JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Effects of Elicitors on the Production of Resveratrol and Viniferins in Cell Cultures of *Vitis vinifera* L. cv Italia

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ABSTRACT: Methyl jasmonate, jasmonic acid and chitosan were tested as elicitors on cell suspension cultures obtained from *Vitis vinifera* cv Italia to investigate their effect on stilbene production. Stilbene accumulation in the callus, grown under nonelicited conditions, was also investigated. Calli and cell suspensions were obtained in a B5 culture medium supplemented with 0.2 mg L^{-1} NAA and 1 mg L^{-1} KIN. Stilbene determination was achieved by HPLC/DAD/MS. Whereas callus biosynthesized only piceid, cell suspensions elicited with jasmonates produced several stilbenes, mainly viniferins. In suspended cells, methyl jasmonate and jasmonic acid were the most effective in stimulating stilbene biosynthesis, whereas chitosan was less effective; in fact, the amount of stilbenes obtained with this elicitor was not significantly different from that obtained for the control cells. The maximum production of total stilbenes was at day 20 of culture with 0.970 and 1.023 mg g^{-1} DW for MeJA and JA, respectively.

KEYWORDS: elicitation, chitosan, jasmonic acid, methyl jasmonate, *Vitis vinifera* cv Italia, cell cultures, viniferins

INTRODUCTION

In *Vitis vinifera* L., the most frequently observed chemical defense response to biotic and abiotic stress is the accumulation of phytoalexins, a restricted group of phenolic compounds belonging to the family of stilbenes, which derive primarily from *trans*-resveratrol (3,5,4′-trihydroxystilbene).[1,2] Phytoalexins are *de novo* synthesized in the plant in response to microorganism attacks, and they accumulate rapidly at the site of attack.[3] Phytoalexin synthesis can also be induced by other environmental elicitors, both physical (e.g., ultraviolet radiation, heat shock, and wound stress) and chemical (e.g., heavy metals, yeast extract, and chitosan).

Elicitors may act as either biotic or abiotic agents. Biotic agents are signal molecules that are directly involved in the host−pathogen interaction, whereas abiotic agents activate the biosynthesis of endogenous biotic agents that are normally inactive. To enhance phytoalexin production in plant cell cultures, a variety of molecules can be used as elicitors, including oligo- and polysaccharides, peptides, proteins and lipids.[4] Elicitor perception triggers various signaling pathways, usually beginning with an influx of calcium and an oxidative burst, followed by the synthesis of signal molecules such as salicylic acid, jasmonic acid (JA) and ethylene. Elicitation mechanisms lead to a broad spectrum of metabolic modifications, such as cell-wall reinforcement, accumulation of antimicrobial compounds (e.g., phytoalexins), synthesis of pathogenesis-related protein for plant protection, lignification, and, frequently, a hypersensitive response that is a form of programmed cell death at the infection site and which hinders pathogen development.[5] The involvement of JA in the signal transduction cascade has led to the use of this molecule and its more active derivative, methyl jasmonate (MeJA), as inducers of defense mechanisms in a number of *in vitro* systems.[6]

V. vinifera cell suspensions are a good model for studying defense mechanisms and the accumulation of stilbenes after elicitation. This simple system has also been used to study the induction of the phenylpropanoid pathway and the transient expression of genes controlled by stilbene synthase gene promoters.[7,8] In studies that aimed at optimizing the *in vitro* production of stilbenes, several elicitors have been shown to induce and/or modify stilbene biosynthesis and metabolism in *V. vinifera* cell cultures.[3,9−14] For example, in cell suspensions of cv Barbera, MeJA[15] and chitosan[16] have been found to increase the accumulation of *trans*- and *cis*-resveratrol and protein expression. In the same cell suspension, chitosan has also been shown to be effective in inducing stilbene production in a bioreactor system.[17] In *V. vinifera* cv Gamay Fréaux var. Teinturier cell suspensions, a study showed that MeJA, in combination with carbohydrates, increased the expression of genes that encode pathogenesis-related proteins (i.e., chitinases and β-1,3-glucanases) and consequently the accumulation of stilbenes and anthocyanins.[18] In cell cultures of *V. vinifera* cv Cabernet Sauvignon treated with MeJA, the relationship between stilbene production and signaling pathways involving calcium, phosphorylation and active oxygen species (ROS) was investigated.[19] In a recent review, the production of stilbenes, mainly *trans*-resveratrol and piceid, from *V. vinifera* cell cultures was evaluated.[20] Nonetheless, only a few types of stilbenes were identified in these studies, and only in some cases the viniferins have been quantified.[21] Recently we identified more than 20 types of stilbenes in elicited cells of cv Malvasia.[22] In another study, conducted using cell lines of the cvs Red Globe and Michele Palieri, we showed that the total quantity of stilbenes was genotype-dependent and that MeJA enhanced stilbene accumulation.[23]

In these years, much attention has been devoted to the effects of *trans*-resveratrol, which is considered to be the most bioactive stilbene on human health. This molecule possesses antioxidant

Received: March 24, 2011
Revised: July 7, 2011
Accepted: July 14, 2011
Published: July 14, 2011

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properties that could protect against cardiovascular diseases such as arteriosclerosis by modulating the metabolism of lipids, reducing cholesterol, and inhibiting the oxidation of low-density lipoproteins (LDL).[24−26] *trans*-Resveratrol also acts as a chemopreventive agent for several types of cancer by inhibiting promotion and progression and/or by suppressing the final steps of carcinogenesis.[27−29]

trans-Resveratrol is an end product of the phenylpropanoid pathway, obtained by condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA in a reaction catalyzed by the enzyme stilbene synthase.[30] There are several monoglucosylated derivatives of *trans*-resveratrol, such as resveratroloside (3,5,4′-trihydroxystilbene-4′-O-β-D-glucopyranoside)[9,11] and piceid (resveratrol-3-β-D-glucopyranoside), which is present in quantities comparable to free resveratrol in grape berries, wines, and cell suspensions.[31,32] Several oxidation products of *trans*-resveratrol have been identified, such as δ- and ε-viniferin (dehydrodimers), α-viniferin (dehydrotrimer), β-viniferin (a cyclic resveratrol tetramer), and more highly polymerized oligomers such as γ-viniferins.[1,2,33,34] Grapevine leaves infected by *Plasmopara vinifera* or following UV irradiation have been shown to produce δ-viniferin, which unlike its analogous ε-viniferin is not widespread in nature.[34,35]

Although considerable progress has been made in the study of *trans*-resveratrol biosynthesis, the knowledge of viniferin biosynthesis in *V. vinifera* is incomplete. To date, peroxidase is the only plant enzyme known to be involved in the oxidation of *trans*-resveratrol and its transformation into viniferins.[36] Although peroxidase is a constitutive enzyme in *V. vinifera*, its levels are strongly modulated during plant-cell development and in response to both biotic and abiotic environmental factors that influence peroxidase isoenzyme expression.

To date, the biological activities of the viniferins have been studied less extensively than *trans*-resveratrol, but *trans*-ε-viniferin has been shown to have hepatoprotective[37] and antioxidant[38] properties and to induce apoptosis of leukemia B cells.[39,40] Moreover, ε-viniferin has been shown to inhibit the uptake of human cytochrome P450 enzymes,[41] noradrenaline and 5-hydroxytryptamine, as well as monoamine oxidase activity.[42]

The objective of the present study was to investigate, by HPLC/DAD/MS, the ability of biotic elicitors (i.e., MeJA, JA, and chitosan) to increase stilbene production, particularly, ε- and δ-viniferins, in cell suspension cultures of *V. vinifera* cv Italia.

MATERIALS AND METHODS

***In Vitro* Plant Material.** To induce callus formation, stem and tendril explants were excised from *V. vinifera* cv Italia plants. The samples were provided by the Experimental Institute for Enology of Velletri (Italy). The explants were surface sterilized for 10 min with 15% (v/v) commercial bleach and one drop of Tween 20 (Sigma) per 100 mL of solution; they were then rinsed three times with sterile distilled water. The explants were cut into 1 cm pieces and cultured in two different media, each of which consisted of 1% (w/v) agarized B5 medium[43] supplemented with α-naphthaleneacetic acid (NAA), kinetin (KIN), and 2% (w/v) sucrose, adjusted to pH 5.7. One medium contained 1 mg L^{-1} NAA and 0.2 mg L^{-1} KIN (medium A); the other contained 0.2 mg L^{-1} NAA and 1 mg L^{-1} KIN (medium B). The explants were maintained in continuous darkness at 26 ± 1 °C. Subcultures of the callus were carried out in medium B every 20 days. Continuous darkness was chosen for the induction and growth of callus on the basis of preliminary experiments (data not shown).

Cell Suspension Cultures. Two grams fresh weight (FW) of calli were inoculated in 250 mL flasks containing 50 mL of the medium that produced the best results in terms of biomass growth (medium B). Cell suspension cultures were maintained on a rotary shaker at 100 rpm at 26 ± 1 °C in continuous light (70 μmol s^{-1} m^{-2}), which are the best conditions for cell growth in liquid medium, as established on the basis of preliminary experiments (data not shown). During the culture period (25 days), cell growth was evaluated by determining the average percent increase in biomass (fresh weight). Measurements were performed until there was no longer an increase in cell growth (stationary phase).

Cell Viability. Cell suspensions were treated for 15 min with a solution of fluorescein diacetate (Sigma) (0.5 mg in 100 mL of absolute acetone). The cells were washed with distilled water, mounted on microscope slides and observed with a Zeiss microscope (Axioscop 2 Plus) using a Zeiss blue filter. Cells with a green fluorescent nucleus and cytoplasm were considered viable.

Elicitation of Cell Cultures. To stimulate stilbene production from cell suspensions, three different elicitors were tested (MeJA, JA, and chitosan). The elicitation treatments were carried out on cell suspensions at the second subculture. Before testing the elicitors, we first determined the moment of maximum stilbene production, based on an experiment using MeJA. In the first experiment, cell suspensions were inoculated at a 20% concentration (3 g fresh weight in 20 mL of medium B). Nineteen flasks were maintained in continuous light at 26 ± 1 °C on a rotary shaker at 100 rpm. On day 10 of culture, which corresponded to the midpoint in the exponential growth phase, 9 flasks were treated with MeJA (25 μM, dissolved in 100% ethanol), added through filter-sterilization; another 9 flasks were treated with ethanol; and 1 flask was untreated. Cells were collected immediately (time 0) and at 1, 3, 5, 9, 24, 48, 72, 120, and 240 h after treatment. The cells were harvested by vacuum filtration, weighed, and stored at −20 °C until extraction for chemical analysis. In a second experiment, cell suspensions were inoculated at 20% concentration (3 g fresh weight in 20 mL of medium B). Twenty-four flasks were maintained in continuous light at 26 ± 1 °C on a rotary shaker at 100 rpm. On day 10 of culture, MeJA (25 μM, dissolved in 100% ethanol), JA (25 μM, dissolved in 100% ethanol) and chitosan with medium molecular weight (50 μg mL^{-1} dissolved in acetic acid 0.1%) (all from Sigma-Aldrich, Italy) were added to the cell suspensions (6 flasks each), through filter-sterilization. As controls, cell suspensions were treated with the solvent only (ethanol for MeJA and JA and acetic acid for chitosan) (2 flasks each). The elicited and control cells were collected 2 (48 h) and 10 days (240 h) after treatment, which corresponded to, respectively, days 12 and 20 of the growth cycle. The cells were harvested by vacuum filtration, weighed, and stored at −20 °C until extraction for chemical analysis.

Extraction of Stilbenes. Cell suspensions (3 g fresh weight for each sample) and calli (50 g fresh weight) were extracted with ethanol/acid water (7/3 v:v) at pH = 3.0 by formic acid, in three successive 24 h steps; the final ratio was 1 g fresh weight/10 mL of extractive mixture. The hydroalcoholic extracts were dried under vacuum (about 30 °C), redissolved in the extractive mixture, and then filtered through a 0.22 μm PTFE membrane (Whatman, New Jersey, USA) and analyzed by HPLC/DAD/MS. The corresponding culture media were lyophilized, redissolved in the extractive mixture, filtered, and analyzed by HPLC/DAD/MS. The dry weight was determined for both calli and cell suspensions as the sum of the extract and cell residue.

HPLC/DAD/MS Analysis of Stilbenes. The analyses were carried out using an HP 1100L liquid chromatograph equipped with a DAD detector and managed by an HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA). The elution method was a multistep linear solvent gradient, passing from 87% H_2O to 85% H_2O in 10 min, to 75% H_2O in 10 min; plateauing at 75% H_2O for 3 min, passing to 5% H_2O in 2 min, and plateauing at 5% H_2O for 3 min. The total time of analysis was 28 min; the equilibration time was 10 min, and the flow rate

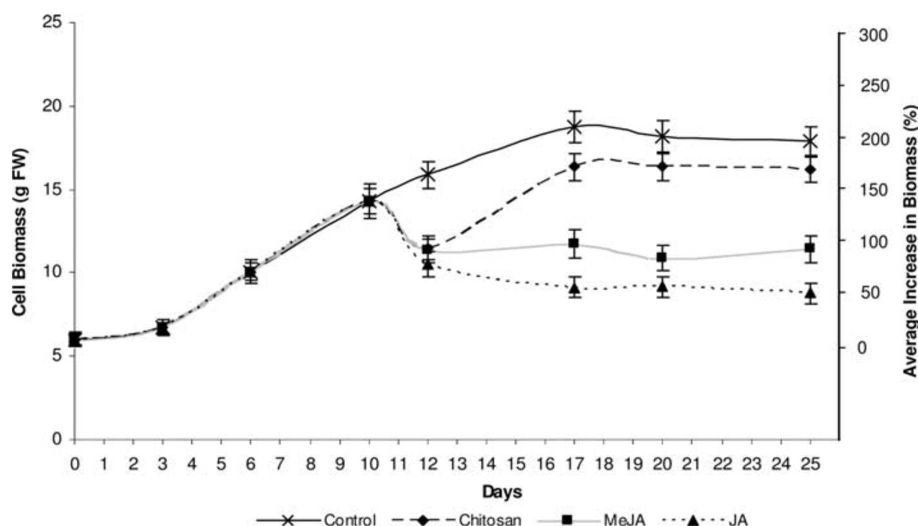

Figure 1. Growth curve of cv Italia cell suspensions nonelicited (control) and elicited with chitosan, methyl jasmonate (MeJA) and jasmonic acid (JA). Cell growth is expressed as fresh weight (left) and average percent increase (right). Each point represents the mean of three different samples ± SD.

was 0.4 mL min^{-1}. The column was a Synergy max RP-12 (150 × 3 mm i.d., 5 μm) maintained at 27 °C with a precolumn of the same phase (Phenomenex, Castel Maggiore, Bo, Italy). Eluents: H_2O (pH 3.2 by HCOOH) and CH_3CN, both HPLC grade.

ESI-MS/MS Analysis. An Applied Biosystems-Sciex (Toronto, Canada) API 3000 triple-quadrupole mass spectrometer equipped with a TurboIonSpray source was used for this study. The TurboIonSpray source operated in negative ion mode with a needle potential of −4500 V and with a "turbo" gas flow of 10 L/min of air heated to 150 °C (nominal heating-gun temperature).

Mass calibration and resolution adjustments on the resolving quadrupoles were performed automatically by using a PPG (polypropylene glycol) 10^{-5} mol/L solution introduced via a built-in infusion pump. The peak width was set on both resolving quadrupoles at 0.7 Th (measured at 1/2 height) for all MS and MS/MS experiments. MS and MS/MS spectra were collected in continuous flow mode by connecting the infusion pump directly to the TurboIonSpray source. The acquired data were processed using the Analyst 1.4 proprietary software including the "Explore" option for spectral interpretation.

Quantitative Determination of Stilbenes. *trans*-Resveratrol, piceid and the other stilbenes were quantified by a four-point calibration curve at 307 nm (r^2 = 0,9999) using as external standards pure *trans*-resveratrol (Extrasynthese Gene-France) from 0 to 1.98 μg.

RESULTS AND DISCUSSION

Optimization of the *in Vitro* Cultures. In both medium A and medium B, stem and tendril explants produced calli after 10 days. At day 20 of culture, 90−92% of the explants had produced calli in the medium with the higher KIN concentration (medium B), compared to 34−62% of the explants in the medium with the lower concentration. There were no important differences in callus production between stem and tendril explants. The calli, separated from the explants, were inoculated in the medium that gave the best results (medium B). After a few subcultures, the calli grew rapidly and appeared sufficiently friable to be transferred to the liquid medium.

To determine the ability of cultured cells to maintain productivity over time, the calli obtained from the same explants (and thus belonging to the same cell line) were harvested and analyzed 3 months and 3 years after inoculation in the callogenic medium. In the young calli, at day 22 of culture, the only stilbene was piceid, with a concentration of 0.054 mg g^{-1} DW, and the concentration increased up to 0.096 mg g^{-1} DW after 3 years (for determination see the next paragraph). It has been hypothesized that piceid could be a form of storage of resveratrol,[6] which is converted into more bioactive derivatives (i.e., *trans*-resveratrol and viniferins) in response to abiotic and biotic stress.

To test the effect of elicitors on suspended cells, cell growth was monitored by measuring the fresh weight at days 0, 3, 6, 10, 12, 17, 20, and 25 (Figure 1).

At day 12 (i.e., two days after elicitation), a decrease in growth was observed for the elicited cells. In particular, the average percent increase in biomass for the cells treated with chitosan, MeJA, and JA, which had reached 138% at day 10, was 80% at day 12. Afterward, the cell growth was more or less constant for MeJA and JA, whereas for chitosan it began to increase again after day 12, reflecting the trend observed for the control cells. The control cells observed by light microscopy showed morphological differences during the growth cycle. During the exponential phase, they were spherical and small in size, whereas during the stationary phase, they were elongated and aggregated into clumps.

The vitality test with fluorescein diaceate (FDA) showed that about 80% of the cells were viable. A high percentage of viable cells was considered as a prerequisite for subjecting the cells to elicitor treatment.

Detection of the Stilbenes. To recognize all of the stilbenes biosynthesized in the samples of cv Italia, all extracts were analyzed by applying the previously optimized chromatographic method.[22] To quantify the target molecules (Figure 2), *trans*-resveratrol was used as external standard. Figure 3 shows the chromatographic profile at 307 nm of the elicited cells with MeJA, collected 240 h days after elicitation, and the UV−vis spectrum of δ-viniferin, the main stilbene in this sample. The total ion current (TIC) and some selected extract ion (EI) profiles obtained in negative polarity are shown in Figure 4. This approach allowed us to detect different classes of stilbenes, even if present in trace amounts in the extract. In particular, 453 m/z was chosen to detect the dimeric stilbenes, 471 m/z for the corresponding oxidized dimers and 227 m/z for *trans*-resveratrol.

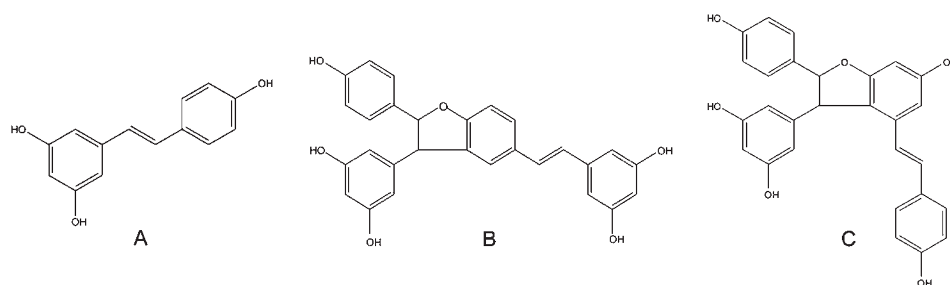

Figure 2. Chemical structures of the main identified stilbenes: (A) *trans*-resveratrol; (B) *trans*-δ-viniferin; (C) *trans*-ε-viniferin.

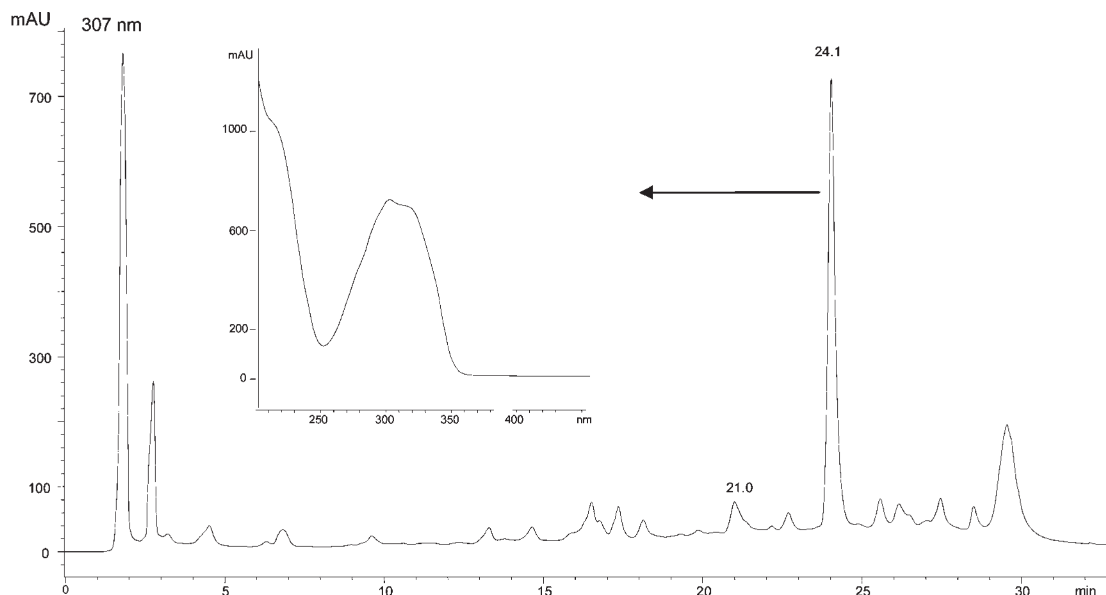

Figure 3. Chromatographic profile at 307 nm with the UV−vis spectra of the main stilbene, *trans*-δ-viniferin, detected in elicited cells of cv Italia.

As found in a previous study,[22] the mass spectra allowed us to recognize, in addition to *trans*-δ-viniferin (D_4), *trans*-ε-viniferin (D_2) as a main dimer. Other minor stilbenes, such as a diglycosidic dimer (D_1), *cis*-δ-viniferin (D_5) and six oxidized dimers (D_{ox}), were also observed (Figure 4B), mainly because of the use of the mass detector.

Content in Stilbenes after Elicitation. To establish the moment of maximum stilbene production after elicitation, a first experiment was carried out to measure stilbene production at different times after elicitation with MeJA. This molecule is a well-known elicitor which is frequently used to induce defense mechanisms in a number of *in vitro* systems. Numerous studies have shown that jasmonates trigger the accumulation of secondary metabolites in cell suspensions of *V. vinifera* cell cultures.[10,18,14,19,21]

As shown in Figure 5, stilbene production began to increase as early as 24 h after elicitation (360.58 μg g^{-1} DW); at 48 h it began to increase consistently; it remained almost unaltered up to 120 h and reached the maximum production at 240 h (1166.82 μg g^{-1} DW). Our purpose was to obtain good yields in terms of stilbenes in a relatively short time, consequently, and in agreement with previous works,[18,15] only the production up to 10 days after elicitation was taken in account.

This first experiment with MeJA showed that this cultivar responded to the elicitation and that *trans*-δ-viniferin was the main dimeric stilbenoid biosynthesized in all of the elicited samples, whereas *trans*-ε-viniferin was produced in consistently lower quantities (Figure 5). The highest content of *trans*-δ-viniferin was found at 240 h (997.40 μg g^{-1} DW), though appreciable quantities were also detected after 48 h (701.13 μg g^{-1} DW). Moreover, the analyses of the culture media of the MeJA-elicited cells conducted between 48 and 240 h showed that δ-viniferin was the main stilbene (0.25−0.37 mg L^{-1}), whereas the medium in which control cells were cultured contained a relatively lower concentration (0.02 mg L^{-1}) of this metabolite.

To increase the yield of stilbenes, other well-known elicitors were also tested (i.e., chitosan and JA). It is known that jasmonates are components of a network of a signal transduction pathway which induces the plant's defense response against biotic and abiotic stress,[44] and JA is known to act as a secondary messenger, like other small molecules such as salicylic acid and ethylene. Chitosan, a linear D-(1,4)-glucosamine, which is an important structural component of several plant fungi cell walls,[45] can mime fungal pathogen attack.

Little is known about the signal transduction pathways implicated in the activation of the defense responses mediated by jasmonates in grapevine. One study[19] investigated the involvement of a variety of early signal transduction pathways, such as calcium, phosphorylation and active oxygen species in methyl jasmonate-induced defense responses in *V. vinifera* cell cultures.

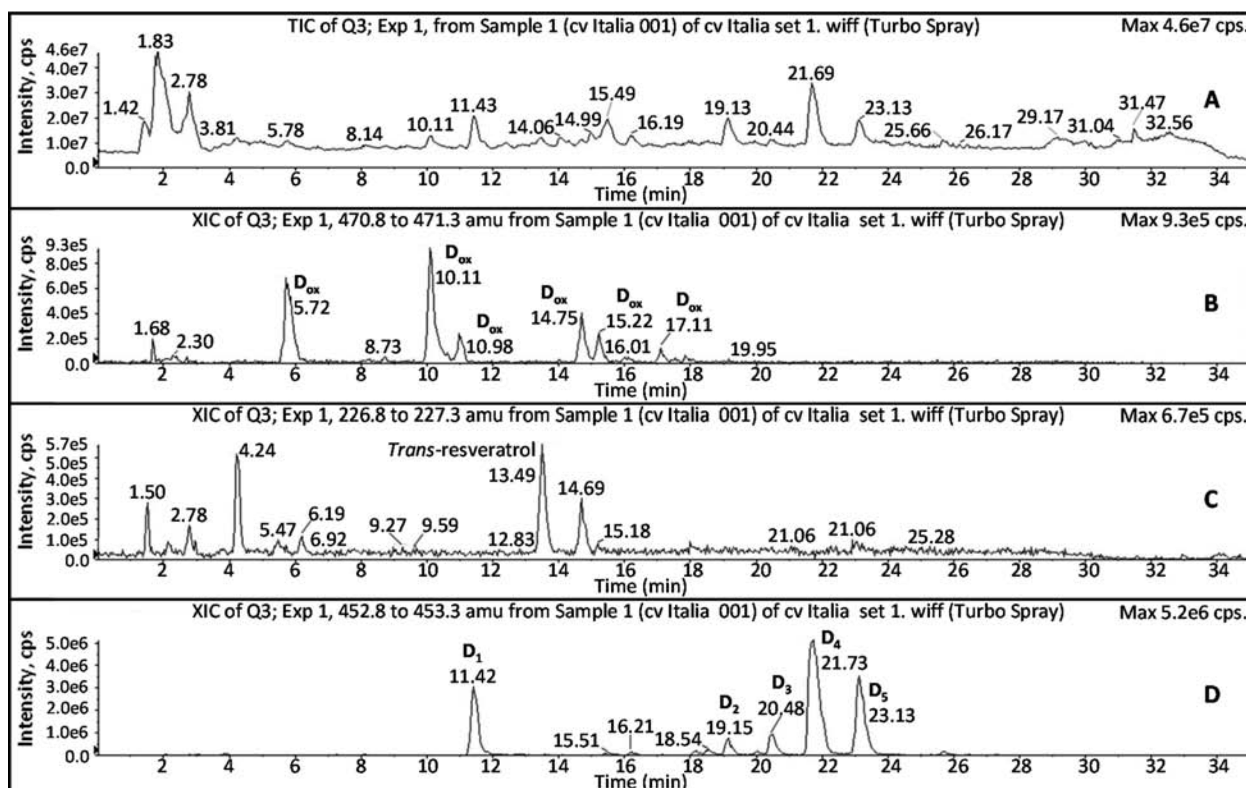

Figure 4. (A) Total ion current (TIC) of the cell extracts obtained after elicitation with MeJA, collected at 240 h. (B) Extracting ion current (XIC) profile at 471 m/z relative to oxidized dimers (D_{ox}). (C) XIC profile at 227 m/z corresponding to resveratrol. (D) XIC profile at 453 m/z for the dimers (D_1 = diglucoside dimer; D_2 = *trans*-ε-viniferin; D_3 = dimer; D_4 = *trans*-δ-viniferin; D_5 = *cis*-δ- viniferin).

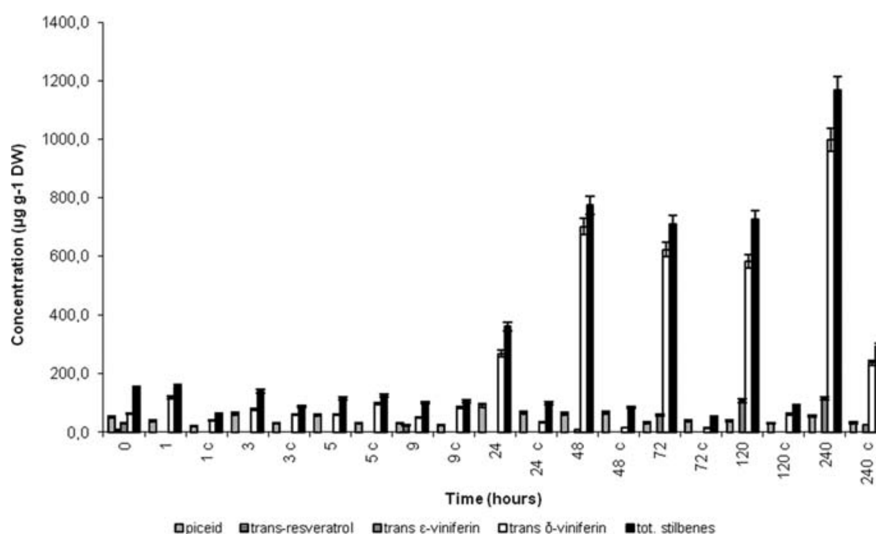

Figure 5. Stilbene production over time in cells of cv Italia elicited with MeJA; the total amount refers only to the sum of *trans*-resveratrol, piceid, ε- and δ-viniferins. Data (μg g^{-1} DW) are expressed as the mean of three independent experiments. The letter "c" indicates the control.

Several studies on different species have reported that chitosan is effective in inducing various plant defense responses and in promoting the production of phytoalexins, such as stilbenes[45] and other phenolic compounds.[46−48] Although the exact mechanism of action of chitosan is still unknown, its effectiveness seems to depend greatly on its degree of polymerization.[45] In light of these considerations, a second experiment was performed to evaluate and compare the ability of MeJA, JA and chitosan to induce the biosynthesis of stilbenes in this cultivar.

The results (Figure 6) show that MeJA and JA increase significantly stilbene production, compared to chitosan and the control. The cells elicited with the jasmonates synthesized a similar amount of total stilbenes, with maximum production at day 20 of culture (0.970 and 1.023 mg g^{-1} DW with MeJA and

dx.doi.org/10.1021/jf201181n |J. Agric. Food Chem. 2011, 59, 9094–9101

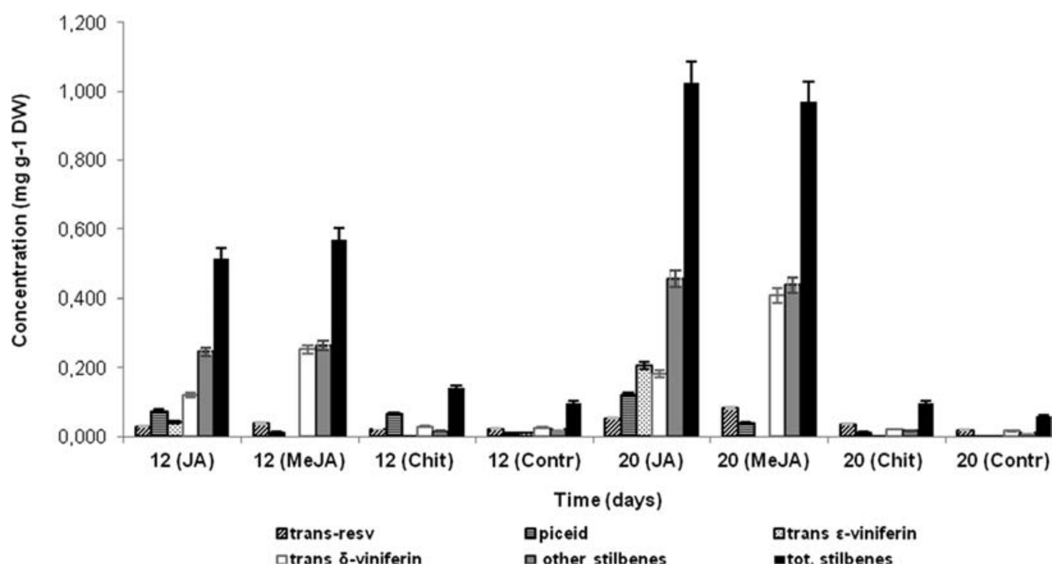

Figure 6. Stilbene production in cells of cv Italia elicited with jasmonic acid (JA), methyl jasmonate (MeJA) and chitosan (Chit), evaluated after 12 and 20 days of culture. Each point represents the mean of three different samples ± SD.

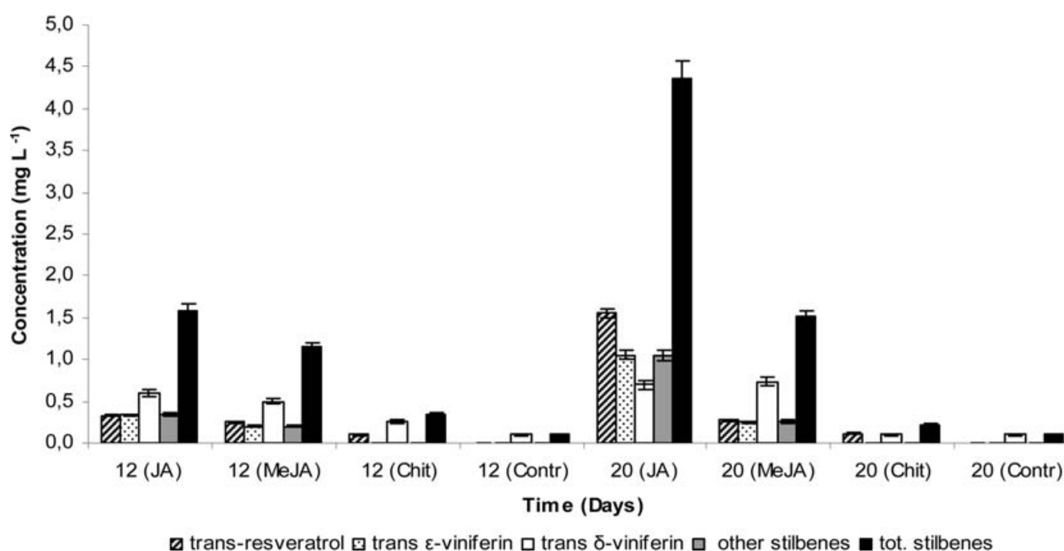

Figure 7. Release of stilbenes in the culture medium of cells treated with jasmonic acid (JA), methyl jasmonate (MeJA), and chitosan (Chit) cv Italia in comparison with the control, evaluated after 12 and 20 days of culture. Each point represents the mean of three different samples ± SD.

JA, respectively). Of note are the different effects of MeJA and JA on the biosynthesis of δ- and ε-viniferin: MeJA at 20 days of culture stimulated δ-viniferin biosynthesis (0.409 mg g^{-1} DW), and only trace amounts of ε-viniferin were found, whereas JA stimulated the biosynthesis of both δ- and ε-viniferin (0.182 and 0.207 mg g^{-1} DW, respectively). Most published studies have described the production of only resveratrol and glycosylated derivatives, whereas few studies have reported viniferin production in cell cultures after elicitation. Recently,[21] δ- and ε-viniferin have been identified in *V. vinifera* cv Chasselas x *Vitis berlandieri* cell cultures elicited with MeJA, yet these viniferins were not quantified. In *V. vinifera* cvs Red Globe and Palieri cell cultures treated with MeJA,[23] ε-viniferin was identified and quantified in both the biomass and the medium, yet in a much lower amount with respect to the cultivar investigated in the present study.

As shown in Figure 6, at day 20 of culture, a peak in production was observed for *trans*-resveratrol (0.055 and 0.084 mg g^{-1} DW with JA and MeJA, respectively) and piceid (0.123 and 0.039 mg g^{-1} DW with JA and MeJA, respectively), albeit in lower quantities with respect to viniferins. Even for these stilbenes, jasmonates were more effective than chitosan. In fact, when using chitosan, the maximum *trans*-resveratrol production (0.141 mg g^{-1} DW) was similar to that for the control cells (0.098 mg g^{-1} DW). By contrast, in another study[15] in which MeJA and chitosan were tested on cultured cells of *V. vinifera* cv Barbera, chitosan induced the best response in terms of *trans*-resveratrol production (0.234 mg g^{-1} DW vs 0.039 mg g^{-1} DW for MeJA). This suggests that in this species the response to different elicitors may be cultivar-dependent as already observed also by other authors. In light of this, it would be interesting to extend the

studies on the effect of elicitation to as broad a spectrum as possible of grape genotypes.

According to the data shown in Figure 6, also in the medium, the highest amount of stilbenes released was observed after 20 days of culture for both jasmonates, whereas the lowest amount, as expected, was observed for chitosan (Figure 7). In particular, JA was the most effective in inducing the release in the medium of *trans*-reveratrol, ε-viniferin and other not characterized stilbenes (1.55, 1.05, 1.05 mg L^{-1}, respectively), in comparison to MeJA (0.27, 0.25, 0.25 mg L^{-1} respectively). With regard to δ-viniferin, both jasmonates induced a similar release (0.73 and 0.70 mg L^{-1} with MeJA and JA, respectively). Chitosan treatment led to a release of a very low amount of *trans*-reveratrol and δ-viniferin (0.12 and 0.10 mg L^{-1}, respectively).

The release of stilbenes in the culture medium, in particular *trans*-resveratrol, has been already reported,[17,14,18] nevertheless the excretion mechanism through the plant cell wall has yet to be elucidated. Some studies focused to evaluate the production of other phenolic compounds suggest that ABC transporters or H^+ gradient-dependent mechanisms are involved.[49,50] They hypothesized that in *V. vinifera*, during berry development, the phenylpropanoid metabolism branch leading to stilbene biosynthesis is located in the cell wall. Moreover, in the same study, stilbene synthase was found mainly in vesicles along the plasma membrane and cell wall, suggesting that this enzyme is secreted in the apoplast and that stilbene synthesis takes place mainly within the cell wall. This is the first compartment with which the pathogen comes into contact; for this reason, stilbene synthesis in the cell wall would render the plant defense response more effective and, at the same time, allows the autotoxicity effect to be avoided. It has been suggested that the localization of the stilbene synthase in the cell wall in grape berries of *Vitis* spp. is linked to an excretion mechanism, which is of practical importance for cell cultures because the excretion of most of the resveratrol into the culture medium could facilitate its recovery. Moreover, the presence in the apoplast of peroxidase isoenzymes in *Vitis* suggests that the oligomerization of resveratrol and the consequent formation of viniferins take place in this compartment, and this could explain their release in the culture medium.[2]

CONCLUSIONS

We demonstrate that treatment with MeJA, JA and chitosan in *V. vinifera* cv Italia cell suspensions induces different responses in terms of stilbene production. In previous studies on cell cultures of other cultivars of *V. vinifera* in which the synthesis of stilbenes was stimulated using different elicitors, only the production of *trans*-resveratrol and its glucosides *trans*- and *cis*-piceids were considered. To the best of our knowledge, only one previous study[23] has evaluated the production of stilbene dimers in cell cultures of *V. vinifera* cvs Red Globe and Michele Palieri elicited with MeJA. In that study, cell cultures produced mainly *trans*-piceid and ε-viniferin. Under MeJA treatment, the production of these molecules began after 24 h and it more than doubled between 48 and 120 h. Our results show that both jasmonates represent suitable elicitors for enhancing stilbene production, but with a different selectivity: JA enhances simultaneously δ- and ε-viniferin biosynthesis, whereas MeJA stimulates preferentially δ-viniferin production. It is known that δ-viniferin, unlike ε-viniferin, is not widespread in nature and has not been extensively studied for its biological activities. For this reason, MeJA was chosen as the elicitor for future research. In light of our results, cell suspension cultures of the cv Italia could be proposed as a potential system for the production of this molecule.

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Funding Sources

This work was supported by the funds from the Ministry of Agricultural and Forestry Policy (MIPAF), Italy, in the framework of a program on grape quality (VANSUT project).

ACKNOWLEDGMENT

The authors are grateful to Ente Cassa di Risparmio di Firenze for supplying a part of the instrumentation used for this research.

REFERENCES

(1) Langcake, P.; Pryce, R. J. New class of phytoalexins from grapevines. *Experientia* **1977**, *33*, 151–152a.

(2) Jeandet, P.; Douillet-Breuil, A. C.; Bessis, R.; Debord, S.; Sbaghi, M; Adrian, M. Phytoalexins from the Vitaceae: Biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. *J. Agric. Food Chem.* **2002**, *50*, 2731–2741.

(3) Zamboni, A.; Vrhovsek, U.; Kassemeyer, H.-H.; Mattivi, F.; Velasco, R. Elicitor-induced production in cell cultures of different grape genotypes (*Vitis* spp.). *Vitis* **2006**, *45*, 63–68.

(4) Namdeo, A. G. Plant cell elicitation for production of secondary metabolites: a review. *Pharmacogn. Rev.* **2007**, *1*, 69–79.

(5) Huckelhoven, R. Cell Wall—Associated Mechanisms of Disease Resistance and Susceptibility. *Annu. Rev. Phytopathol.* **2007**, *45*, 101–127.

(6) Belhadj, A.; Saigne, C.; Telef, N.; Cluzet, S.; Bouscaut, J.; Corio-Costet, M. F.; Mérillon, J. M. Methyl jasmonate induces defense responses in grapevine and triggers protection against Erysiphe necator. *J. Agric. Food Chem.* **2006**, *54*, 9119–9125.

(7) Liswidowati, F. M.; Melchior, F.; Hohmann, F.; Schwer, B.; Kindl, H. Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. *Planta* **1991**, *183*, 307–314.

(8) Melchior, F.; Kindl, H. Coordinate-dependent and elicitor dependent expression of stilbene synthase and phenylalanine ammonia-lyase genes in *Vitis* cv optima. *Arch. Biochem. Biophys.* **1991**, *288*, 552–557.

(9) Waffo-Teguo, P.; Fauconneau, B.; Deffieux, G.; Huguet, F.; Vercauteren, J.; Merillon, J. M. Isolation, identification, and antioxidant activity of three stilbene glucosides newly extracted from *Vitis vinifera* cell cultures. *J. Nat. Prod.* **1998**, *61*, 655–657.

(10) Krisa, S.; Larronde, F.; Budzinski, H.; Decendit, A.; Deffieux, G.; Mérillon, J. M. Stilbene production by *Vitis vinifera* cell suspension cultures: Methyl jasmonate induction and ^{13}C biolabeling. *J. Nat. Prod.* **1999**, *62*, 1688–1690.

(11) Decendit, A.; Waffo-Teguo, P.; Richard, T.; Krisa, S.; Vercauteren, J.; Monti, J.-P.; Deffieux, G.; Mérillon, J.-M. Galloylated catechins and stilbene diglucosides in *Vitis vinifera* cell suspension cultures. *Phytochemistry* **2002**, *60*, 795–798.

(12) Aziz, A.; Poinssot, B.; Daire, X.; Adrian, M.; Bezier, A.; Lambert, B.; Joubert, J. M.; Pugin, A. Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola*. *Mol. Plant—Microbe Interact.* **2003**, *16*, 1118–1128.

(13) Righetti, L.; Franceschetti, M.; Ferri, M.; Tassoni, A.; Bagni, N. Resveratrol production in *Vitis vinifera* cell suspensions treated with several elicitors. *Caryologia* **2007**, *60*, 169–171.

(14) Lijavetzky, D.; Almagro, L.; Belchi-Navarro, S.; Martínez-Zapater, J. M.; Bru, R.; Pedreño, M. A. Synergistic effect of methyljasmonate and cyclodextrin on stilbene biosynthesis pathway gene expression and resveratrol production in Monastrell grapevine cell cultures. *BMC Res. Notes* **2008**, *1*, 132–140.

(15) Tassoni, A.; Fornalè, S.; Franceschetti, M.; Musiani, F.; Michael, A. J.; Perry, B.; Jasmonates, Bagni N. and Na-orthovanadate promote resveratrol production in *Vitis vinifera* cv Barbera cell cultures. *New Phytol.* **2005**, *166*, 895–905.

(16) Ferri, M.; Tassoni, A.; Franceschetti, M.; Righetti, L.; Naldrett, M. J.; Bagni, N. Chitosan treatment induces changes of protein expression profile and stilbene distribution in *Vitis vinifera* cell suspensions. *Proteomics* **2009**, *9*, 610–624.

(17) Ferri, M.; Dipalo, S. C. F.; Bagni, N.; Tassoni, A. Chitosan elicits mono-glucosylated stilbene production and release in fed-batch bioreactor cultures of grape cells. *Food Chem.* **2010**, *124*, 1473–1479.

(18) Belhadj, A.; Telef, N.; Saigne, C.; Cluzet, S.; Barrieu, F.; Hamdi, S.; Mérillon, J.-M. Effect of methyl jasmonate in combination with carbohydrates on gene expression of PR proteins, stilbene and anthocyanin accumulation in grapevine cell cultures. *Plant Physiol. Biochem.* **2008**, *46*, 493–499.

(19) Faurie, B.; Cluzet, S.; Mérillon, J.-M. Implication of signaling pathways involving calcium, phosphorylation and active oxygen species in methyl jasmonate-induced defense responses in grapevine cell cultures. *J. Plant Physiol.* **2009**, *166*, 1863–1877.

(20) Donnez, D.; Jeandet, P.; Clément, C.; Courot, E. Bioproduction of resveratrol and stilbene derivatives by plant cells and microorganisms. *Trends Biotechnol.* **2009**, *27*, 706–713.

(21) Donnez, D.; Kim, K. H.; Antoine, S.; Conreux, A.; De Luca, V.; Jeandet, P.; Clément, C.; Courot, E. Bioproduction of resveratrol and viniferins by an elicited grapevine cell culture in a 2L stirred bioreactor. *Process Biochem.* **2011**, *46*, 1056–1062.

(22) Mulinacci, N.; Innocenti, M.; Santamaria, A. R.; la Marca, G.; Pasqua, G. 2010. HPLC-API/ES/MS/MS investigation of stilbenoids in *Vitis vinifera* L. cell cultures, cv Malvasia. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2065–2073.

(23) Santamaria, A. R.; Antonacci, D.; Valletta, A.; Caruso, G.; Cavaliere, C.; Gubbiotti, R.; Laganà, A.; Pasqua, G. Stilbene production in cell cultures of *Vitis vinifera* L.cv Red Globe and Michele Palieri elicited by methyljasmonate. *Nat. Prod. Res.* **2010**, *24*, 1488–1498.

(24) Lin, J. K.; Tsai, S. H. Chemoprevention of cancer and cardiovascular disease by resveratrol. *Proc. Natl. Sci. Counc., Repub. China* **1999**, *23*, 99–106.

(25) Bradamante, S.; Barenghi, L.; Villa, A. Cardiovascular protective effects of resveratrol. *Cardiovasc. Drug Rev.* **2004**, *22*, 169–188.

(26) Das, S.; Das, D. K. Resveratrol: A Therapeutic Promise for Cardiovascular Diseases. *Recent Pat. Cardiovasc. Drug Discovery* **2007**, *2*, 133–138.

(27) Jannin, B.; Menzel, M.; Berlot, J. P.; Delmas, D.; Lancon, A; Latruffe, N. Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: Plasmatic protein binding and cell uptake. *Biochem. Pharmacol.* **2004**, *68*, 1113–1118.

(28) Delmas, D.; Lancon, A.; Colin, D.; Jannin, B. Resveratrol as a chemopreventive agent: A promising molecule forfighting cancer. *Curr. Drug Targets* **2006**, *7*, 423–442.

(29) Udenigwe, C. C.; Ramprasath, V. R.; Aluko, R .E .; Jones, P. J. H. Potential of resveratrol in anticancer and anti-inflammatory therapy. *Nutr. Rev.* **2008**, *66*, 445–454.

(30) Schroder, J.; Schroder, G. Stilbene and chalcone synthases related enzymes with key functions in plant-specific pathways. *Z. Naturforsch. C* **1990**, *45*, 1–8.

(31) Ribeiro de Lima, M. T.; Waffo-Teguo, P.; Teissedre, P. L.; Pujolas, A.; Vercauteren, J.; Cabanis, J. C.; Mérillon, J. M. Determination of stilbenes (*trans*-astringin, *cis*- and *trans*-piceid, and *cis*- and *trans*-resveratrol) in Portuguese wines. *J. Agric. Food Chem.* **1999**, *47*, 2666–2670.

(32) Romero-Perez, A. I.; Ibern-Gomez, M.; Lamuela-Raventos, R. M.; de La Torre-Boronat, M. C. Piceid, the major resveratrol derivative in grape juices. *J. Agric. Food Chem.* **1999**, *47*, 1533–1536.

(33) Langcake, P.; Pryce, R. J. Production of resveratrol and viniferins by grapevines in response to UV irradiation. *Phytochemistry* **1977**, *16*, 1193–1196.

(34) Pezet, R.; Perret, C.; Jean-Denis, J. B.; Tabacchi, R.; Gindro, K.; Viret, O. Delta-viniferin, a resveratrol dehydrodimer: one of the major stilbenes synthesized by stressed grapevine leaves. *J. Agric. Food Chem.* **2003**, *51*, 5488–5492.

(35) Waffo-Teguo, P.; Lee, D.; Cuendet, M.; Pezzuto, J. M.; Kinghorn, A,. D. Two new stilbene dimmer glucosides from grape (*Vitis vinifera*) cell cultures. *J. Nat. Prod.* **2001**, *64*, 136–138.

(36) Morales, M.; Alcantara, J.; Ros-Barcelo', A. Oxidation of *trans* resveratrol by a hypodermal peroxidase isoenzymes from Gamay rouge grape (*Vitis vinifera*) berries. *Am. J. Enol. Vitic.* **1997**, *48*, 33–38.

(37) Oshima, Y.; Namao, K.; Kamijou, A.; Matsuoka, S.; Nakano, M.; Terao, K.; Ohizumi, Y. Powerful hepatoprotective and hepatotoxic plant oligostilbenes, isolated from the Oriental medicinal plant *Vitis coignetiae* (Vitaceae). *Experientia* **1995**, *51*, 63–66.

(38) Privat, C.; Telo, J. P.; Bernardes-Genisson, V.; Vieira, A.; Souchard, J. P.; Nepveu, F. Antioxidant properties of *trans*-viniferin as compared to stilbene derivatives inaqueous and nonaqueous media. *J. Agric. Food Chem.* **2002**, *50*, 1213–1217.

(39) Billard, C.; Izard, J. C.; Roman, V.; Kern, C.; Mathiot, C.; Mentz, F.; Kolb, J. P. Comparative antiproliferative and apoptotic effects of resveratrol, ε-viniferin and vine-shots derived polyphenols (vineatrols) on chronic B lymphocytic leucemia cells and normal human lymphocytes. *Leuk. Lymphoma* **2002**, *43*, 1991–2002.

(40) Quiney, C.; Dauzonne, D.; Kern, C.; Fourneron, J. D.; Izard, J. C.; Mohammad, R. M.; Kolb, J. P.; Billard, C. Flavones and polyphenols inhibit the NO pathway during apoptosis of leukemia B-cells. *Leuk. Res.* **2004**, *28*, 851–861.

(41) Piver, B.; Berthou, F.; Dreano, Y.; Lucas, D. Differential inhibition of human cytochrome P450 enzymes by ε-viniferin, the dimmer of resveratrol: comparison with resveratrol and polyphenols from alcoholized beverages. *Life Sci.* **2003**, *73*, 1199–1213.

(42) Yanez, M.; Fraiz, N.; Cano, E.; Orallo, F. (-)*Trans*-ε-viniferin, a polyphenol present in wines, is an inhibitor of noradrenaline and 5-hydroxytryptamine uptake and of monoamine oxidase activity. *Eur. J. Pharmacol.* **2006**, *542*, 54–60.

(43) Gamborg, O. L.; Miller, R.; Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **1968**, *50*, 151–158.

(44) Wasternack, C. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann. Bot. (London)* **2007**, *100*, 681–97.

(45) Hadwiger, L. A. In *Chitin and Chitosanes*; Jolles, P., Muzzarelli, R. A. A., Eds.; Birkhauser Verlag: Basel, Switzerland, 1999; pp 185—200.

(46) Kim, H. J.; Chen, F.; Wang, X.; Rajapakse, N. C. Effect of chitosan on the biological properties of sweet basil (*Ocinum basilicum* L.). *J. Agric. Food Chem.* **2005**, *53*, 3696–3701.

(47) Lo, S. C. C.; Nicholson, R. L. Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls. Implications for a compensatory role in the defense response. *Plant Physiol.* **1998**, *116*, 979–989.

(48) Bézier, A.; Lambert, B.; Baillieul, F. Study of defense-related gene expression in grapevine leaves and berries infected with *Botrytis cinerea*. *Eur. J. Plant Pathol.* **2002**, *108*, 111–120.

(49) Yazaki, K. ABC transporters involved in the transport of plant secondary metabolites. *FEBS Lett.* **2006**, *580*, 1183–1191.

(50) Fornara, V.; Onelli, E.; Sparvoli, F.; Rossoni, M.; Aina1, R.; Marino, G.; Citterio, S. Localization of stilbene synthase in *Vitis vinifera* L. during berry development. *Protoplasma* **2008**, *233*, 83–93.