trans-Resveratrol and Grape Disease Resistance. A Dynamical Study by High-Resolution Laser-Based Techniques[1]

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Two modern laser-based techniques were synchronously applied to study the dynamics of the trans-resveratrol activity in *Botrytis cinerea*-infected grapes. Direct analysis of trans-resveratrol in both infected and noninfected grapes (*Vitis vinifera*, Aledo variety) was performed by using an analytical technique incorporating laser desorption coupled with laser resonant ionization and time-of-flight mass spectrometry. On the other hand, one of the most sensitive on-line methods for trace gas detection, laser photoacoustic spectroscopy, was used to investigate the involvement of the plant hormone ethylene (C_2H_4) in the *B. cinerea* grapes interaction and its temporal relationship with the trans-resveratrol content upon infection. The trans-resveratrol content and the ethylene released by noninfected grapes showed an opposite behavior. In this case, a high trans-resveratrol content corresponds to a low ethylene emission. For the *B. cinerea*-infected grapes, ethylene emission rises up after 48 h when the analogous content of trans-resveratrol started to decrease irreversibly. Moreover, the activity of trans-resveratrol as natural pesticide has been investigated by exogenous application on grapes. A short submerge (5 s) of the grapes in 1.6×10^{-4} M solution of trans-resveratrol delays the increase of C_2H_4 emission with about 48 h and produces a decrease of the C_2H_4 concentration and its emission rate. The treatment has positive effects on fruit conservation during storage; it doubled the normal shelf-life of grapes at room temperature, maintaining their post-harvest quality within 10 d.

The fungus *Botrytis cinerea* is a plant necrotrophic pathogen that colonizes senescent or dead plant tissues and causes softening in fruits. Fungal hyphae can penetrate through wounds or natural openings of the plant tissue and spread from previously colonized dead tissues into healthy tissues. *B. cinerea* attacks different plant tissues and has a broad host range. It is a major cause of post-harvest rot of perishable plant products, including grapes (*Vitis vinifera*) at harvest and in storage. Because it is also able to infect at low temperatures, it can result in important economic losses, either in pre- and post-harvest crops (Mansfield, 1980).

Under a pathogen attack, plants evolve sophisticated systems of detection and response to decipher the pathogen signals and to induce appropriate defenses. These systems include specific networks that operate through the action of signaling molecules such as salicylate, jasmonate, and ethylene and generate the accumulation of pathogenicity-related proteins, phytoalexins, or other phenolic compounds (Elad, 1997; Dong, 1998; Feys and Parker, 2000).

trans-Resveratrol (3,5,4′-trihydroxystilbene) is an antioxidant compound naturally produced in a huge number of plants, including grapevine where it is the major component of the phytoalexin response of the plant. It is accumulated in vine leaves and grape skin in response to various fungal infections, UV radiation, or chemicals (Langcake, 1981; Jeandet et al., 1995b; Adrian et al., 1997), and it has been found in wines in concentrations depending on viticultural and enological practice (Soleas et al., 1997a).

Since it was reported that *B. cinerea* can act as elicitor toward the production of trans-resveratrol in grapevines (Langcake and Pryce, 1976), many investigations have been carried out on this host-pathogen interaction (Stein and Blaich, 1985; Jeandet et al., 1995b; Adrian et al., 1998; Breuil et al., 1998) mainly by monitoring the trans-resveratrol production in grapevine leaves.

Analytical interest in trans-resveratrol was attributed to its natural pesticide properties. Recent study showed that trans-resveratrol is fungitoxic at physiological concentrations against *B. cinerea* (Adrian et al., 1998). However trans-resveratrol has also proven to enhance the resistance of vineplants to other pathogens, such as *Plasmopara viticola* (Dai et al.,

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1995), *Phomopsis viticola* (Hoos and Blaich, 1990), or *Rhizopus stonifer* (Sarig et al., 1997). This rather unspecific antifungal character and the selective accumulation of trans-resveratrol in grape skin make it a good candidate as a natural pesticide against pathogen attack for improving the natural resistance of grapes to fungal infection. In addition, because of its antioxidant properties, trans-resveratrol can also have positive effects on fruit conservation during storage. As a consequence, both endogenous enhancement and exogenous application could be exploited to reduce grape spoilage.

Another compound that was proven to be produced in the interaction of fruits with *B. cinerea* is ethylene (C_2H_4). Increase of ethylene (C_2H_4) production is a frequently observed phenomenon during the interaction between host and pathogen (Abeles et al., 1992). It has been suggested that ethylene released during infection represents an early response of plants to the perception of a pathogen attack and can be associated with the induction of a defense reaction (Boller, 1991). On the other hand, ethylene is considered to have a major importance in the development of the disease symptoms (Mattoo and Suttle, 1991). However, its role in pathogenesis and resistance is far from clear (Boller, 1991; Dong, 1998; Feys and Parker, 2000).

Ethylene is also involved in the ripening process and therefore its role during pathogenesis in harvested fruits is essential to determine the fruit quality. During the ripening phase of the climacteric fruits (e.g. apples, tomatoes, etc.) both CO_2 and ethylene are emitted at elevated levels as opposed to non-climacteric fruits (e.g. citrus). Grapes are classified as non-climateric fruits and can also produce ethylene, although at very low emission rate.

Within this study, we focused on monitoring trans-resveratrol and ethylene evolution during the *B. cinerea*-grapes interaction. We investigated the possible involvement of ethylene in the infection of grapes with *B. cinerea* and its temporal relationship with the trans-resveratrol content upon infection.

Some micro-organisms, including phytopathogenic fungi like *B. cinerea*, can synthesize ethylene themselves (Fukuda et al., 1993; Cristescu et al., 2002). Because both the grapes and the fungus *B. cinerea* can release ethylene, analysis of ethylene emission during the grape-fungus interaction becomes a complex problem. The investigation of these compounds requires highly sensitive, fast and, if possible, on-line analytical techniques. Among the current available techniques, the most promising are those based on laser technologies, with emphasis on analysis of volatile (ethylene) and non-volatile (trans-resveratrol) compounds.

Laser photoacoustic spectroscopy (LPAS) is one of the most sensitive on-line methods used for trace gas detection of volatile compounds released by the plants. It allows accurate and real-time identification of the natural plant stress-signal and defense molecules such as ethylene, acetaldehyde, ethane, etc. (Harren et al., 1990; Oomens et al., 1998; Leprince et al., 2000). Especially in the case of the non-climacteric fruits like grapes, which release very low amounts of ethylene, this technique represents a powerful tool for on-line measuring of ethylene production. The resulting detection limit for ethylene is 10 pL L^{-1} (Harren and Reuss, 1997), which makes the photoacoustic method 3 orders of magnitude more sensitive than traditional gas chromatography analysis.

On the other hand, a new technique based on laser desorption (LD) coupled with laser resonant multiphoton ionization with time-of-flight mass spectrometric detection (REMPI-TOFMS) has been recently developed, which allows the performance of fast and direct analysis of non-volatile compounds in fruits, such as trans-resveratrol in grapes (Montero et al., 2000a, 2000b; Orea et al., 2001).

The present work reports the synchronous use of these two laser-based techniques to study the dynamics of the trans-resveratrol activity in *B. cinerea*-infected grapes. *B. cinerea*-infected and noninfected grapes were monitored (a) by LD coupled with REMPI-TOFMS for their trans-resveratrol evolution and (b) by LPAS for the ethylene release. Moreover, the activity of trans-resveratrol as natural pesticide has been investigated by its exogenous application on grapes. The reader interested in a comprehensive description of the physical principles behind both techniques and their applicability in chemical and biological studies is addressed to a recent review in which these subjects are included (Orea and González Ureña, 2002).

RESULTS AND DISCUSSION

Non-Volatile Analysis: trans-Resveratrol Elicitation by *B. cinerea*

Analysis of trans-resveratrol is generally carried out by gas chromatography (Jeandet et al., 1995a; Soleas et al., 1997c), HPLC (Jeandet et al., 1997; Juan et al., 1999; Sobolev and Cole, 1999), or capillary electrophoresis (Arce et al., 1998; Berzas Nevado et al., 1999). Regardless of the separation technique, its analysis in grapes and wines requires the use of preconcentration and/or multisolvent extraction techniques because of the complexity of the matrices and the low concentration of the analyte. The combination of LD followed by REMPI-TOFMS detection can overcome these error sources.

Figure 1 shows a time-of-flight spectrum obtained from a sample of grape skin corresponding to a desorption area with 48 mg of grape skin and 79 mg of Zn. The trans-resveratrol peak is clearly noticeable. For this sample, the trans-resveratrol content has been determined using the standard additions method, i.e. adding known quantities of trans-resveratrol to several identical samples of grape skin;

the value obtained for the intercept with the concentration axis gives the quantity of analyte in the blank. A value of 16.0 ± 0.5 μg trans-resveratrol g^{-1} grape skin was obtained, which corresponds to 16 μL L^{-1} of trans-resveratrol. The trans-resveratrol content in grape flesh was also investigated, however no significant signal was found (i.e. content below 2 nL L^{-1}). This finding proves that the main content of trans-resveratrol selectively accumulates in grape skin, which it is consistent with previous investigations (Jeandet et al., 1991).

To investigate the post-harvest elicitation of trans-resveratrol in grapes upon *B. cinerea* infection, three batches of samples were monitored for their trans-resveratrol content: noninfected, mock-infected, and *B. cinerea*-infected. Figure 2 displays the evolution of the trans-resveratrol content in each case. Whereas the noninfected grapes show a constant trans-resveratrol content during the experiment, in the mock-infected grapes, a sudden decrease is observed the 1st d after the buffer inoculation with a smooth diminution during the next days. For the *B. cinerea*-infected group, a significant increase in the trans-resveratrol content is observed with respect to the mock-infected group by the 2nd d after the infection; afterwards, the trans-resveratrol shows a rapid decrease leading to the disappearance of the compound by the 5th d after infection. This decrease is probably attributable to the degradation of the compound by a laccase-like stilbene oxidase produced by *B. cinerea* (Pezet et al., 1991; Sbaghi et al., 1996). As it has been already well established, this extracellular enzyme produced by the fungus is capable of oxidizing the trans-resveratrol, leading to the degradation of the compound by the synthesis of its trans-dehydrodimer (Adrian et al., 1998; Breuil et al., 1998; Cichewicz et al., 2000).

Previous investigations on the production of trans-resveratrol by grapes in response to *B. cinerea* infection (Jeandet et al., 1995b; Adrian et al., 2000) found that its elicitation occurred predominantly in the noninfected grapes surrounding the infected ones, whereas in the latter, the trans-resveratrol content was always lower than in the noninfected grapes. Although the authors gave no information on the infection method (spray, punching, etc.), conidial concentration, time of analysis after the infection, etc., this apparent contradictory result is not so when the different time scale of both experiments is considered; it seems that in a previously reported case, the trans-resveratrol analysis was done several days after the *B. cinerea* infection, and (as Jeandet et al., 1995b claim) the low trans-resveratrol content then found was attributable to the degradation of the compound by the fungus (as it happened in the present case after the 2nd d).

Moreover, the trans-resveratrol evolution found in the present work is consistent with previous in vitro investigations on the induction of trans-resveratrol by *B. cinerea* in leaves (Paul et al., 1998) where the maximum yield of trans-resveratrol was reported in the 3rd d after infection, followed by a rapid reduction on the trans-resveratrol content by the 5th d.

It is out of the scope of this paper to consider the biochemical and molecular mechanisms of both the production of trans-resveratrol by the grape as response to the fungal infection and the metabolism of the compound by the fungus. This subject, i.e. the interaction between *B. cinerea* and trans-resveratrol, has been extensively studied in the past years by several groups; thus, interested readers are directed to reviews for further information on this matter (for example, see Cassidy et al., 2000; Soleas et al., 1997b).

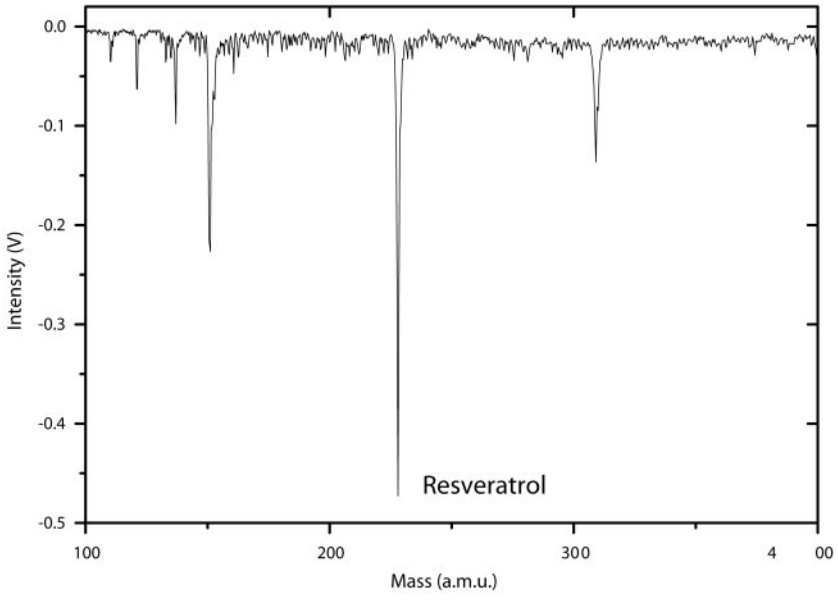

Figure 1. TOFMS spectrum of a grape skin sample obtained at normal experimental conditions. See text for comments.

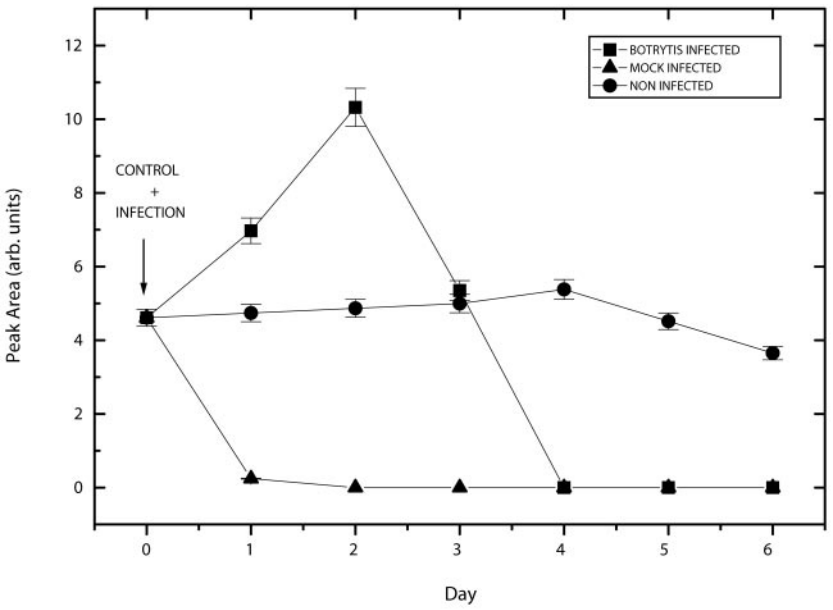

Figure 2. Evolution of the trans-resveratrol content in grape skin in noninfected, mock-infected, and *Botrytis*-infected grapes. The clear elicitation of trans-resveratrol by the *Botrytis cinerea* can be noticed. See text for details.

Ethylene Emission by Grapes: External Application of trans-Resveratrol

As non-climacteric fruits, grapes release ethylene at very low production rate (Archbold et al., 1997) almost undetectable with standard procedures. The use of the LPAS enables us to reveal it. Ethylene released by noninfected and *B. cinerea*-infected grapes with and without exogenous trans-resveratrol application was monitored on-line by LPAS.

In Figure 3, the evolution of ethylene emission from noninfected (Fig. 3A) and mock-infected (Fig. 3B) grapes is presented compared with that from grapes treated with trans-resveratrol. Ethylene release of the noninfected grapes showed an increase during the first 48 h followed by a slow decrease. For the mock-infected grapes, C_2H_4 production increased within the first 24 h up to 13.65 ± 0.5 pL $h^{-1}g^{-1}$ fresh weight and then remained constant during the measurements. In comparison, exogenous application of trans-resveratrol caused a decrease of ethylene emission of at least three times for both noninfected and mock-infected fruits. Its inhibitory effect on ethylene production became evident after 10 to 12 h from the application (Fig. 3A).

trans-Resveratrol had a significant effect also on the ethylene released by the *B. cinerea*-infected grapes (Fig. 4). There are two aspects that have to be considered here. First, the trans-resveratrol treatment determined a delay of increasing ethylene emission of about 2 d. After 48 h from inoculation, ethylene released by the untreated grapes started to increase from 10.5 to 80 pL h^{-1} g^{-1} fresh weight in the d 8 of measurement, whereas from the trans-resveratrol-treated fruits, a constant production of about 8 pL h^{-1} g^{-1} fresh weight was monitored during the first 96 h. Second, the enhanced formation of ethylene for the treated grapes is two times less and presented a slower rate than that corresponding to the untreated ones.

It was demonstrated that the fungus produces ethylene itself when grown on potato dextrose agar media with or without the addition of ethylene precursor, L-Met (Cristescu et al., 2002; Qadir et al., 1997). We reported that ethylene released by *B. cinerea* under in vitro conditions is associated with the hyphal growth (Cristescu et al., 2002). On the other hand, previous studies indicated that trans-resveratrol has real inhibitory effects on conidial germination of *B. cinerea* liquid cultures and also on the mycelia growth (Adrian et al., 1997). Our data indicate the correlation between these observations and show that trans-resveratrol acts indirectly on the ethylene production by playing an active antifungal role in the *B. cinerea* grapes interaction. To our knowledge, this paper is the first report on real-time ethylene monitoring by *B. cinerea*-infected grapes in regard to trans-resveratrol activity.

Moreover, we found a strong inverse relationship between ethylene production by grapes and trans-resveratrol content measured by LD and REMPI-TOFMS. The trans-resveratrol content from the noninfected fruits (Fig. 2) was higher than that corresponding to the mock-infected, which drastically decreased to zero during the 1st d. In correlation, ethylene released by mock-infected grapes increased in the 1st d up to a certain level and showed higher values compared with the noninfected (Fig. 3). For the *B. cinerea*-infected fruits, ethylene emission rises up after 48 h (Fig. 4) when the analogous content of trans-resveratrol started to decrease irreversibly (Fig. 2).

Ethylene is not only a plant hormone, but can be also biosynthesized by various micro-organisms including bacteria and fungi (Fukuda et al., 1993). This

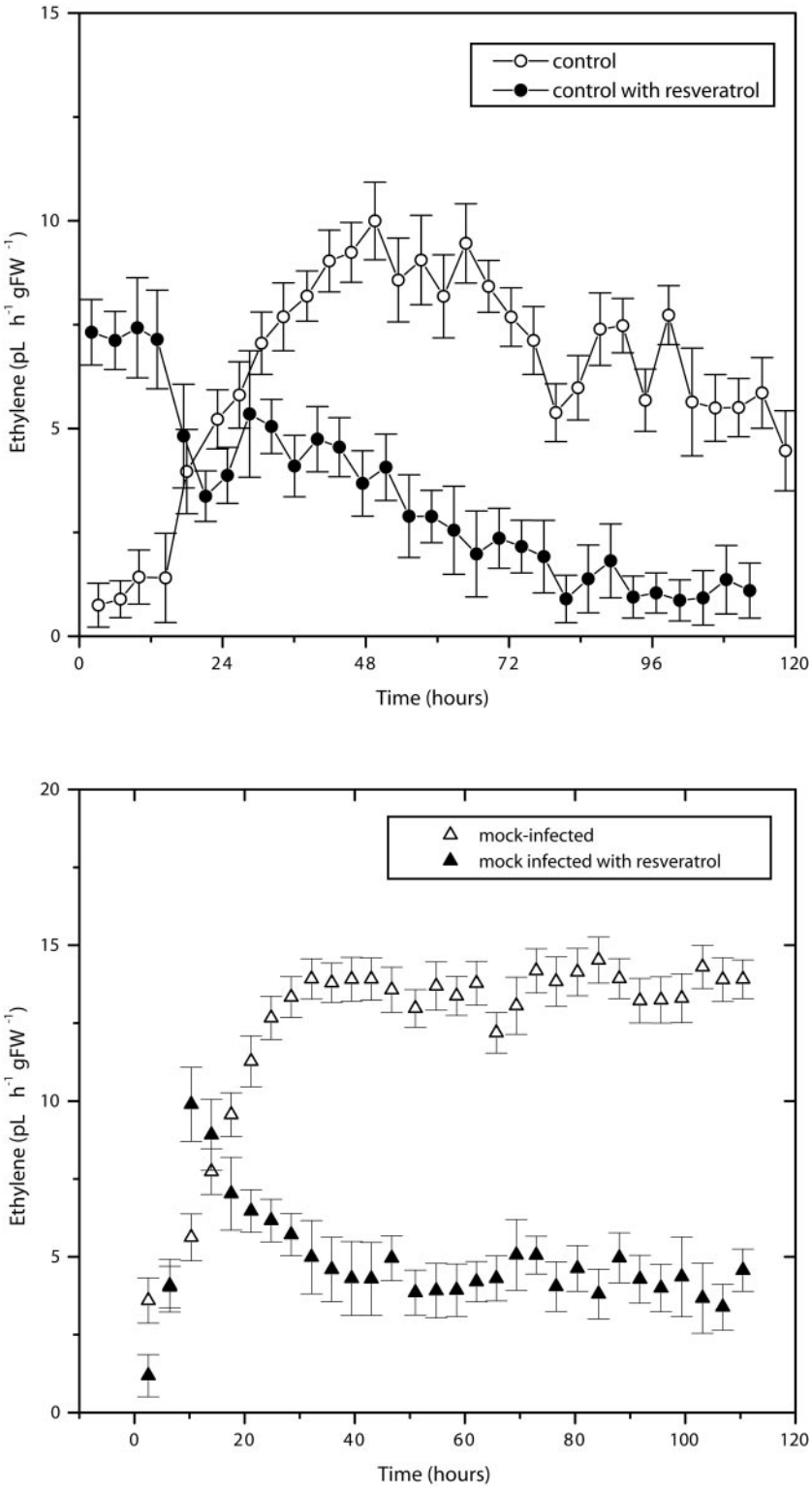

Figure 3. A, Ethylene emission from noninfected grapes (○) compared with the trans-resveratrol-treated (concentration of 1.6×10^{-4} M trans-resveratrol in water) (●); B, mock-infected grapes (△) compared with mock-infected previously treated with trans-resveratrol (△).

significant decrease of the ethylene production in the treated grapes can be attributed to the action of trans-resveratrol on different micro-organisms (bacteria and fungi) present on the grapes. This hypothesis is supported by a recent work on the effects of *B. cinerea* inoculation on grapes (Dorado et al., 2001), performed in similar experimental conditions and with the same variety of grapes as in the present study. According to this report, other micro-organisms like bacteria and fungi, distinct from the inoculated *B. cinerea*, were

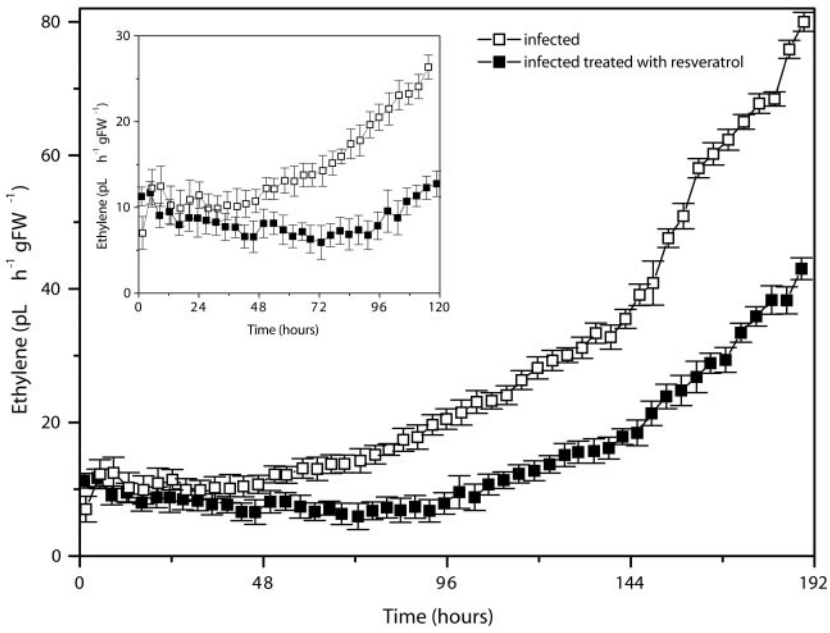

Figure 4. Ethylene production by grapes infected with *Botrytis cinerea* (5 μL of the suspension at 10^3 conidia mL^{-1} per grape). At 0 h, grapes were inoculated and immediately placed into cuvettes under continuous air flow of 2 L h^{-1}. The insets show the ethylene emission from untreated fruits (□) compared with trans-resveratrol-treated ones (■) for the first 4 d.

developed during the incubation period of grapes and caused the deterioration of the fruits. Moreover, growth of yeasts and molds, including *B. cinerea* (which could be detected after 6 d), was observed on the mock-infected fruits. The identified noninoculated micro-organisms present on grapes were mainly yeasts and imperfect fungi such as *Penicillium*, *Aspergillus*, and *Alternaria* spp., which are known that include ethylene-producing species (Fukuda et al., 1993).

The availability of the new molecular tools to study the *B. cinerea* biology described in the late 90s (ten Have et al., 2001; Wubben et al., 2000) will make the characterization of fungus-produced ethylene in pathogenesis possible, and it will provide more answers on the physiology of the event of *B. cinerea* infection in grapes with respect to the trans-resveratrol synthesis.

Improving the Post-Harvest Quality of Grapes by trans-Resveratrol Exogenous Application

Following the preferred embodiment of the invention included in the patents cited in "Materials and Methods," several experiments were performed to prove the antioxidant properties of trans-resveratrol on grapes conservation as well as its pesticide activity against the fungal attack. Different concentrations of trans-resveratrol were tested to find the minimum concentration of this compound that allows the optimum conservation of the fruits. The fruits were immersed into a solution containing 1.6×10^{-4} M trans-resveratrol for few seconds, as indicated in "Materials and Methods." As shown in Figure 5, significant differences between the untreated grapes and those treated with trans-resveratrol were obtained after 10 d from the treatment. The trans-resveratrol treated bunches clearly still maintained a physical aspect with no sign of losses or deterioration, whereas the untreated ones were not only dehydrated, but significantly infected and deteriorated, showing local development of fungi, as one would expect after this period of time because normal shelf-life of grapes at room temperature is about 5 d. Although the antifungal character of trans-resveratrol has been already described, mainly by in vitro investigations, to the best of our knowledge, this is the first time in which its direct application to fruits as natural pesticide is reported.

This interesting result opened the way to subsequent investigations to get insight into the dynamics of the process. To this end, both microbiological and biochemical analysis, as well as a sensory test, have been carried out. Although these results are the subject of two forthcoming papers (see below), here, we can anticipate the main findings. The development of the fungi growth was monitored over a period of 12 d on treated and nontreated grapes, showing significant differences between both groups regarding the number of the microorganisms counted as colony formation units per gram of grape (C. Montero, J.M. Orea, J.B. Jiménez, A. González Ureña, K. Slowing, M.P. Gómez-Serranillos, and E. Carretero, unpublished data). On the other hand, it was also demonstrated that the resveratrol application to several fruits does not alter their organoleptic and biochemical properties (A. González Ureña, J.M. Orea, C. Montero, J.B. Jiménez, J.L. González, A. Sánchez, and M. Dorado, unpublished data).

Finally, it is interesting to notice that although some authors have claimed that the risks for the human health related to the consumption of natural

Figure 5. Top, Bunch of grapes immersed 5 s in water after 10 d of storage at room temperature. Bottom, Bunch of grapes immersed 5 s in a 1.6×10^{-4} M of trans-resveratrol and storage under the same conditions. Their different heath status is evident. See text for comments.

chemicals in foods are even greater than the risks from pesticide residues (Pimentel et al., 1996; Swirsky et al., 1997), the lack of toxicity of the resveratrol has already been demonstrated. One of the main stages in the development of new natural pesticides is the study of the toxicological and environmental properties of the compound to be used (Duke, 1990). Biological control agents are one of the more interesting alternatives to the use of harmful chemical pesticides, but it has to be demonstrated that they are safe for human consumption. As stated above, in the case of resveratrol, a considerable number of investigations are currently focussed on the health benefits of resveratrol consumption (for recent reviews on this subject, see Frémont, 2000; German and Walzem, 2000; Parr and Bolwell, 2000) giving it an additional value as candidate for bio-control experiments against *B. cinerea*.

CONCLUDING REMARKS

The capability of the LD coupled with resonant ionization spectrometry to monitor natural pesticides, in this case trans-resveratrol content in grapes, has been evidenced. The technique allows fast, direct, and high sensitive analysis of trans-resveratrol in grapes with great sensitivity and resolution and demonstrated the post-harvest elicitation of trans-resveratrol by *B. cinerea* in grape skin. trans-Resveratrol content shows a maximum on the 2nd d after infection, followed by a rapid decrease attributable to the metabolism of the compound by the fungus.

On the other hand, the LPAS technique was used to monitor ethylene production from noninfected and *B. cinerea*-infected grapes over a period of several days. For the noninfected grapes, we found an inverse relationship between the C_2H_4 emission and the trans-resveratrol content; low values of C_2H_4 release correspond to high trans-resveratrol content. Exogenous application of trans-resveratrol had an indirect effect on ethylene production and determined its decrease of at least three times. This significant decrease of the ethylene production in the treated grapes can be attributed to the action of trans-resveratrol on different micro-organisms (bacteria and fungi) present in the grapes.

Using quantitative measurements of ethylene release, we demonstrated the antifungal character of exogenous trans-resveratrol for *B. cinerea*-infected grapes. The trans-resveratrol treatment modified the ethylene release in the grapes, in relation to the untreated ones, in two ways: (a) delaying the increase of C_2H_4 emission with about 48 h and (b) decreasing the C_2H_4 concentration and its rate of emission.

Finally, the effect of trans-resveratrol as natural pesticide by its exogenous application to grapes has been proven; treated grapes doubled their normal shelf-life at room temperature, maintaining their post-harvest quality within 10 d in comparison with the untreated grapes. This result offers a new, simple, and inexpensive modality, which can be used to improve the shelf-life of fruits and to preserve their natural post-harvest quality.

MATERIALS AND METHODS

LD and REMPI-TOFMS

A new laser technique for fast and direct analysis of non-volatile compounds in fruits, particularly trans-resveratrol in grapes (*Vitis vinifera*), has been developed in our lab (Montero et al., 2000b; Orea et al., 2001) by the combination of LD with REMPI coupled to TOFMS detection. The experimental set-up has been already described elsewhere (Orea et al., 1998), so only a brief report is given here.

Essentially, it consists of two independent high vacuum chambers; the first chamber is used for both LD and laser post-ionization of the sample followed by the ions acceleration toward the second chamber, basically a time-of-flight unit with a two-microchannel plate detector. A few nanosecond laser pulses from the fundamental emission of a Nd:YAG laser are used for sample desorption. A frequency-doubled dye laser is then used to selectively ionize the desorbed neutrals by REMPI. To this end, active

wavelength laser scanning is achieved with tunability from 230 up to 730 nm: trans-resveratrol is ionized through a one color-two photons process, and the resonant ionization region lies between 301.8 and 307.5 nm with the maximum at 302.1 nm, which is the optimal wavelength for trans-resveratrol analysis in complex samples. In addition to the selective ionization due to REMPI, additional selectivity is provided by the use of mass spectrometry, i.e. providing mass identification and making the technique more sensitive and universal.

A basic feature of the technique is the absence of any separation method for sample preparation. The samples were prepared by cold-pressing the grape skin by means of a hydraulic press, after verification that with this easy procedure, all of the trans-resveratrol is extracted from the skin. Thus, the combination of LD followed by REMPI-TOFMS detection can overcome the main error sources, present in the chromatographic methods generally employed for trans-resveratrol analysis.

After the optimization of the experimental conditions and the location of the resonant wavelength of the analyte, the validation of the method has been carried out with excellent results, including: a variation of the signal with the concentration giving a linear fit with a regression coefficient of 0.9997 in the range of interest, a precision better than 5% in both repeatability and reproducibility studies, and an accuracy of 96%. The combination of laser resonant ionization and mass spectrometry detection reaches a detection limit of 2 nL L^{-1} and a sensitivity on the order of 20 ng per single laser shot.

LPAS

In photoacoustic spectroscopy, the infrared absorption spectra of molecular gases are used to detect very small quantities of these gases (Zharov and Letokhov, 1986; Mandelis and Hess, 1997; Harren et al., 2000). Different molecules present absorption of light at different wavelengths. The absorbed photon energy is transformed into translational energy by collisions, resulting in a rise in gas-temperature. For a gas placed into a confined space (e.g. the photoacoustic cell), the temperature-rise causes an increase of pressure.

Modulating the radiation source at an acoustic frequency results in a periodical pressure variation that can be observed as a photoacoustic signal; in the gas phase, the effect can be detected with a sensitive microphone. This photoacoustic signal is directly related to the concentration of absorbing molecules in the cell. Using a sensitive microphone to measure this signal, very low concentrations can be detected. When the absorption coefficients of possibly present gases in the infrared region are known, different trace gases can be distinguished by measuring the photoacoustic signal at various wavelengths.

Laser-based photoacoustic detectors are able to monitor trace gas concentrations under atmospheric conditions with orders of magnitude of better sensitivity than conventional scientific instrumentation; in addition, they are able to monitor noninvasively and on-line under dynamic conditions.

Ethylene production from grapes was measured in real time using a sensitive laser-based photoacoustic detector in combination with a gas flow through system developed in the laboratories of the University of Nijmegen (The Netherlands; Bijnen et al., 1996). A detailed description of the system was given elsewhere (Harren and Reuss, 1997; te Lintel Hekkert et al., 1998). In brief, the detector consists of a line-tunable CO_2 laser emitting radiation in the 9- to 11-μm infrared wavelength region and a photoacoustic cell, in which the gas is detected.

Photoacoustic detection of C_2H_4 is based on its strong and distinct absorption pattern in the CO_2 laser wavelength region (Brewer et al., 1982). By modulating the laser beam with a chopper, pressure waves are generated and detected with a sensitive miniature microphone (type Knowles electret BT-1754) placed inside the photoacoustic cell. Trace gases released by the grapes under specific conditions were transported to the photoacoustic cell through a flow system using air as carrier gas.

The gas flow through the measuring system can be controlled using electrical three-way valves that switch a particular gas stream to the photoacoustic cell. In this way, the gas emission from a number of cuvettes (up to six per experiment) containing the grapes is transported to the photoacoustic cell alternately and at controlled flow rates (2 L h^{-1}), preventing accumulation induced effects. The flow is adjusted by a flow controller and continuously monitored by a mass flow sensor (type 5850 S, Brooks Instrument, Veenendaal, The Netherlands).

The laser-based ethylene detector and the electric three way valves are operated fully automatically by computer program, and it can be used to perform continuous measurements for periods of up to several weeks.

To eliminate other interfering gases (H_2O, CO_2, etc.) that may influence the results because of the overlap between their spectral absorption and the CO_2 laser wavelengths, a number of filters and scrubbers are introduced in the measuring system.

The C_2H_4 concentration is calculated from the photoacoustic signal by comparing the corresponding signal at a laser line where ethylene possesses the strongest absorption (10P14 line; wavelength, 10.53 μm; absorption strength, 30.4 atm^{-1} cm^{-1}) with the background signal at a laser line with much weaker absorption (10P12 line; wavelength, 10.51 μm; absorption strength, 4.319 atm^{-1} cm^{-1}).

The time response is determined mainly by the time needed to switch the grating between the two laser lines. In the present set-up, the sampling rate is 40 s. From the obtained ethylene emissions the levels corresponding to an empty cuvette are subtracted. The C_2H_4 production from the grapes was related to the emission rate by multiplying the measured value with the flow rate and divided by the fresh weight; the results were expressed in picoliters per hour per gram fresh weight. For a better overview of the ethylene emission rates we displayed the results of the measurements by the average of the sampling rate every 3 h (the errors attributable to averaging were smaller than the symbol size). Each measurement was repeated at least three times; representative data are shown in "Results and Discussion."

Preparation of *Botrytis cinerea* Conidial Suspension and Inoculation of Grapes

B. cinerea-infected and noninfected grapes were monitored for their trans-resveratrol evolution by LD + REMPI-TOFMS (Madrid) and C_2H_4 emission by using LPAS (Nijmegen). The experiments were conducted synchronously in both laboratories. The grapes (var Aledo) were directly purchased in Madrid and also sent to Nijmegen for ethylene detection.

The strain (*B. cinerea* 2100 from a Spanish Culture Collection) was grown on potato dextrose agar at 24°C and high humidity with 14-h light photoperiods. To prepare the conidia suspension, the fungus was removed from the cultured plates by gently brushing on the plate surface with a sterile platinum loop and suspended in 2 mL of distilled water. Fungal suspension was filtered through two layers of gauze to separate the conidia. The final concentration of 10^3 conidia mL^{-1} was determined with a Neubauer counter of 0.0025 mm^2 under a light microscope.

The first set of experiments investigated trans-resveratrol content from three groups of grapes (var Aledo): noninfected, mock-infected, and *B. cinerea*-infected grapes, respectively. In the *B. cinerea*-infected group, 5 μL of the conidial suspension at 10^3 conidia mL^{-1} was inoculated in each grape at the equator site with a needle head (0.8 mm in diameter) at constant depth of 4 mm below the fruit skin. The mock-infected fruits were injected similarly with 5 μL of buffer (0.11 M Glc and 67 mM KH_2PO_4).

To prepare the samples for the trans-resveratrol content measurements, all of the grapes were individually placed on a grid with wet paper below the grid and covered by a plastic film to maintain a high humidity (80%–85% relative humidity); they were incubated at room temperature. The evolution of the trans-resveratrol content was monitored in these grapes using the LD and REMPI-TOFMS technique. Each group consisted of 40 grapes of a similar size; every day, seven grapes of each group were peeled-off, and their skin was analyzed for the trans-resveratrol content. Experiments were repeated three times. Representative data are shown in "Results and Discussion."

Exogenous Application of trans-Resveratrol in Fruits

The second set of experiments investigated the effect of the exogenous application of trans-resveratrol on ethylene emission from the noninfected, mock-infected, and *B. cinerea*-infected grapes, respectively, in comparison with the untreated fruits. EU regulations prohibit the full description of the methodology currently patent pending (González Ureña et al., 1999, 2000), but the essential procedure for the treatment of the fruits with trans-resveratrol is given below. The treatment consisted of submerging the fruits in solution of trans-resveratrol in water (1.6×10^{-4} M) for few seconds (5 s). The mock-infected and *B. cinerea*-infected grapes were then injected in the way described in "Preparation of *Botrytis cinerea* Conidial Suspension and

Inoculation of Grapes." Immediately after treatment, fruits were placed inside closed glass cuvettes (0.7-L volume; fresh weight of 100 g grapes per cuvette) connected to the laser-based ethylene detector and flushed with humidified air (80%–85% relative humidity) at a continuous flow of 2 L h^{-1} and at atmospheric pressure. All experiments were conducted in normally illuminated laboratory conditions at constant temperature of 22°C.

In addition, we investigated the effect of trans-resveratrol on the shelf-life of the fruits by using two groups of grapes directly purchased from the market. To avoid effects of different maturity stage between bunches, they were cut in two similar moieties and each one was incorporated into the groups. One group was treated with trans-resveratrol at 1.6×10^{-4} M concentration during the same day. The second group was immersed in water for approximately 5 s and served as control. Because of the slight solubility of trans-resveratrol in water and to ensure a homogeneous application, the solution was stirred during the treatment. After the treatment, the fruits were kept in open air at constant room temperature. Each experiment contained three half-bunches per group.

The grapes used in all the experiments were directly purchased from the market and no additional cleaning was performed. The commercial trans-resveratrol was provided by Sigma-Aldrich (St. Louis).

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