans-cinnamic acid at 270 nm (Reignault *et al.*, 2001).

Data analysis

Ten individual immobilized cell cultures from each different treatment (± ascorbate) were weekly assayed for RA accumulation and PAL activity. The duration of the culture period was five weeks in immobilization. Experiments were set-up in a completely randomized design. Statistical analysis was based on analysis of variance (ANOVA). Significant differences ($p < 0.01$) among the means were determined by Duncan's multiple range test. Correlations among different treatments and the investigated physiological parameters were calculated using EXCEL software (Microsoft Inc., USA).

Chemicals

Unless otherwise stated, all solvents and chemicals used were of analytical quality and were obtained from Sigma Company, St. Louis. Water was double distilled.

RESULTS AND DISCUSSION

Effect of ascorbate and oxygen on RA accumulation

Continuous RA accumulation in immobilized *O. basilicum* cells was observed throughout the culture period, which depended significantly on the addition of ascorbate to the nutrient medium and the provision of air to the immobilized cultures; when no oxygen was pumped into the cultures (i.e. oxygen was limited), the average RA production (over the five-week culture period) from cells supplemented with ascorbate was three-fold higher than from cells cultured in an ascorbate-free medium. This effect was not repeated when cultures were aerated; on the contrary, addition of ascorbate was associated with a reduction of RA accumulation during weeks 2 to 5. In non-aerated cultures, the observed effect of ascorbate on elicitation of RA biosynthesis might have been associated with the activity of components of the phenylpropanoid pathway in *O. basilicum*. For example, Wang *et al.* (1997) isolated a 4-coumaroyl-CoA 3-hydroxylase from cell cultures of *Lithospermum erythrorhizon*, which required ascorbate, NADH or NADPH as cofactors, with ascorbate being most effective. Ascorbate also influences the $NADP^+$/NADPH ratio, which in turn has been found to control secondary metabolite biosynthesis via the oxidative pentose phosphate pathway (Lattanzio *et al.*, 2009). It is possible that, under conditions of forced aeration, primary metabolism is overwhelmingly favored against the biosynthesis of secondary metabolites. Primary metabolism could also have been promoted by the addition of the electron-donating ascorbate, as reported elsewhere (Barberaki & Kintzios, 2002; Papanastasiou *et al.*, 2008). Definitely, the complex association between oxygen transfer, ascorbate and secondary biosynthetic pathways needs to be further investigated (see also below about PAL activity).

Effect of the immobilation volume on RA accumulation

The highest RA concentration (20 mg g^{-1} d.w.) was observed during the first week of culture, and declined slightly thereafter (Fig. 2A). This concentration is the maximum reported for an *Ocimum* species: Rady & Nazif (2005) have reported previously the synthesis of 3 mg g^{-1} d.w. RA in *O. americanum* plantlets regenerated from shoot cultures grown on MS medium supplemented with BA (1 mg l^{-1}) and NAA (0.25 mg l^{-1}). By increasing the volume of the immo-

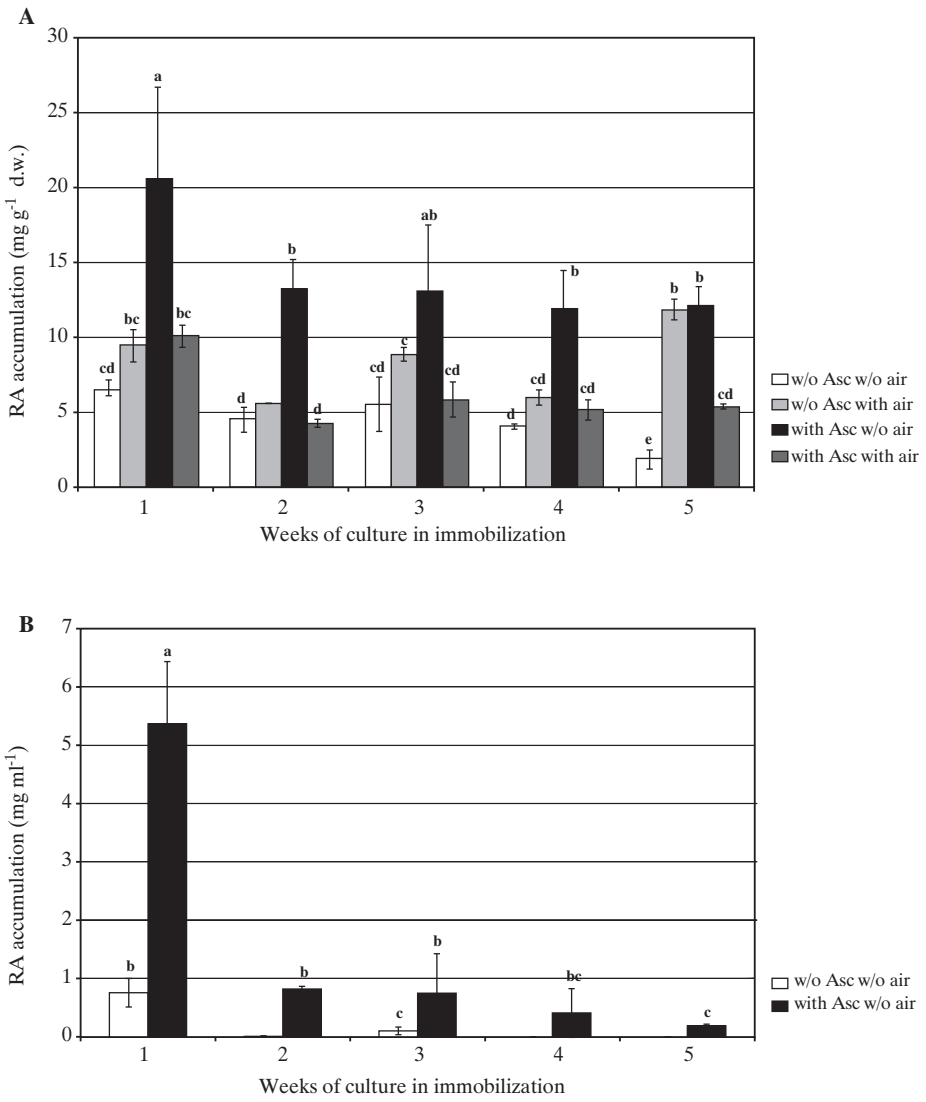

FIG. 2. A) RA accumulation in immobilized *O. basilicum* cells over a culture period of five weeks. White columns: non-aerated, control cultures; black columns: non-aerated, ascorbate-treated cultures; gray columns: aerated, control cultures; shaded columns: aerated, ascorbate-treated cultures. B) RA accumulation in nutrient medium over a culture period of five weeks (non-aerated cultures only). White columns: control cultures; black columns: ascorbate-treated cultures. Vertical bars correspond to standard deviations (n = 10). Columns marked with different letters indicate statistically different values ($p < 0.01$).

bilization matrix, RA accumulation in immobilized cells (20 + 6 mg g^{-1} d.w.) increased approximately 1400 times compared to cells immobilized in spherical beads (which accumulated RA at an average concentration of 14 μg g^{-1} d.w.). The achieved concentration was also much higher than in 2.5-l bioreactors (29 ± 4 μg g^{-1} d.w.) and in sweet basil plants regenerated in 2.5-l bioreactors (178 ± 52 μg g^{-1} d.w.) (Kintzios *et al.*, 2004).

Similarly to immobilized cells, a high RA concentration (5 mg ml^{-1}) in the nutrient medium was also determined during the first week of culture, but decreased considerably during the rest of the culture period (Fig. 2B). The highest RA concentration was determined in the medium of non-aerated, ascorbate-supplemented cultures (conditions of oxygen limitation), whereas considerably lower RA levels were determined in the medium of aerated cultures (only results from non-aerated cultures are presented). Hence, RA accumulation in immobilized cells and its exudation to the culture medium were negatively correlated with the duration of the culture, with or without

the addition of ascorbate (average $r^2 = -0.80$ and -0.79, respectively, for non-aerated cultures). On the other hand, the pattern of RA exudation in the nutrient medium was highly positive correlated with RA accumulation in immobilized cells ($r^2 = 0.72$ and 0.99 for control and ascorbate-treated cultures, respectively, for non-aerated cultures).

Effect of culture growth on RA accumulation

In our experiments, sweet basil cells were grown for one week in liquid medium prior to immobilization. As previously determined under identical culture conditions (Kintzios *et al*., 2003), maximum RA accumulation took place in sweet basil cell suspensions during the first week of culture in liquid medium, concomitantly with the exponential phase of culture growth. RA concentration declined rapidly thereafter. This pattern is in full agreement with the observed maximum in RA accumulation during the first week of culture in immobilization (which corresponds to the fifth week since culture establishment, i.e. 3 weeks after callus induction and after one week in suspension culture) and the reduced concentration during the rest of the culture period. Increased RA accumulation might have been associated with alteration of the growth of immobilized sweet basil cells. Reports on the growth-dependence of RA biosynthesis *in vitro* are frequently contradictory to each other. For example, suspension cultures of *Coleus blumei* synthesize and accumulate all their RA during only a few days at the end of the growth phase (Horn *et al.*, 2004). However, Bauer *et al.* (2004) demonstrated that different lines showed different RA accumulation in relation to their growth rate; it was either parallel or inversely related to the tissue growth. In a number of parallel-run experiments, Kintzios *et al.* (1998) observed that RA accumulation in *Salvia officinalis* was inversely related to callus growth whereas, in contrast, RA accumulation in *S. fruticosa* callus continuously increased in a parallel fashion to callus growth.

It is also possible that biosynthesis of RA is stimulated as a cellular response to induced stress. For example, perturbation of proline metabolism in shoot cultures of *Origanum vulgare* is associated with a redirection of metabolites from the pentose phosphate pathway toward phenolic acid synthesis (Yang & Shetty, 1998; Lattanzio *et al.*, 2009). Mass-transfer phenomena occurring in gel-immobilized cell systems have been thoroughly investigated, however the relationship of immobilization to cell physiology through the creation of nutrient transport gradients within the gel matrix has not been fully explored (Radovich, 1985; Nguyen & Luong, 1986). Basil cells might have experienced an increased stress under conditions of nutrient and oxygen restriction, such as those applied with the immobilization procedure described in the present study. However, restriction of nutrient availability depends on the size of the calcium alginate gel matrix. With the exception of cells bordering the upper or the lower nutrient medium reservoirs, mass and oxygen transfer to the majority of cells immobilized within the 4-ml gel matrix was severely inhibited, which might have resulted to the induction of considerable stress. On the contrary, small (0.27 ml) spherical beads exhibited a lower external mass transfer resistance, thus enabling increased nutrient and oxygen flow to immobilized cells.

Patterns of PAL activity

In non-aerated cultures, PAL activity in immobilized cells increased gradually between the first and the fourth weeks, but decreased during the fifth week (Fig. 3A). Furthermore, in ascorbate-treated cultures PAL activity was negatively correlated with RA concentration in immobilized cells ($r^2 = -0.78$) and the nutrient medium ($r^2 = -0.75$). In similar fashion, we have previously reported that PAL activity in bioreactor-cultured tissues of sweet basil was inversely related to rosmarinic acid accumulation (Kintzios *et al*., 2004). Although the first step to rosmarinic acid biosynthesis is the deamination of L-phenylalanine to *trans*-cinnamic acid, the whole process is quite complex, requiring additional reactions, such as the transamination of L-tyrosine and *trans*-cinnamate hydroxylation to 4-coumaric acid in an absolute manner (Petersen & Simmonds, 2003). Therefore, a decrease in PAL activity of immobilized *O. basilicum* cultures may have been counterbalanced by an overexpression of other enzymes in the rosmarinic acid biosynthetic pathway, a hypothesis that is currently under investigation in our laboratory. On the other hand, when immobilized cell cultures were aerated, a fluctuating PAL activity was observed over the culture period, which was five- to ten-fold higher than in non-aerated cultures (Fig. 3B). Contrary to non-aerated cultures, PAL activity was highly correlated with RA concentration in immobilized cells ($r^2 = 0.73$), but only when no ascorbate was added to the culture medium (i.e. no correlation was identified in ascorbate-treated,

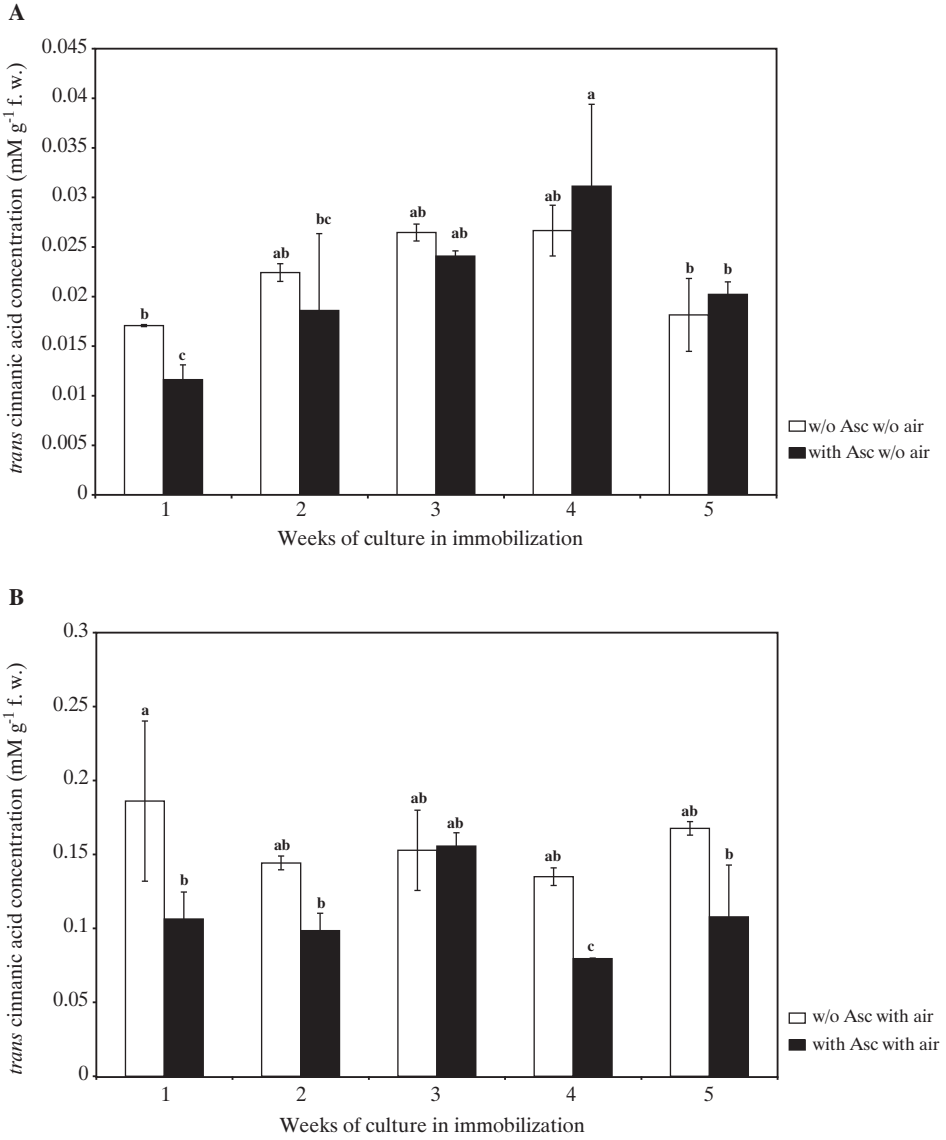

FIG. 3. PAL activity (expressed as *trans*-cinnamic acid concentration) in immobilized *O. basilicum* cells over a culture period of five weeks. A) Non-aerated cultures. B) Aerated cultures. White columns: control cultures; black columns: ascorbate-treated cultures. Vertical bars correspond to standard deviations (n = 10). Columns marked with different letters indicate statistically different values ($p < 0.01$).

aerated cultures). Once again, the complex relationship between oxygen transfer, ascorbate and RA biosynthesis was observed, with PAL activity appearing to be a critical common link.

CONCLUSION

Cell immobilization in alginate gels is quite popular due to low cost, natural origin and easy handling (Douméche *et al.*, 2004). The present study demonstrates that immobilization of *O. basilicum* cells in a 4-ml gel matrix could be utilized as an approach for scaling-up rosmarinic acid production in a mini-bioreactor. Mini-bioreactors (an example of which is the test-tube reactor, similar to the one presented here) have a volume less than 100 ml and are available for various purposes, since they perform like large-scale bioreactors as far as most of the process parameters are concerned. Thus, they offer the advantage for fast and direct scale-up, which reduces development time and costs (Tanaka *et al.*, 1993; Kumar *et al.*, 2004; Matkowski, 2008). In addition, achieving secretion of the product from living, immobilized cells is critical to efficient operation of perfusion systems. Further ex-

periments are focused on investigating the effect of different mass transfer rates on RA accumulation, by incorporating the immobilized culture in an appropriate bioreactor system.

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