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Enhancement of Viniferin Production in *Vitis vinifera* L. cv. Alphonse Lavallée Cell Suspensions by Low-Energy Ultrasound Alone and in Combination with Methyl Jasmonate

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ABSTRACT: This study examined for the first time the effect of low-energy ultrasound (US), used alone or in combination with methyl jasmonate (MeJA), on viniferin production in cell cultures of *Vitis vinifera* L. cv Alphonse Lavallée. Cell suspensions were exposed for 2 min to US (power 30, 60, and 90 mW cm^{-3}). The highest viniferin production was obtained at 30 mW cm^{-3}. When sonication was performed twice, the effect on viniferin production was negligible, whereas triple sonication slightly increased production. US treatment at 30 mW cm^{-3} for 5 min decreased viniferin production and induced cellular death. The combined use of MeJA and US (2 min) increased the production of δ-viniferin, the dominant stilbene, more than each elicitor used alone. These results suggest that low-energy US, alone and in combination with MeJA, can act as a physical elicitor to stimulate viniferin production in *V. vinifera* cell cultures.

KEYWORDS: *cell cultures, Vitis vinifera L. cv. Alphonse Lavallée, δ- and ε-viniferins, ultrasound sonication, methyl jasmonate, chemical elicitor, physical elicitor*

INTRODUCTION

Cell cultures represent a promising methodological approach for producing secondary metabolites, the accumulation of which is part of a plant's defense response against pathogen infection and environmental stimuli. One of the most effective means of improving the yield of secondary metabolites is the treatment of plant cell cultures with biotic and abiotic elicitors.[1−3] The most frequently used elicitors include fungal elicitors, carbohydrates, yeast extract, jasmonates, and chitosan.[4] Elicitors activate a complex network of multiple signal pathways, initiating a cascade of physiological events in the cells, such as changes in membrane potential and Ca^{2+} flux; the production of reactive oxygen species (ROS) followed by the synthesis of signal molecules such as ethylene, salicylic acid, jasmonic acid (JA), and its derivative methyl jasmonate (MeJA); the activation of defense-related genes; and the synthesis of antimicrobial compounds (e.g., phytoalexins).

Ultrasound (US) is an abiotic elicitor that can stimulate the biosynthesis of secondary metabolites in cell cultures, yet few studies have evaluated its use.[5−8] Although high-energy US is generally destructive to cells, mild- and low-energy US may stimulate cellular biosynthesis.[9] Low-energy US has been applied to in vitro systems to improve the production of saponins in cell suspension cultures of *Panax ginseng*,[5,6] to stimulate shikonin biosynthesis in cell suspension cultures of *Lithospermum erythrorhizon*,[10] and to increase the biosynthesis of taxol in cell suspension cultures of *Taxus chinensis*.[7,8] US has also been used to increase metabolite release in the culture medium, given that it enhances the permeability of the cell membrane.[10]

Vitis vinifera L. cell cultures have been used in diverse studies to explore the effect of different elicitors on the regulation of stilbene biosynthesis (in particular, phytoalexins) and stilbene production and release.[11−21] Moreover, *V. vinifera* cell cultures are a good model for studying the effects of abiotic and biotic elicitors on the accumulation of stilbenes, allowing for good repeatability in terms of production.[18−20] However, to the best of our knowledge, no studies have been published on the use of US in *V. vinifera* cell cultures.

In *V. vinifera* plants, the most frequently observed chemical defense response to environmental stimuli and pathogen infection is the production of the stress metabolites, stilbenes.[22] When attacked by fungal diseases such as *Botrytis cinerea* and *Plasmopara viticola*,[23,24] grapevine plants synthesize the stilbene *trans*-resveratrol (*trans*-3,5,4′-trihydroxystilbene), which is controlled by the key enzyme stilbene synthase, which catalyzes the condensation of three malonyl-CoA molecules and one 4-coumaroyl-CoA molecule.[25] A derivative of *trans*-resveratrol is pterostilbene, a double-methylated product with fungitoxic activity. Other oxidation products of *trans*-resveratrol include δ- and ε-viniferin (dehydrodimers), α-viniferin (dehydrotrimer), β-viniferin (a cyclic resveratrol tetramer), and more highly polymerized oligomers such as γ-viniferins. At present, knowledge of viniferin biosynthesis in *V. vinifera* is incomplete. Stress-induced peroxidase is the only plant enzyme known to be involved in the oxidation of *trans*-resveratrol and its transformation into viniferins.[26,27] The biosynthesis of δ- and ε-viniferin is also catalyzed by fungal laccase-like stilbene oxidase.[28,29]

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trans-Resveratrol itself could be considered as a moderate antifungal stilbene, whereas δ- and ε-viniferins, as well as pterostilbene, possess fungitoxic activity that is greater than that of *trans*-resveratrol. In particular, the antifungal activity of ε-viniferin has been shown to be very similar to that of pterostilbene on *B. cinerea* conidia germination.[30] Pterostilbene activity, in terms of inhibiting fungal growth in vitro, has been found to be 5 times greater than the activity of *trans*-resveratrol, indicating that the methylation of hydroxyphenyl groups could increase the biocidal activity of phenolics.[30] The mechanisms of pterostilbene fungitoxicity could be related to its capacity to penetrate lipophilic membranes[31] and cause a rapid destruction of *B. cinerea* conidia.

In a recent study on the leaves, shoots, and flowers of two *V. vinifera* cultivars (i.e., Merlot and Cabernet Sauvignon), the distribution of viniferins was evaluated following inoculation with *B. cinerea* at prebloom, bloom, and postbloom stages.[24] The δ-viniferin content was negligible in the leaves, shoots, and flowers of the Merlot cultivar at all stages of development, whereas Cabernet Sauvignon flowers showed high δ-viniferin content at the prebloom and bloom stages. δ-Viniferin was also found in leaves but not in shoots.[24] In light of these results, an in vitro system could be developed to achieve reproducible long-term productivity of bioactive molecules.

Of interest is the finding that viniferins have been shown to have different biological activities in humans.[32−38] Hepatoprotective[38] and antioxidant[32] properties and the ability to induce apoptosis of leukemia B cells[33] have been demonstrated for ε-viniferin. Moreover, ε-viniferin has been shown to inhibit human cytochrome P450 enzymes,[34] monoamine oxidase activity, and the uptake of noradrenaline and 5-hydroxytryptamine.[36] It has also been shown that α-viniferin inhibits the growth of human colon cancer cells.[37]

The objective of the present study was to evaluate the production of stilbenes, mainly viniferins, in *V. vinifera* cv. Alphonse Lavallée cell cultures after elicitation with US and MeJA, alone or combined. Alphonse Lavallèe cell cultures were chosen because according to our preliminary results they were the most productive in terms of stilbenes after elicitation with MeJA. Moreover, to accurately quantify the viniferin content, a General Atomic and Molecular Electronic Structure System (GAMESS) study was performed to compare the theoretical molar epsilon of δ- and ε-viniferins to that of *trans*-resveratrol.

MATERIALS AND METHODS

In Vitro Plant Cell Cultures. The cell cultures were obtained by culturing young stems and tendrils of *V. vinifera* cv. Alphonse Lavallèe. The plant material were provided by the Experimental Institute for Enology of Velletri (Italy). The cell cultures had been propagated in solid medium for >3 years. The experiments for studying the effects of low-energy US and MeJA were performed in cell suspension cultures obtained by inoculating 2 g fresh weight (FW) of calli in 250 mL flasks containing 50 mL of B5 medium[39] supplemented with 0.2 mg/L α-naphthaleneacetic acid (NAA), 1 mg/L kinetin (KIN), and 2% (w/v) sucrose, adjusted to pH 5.7. Subcultures were carried out every 20 days and were maintained in continuous light (70 $\mu mol^{-1}\ m^{-2}$) at 26 ± 1 °C. The cell suspensions were inoculated at a 15% concentration (3 g fresh weight in 20 mL of liquid medium). During the culture period (20 days), cell growth was evaluated by determining the average percentage increment of packed cell volume (PCV) and expressed as percent growth index (I%):

$$I\% = \frac{\text{final PCV} - \text{initial PCV}}{\text{initial PCV}} \times 100$$

Measures were performed until there was no longer an increase in cell growth (stationary phase). The cell suspensions had been maintained in shake-flask culture for >2 months before any experiments were performed.

Exposure of *V. vinifera* Cell Cultures to Low-Energy Ultrasound. In a first experiment, the effect of different energy levels of US on cell suspension cultures was evaluated. An ultrasonic cleaning bath (Elmasonic S 60/(H)) with a fixed frequency of 37 kHz and a variable energy level [low (30 mW/cm^3), medium (60 mW/cm^3), and high levels (90 mW/cm^3)] was used to insonate the cell suspensions. For exposure, the flasks were dipped in the ultrasonic bath to a depth at which the liquid in the flasks was about 1.0 cm below the liquid in the bath. The data are expressed as the mean of three replicate measurements. The ultrasonic bath temperature was maintained at 25 ± 0.5 °C during the exposure. The exposure period was fixed at 2 min at all energy levels. The cells were treated at day 7 postinoculation; at day 12 of culture, both the treated cells and the control cells were harvested and separated from the medium by vacuum filtration, weighed, and stored at −20 °C until the analyses. In a second experiment, the effects on cell suspension cultures of three successive sonications at low energy (30 mW/cm^3), each lasting 2 min, were evaluated. The cells were treated at day 7 (single treatment), at days 7 and 14 (double treatment), and at days 7, 14, and 21 (triple treatment) postinoculation. The cells were harvested after 5 days from each treatment. In a third experiment, the effects of the time of treatment (time of exposure to US of 2 and 5 min) and MeJA (25 μM), used alone and in combination with US (30 mW/cm^3), were evaluated. MeJA (Sigma-Aldrich, Italy) was dissolved in 100% ethanol and added to the cells through filter sterilization. On the basis of the results of the experiments described above, the cells were treated only at day 7 postinoculation (single treatment). The treated and control cells were collected at day 12 postinoculation, separated from the medium, weighed, and stored at −20 °C until the analyses. In each experiment, the treated and control cells were obtained from the same cell suspensions to avoid deviations of the results due to somaclonal variation.

Evaluation of Cell Viability. Cell viability was investigated in samples treated with low-energy US (30 mW/cm^3) for 2 and 5 min. Cell suspensions, harvested immediately after the US elicitation, were treated for 15 min with a solution consisting of 0.5 mg of fluorescein diacetate (FDA) (Sigma) 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 excitation at λ 386 nm. Cells with a green fluorescent nucleus and cytoplasm were considered to be viable.

Extraction of Stilbenes. Cell suspensions (3 g fresh weight for each sample) 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, Piscataway, NJ, USA) and analyzed by HPLC/DAD/MS. The dry weight was determined as the sum of the extract and cell residue. A liquid/liquid extractive procedure using ethyl acetate was applied at the culture media.

HPLC/DAD/MS Analysis of Stilbenes. The analyses were carried out using an HP 1100 L 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 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 were H_2O (pH 3.2 by HCOOH) and CH_3CN, both of HPLC grade.

Quantitative Determination of Stilbenes. *trans*-Resveratrol, piceid, and the other stilbenes were quantified by a five-point calibration curve at 307 nm (r^2 = 0.9999) using pure *trans*-resveratrol

as external standard (Extrasynthese Geney, France) from 0 to 1.98 μg. The multiplicative correction factor 2 was applied to express the concentration of the viniferins and the other dimers of resveratrol in the data shown in Figures 3, 5, and 6.

Quantum Chemical Computations (Orbital Calculations). The calculations were performed with GAMESS, Gamess version of June 6, 1999, from Iowa State University [A; B]. The main calculation parameters are restricted Hartree−Fock (RHF); the optimization of the molecular geometry was obtained using analytic energy gradients: maximum number of steps to take = 1000; gradient convergence tolerance 0.0001(Hartree/Bohr); Gaussian basis set, N21 (Pople's N-21G split valence basis set), number of Gaussians (N) = 3 (H−Xe).

Statistics. All experiments were performed in triplicate, and each datum is the mean of three independent measures. The statistical significance of a treatment effect was evaluated by ANOVA test at a probability limit of $P < 0.05$.

RESULTS AND DISCUSSION

To optimize the response of this in vitro system in terms of the energy level of US, time of exposure, and combination with MeJA, three different experiments of elicitation by US were performed. Stilbene production was then evaluated by comparing treated and control samples through HPLC/DAD/MS analysis.

Before the experiments were performed, the cell suspension growth curve was studied. The cell growth was monitored by measuring the average percentage increment of PCV from 0 to 20 days (Figure 1); maximum cell growth (60%) was observed at day 15.

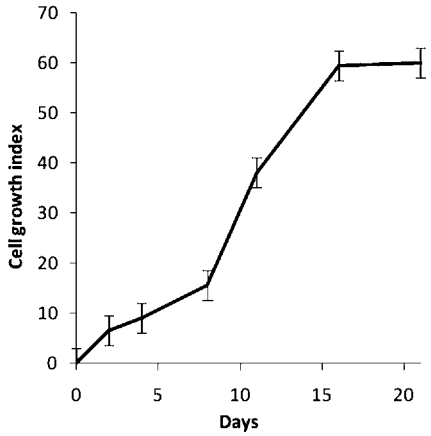

Figure 1. Growth curve of *V. vinifera* cv. Alphonse Lavallée cell suspensions. Each point represents the mean of three replicates ± SD.

To examine the effects of US exposure on stilbene production, the cell suspensions were exposed to three different levels [30 mW/cm^3 (low), 60 mW/cm^3 (medium), and 90 mW/cm^3 (high)], all on day 7 of culture. In the few studies published to date on the elicitation of cultured cells with US, the energy levels ranged from 3.4 to 113.7 mW/cm^3.[5−7,10] Our preliminary data obtained on these cell cultures showed that levels <30 mW/cm^3 had a negligible effect on both stilbene production and cell viability, whereas at levels >90 mW/cm^3 almost all of the cells died (data not shown).

The chromatographic profiles at 307 nm of the extracts obtained from the cell cultures exposed to the different US energy levels are reported in Figure 2; in the figure the profiles are compared with those of the control sample, applying the same scale for the signal intensity. As seen in Figure 3, all US levels induced an increase in total stilbenes. The highest total stilbene content (1.29 mg g^{-1} DW), which was nearly 3 times greater than that for the control cells (0.45 mg g^{-1} DW), was obtained with the low US energy (30 mW/cm^3). The main *trans*-resveratrol dimer present in all of the samples was δ-viniferin, followed by ε-viniferin.

In cells elicited with low-energy US, the δ-viniferin content (1.06 mg g^{-1} DW) was >3 times greater than that for the control. The highest ε-viniferin content (0.34 mg g^{-1} DW), which was 8.5 times higher than that for the control, was observed in cells elicited with medium US energy (60 mW/cm^3). Interestingly, in these cells, US was less effective in increasing δ-viniferin content (0.57 mg g^{-1} DW, which was less than twice that found for the control). The cells exposed to the highest energy (90 mW/cm^3) showed the lowest total stilbene content (0.85 mg g^{-1} DW); however, the δ-viniferin content (0.56 mg g^{-1} DW) was similar to that obtained with medium level, whereas the ε-viniferin content (0.13 mg g^{-1} DW) was about 3 times lower than that observed with medium US. The chemical analyses also revealed the copresence of a *trans*-resveratrol glycosylated dimer and a *cis*-viniferin, the structures of which have not yet been determined. Independent of the US energy level, low contents of the glycosylated dimer were obtained in elicited cells (0.05−0.06 mg g^{-1} DW), although the contents were higher than that for the control. The *cis*-viniferin content was not affected by low and medium US (0.07 mg g^{-1} DW), whereas there was a slight increase in response to elicitation with high US (0.11 mg g^{-1} DW).

The obtained results show that each viniferin responds differently to US, which is consistent with recently published results[19] on the treatment of cell cultures of *V. vinifera* cv. Italia with chemical elicitors (i.e., jasmonates). In these cells, δ- and ε-viniferins were the main dimeric stilbenes biosynthesized in response to elicitation, yet MeJA was more efficient in enhancing δ-viniferin biosynthesis, whereas the elicitation with JA increased the ε-viniferin content. These data suggest that the type of elicitor used and its concentration/intensity could be modulated on the basis of the objectives (e.g., increased production of δ- or ε-viniferin).

With the objective of improving stilbene production, the second experiment was carried out to determine the effect of multiple treatments with US. In light of the results shown in Figure 3, the low-energy level was chosen for the successive experiments as the most effective in promoting stilbene biosynthesis. During cell growth, the cell suspensions were exposed to single, double, and triple US treatments, each lasting 2 min, and the quantitative results in terms of viniferin content and total stilbenes are shown in Figure 4.

The single treatment caused an increase for all of the stilbenes, with the greatest increase found for δ-viniferin (0.45 mg g^{-1} DW) with respect to the control (0.32 mg g^{-1} DW). When the results obtained with single treatment were compared to the results of the first experiment (applying the same US energy), a similar qualitative response was observed (i.e., higher increase for δ-viniferin). However, in the second experiment the δ-viniferin content was consistently lower and the increment with respect to the control was less evident. The cells subjected to triple treatment showed a very small increase in the δ-viniferin concentration (0.25 mg g^{-1} DW) and a marked increase in ε-viniferin (0.27 mg g^{-1} DW) compared to the controls (0.22 and 0.10 mg g^{-1} DW, respectively). Again, it should be stressed that the production of ε- and δ-viniferins differed depending on the specific treatment.

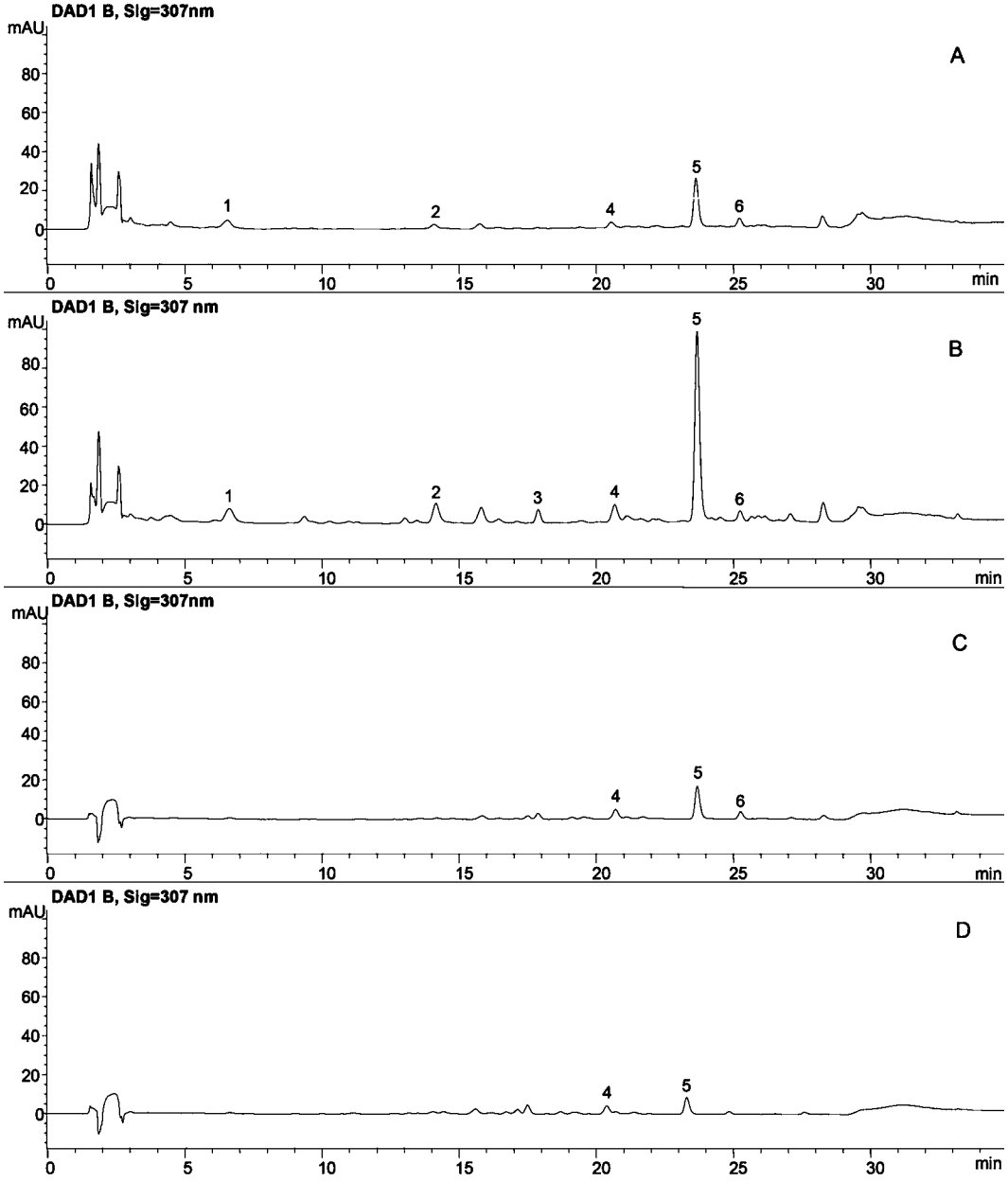

Figure 2. Chromatographic profiles at 307 nm (scale in mAu) of samples treated with three different US powers: (A) not elicited sample (control); (B) low-power US; (C) medium-power US; (D) high-power US (1, piceid; 2, *trans*-resveratrol; 3, monoglycosylated dimer; 4, *trans*-ε-viniferin; 5, *trans*-δ-viniferin; 6, *cis*-viniferin). Different volumes were used to solubilize samples A–D, whereas the injected volumes are the same; this figure cannot be used for a semiquantitative evaluation.

To evaluate the effectiveness of different treatments in promoting δ-viniferin production in grape cells, a third experiment was carried out. The effect of exposure to low-energy US for >2 min (5 min) and the effect of MeJA alone and in combination with US for 2 min were investigated. As shown in Figure 5, following 2 min of exposure, the δ-viniferin content (0.72 mg g^{-1} DW) was more than twice as high as that for the control (0.34 mg g^{-1} DW), whereas 5 min of exposure caused the δ-viniferin concentration (0.20 mg g^{-1} DW) to decrease by nearly half. This finding appeared to be mainly related to the high degree of cell death caused by prolonged sonication. In fact, the FDA viability test showed that in the control about 79% of the cells were viable, whereas after 2 and 5 min of US treatment, the cell viability was 22 and 7%, respectively.

To date, no studies have been published on the combined effect of US and chemical elicitors on *V. vinifera* cell cultures. With regard to the few studies performed on other species, in *T. chinensis* cell cultures, the simultaneous elicitation with US and MeJA increased the taxol content by 1.2- and 1.5-fold, with respect to each treatment used alone.[7] In a recent study, the combined use of US and the signal molecule salicylic acid (SA) led to increases in taxol production of 1.2- and 4-fold in cell cultures of *Taxus baccata*[40] and increases of 7- and 3-fold in *Corylus avellana* cells, compared to the single treatments.[41]

In light on these encouraging data, we decided to investigate the possible synergistic effects of MeJA and US on grape cell cultures. On the basis of the previously obtained results, the cells were subjected to low US energy (30 mW/cm^3) for 2 min. As expected, in our study, treatment with MeJA alone induced

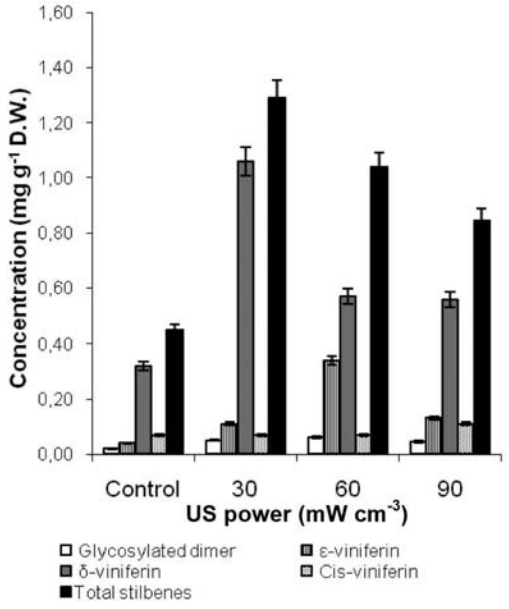

Figure 3. Stilbene production in cell suspensions of *V. vinifera* cv. Alphonse Lavallée exposed to low (30 mW cm^{-3}), medium (60 mW cm^{-3}), and high (90 mW cm^{-3}) US power. Each point represents the average of three values ± SD.

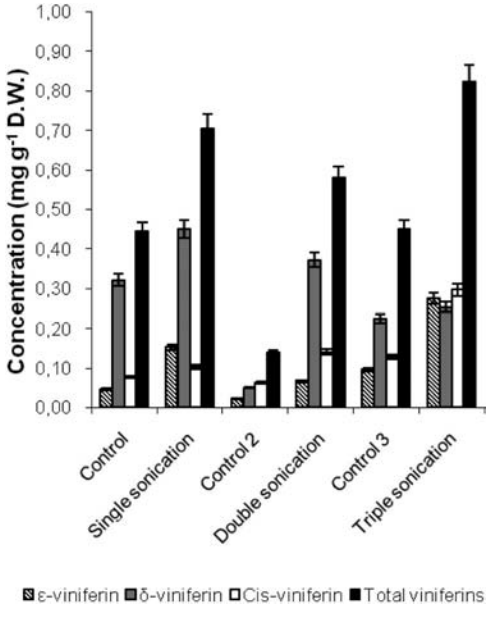

Figure 4. Stilbene production in cells exposed to single, double, or triple successive sonications (respectively, at days 7, 14, and 21 of culture) by low US power (30 mW cm^{-3}). Each point represents the average of three values ± SD.

an increase in *δ*-viniferin; in particular, it increased by 2.6-fold (0.88 mg g^{-1} DW) with respect to the control (0.34 mg g^{-1} DW). In fact, the chemical elicitor MeJA was previously proven to be effective in inducing stilbene production in *V. vinifera* cv. Italia.[19,20]

As shown in Figure 5, of the treatments tested in this study, the combination of MeJA and US was the most effective in promoting *δ*-viniferin production. The simultaneous chemical and physical elicitation increased *δ*-viniferin production by 4.2-fold (1.43 mg g^{-1} DW) compared to the control and by 2- and 1.6-fold compared to, respectively, US and MeJA alone. These

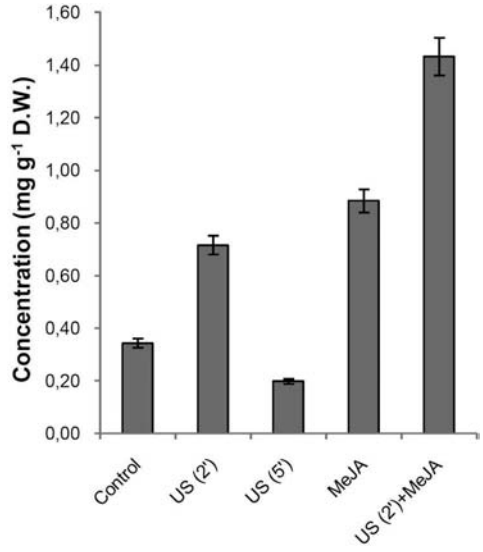

Figure 5. *δ*-Viniferin production in cells exposed to different times of sonication treatments with low-power US alone or in combination with MeJA. Each point represents the average of three values ± SD.

results, together with those of the above-mentioned studies,[7,40,41] demonstrate that low-energy US combined with MeJA or SA can act synergistically in inducing defense responses, although this phenomenon needs to be explored more extensively.

Because the third experiment provided the highest yield in terms of viniferin production, the release in the culture medium was also analyzed. First, the medium was directely analyzed without extraction by HPLC/DAD/MS, but no stilbenes were detected. A liquid/liquid extractive procedure was then applied using ethyl acetate[21] to concentrate the stilbenes, obtaining a final extract that was 40 times more concentrated with respect to the culture medium. Only a few samples showed a small amount of stilbenes, particularly *δ*-viniferin, with a concentration of 0.157 mg L^{-1} for the sample treated with MeJA. The release in small amounts of viniferins in culture medium of *V. vinifera* cv. Chasselas × *Vitis berlandieri* cell cultures has been reported by Donnez et al.[21] *trans*-Resveratrol was not detected in the culture media or in the cells (Figures 4 and 5). In contrast with our results, *trans*-resveratrol and piceid from *V. vinifera* cell cultures were reported.[42] Our results suggest that the US treatments induce a rapid conversion of *trans*-resveratrol in its dimer forms, mainly *δ*-viniferin.

To date, little is known about the mechanisms through which US acts as an elicitor of the plant's defense responses, including secondary metabolite production. Wu and Lin[5−7] hypothesized that low-energy US mainly acts on the cells as a mechanical stress similar to wounding. In fact, the exposure of cells cultivated in liquid medium to US induces energy-intense hydrodynamic events such as acoustic cavitation and microstreaming, which cause mechanical damage and shear stress to the cells.[7]

At present, the specific events involved in the cascade that begins with US exposure and culminates in the production of secondary metabolites are not yet fully known. The first paper on this topic was published by Wu and Lin,[5] who studied the effects of US elicitation on *P. ginseng* cell cultures. Immediately after elicitation, they observed an increase in cross-membrane ion fluxes (Ca^{2+} influx and K^+ efflux/H^+ influx), which is also one of the early events of a normal signal transduction cascade.

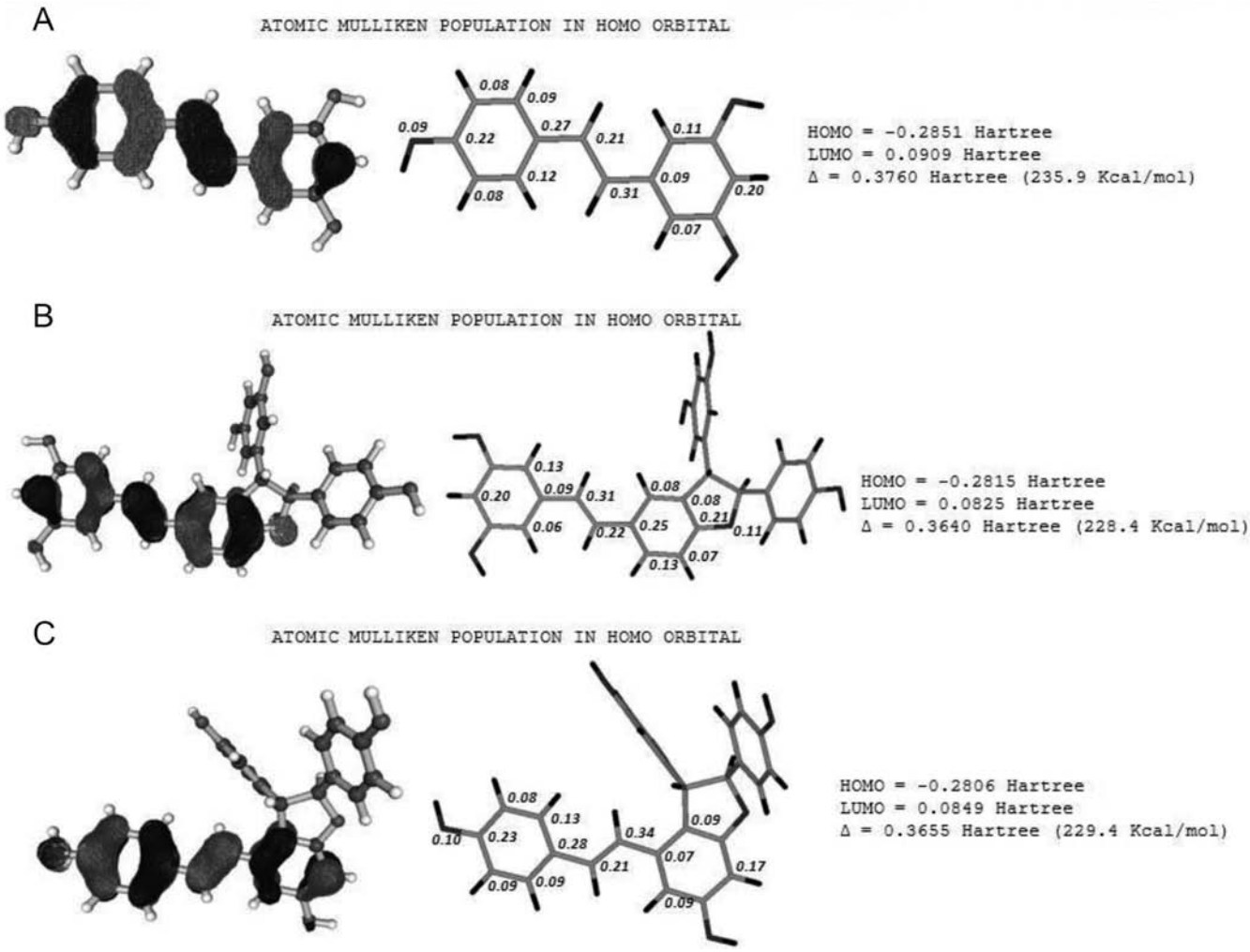

Figure 6. Results from the molecular dynamic simulation at 300 K in implicit solvent: the shown structures are related to *trans*-resveratrol (A), *trans*-*δ*-viniferin (B), and *trans*-*ε*-viniferin (C).

They also observed the production of the ROS hydrogen peroxide (H_2O_2). In US-elicited cell cultures of *T. chinensis*, Wu and Ge[8] also observed H_2O_2 and superoxide anion ($O_2^{\bullet-}$) production. A rapid and transient increase in intracellular ROS concentration, termed an "oxidative burst", is known to be often associated with cell responses to both biotic and abiotic stresses.[43] In *T. chinensis*, it was also observed that US induced an increase in the intracellular content of JA, as well as in the activities of lipoxygenase and allene oxide synthase, enzymes for JA synthesis.[8] Jasmonates are involved in plant defense responses mainly to biotic stress, such as herbivores, necrotrophyc phytopatogens, foliar biotrophs, and biotrophic root pathogens and symbionts.[44]

On the basis of the results of this study, the objective of which was to increase the yield of stilbenes in cell suspensions of *V. vinifera* cv. Alphonse Lavallée, we can conclude that stimulation with the physical elicitor US at a low frequency for a few minutes results in an increase in viniferin production. The combined use of a chemical elicitor (MeJA) with a physical elicitor (US) increased *δ*-viniferin production by nearly 1.6-fold, when compared to MeJA alone, suggesting that the two elicitors act in synergy. The use of higher US power and/or longer times of sonication, which resulted in decreased cellular vitality, significantly reduced the yield of stilbenes. In all of the experiments, sonicated cells were more productive than the controls, the dimeric forms were dominant, and *δ*-viniferin was the main product. To determine the specific mechanisms of the US-induced effects in plant cell cultures and to evaluate the possibility of the large-scale application of this method, additional experiments are needed.

Molecular Dynamics Simulation and Quantum Chemical Computations. This study was carried out to calculate the theoretical molar epsilon of *δ*- and *ε*-viniferins with respect to that of *trans*-resveratrol, with the aim of accurately quantifying these stilbenes in our samples. In fact, these molecules are difficult to find as commercial pure standards, and particularly *δ* viniferin is not available. The main results of this study are shown in Figure 6.

The molecular dynamic simulation at 300 K in implicit solvent carried out for 2000 ps (2 ns) showed the almost complete planarity of the chromophore in both viniferins.

The HOMO of the *δ*- and *ε*-viniferins in most cases involves about half of the molecule, in particular, the portion that maintains the structure of the *trans*-resveratrol. Consequently, the electronic distribution is comparable to that found in the HOMO of the monomer, *trans*-resveratrol. Given that the chromophore has an equal extension for the three molecules, whereas the dimension of the two viniferins is around double with respect to *trans*-resveratrol, it can be assumed that the

coefficient of molar extinction for the δ- and ε-viniferins is half of that known for *trans*-resveratrol.

The energy levels of HOMO and LUMO of the δ- and ε-viniferins differed by only 1 kcal/mol, whereas a greater difference was found (close to 8 kcal/mol) with respect to *trans*-resveratrol. These findings are consistent with the observed values of the maximum wavelengths in their UV−vis spectra: for δ- and ε-viniferins, about the same value (i.e., close to 320 nm) was recorded, whereas for *trans*-resveratrol a lower wavelength was measured (307 nm). In light of these findings, we chose 2 as a multiplicative factor to be applied to all of the quantitative data, calculated by the curve of *trans*-resveratrol but referring to the viniferins.

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Notes

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