0273-2289/96/5602—0169$08.00

Biotransformation from Geraniol to Nerol by Immobilized Grapevine Cells (*V. vinifera*)

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Received November 1, 1993;
Revised November 16, 1994; Accepted December 12, 1994

ABSTRACT

The abilities of two grapevine cell suspensions (*Vitis vinifera* L. cv. Gamay Fréaux and *Vitis vinifera* L. cv. Monastrell) to biotransform geraniol into nerol in a biphasic system based on the culture medium and Miglyol 812 were compared. The Gamay grape cell suspension was able to transform higher concentrations of geraniol into nerol than the Monastrell one. Gamay grape cells were immobilized in both calcium alginate beads and polyurethane foams. The cytotoxic effect of increasing concentrations of geraniol, as well as the ability of the immobilized cells to biotransform geraniol into nerol, was checked. Immobilization proved to be advantageous in protecting cells against the toxicity of the substrate. Furthermore, immobilization also seemed to have an effect on the secondary metabolism, the cells immobilized in polyurethane foams being more efficient at performing the isomerization process (40% conversion of geraniol into nerol) than both the freely suspended and calcium alginate immobilized cells (20% conversion).

Index Entries: Immobilized plant cells; biotransformations; *Vitis vinifera*; monoterpenes; biphasic systems; cell viability.

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INTRODUCTION

In recent years, the ability of cultured plant cells to metabolize and/or transform exogenous compounds has been intensively studied. In general, many of the cell cultures tested so far are able to perform biotransformations of foreign substrates with the possibility of obtaining more useful substances (*1*). Thus, plant cells can be an alternative source of high-value compounds that are difficult to obtain by means of chemical and microbiological synthesis. Plant cells are normally very sensitive to chemical and physical stress, and a number of biological and technological factors must be considered if industrial use is envisaged.

Many of the problems concerning the use of plant cells to produce fine chemicals can be alleviated by cell-immobilization techniques (*2*). Immobilization allows the reuse of the cell culture in continuous systems, protecting cells against physical and chemical stress and increasing the cell line stability and the secondary metabolism (*3*).

Among the natural compounds of industrial interest, monoterpenes are well known because of their use as flavors and fragances in the food and cosmetics industries. Nowadays, these compounds are principally obtained either by chemical synthesis or from their natural sources. Nevertheless, the use of in vitro cultures of plant cells is an alternative means that is being considered (*4*). The toxicity of monoterpenes is a handicap to be taken into account when deciding their biotransformation by plant cell cultures. Monoterpenes have been shown to be toxic for plant cells, lowering the mitochondria number in seedling roots, inhibiting the photosynthesis and respiration processes, and decreasing cell membrane permeability (*5*). The use of biphasic systems containing nonmiscible solvents within the culture medium seems to exert a certain degree of protection on plant cells, the extraction of the product being performed meanwhile (*6*).

Since grapevine characteristic aroma comes from certain monoterpenes as linalool, nerol, geraniol, and α-terpineol, the aim of the present work has been the biotransformation of geraniol into nerol by grapevine cells (*V. vinifera*). The biotranformation process was carried out not only with the freely suspended cells, but also immobilized in two different supports (calcium alginate beads and polyurethane foams) placed within biphasic systems consisting of culture medium and Miglyol 812 (a mixture of water-nonsoluble triglycerides).

This biotransformation process was chosen as a model system with plant cells, since an isomerization reaction of this kind by chemical means presents certain difficulties. The immobilized cell systems were checked not only for the capacity to biotransform geraniol into nerol, but also to determine the advantages of every system to protect plant cells from cytotoxic effects exerted by the substrate. Therefore, this work attempts to further the knowledge of the possibilities offered by the combined use of immobilized plant cells and biphasic systems to carry out these biotransformations.

MATERIALS AND METHODS

Cell Cultures

Two different grape cell suspensions were employed: *V. vinifera* L. cv. Gamay Fréaux, established by J. C. Pech (ENSAT, Toulouse, France) in 1978, and *V. vinifera* L. cv. Monastrell, established by A. Ros-Barceló (University of Murcia, Murcia, Spain) in 1988. Both cell lines were initiated from pulp fragments of young fruits.

Gamay grape suspension cultures were maintained in Murashige-Skoog medium, pH 6.0, supplemented with 30 g/L sucrose, 2 mg/L kinetin, 0.1 mg/L 1-naphthalenacetic acid and 250 mg/L acid-hydrolysed casein, under 16-h photoperiods (1.7 W/m^2). Monastrell grape suspension cultures were maintained in Gamborg B5 medium, pH 6.0, supplemented with 30 mg/L sucrose, 0.2 mg/L kinetin, 0.1 mg/L 1-naphthalenacetic acid, and 250 mg/L acid-hydrolyzed casein in the dark.

Both cell cultures were kept at 27°C on gyratory shakers (120 rpm) and subcultured by transferring 20 mL of 20-d-old cell suspension to 80 mL of fresh medium. Biomass was estimated by measurement of packed cell volume (PCV) after centrifugation at 1000*g* for 5 min.

Immobilization Procedures

Calcium Alginate

Twenty milliliters of PCV from a suspension culture at the end of the lag-growth phase were resuspended in 60 mL of 1.6% (w/v) sodium alginate solution containing 0.86% (w/v) of sodium chloride. The cells-alginate suspension was dripped through an injector (0.1 mm of needle diameter) into a 0.05*M* calcium chloride solution. Bead diameter was adjusted to 2.0 mm by controlling the air flow through the injector. Prior to transfer to the fresh medium, beads were maintained into the calcium chloride solution until complete gelation and washed three times with Hank's solution. Assays employing these immobilized cells were carried out immediately after immobilization.

Polyurethane Foam

A polyurethane foam matrix of an average pore size of 0.75 mm and 30 pores/cm^2 (Polyol International BV, Switzerland) was cut into 2 × 2.5 × 3.5 cm pads, washed in ethanol, later in water, and autoclaved in a 50-m*M* sodium phosphate buffer, pH 6.5. Four of these empty foam matrices were fixed at the bottom of a 250-mL Erlenmeyer flask (adapted from ref. 7), and then 100 mL of a 10% PCV grape cells suspension containing 1 m*M* cysteine were placed in the flask. Experiments employing these immobilized cells were performed after a period of 3–4 wk, once most of the void space of the foam pads was colonized by the cells. Biomass immobilized into the polyurethane foam matrices was estimated by determining the PCV, after cells removal by means of a vigorous shaking.

Viability Assay

Viability of the freely suspended and calcium alginate-immobilized cells was estimated by the 2,3,5-triphenyhltetrazolium chloride (TTC) reduction method adapted as described in ref. (*8*). Viability of polyurethane-immobilized cells was estimated by the same method, after cell removal from the foam matrix by means of vigorous shaking of the culture medium. Assays were performed in triplicate.

Cytotoxicity of Geraniol

Both freely suspended and immobilized cells (5 mL PCV) were placed in 35 mL of Murashige-Skoog medium. Increasing concentrations of geraniol were added, either directly into the medium, monophasic system, or as a solute in 4 mL of Miglyol 812 (Huls Troisdorf AG, Germany) biphasic system. Cell viability was estimated as described above, 24 h after geraniol addition. All figures show typical results from three independent experiments.

Biotransformation Assay

In order to biotransform all added geraniol within a reasonable reaction time, different concentrations for both substrate and cells were employed in the experiments. Hence, 50 mL PCV of freely suspended cells, 40 mL PCV of polyurethane foam-immobilized cells, and 10 mL PCV of calcium alginate-immobilized cells were resuspended each in fresh medium to reach a final volume of 100 mL. Then, geraniol was added as a solute in 10 mL of Miglyol 812; this solution was previously sterilized by means of 0.22-μm filters. Throughout the experimental time, 0.2-mL aliquots were removed from the organic phase, centrifuged (1000*g*, 5 min), and analyzed by gas chromatography. In the same way, a volume of 1 mL PCV of freely suspended or immobilized cells was removed from the flasks every 2 d, washed with Hank's solution, and homogenized with 10 mL of *n*-pentane. These organic extracts were then centrifuged, concentrated in a vacuum evaporator, and subjected to chromatographic analysis. Typical results from two independent experiments are shown.

Monoterpene Analysis

Concentrations of geraniol and biotransformation products, both within the organic phase (Miglyol 812) and the cell extracts, were determined using a Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector (FID) and a 30-m Supelco SPB-1 capillary column. Hydrogen and air flows were adjusted to 38 and 480 mL/min, respectively. Injector and detector temperatures were set at 300°C. In order to quantify the reaction intermediates, every sample was analyzed under two different conditions.

First set of conditions: 40 cm/min nitrogen flow, oven temperature set at 80°C for 10 min, then increasing at a rate of 1°C/min. Under these conditions, the retention times were as follows: 43.00 min for nerol plus citronellol; 43.32 min for neral, 45.75 min for geraniol, and 46.09 min for geranial.

Second set of conditions: 200 cm/min nitrogen flow, oven temperature set at 60°C. Under these conditions, the retention times were as follows: 38.83 min for nerol plus neral, 40.7 min for citronellol, and 48.7 min for geraniol plus geranial.

Analyses were carried out in triplicate. Linalool was employed as internal standard once it was demonstrated that this compound was not involved in the geraniol metabolization.

RESULTS

Figures 1 and 2 present the ability of two suspensions cultures of grape cells (*V. vinifera* L. cv. Monastrell and *V. vinifera* L. cv. Gamay Fréaux) to biotransform geraniol into nerol using biphasic systems based on culture medium and Miglyol 812 (10/1, v/v). None of the plant cell cultures were able to biotransform stoichiometrically geraniol into nerol, this latter being the main product detected within the reaction medium. When the organic phase and the cell extracts were analyzed, any other monoterpenic alcohol was not observed (i.e., citronellol, terpineol, linalool, and so on), except for small amounts of geraniol and neral (< 2% of the amount of geraniol added). As can be seen, the Gamay grape suspension culture was able to metabolize geraniol after 80 h of study, whereas in the Monastrell grape suspension, only 80% was metabolized after 200 h of experiment. In addition, the Gamay grape performed a faster biotransformation, even though geraniol concentration used in this case was fivefold (0.5 mL/L suspension culture) that used for the Monastrell grape transformation (0.1 mL/L suspension culture). It has to be remarked that the former took half of the time taken by the latter (40 and 80 h, respectively) to achieve 20% of nerol conversion. We also observed some differences with respect to the biotransformation equilibrium. When the process was carried out by the Gamay grape cell suspension (Fig. 2), it took 60 h to reach a 21% conversion, and 48 h later, the product concentration within the organic phase was nearly the same. On the contrary, when the process was performed by the Monastrell cells (Fig. 1), it took 144 h to achieve 25% of conversion, and there was a 25% substrate left. From this time on, the concentration of geraniol and nerol within the organic phase went down at similar rate. Thus, Gamay grape cells were able to transform higher concentrations and were quicker to achieve the same percentage of the total amounts added. These facts made us use the Gamay cell line from that moment on.

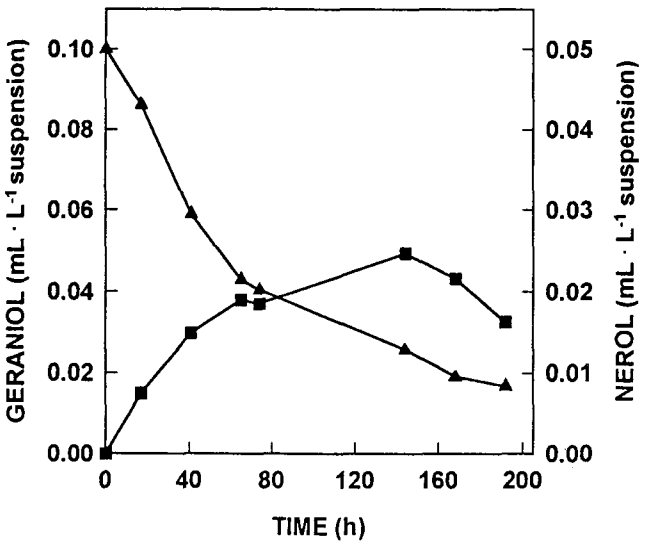

Fig. 1. Biotransformation of geraniol catalyzed by the Monastrell grape cell suspension in the biphasic system. Geraniol (▲); nerol (■).

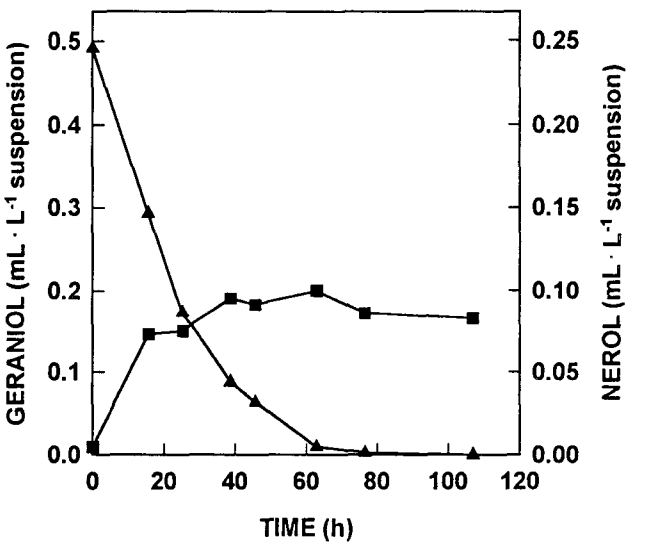

Fig. 2. Biotransformation of geraniol catalyzed by the Gamay grape cell suspension in the biphasic system. Geraniol (▲); nerol (■).

The effect of geraniol on the viability of Gamay grape cells, both freely suspended and immobilized, is depicted in Fig. 3. The higher viability percentages were for cells immobilized into calcium alginate. On the contrary, freely suspended cells were far more affected by increasing concentrations of this monoterpene. Thus, geraniol concentrations higher than 0.2 mL/L of suspension culture affected cells immobilized in calcium alginate, whereas for polyurethane-immobilized cells, this concentration was lower (0.1 mL/L of suspension) and even lower for freely suspended cells.

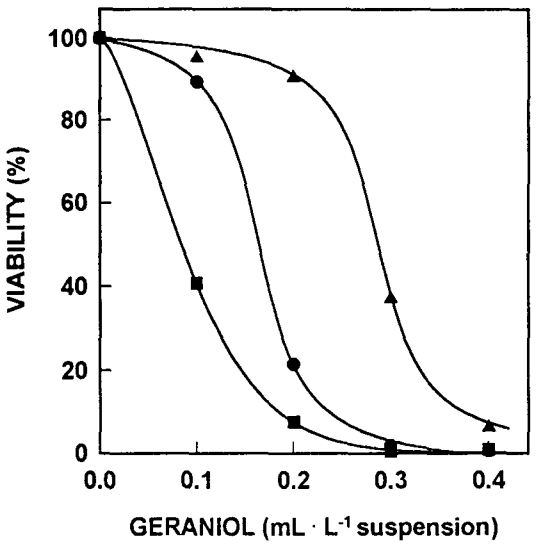

Fig. 3. Effect of increasing concentrations of geraniol on the viability of Gamay grape cells, freely suspended (■) and immobilized into calcium alginate beads (▲) and polyurethane foams (●) in the monophasic system.

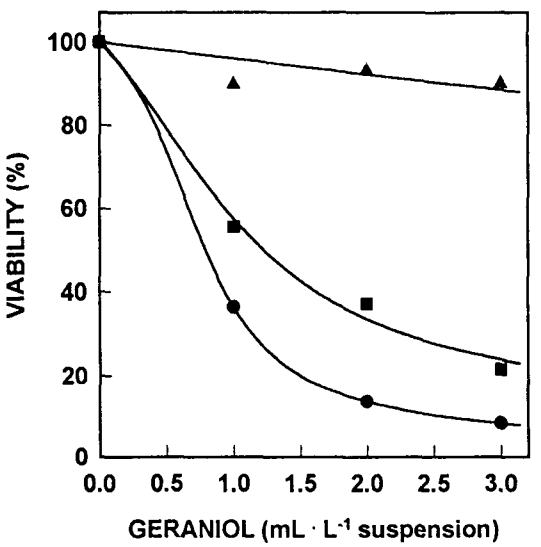

Fig. 4. Effect of increasing concentrations of geraniol on the viability of Gamay grape cells, freely suspended (■) and immobilized into calcium alginate beads (▲) and polyurethane foams (●) in the biphasic system.

The effect of increasing concentrations of geraniol on Gamay grape cells, both freely suspended and immobilized, when this monoterpene was added dissolved in Miglyol 812 is presented in Fig. 4. As in the previous case, the higher viability percentages were those of calcium alginate-immobilized cells. The viability assay showed percentages higher than 90% when Miglyol 812 contained a geraniol concentration of 3 mL/L of suspension culture.

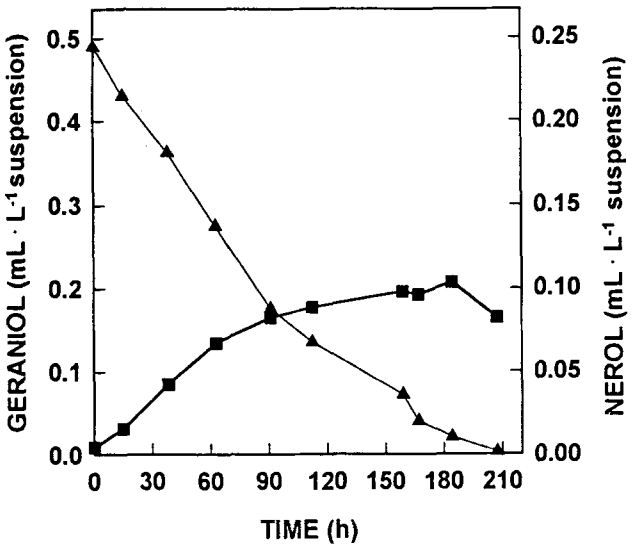

Fig. 5. Biotransformation of geraniol catalyzed by the calcium alginate-immobilized Gamay grape cells, in the biphasic system (culture medium/Miglyol 812, 10/1, v/v). Geraniol (▲); nerol (■). Initial geraniol concentration: 0.5 mL/L of suspension.

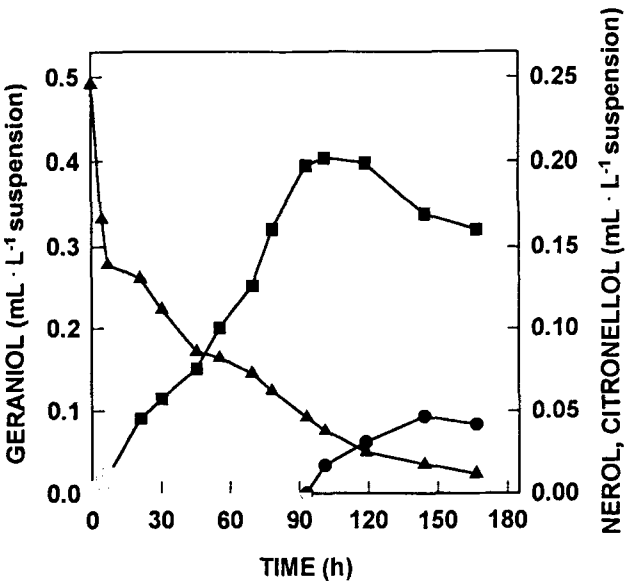

Fig. 6. Biotransformation of geraniol catalyzed by the polyurethane foam-immobilized Gamay grape cells, in the biphasic system (culture medium/Miglyol 812, 10/1, v/v). Geraniol (▲); nerol (■); citronellol (●).

The ability of *V. vinifera* L. cv. Gamay Fréaux cells, immobilized both in calcium alginate and polyurethane foams, to biotransform geraniol into nerol in the biphasic system is presented in Figs. 5 and 6, respectively. As in the previous studies, the calcium alginate system showed that nerol was the only detected product in the organic phase, the maximum con-

version after 180 h of incubation being 20%. On the contrary, in the polyurethane foam-immobilized cell system, not only was conversion 40%, but citronellol appeared as another biotransformation product. This fact took place after 100 h of incubation. It has to be remarked that none of the experiments showed any other monoterpenic alcohols (i.e., linalool, terpineol, and so forth), and only aldehydes as geranial and neral were found at concentrations lower than 3% of the amount of geraniol initially added.

DISCUSSION

When comparing the ability of the two suspension cultures of grape cells to biotransform geraniol into nerol (Figs. 1 and 2), it was observed that the latter was the only main product deleted within the reaction medium.

Ambid et al. reported interconversions of monoterpenes catalyzed by cell suspensions of *V. vinifera* L. cv. Muscat de Frontignan, in which geranial and neral were biotransformed into geraniol and nerol, as well as geraniol into geranyl acetate (*9*) and geraniol into neral, nerol, and citronellol (*10*). When comparing the ability of plant cell suspensions from different sources to biotransform exogenous geraniol, Carriere et al. (*11*) reported a maximum conversion of 25% of geraniol into nerol for *Euphorbia characias* and 40–60% of geraniol into geranial and neral for *Glycine maximus*. In general, geraniol was biotransformed into geranial, neral, and nerol, and further metabolized into other compounds. Gbolade and Lockwood (*12*) observed maximum conversion values of 32–36% of geraniol into nerol when using plant cell suspensions of *Petroselinum crispum* of the "Paramount" and "Plain-leaved" varieties.

None of the above-mentioned experiments presented total biotransformation. Furthermore, the reaction products disappeared from the culture medium owing to the fact that cells metabolized them into other compounds. Thus, Croteau et al., using *Mentha piperita* cells and *l*-menthone as substrate, demonstrated that this compound was metabolized into esters (*13*) or even degraded by β-oxidation (*14*). Other studies with *V. vinifera* L. cv. Muscat de Frontignan showed that added geraniol was metabolized into glycosides and geranic acid (*15*).

Cormier and Ambid (*16*) also studied geraniol metabolization by cell suspensions of *V. vinifera* L. cv. Muscat de Frontignan within a biphasic system culture medium-Miglyol 812. They proposed different metabolization pathways, showing that Miglyol 812 improved the persistence of the products and limited the amount of geraniol within the organic phase.

On the other hand, it seems that there is not any clear relationship among the monoterpene toxicity, its structure, and culture medium solubility. Brown et al. stated that geraniol concentrations higher than 0.5 g/L were lethal for suspension cultures of *Pelargonium fragrans* cells within all of the growth phases (*5*). In our study, higher viability percentages were obtained for cells immobilized into calcium alginate. In contrast, freely

suspended cells in the monophasic system and polyurethane-immobilized cells in the biphasic system were far more affected by increased geraniol concentrations. Although there are no studies, to our knowledge, on the possible protective effect of the immobilization support on plant cells subjected to the presence of cytotoxic compounds, this effect has been shown for microorganisms. Thus, Barros et al. (*17*) observed higher viability levels for *Saccharomyces bayamus* cells immobilized into κ-carragenan when subjected to the cytotoxic effect of oleic acid. Schueler et al. (*18*) also observed higher cell stability, for *Pseudomonas oleovorans* to biotransform 1-octene into 1,2-epoxyoctane when immobilized within a solid support. In our study, the higher protective effect of calcium alginate in comparison with the polyurethane foam results can be explained considering the different nature of the support. Thus, the polyurethane foam hydrophobicity makes geraniol stick on this support. On the other hand, when comparing the monophasic and biphasic systems, it was observed that the organic phase allows higher concentrations of monoterpenes without causing any detrimental effect to the cell suspension (Figs. 3 and 4). In fact, biphasic systems have been used successfully in plant cell studies for *in situ* extraction of the nonpolar neoformed compounds (*5,19*). The lower cytotoxic effect on the cell suspension cultures can be explained by considering the partition of compounds between both phases.

With respect to the reaction kinetics (Figs. 5 and 6), it was observed that the immobilization process did not affect, in a significant manner, the rate of conversion, since it has to be taken into account that different cell concentrations were used in the various experiments. Thus, the rate of biotransformation of the calcium alginate-immobilized cell systems seems to be lower than that of both the freely suspended and the polyurethane-immobilized cell systems. However, the former system cell concentration was also lower (10% PCV) than those used in the other systems (50% and 40% PCV, respectively).

When comparing the ability of Gamay grape cells immobilized both in calcium alginate and polyurethane foam to biotransform geraniol into nerol (Figs. 5 and 6), it was observed that within the calcium alginate system, nerol was the only biotransformation product, whereas in the polyurethane foam-immobilized one, not only nerol, but citronellol appeared as another biotransformation product. This monoterpenic alcohol can be obtained from geraniol and nerol in a *V. vinifera* L. cv. Muscat suspension culture catalyzed reaction (*10*). In our studies with the Gamay grape cells, citronellol was only produced from nerol, once the geraniol to nerol biotransformation achieved a 40% conversion. This was only observed with the polyurethane-immobilized cells.

Other studies concerning the biotransformation of various substrates using polyurethane foam- and calcium alginate-immobilized cells have been performed resulting in different behaviors (*20–22*). Gbolade and Lockwood (*23*) used *P. crispum* cv. Paramount and Plain-leaved cells immobilized in polyurethane foam to biotransform monoterpenes. They

found that the immobilized system was more efficient than the freely suspended with respect to the geraniol into nerol isomerization, even though it was less efficient when reducing the citral and citroneral aldehydes.

CONCLUSIONS

In our study, the use of immobilization techniques in combination with a biphasic system minimizes the cytotoxic effects generated by those compounds involved within the biotransformation. The fact that the calcium alginate-immobilized cells were more protected than those immobilized within the polyurethane foams (in both the mono- and biphasic systems) can be the result of the hydrophobic nature of the foams. However, the maximum conversion of geraniol into nerol was observed for the polyurethane-immobilized system (40% compared with 20% for the freely suspended and calcium alginate-immobilized cells, respectively). Further, in this system, once the maximum conversion of geraniol into nerol was achieved, citronellol appeared as a second product of the biotransformation process.

ACKNOWLEDGMENTS

This work was partially supported by the grant no. BIO89/0297 from CICYT, Spain. J. Guardiola is a fellow of the Instituto de Fomento de la Región de Murcia. We are grateful to A. Ros Barceló and R. Muñoz for the generous supply of the grape cell lines. We are also grateful to Polyol International BV for the gift of the polyurethane foam and to Hüls Española SA for the Miglyol 812 samples.

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