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Modified Cyclodextrins Are Chemically Defined Glucan Inducers of Defense Responses in Grapevine Cell Cultures

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In grapevine (*Vitis vinifera* L.), defense responses after microbial infection or treatment with elicitors involve accumulation of phytoalexins, oxidative burst, and the synthesis of pathogenesis-related proteins. Oligosaccharide fractions from fungal or algal cell walls efficiently induce the defense responses, but a detailed analysis of the elicitor−plant cell surface interaction at the molecular level is precluded by the lack of chemically pure oligosaccharide elicitors. A grapevine liquid cell culture system was used to examine the properties of cyclodextrins (CDs) as inducers of defense responses. This work shows that the chemically pure heptakis(2,6-di-*O*-methyl)-βCD caused a dramatic extracellular accumulation of the phytoalexin resveratrol and changes in peroxidase activity and isoenzymatic pattern. Other modified CDs tested on several grapevine cell lines resulted in different eliciting capacities of CDs and different sensibilities of the cell lines. The spent medium of elicited cultures was shown to disturb *Botrytis cinerea* growth in a plate assay.

KEYWORDS: Grapevine; cell culture; elicitor; peroxidase; cyclodextrin; DIMEB; resveratrol

INTRODUCTION

In grapevine, the most frequently observed and most thoroughly characterized inducible defense mechanisms are the accumulation of phytoalexins and the synthesis of pathogenesis-related proteins (PR) (*1*). These responses are activated after infection by pathogenic microorganisms, although it has been described that abiotic stress, such as UV light (*2*), fungal oligosaccharides prepared from cell wall fragments, or oligosaccharides resulting from the action of fungal-degrading enzymes on host cell walls, may also elicit the same responses (*3*, *4*).

Phytoalexins from *Vitis* species are constituted of a restricted group of molecules belonging to the stilbene family (*5*), which derive primarily from *trans*-resveratrol (*t*-R, 3,4′,5-trihydroxystilbene), the presence of which in planta has been related to grapevine disease resistance. *t*-R is synthesized as an end product of the phenylpropanoid pathway by condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA in a reaction catalyzed by stilbene synthase (*6*). The subsequent oxidative coupling of *t*-R leads to several oligomers such as ϵ- and α-viniferins (*2*). Thus, the accumulation of *t*-R has been correlated with various degrees of disease resistance (*7*). For example, transgenic tobacco plants transformed with stilbene synthase genes from grapevine displayed enhanced resistance to fungal infection and accumulated *t*-R in the infection sites (*8*).

Although considerable progress has been made in the study of *t*-R biosynthesis, the enzymology of viniferins synthesis in grapevine is still incomplete. Until now, peroxidase has been considered the only plant enzyme to be associated with the oxidation of *t*-R to viniferins through a process similar to that involved in the formation of oligomeric lignans (*9*). However, to date, all experiments performed using *t*-R and peroxidase to obtain resveratrol oligomers with the natural configuration (coupling pattern) found in planta have been unsuccessful. Thus, Langcake and Pryce (*10*) demonstrated the formation of a grapevine phytoalexin mimic, analogous in structure and biological properties to ϵ-viniferin with a different oxidation coupling by using horseradish peroxidase.

The oxidation of 4-hydroxystilbenes (including *t*-R) has been studied extensively (*10*−*12*). The most significant results showed that *t*-R oxidation in grapevine is controlled by three peroxidase isoenzymes, one of an acidic nature (named A_1), another basic peroxidase of low p*I*, B_3 (both located bound to the cell wall in equilibrium with the cell wall free spaces), and a strongly basic peroxidase isoenzyme, B_5 (located at the vacuolar level). These isoenzymes of peroxidase are linked to both constitutive and inducible defenses of grapevine against biotic and abiotic elicitors (*12*).

Grapevine peroxidases, as members of the class III peroxidase group, are protohemin IX-containing glycoproteins having molecular masses usually in the range from 35 to 45 kDa (*13*, *14*), depending on the glycosylation degree. They are able to oxidize a wide variety of natural phenolics (*15*) in accordance

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with their role in determining both the final cell wall architecture (especially with regard to lignin deposition and extensin insolubilization) and the turnover of vacuolar phenolic metabolites, a task that also forms part of the molecular program of disease resistance (*16*). Although peroxidase is a constitutive enzyme in grapevines, its levels are strongly modulated during plant cell development and in response to both biotic and abiotic environmental factors that also influence peroxidase isoenzyme expression (*12*, *17*).

Grapevine cell suspension has been shown to be a good model system of reduced complexity for studying the defense mechanisms because it has been demonstrated that key enzymes leading from L-phenylalanine to *t*-R are induced in response to glucan elicitors prepared from *B. cinerea* cell walls, the final product *t*-R being accumulated inside the cells and, to a certain extent, in the extracellular medium (*18*). This simplified system of grapevine cell cultures elicited with fungal cell wall fragments has also been used to study the induction of the phenylpropanoid pathway (*18*, *19*) and the transient expression of genes controlled by stilbene synthase gene promoters (*20*). Laminarin, a mixture of linear β-1,3-glucan with an average polymerization degree of 33 (*21*), derived from brown algae has recently been shown to elicit defense responses in grapevine cell suspensions, such as the expression of defense-related genes and stilbene phytoalexin accumulation, among others. In addition, the infection of grapevine plants by *B. cinerea* and *P. viticola* was reduced when plants were treated with laminarin (*22*). However, the investigation of other molecular events such as plant plasma membrane signal reception and the transduction involved in the response of the cells to elicitor molecules has been precluded by the lack of a chemically defined carbohydrate elicitor.

Cyclodextrins (CDs) are cyclic $\alpha(1-4)$-linked oligosaccharides of six to eight α-D-glucopyranose residues containing a relatively hydrophobic central cavity and a hydrophilic outer surface. These natural sugars derive from starch by the action of microbial cyclodextrin glycosyl transferase (*23*), the major product of which is the seven-membered ring called βCD. Modified CDs (alkylated, esterified, glycosylated, or substituted) are prepared by chemical modification of the free hydroxyl groups at positions 2, 3, and/or 6 of the glucose residues. There exists a whole range of commercially available modified CDs with a chemically defined structure. The hydrophobic central cavity can be filled with poorly water-soluble compounds of low molecular weight to form the so-called inclusion complexes that become as water-soluble as the CD itself by virtue of the external hydrophilic surface of the CD molecule (*23*). It has been shown that stilbene compounds, such as *trans*-resveratrol (*24*) and diethylstilbestrol (*25*), are able to form inclusion complexes with CDs, greatly enhancing its solubility in an aqueous medium.

In a previous work (*24*), we investigated the effect of DIMEB, the doubly methylated βCD in hydroxyls 2 and 6, on resveratrol metabolism in grapevine cell cultures inoculated with the grapevine pathogenic bacteria *Xylophilus ampelinus*. The inoculated cell cultures accumulated significant levels of *trans*-piceid in the cells, whereas no *t*-R was observed in the extracellular medium. When the culture medium was supplemented with 5 mM DIMEB, both infected and noninfected cell cultures accumulated *trans*-piceid in the cells and secreted *t*-R in the spent medium. It was suggested that DIMEB might be acting as an elicitor independent of the presence of the bacterial pathogen.

In this work, we have investigated in detail the properties of CDs as elicitors of the defense responses in grapevine cell cultures and have shown that certain modified CDs are able to elicit some of these responses, as evidenced by the high levels of the *t*-R and stilbene-like compounds accumulated, changes in extracellular cell wall-like peroxidase, and their isoenzyme expression pattern, as well as the effect on *B. cinerea* growth.

MATERIALS AND METHODS

Plant Material. *Vitis vinifera* L. cv. Gamay calli was kindly supplied by Drs. J. C. Pech and A. Latché (ENSA, Toulouse, France) in 1989, whereas the cv. Monastrell green and albino calli were established as described by Calderón et al. (*26*). These cell lines were maintained at 25 °C, with a 16 h light/8 h dark photoperiod of 6 W m^{-2} (except for albino Monastrell, which was maintained in the dark) in 250 mL flasks containing 100 mL of Gamborg B_5 basal medium supplemented with Morel vitamins (*27*), 250 mg L^{-1} casein hydrolysate, 20 g L^{-1} sucrose, 0.2 mg L^{-1} kinetin, 0.1 mg L^{-1} α-naphthalene acetic acid, and 8 g L^{-1} agar, by periodical subculturing every 25 days. Grapevine cell suspensions were initiated by inoculating friable callus pieces in 250 mL Erlenmeyer flasks containing 100 mL of the same medium without agar and rotary shaking (110 rpm) at 25 °C with a 16 h light/8 h dark photoperiod of 6 W m^{-2} (except for albino Monastrell, which was maintained in the dark). These cell suspensions exhibited a typical S-shaped growth curve: initial cell density, measured as packed cell volume (PCV), of 30% increased to 60% in 14 days and then leveled off, reaching the stationary phase. Thus, cell suspension lines were routinely maintained by periodical subculturing duplicating the culture flasks every 14 days. Cell viability was inspected by vital dye staining and microscopical observation: fluorescein diacetate was used for Gamay and neutral red for Monastrell (*12*, *24*).

Chemicals. G2-βCD was a gift from Ensuiko Sugar Refining Co., Ltd., Tokyo, Japan. βCD, DIMEB, HYPROB, sulfo-βCD, *trans*-resveratrol, 4-methoxy-α-naphthol, cell culture Gamborg B_5 basal medium, and other general reagents were from Sigma-Aldrich (Madrid, Spain).

Elicitation of Grapevine Cell Cultures. Seven-day-old grapevine cell suspensions were used in the experiments. Four grams of washed cells was transferred to a 50 mL Erlenmeyer flask and suspended in 10 mL of sterile fresh standard medium containing a given concentration of CDs. The cell suspension was incubated for up to 168 h with continuous orbital shaking at 100 rpm, at 25 °C and with the photoperiod indicated in each experiment. Only albino Monastrell was kept in darkness. After incubation, cells were separated from the spent media by filtration under partial vacuum, rapidly washed with cold distilled water, weighed, and then frozen at −20 °C until use. The spent media were used for analysis of *t*-R, peroxidase activity, peroxidase isoenzyme, and protein electrophoresis. Elicitation experiments were made in triplicate.

Analytical Determinations. Aliquots of the spent media were extensively dialyzed against 5 mM sodium acetate buffer (pH 5.0) to remove the culture medium remaining salts.

Enzyme Assay. Peroxidase activity was determined using 4-methoxy-α-naphthol (4MN) and H_2O_2 as substrates, as described by Ferrer et al. (*28*). For this, the reaction was started by adding 10 μL of the dialyzed spent medium to 990 μL of reaction medium containing 50 mM sodium acetate (pH 5.0), 0.45 mM H_2O_2, and 1 mM 4MN. The appearance of the dye product was monitored at 593 nm ($\epsilon_{593} = 21000$ M^{-1} cm^{-1}) in a Kontron Uvikon 940 spectrophotometer equipped with thermostated cells at 25 °C. One unit of peroxidase is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of 4MN per minute.

Isoelectric Focusing (IEF). IEF in nonequilibrium conditions was performed on 5% polyacrylamide 1 mm thickness slab gels in 3.5−10.0 pH gradients using a Mini-Protean II (Bio-Rad) electrophoresis cell as described by Ros-Barceló et al. (*29*). Samples were prepared by mixing equal amounts of the extensively dialyzed spent medium and sample buffer consisting of 30% glycerol and 15% ampholines in ultrapure water. IEF was run at 4 °C, first at 200 V constant voltage for 90 min and then at 400 V constant voltage for 15 min; 20 mM acetic acid and 25 mM sodium hydroxide were used as electrode solutions.

In-Gel Peroxidase Detection. After IEF, the slab gels were equilibrated in 50 mM sodium acetate (pH 5.0) buffer for 10 min and then transferred to development buffer consisting of 50 mM sodium acetate (pH 5.0), 0.45 mM H_2O_2, and 1 mM 4MN for 10 min. In-gel reaction was stopped by rinsing the gel three times in distilled water, and the gel was immediately photographed.

Analysis of *t*-R in Culture Media. An aliquot of the spent medium was properly diluted in fresh medium, filtered through a 0.2 μm Anopore filter, and injected in a HPLC (LaChrom, Merck-Hitachi) system with a LiChrospher 100 RP-18 column (250 × 4 mm, mesh particle size = 5 μm). The column was eluted at 1 mL min^{-1} in gradient mode: solvent A, 0.05% trifluoroacetic acid; solvent B, 0.05% trifluoroacetic acid in methanol/acetonitrile 60:40 v/v, according to the method of Dalluge et al. (*30*). *t*-R was identified and quantified by comparison with the authentic compound from Sigma-Aldrich (Madrid, Spain) and by its characteristic UV spectrum.

Antifungal Assay. *B. cinerea* hyphal growth determination was performed in a radial growth plate assay. For this, 0.85 g of CMA (Oxoid) in 25 mL of distilled water was sterilized in an autoclave. Twenty-five milliliters of grapevine cell suspension spent medium was infiltrated in sterile conditions (0.22 μm HT Tuffryn low protein binding membrane filter) into the CMA medium previously cooled to 40 °C. The medium was poured into 90 mm diameter sterile Petri dishes, allowed to solidify, and inoculated after 12 h in the center with 20 μL of *B. cinerea* spores (5 × 10^4 spores/mL). After 1 week of incubation in the dark at 30 °C, the extent and type of growth were observed, and the morphology of hyphae was examined by dark field microscopy.

RESULTS AND DISCUSSION

Qualitative and Quantitative Analyses of Culture Media from DIMEB-Elicited Grapevine Cells. Three different grapevine cell lines, namely, Gamay, green Monastrell, and albino Monastrell, were grown in the presence of the cyclodextrin DIMEB as an elicitor and, after different incubation times, the culture media were analyzed for the appearance of phytoalexin-like stilbene compounds. In all three cell lines, the addition of DIMEB to the cell cultures did not seem to affect the growth and viability of the cultured cells during treatment with respect to controls. PCV underwent negligible changes during the incubation (e.g., for Gamay control was 53.3 ± 2.2 at 24 h and 55.1 ± 1.6 at 96 h and 50 mM DIMEB-elicited was 54.4 ± 0.8 at 24 h and 52.9 ± 1.9 at 96 h), whereas the pH remained almost unaltered, close to 6.0, the same as that of the control cells. Microscopical inspection of the suspension along the incubation stained with vital dyes displayed similar proportion of live cells between control and elicitor-treated. Only the aging of the elicited cultures beyond 200 h resulted in massive cell death, probably due to carbon starvation.

As shown in **Figure 1a**, the control medium chromatograms showed no stilbene accumulation, whereas high levels of these compounds were accumulated in the culture media of grapevine cells incubated in the presence of DIMEB. In all three cell lines tested the major phytoalexin-like stilbene accumulated was *t*-R, although its purity depended on the cell line. Thus, the Gamay cells accumulated mainly *t*-R with the occasional presence of *cis*-R (**Figure 1c**). Albino Monastrell cells, unlike the other two lines, were grown in the dark and always accumulated pure *t*-R (**Figure** 1b), whereas green Monastrell cells accumulated both *t*-R and, in larger amounts than Gamay, *cis*-R (**Figure 1d**). It is well-known that the reaction product of resveratrol synthase is the trans-isomer, whereas the cis-isomer is produced by photoisomerization of the trans one (*31*). The fact that *cis*-R does not appear in albino Monastrell and appears at much lower level in Gamay than in green Monastrell cells could be due to the photoprotection of the media, either because of incubation in darkness or because of the presence of red protecting anthocyanin pigments, respectively.

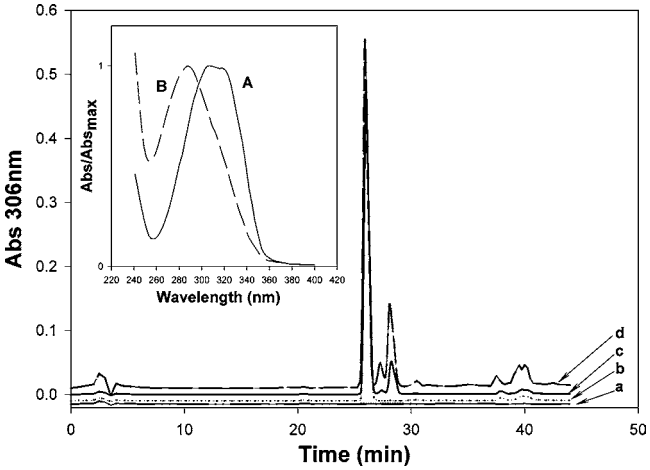

Figure 1. Chromatographic analyses of grapevine cell suspension spent medium. The spent medium is chromatographically analyzed, and compounds are identified from their UV spectra and retention times. Chromatograms shown correspond to nonelicited control of albino Monastrell (**a**), DIMEB-elicited albino Monastrell (**b**), DIMEB-elicited Gamay rouge (**c**), and DIMEB-elicited green Monastrell (**d**) spent media after 96 h of incubation in conditions as indicated under Materials and Methods. Controls of the other two cell lines displayed flat chromatographic profiles as the one shown in (**a**). (**Inset**) Normalized UV–vis spectra at retention times of 26 min (**A**) and 28.2 min (**B**) correspond to *trans*- and *cis*-R, respectively.

Figure 2A describes the effect of different concentrations of DIMEB on *t*-R production by Monastrell albino cell cultures in the spent medium. The results show that *t*-R was produced by cells and secreted outward to the spent medium only when the media were supplemented with DIMEB. The accumulation of *t*-R was dependent on both DIMEB concentration and culture elicitation time. This hydroxystilbene is accumulated in the culture media throughout the incubation period, reaching a plateau after 120 h (in the case of medium supplemented with 50 mM DIMEB). Similar results were obtained with Gamay cell cultures (data not shown). Even when the accumulated *t*-R concentration reached the highest value, the spent medium was fully transparent and homogeneous; no turbidity or precipitation was observed due to the high concentration of *t*-R. We observed that DIMEB dramatically increased the solubility limit of *t*-R (0.2 mM in Gamborg B_5 medium) due to the formation of inclusion complexes (*24*). It is clear, then, that the empty DIMEB molecules which act as elicitors of the *t*-R production serve as adsorbents for the *t*-R as well. This latter function not only leads to the substantial accumulation of this compound but also prevents the possible toxic and/or inhibitory effects of *trans*-resveratrol on the grapevine cells.

In the case of green Monastrell cell cultures (**Figure 2B**), the accumulation of both *trans*- and *cis*-R reached a plateau after 48 h of elicitation, in some cases slightly decreasing afterward. The trans-to-cis ratio varied in each experiment, although the *t*-R isomer concentration was always higher than that of the cis-isomer. Total resveratrol accumulation in the green Monastrell line was ~30-fold lower than in the other cell lines.

Ability of Different Cyclodextrins To Elicit Grapevine Cells. We studied the ability of different types of cyclodextrins, both natural and synthetic, to act as elicitors in grapevine cells. **Table 1** shows the concentration of resveratrol accumulated in culture media of grapevine cells incubated in the presence of commonly used β-type (7-glucose ring) cyclodextrins at a concentration of 50 mM, except for the natural βCD, used at a concentration of 15 mM because its solubility limit in water at

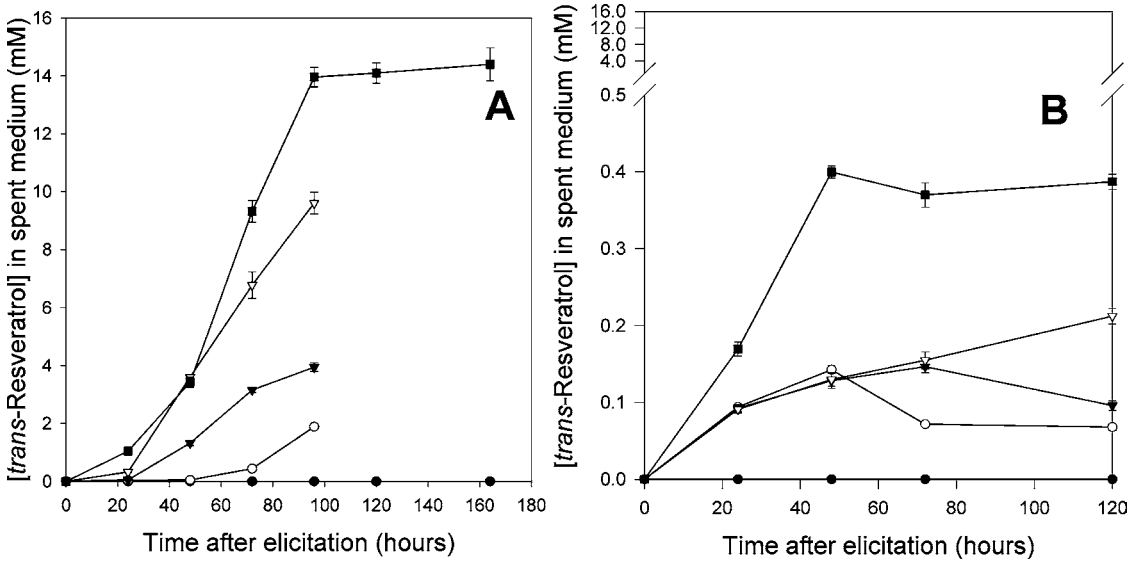

Figure 2. Time course of *trans*-resveratrol accumulation in the spent medium of DIMEB-elicited grapevine cell suspensions. The accumulation of *t*-R in the spent medium was recorded at several DIMEB concentrations [control (●), 5 mM (▼), 10 mM (■), 25 mM (◆), and 50 mM (▲)] in cell suspensions of albino Monastrell (**A**) and green Monastrell (**B**) lines. Each data point corresponds to the mean ± SE of three independent elicitation experiments.

Table 1. Ability of Different CDs To Elicit Grapevine Cells[a]

		trans-resveratrol (mM)		
type of CD	[CD] in culture medium[b] (g L^{-1})	Gamay rouge[c]	Monastrell albine	Monastrell green[d]
none		nd[e]	nd	nd
DIMEB	66.5	13.4 ± 0.6	14.8 ± 0.5	0.39 ± 0.03
HYPROB	69.0	4.36 ± 0.24	13.3 ± 0.4	0.39 ± 0.05
G2-βCD	72.8	1.73 ± 0.11	2.92 ± 0.16	0.09 ± 0.01
sulfo-βCD[f]	92.6	nd	nd	nd
βCD	17.0	0.13 ± 0.01	0.26 ± 0.03	nd

[a] Elicitation experiments were carried out in triplicate as stated under Materials and Methods using the type and concentration of CD indicated. Concentration of *t*-R ± SE in the spent medium after 96 h of incubation of grapevine cells with CDs was determined as an indication of the culture response to CD elicitation. [b] These concentrations correspond to 50 mM except βCD, which corresponds to 15 mM due to its lower solubility. [c] Occurrence of unidentified phenolics. [d] Abundant *cis*-R. [e] Not detected. [f] Data after 24 h of incubation.

25 °C is 18 mM. All of the CDs tested were innocuous to the cells except sulfo-βCD, which was lethal at the concentration used, as seen by microscopic observation of cells and the browning of the culture. As mentioned above, the Gamay spent media accumulated *t*-R and other minor unidentified phenolic compounds, whereas the green Monastrell cultures accumulated both *cis*- and *trans*-R and the albino Monastrell media only pure *t*-R. The best elicitor was DIMEB followed by HYPROB, which contain methoxy and 2-hydroxypropoxy groups, respectively, and share the common feature of being ether derivatives of sugars. Although these CDs are synthetic compounds, their ability to act as elicitors can be explained as follows: (i) the pectin fraction of the cell walls contains ether- and ester-derivatized sugars such as methyl galacturonate, 3-acetyl galacturonate, 2-*O*-methyl xylose, and 2-*O*-methyl-4-acetyl fucose (*32*); (ii) the treatment of a cell suspension with pectolytic enzymes releases pectin oligosaccharides, which act as elicitors of phytoalexin synthesis (*17*). In this sense, the modified CDs may resemble chemically the alkyl-derivatized pectic oligosaccharides naturally released from the cell walls during fungal attack and therefore share the ability to induce the activation of a cascade of cellular events that gives rise to phytoalexin synthesis.

On the other hand, the natural branched G2-βCD was also able to challenge the cells but to a lower extent than the ether derivatives, whereas the effect of the natural nonsubstituted βCD was practically negligible compared with that of the branched and derivatized ones. Thus, for these oligosaccharides to act as elicitors, it seems to be important that some hydroxyl groups should be substituted by an uncharged, nonbulky, alkyl group.

Albino Monastrell cultures accumulated higher levels of *t*-R than the other lines for all of the CDs tested.

Peroxidase Activity and Analysis of Isoenzyme Peroxidase Patterns in the Culture Media of CD-Elicited Grapevine Cells. Figure 3 shows changes in extracellular peroxidase activity during the elicitation of cell cultures by different concentrations of DIMEB. As can be observed, there were slight differences in the peroxidase activity measured in the spent media of Gamay cells (**Figure 3A**) elicited with 5 and 10 mM DIMEB compared with the control, and this activity was maintained at a high level during the 4 days the assay lasted. However, at high DIMEB concentrations (25 and 50 mM), peroxidase activity rapidly fell to significantly lower levels during the first day and subsequently began to increase to reach the original levels. This decline of ~70% was in accordance with that found during the elicitation of suspension culture cells of spruce with a fungal cell wall preparation of the pathogenic fungus *Rhizosphaera kalkhoffii* by Messer and Boll (*33*). These authors demonstrated that extracellular enzymes present in the spent medium, such as peroxidase, β-glucosidase, or acid phosphatase, became inactivated for 1 day upon elicitor-induced release of H_2O_2 by the cells, which lasted for 1 day. A similar effect might be assigned to the higher concentrations of DIMEB, but not to the lower ones.

The changes in peroxidase activity are closely related to the expression of the isoenzyme pattern shown in **Figure 4**. As can be seen from the **Figure 4A**, there was practically no difference at 24 h between the intensity of the main band of the control (**Figure 4A**, lane 1) and of the low cyclodextrin concentrations (lanes 2 and 3, corresponding to 5 and 10 mM); however, at high cyclodextrin concentrations (lanes 4 and 5, corresponding to 25 and 50 mM) the intensity of the band decreased. After 96 h of elicitation, the intensity of the band at low cyclodextrin concentrations (**Figure 4B**, lanes 2 and 3, corresponding to 5

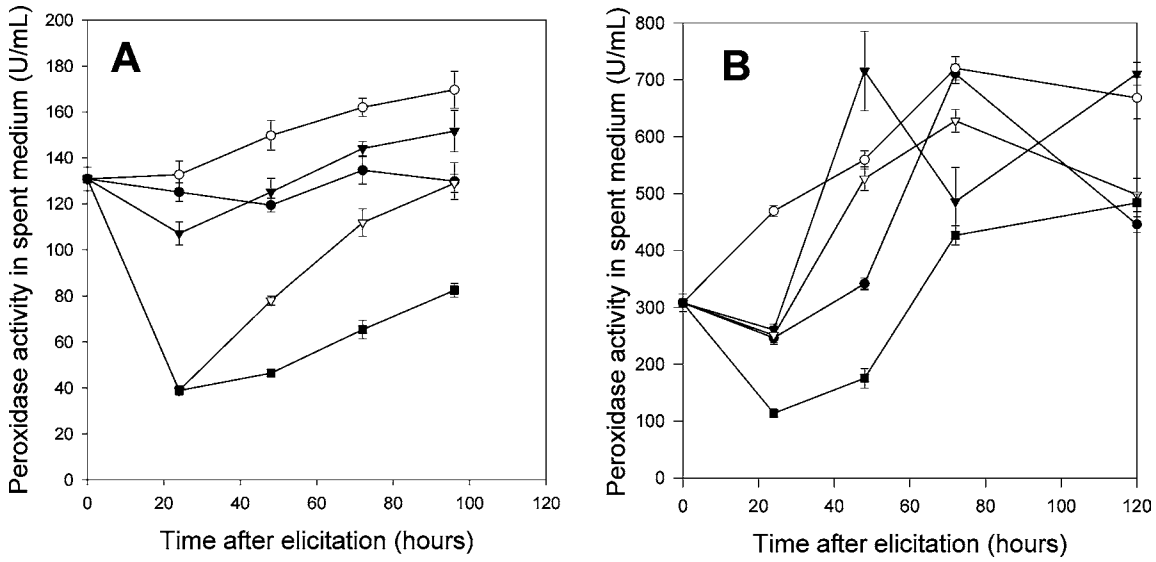

Figure 3. Time course of peroxidase activity in the spent medium of DIMEB-elicited grapevine cell suspensions. The peroxidase activity in the spent medium was determined during incubation of the cell suspension at several DIMEB concentrations [control (●), 5 mM (▼), 10 mM (■), 25 mM (◆), and 50 mM (▲)] in cell suspensions of Gamay (**A**) and albino Monastrell (**B**) lines. Each data point corresponds to the mean ± SE of three independent elicitation experiments.

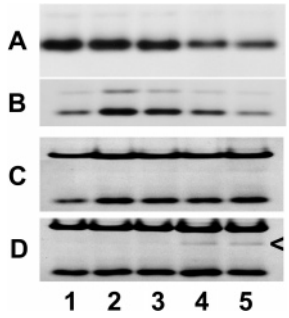

Figure 4. Isoenzymatic pattern of peroxidase in spent medium of DIMEB-elicited grapevine cell suspensions. Isoenzymes of peroxidase were resolved by nonequilibrium IEF as indicated under Materials and Methods. Peroxidase bands were detected by incubation with hydrogen peroxide and 4-methoxy-α-naphthol, as described by Ferrer et al. (*28*). The pattern was recorded 24 h (**A**, **C**), 96 h (**B**), and 120 h (**D**) after DIMEB elicitation for both Gamay (**A**, **B**) and albino Monastrell (**C**, **D**) cell lines, at each DIMEB concentration: control (lane 1), 5 mM (lane 2), 10 mM (lane 3), 25 mM (lane 4), and 50 mM (lane 5).

and 10 mM) increased with respect to the control (lane 1), whereas at high cyclodextrin concentrations (lanes 4 and 5, corresponding to 25 and 50 mM) the intensity was similar to that of the control. According to the classification of peroxidase isoenzyme expression established by Pedreño et al. (*34*), the two bands detected correspond to the basic peroxidase isoenzyme group of high p*I*, the strongly basic peroxidase isoenzyme being the main component of peroxidase polymorphism in both Gamay cell cultures and mature grapes (*35*). Thus, there was a strong correlation between the band intensity of the main basic isoenzyme and the peroxidase activity measurements in Gamay cells. Calderón et al. (*12*) considered this peroxidase isoenzyme group of high p*I* as a constitutive (noninducible) marker of disease resistance in grapevine leaves and stems due to their presence in both sensitive and highly resistant grapevine cultivars and to their capacity to oxidize 4-hydroxystilbenes to a great extent.

On the other hand, changes in extracellular peroxidase activity during the elicitation of albino Monastrell cultures by different concentrations of DIMEB (**Figure 3B**) were difficult to understand because there was no clear difference between the levels of peroxidase activity obtained from elicited and nonelicited cell cultures. However, examination of the isoenzyme peroxidase pattern at short times (during the first 24 h) revealed slight differences in the intensity of the band (**Figure 4C**), which corresponded once more to this strong basic peroxidase isoenzyme. The other band detected in the control and in all different treatments belonged to the basic isoenzyme group of low p*I* (*29*) and was also constitutively expressed during the growth of Monastrell cell cultures (*26*). Therefore, when elicitation was studied at 120 h, we detected the presence of another isoenzyme of low p*I* (**Figure 4D**, lanes 4 and 5, at the arrow), its appearance in the isoenzyme pattern being strictly dependent on elicitor concentration (25 and 50 mM DIMEB). These results are in accordance with those found by Ros-Barceló et al. (*36*), demonstrating that this peroxidase isoenzyme was inducible in cell cultures and grapevine cultivars sensitive to either biotic or abiotic elicitors (*37*) but was constitutive in resistant grapevine cultivars, in which it represented the major constituent of the basic isoperoxidase of low p*I*.

Effect of CD-Elicited Spent Medium on *B. cinerea* Growth. The antifungal activity of the spent medium of grapevine cell suspensions that had been incubated in the presence of DIMEB for 96 h was tested by a radial growth plate assay of the grapevine fungal pathogen *B. cinerea*. The fungus was inoculated in the center of a CMA medium plate that contained spent medium, and the growth of hyphae was observed by dark field microscopy. As seen in **Figure 5A**, the growth of hyphae in plates supplemented with nonelicited control spent medium was normal and similar to that of control plates (only CMA medium or CMA + 12.5 mM DIMEB, not shown), whereas hyphae from plates supplemented with elicited spent medium (**Figure 5B** shows the Gamay spent medium-supplemented CMA) showed abnormal growth. Macroscopically, the fungus showed exploratory behavior, the hyphae occupying the whole plate but loosely. Microscopically, it was often observed that the hyphal septum was absent, vesicles were abundant in the cytoplasm (unsepted hyphae), and the hyphal tips followed a random growth direction (random hyphal tip). Thus, the elicitation of grapevine cells with cyclodextrin causes

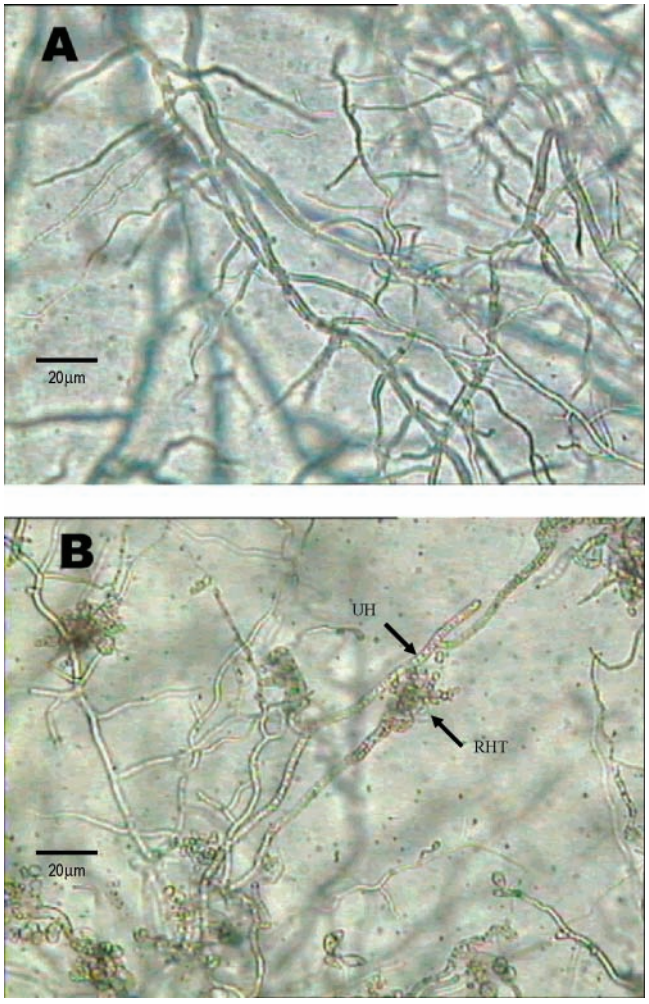

Figure 5. Photomicrographs of *B. cinerea* mycelium growing on spent medium-supplemented corn meal agar. Hyphae are observed directly on the Petri dish by light microscopy, at 250×. Medium was supplemented with (**A**) nonelicited and (**B**) DIMEB-elicited spent medium of Gamay cell suspension. UH indicates unsepted hyphae, and RHT stands for random hyphal tip.

an antifungal response in the culture as evidenced by the interference of the elicited spent medium on the fungus growth and morphology. It is well-known that resveratrol itself inhibits the spread of *B. cinerea* infection (*38*); moreover, strains of *B. cinerea* that degrade resveratrol are more pathogenic to in vitro cultures of grapevine than those unable to degrade this phytoalexin (*39*). However, it cannot be ruled out that other components of the elicited spent medium, such as antifungal proteins, may have an additional effect. This possibility is currently being explored.

Oligosaccharidic fractions from either fungal or plant cell walls are well-known substances that elicit defense responses in both plant cell cultures and tissues. Plant receptors are known to play a role in the recognition of these substances and the transduction of signals; to characterize these surface proteins, chemically defined challenging molecules are basic tools. The oligosaccharidic fractions used so far to elicit plant cell cultures are fragments of a broader or narrower range of lengths and so are not appropriate for the characterization of receptors. In this work, we have shown for the first time that a chemically defined cyclic oligosaccharide, for instance, DIMEB, acts as an elicitor of grapevine cell suspensions, inducing typical defense responses of phytoalexin synthesis and a resistance-marker peroxidase isoenzyme. Therefore, this work opens the possibility of characterizing the receptors involved in the perception of glucan elicitor with a chemically defined structure.

ABBREVIATIONS USED

4MN, 4-methoxy-α-naphthol; *β*CD, cycloheptamylose; *B. cinerea*, *Botrytis cinerea*; CDs, cyclodextrins; CMA, corn meal agar; DIMEB, heptakis(2,6-di-*O*-methyl)-*β*CD; G2-*β*CD, maltosyl-*β*CD; HYPROB, 2-hydroxypropyl-*β*CD; IEF, isoelectric focusing; PCV, packed cell volume; p*I*, isoelectric point; *P. viticola*, *Plasmopara viticola*; PR, pathogenesis-related proteins; rpm, revolutions per minute; sulfo-*β*CD, *β*CD sulfated, sodium salt; *t*-R, *trans*-resveratrol.

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