Changes in the Phytoalexin Content of Various *Vitis* Spp. in Response to Ultraviolet C Elicitation

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The phytoalexin production potential of three American *Vitis* species and that of three cultivars of *Vitis vinifera* were evaluated in response to UV-C irradiation. Time course changes in resveratrol, piceid, ϵ-viniferin, and pterostilbene contents were studied within 3 days after a short UV-C irradiation. Results show that the two major stilbenes accumulated as a response to UV-C elicitation are resveratrol and ϵ-viniferin, a resveratrol dehydrodimer, the concentration of both compounds usually reaching quantities >100 μg/g of fresh weight. In contrast, piceid and pterostilbene were constantly produced in low quantities. Owing to the results obtained, the role of stilbene phytoalexins in the resistance of grapevines to diseases is discussed.

Keywords: *Vitis species; resveratrol; piceid; ϵ-viniferin; pterostilbene; phytoalexin production potential*

INTRODUCTION

Infection by fungi, such as *Botrytis cinerea* Pers., the causal agent for gray mold, UV light irradiation, and heavy metal ions are potential stresses capable of eliciting plant defense responses in *Vitis* spp. In grapevines, such responses include the synthesis of phytoalexins (Langcake and Pryce, 1976, 1977b; Adrian et al., 1996) and several pathogenesis-related proteins (PR proteins; Derckel et al., 1996).

The first compounds are secondary metabolites that are derived biosynthetically from the shikimic−polymalonate pathway. Grapevine phytoalexins belong to the stilbene family (Langcake and Pryce, 1977a), the skeleton of which is based on the *trans*-resveratrol structure (3,5,4′-trihydroxystilbene) (Figure 1). In addition to resveratrol, other compounds considered as oligomers of resveratrol and termed viniferins were also found in grape leaves upon infection or stress, the major component of which appears to be ϵ-viniferin, a resveratrol dehydrodimer. Simple stilbenes have also been identified: *trans*-pterostilbene, a dimethylated derivative of resveratrol (3,5-dimethoxy-4′-hydroxyresveratrol) (Langcake et al., 1979; Guisalberti et al., 1978; Pezet and Pont, 1988), and piceid, a 3-*O*-*β*-glucoside of resveratrol (Waterhouse and Lamuela Raventos, 1994).

Stilbenes have provoked an intense interest due to their antifungal properties (Smith, 1982; Hoos and Blaich, 1990; Adrian et al., 1997a). Specifically, the presence of resveratrol has been shown to be closely related to disease resistance in *Vitis* spp. (Dercks and Creasy, 1989; Sbaghi et al., 1995).

Moreover, resveratrol is a constituent of wine (Goldberg et al., 1995; Jeandet et al., 1995a) and shows interesting biological properties; that is, *trans*-resveratrol and its glucoside inhibit low-density lipoprotein oxidation (Frankel et al., 1993; Waffo Teguo et al., 1998) and reduce platelet aggregation (Bertelli et al., 1995; Pace-Asciak et al., 1995), two major parameters implicated in atherothrombogenesis. Recently, resveratrol has also been shown to reduce tumor initiation, promotion, and progression (Jang et al., 1997).

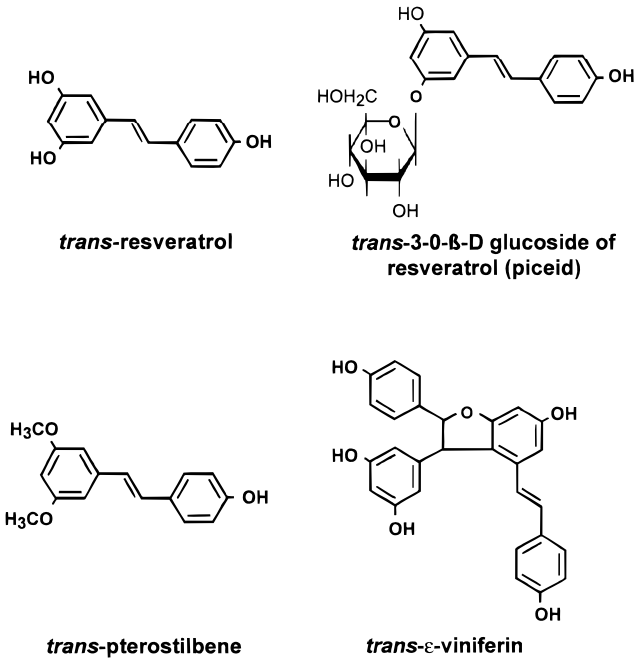

Figure 1. . Chemical structures of phytoalexin-type compounds in grapevine.

Although there are a number of works on the relationship between production of resveratrol and grape disease resistance, the biological significance of the other stilbenes (i.e., ϵ-viniferin, piceid, and pterostilbene) in the vine−*B. cinerea* interaction is poorly understood, largely due to the lack of data concerning

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their chromatographic determination (Jeandet et al., 1997). However, study of pterostilbene and ϵ-viniferin production in *Vitis* species is of particular relevance because both are considered to be more fungitoxic than resveratrol itself. In fact, the ID_{50} values of resveratrol, ϵ-viniferin, and pterostilbene for germination of *B. cinerea* conidia are, respectively, 90, 37, and 20 μg/mL (Langcake and Pryce, 1977b; Pezet and Pont, 1988; Adrian et al., 1997a). Moreover, the determination of the piceid (resveratrol glucoside) content of grape leaves is also of great interest because it is thought to constitute a mobile form of resveratrol in grapevine cells.

Thus, measurement of the phytoalexin production potential of *Vitis* spp. should take in account not only the determination of resveratrol but also the determination of the other major phytoalexins of grapevine leaves with the aim of clarifying their role in the resistance of that plant against fungal attacks.

The aim of this study was to determine the phytoalexin production potential of four *Vitis* species (i.e., the resistant American species *V. rupestris*, *V. cinerea*, and *V. labrusca* and three cultivars of the genetically susceptible species, *V. vinifera*) including the analysis of resveratrol and its β-glucoside, pterostilbene, and ϵ-viniferin using the method previously described by Jeandet et al. (1997). Identification of each phytoalexin was accomplished by on-line spectral comparisons with known standards.

MATERIALS AND METHODS

Standards. A chemically pure standard of *trans*-resveratrol was synthesized by a Wittig condensation (Jeandet et al., 1991). *trans*-Piceid was isolated from dried roots of *Polygonum cuspidatum* as described by Waterhouse and Lamuela Raventos (1994). A resveratrol dehydrodimer analogous to ϵ-viniferin was obtained by oxidative dimerization of *trans*-resveratrol in the presence of horseradish peroxidase and H_2O_2 (Jeandet et al., 1997; Breuil et al., 1998). A chemically pure standard of *trans*-pterostilbene was obtained from Drs. R. Pezet and V. Pont (Pezet and Pont, 1988) and this compound was also synthesized in our laboratory using a Wittig condensation (A. C. Breuil and P. Jeandet, unpublished results).

Plant Material. Correlation has been found between production of resveratrol in grape berries and that of grape leaves (Jeandet et al., 1991); because leaves are available during the vegetative cycle of grapevines, stilbene production has been assayed in the grape leaves only. Cuttings from *V. vinifera* cv. Pinot noir, Mourvedre, and Xarello and from *V. rupestris*, *V. labrusca*, and *V. cinerea* were supplied by Dr. P. Lavie, Institut Jules Guyot. Cultures were performed under controlled conditions, in a climate room at 25 °C with a 16 h light/8 h dark photoperiod. Leaves used were taken from the upper part of 3-month-old shoots.

Elicitation by UV-C Light. Grapevine leaves were irradiated using a UV-C lamp (254 nm, Spectroline, Model ENF-260C/FE, output 400 μW/cm2, 15 cm distant) for 15 min on their abaxial surfaces. Induced leaves and controls (unelicited leaves) were placed at room temperature in the dark, petioles in water, and assayed for stilbene production at regular intervals of time. Following induction detached vine leaves were examined under long-wave UV-C light. *trans*-Stilbenes are very easily detected at 366 nm because they give bright blue fluorescence (Langcake and Pryce, 1976).

Stilbene Extraction. The protocol used for stilbene extraction was adapted from the method previously described by Langcake and Pryce (1976) and Jeandet et al. (1991). Leaves were ground with an Ultraturrax in methanol/water (85:15). After filtration under vacuum to remove plant fragments, the filtrate was prepurified on a Sep-Pak C_{18} Cartridge (Waters, Milford, MA). Elution was performed with methanol/water (85:15), and the eluate was evaporated to dryness at 35 °C.

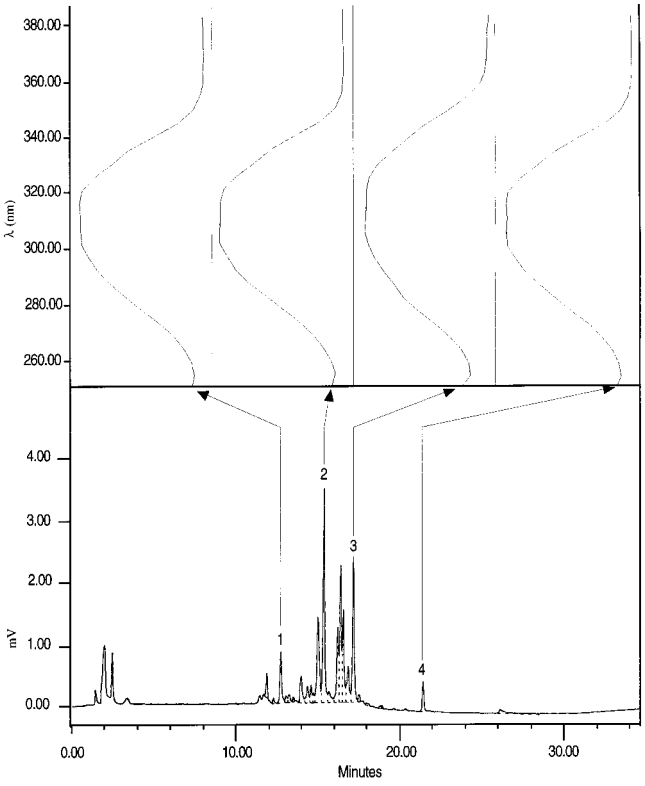

Figure 2. HPLC chromatogram (diode array detection) of a *V. vinifera* cv. Xarello grape leaf extract as induced by UV light irradiation. Peak 1, *trans-piceid*; peak 2, *trans-resveratrol*; peak 3, *trans-ϵ*-viniferin; peak 4, *trans-pterostilbene*. (Inset) UV spectra of the corresponding stilbenes. For chromatographic conditions see Jeandet et al. (1997). Retention times were 13.0, 15.5, 17.3, and 21.3 min for peaks 1, 2, 3, and 4, respectively.

Dried extracts were redissolved in methanol (10 mL/g of fresh weight). All steps during stilbene extractions were done in the dark to avoid photochemical isomerization of *trans*-stilbenes to the less fluorescent *cis* forms.

HPLC Analysis. The chromatographic system used (Waters) comprised a Model W600 system controller, a Model W717 sample injector, a Model W996 photodiode array detector, and a Model 474 fluorometer. Extracts were analyzed on a reversed phase column (column C_{18}, Merck, 250 mm × 4 mm, 5 μm) with a linear gradient elution according to the method developed by Jeandet et al. (1997).

Identification and Quantification of Phytoalexin-Type Compounds. Identification of each phytoalexin (*trans*-piceid, *trans*-resveratrol, *trans-ϵ*-viniferin, and *trans*-pterostilbene) was achieved by on-line spectral comparisons with known standards [see Jeandet et al. (1997)]. Specific detection of all stilbenes was also obtained using fluorometric analysis (λ_{ex} = 330 nm, λ_{em} = 374 nm). Quantification of stilbenes was accomplished using the calibration curves already described in Jeandet et al. (1997).

RESULTS AND DISCUSSION

The phytoalexin production potential of the four *Vitis* spp. considered (i.e., *V. rupestris*, *V. cinerea*, *V. labrusca*, and *V. vinifera* cv. Mourvèdre, Pinot noir, and Xarello) was measured at regular intervals of time up to 72 h after UV-C irradiation. Figure 2 shows a typical chromatogram corresponding to the injection of a UV-C-induced leaf extract of *V. vinifera cv*. Xarello. Four major peaks appeared within this extract (peaks 1−4). They were identified as *trans*-piceid, *trans*-resveratrol, *trans-ϵ* viniferin, and *trans*-pterostilbene, respectively, by the following criteria: Their retention times were identical

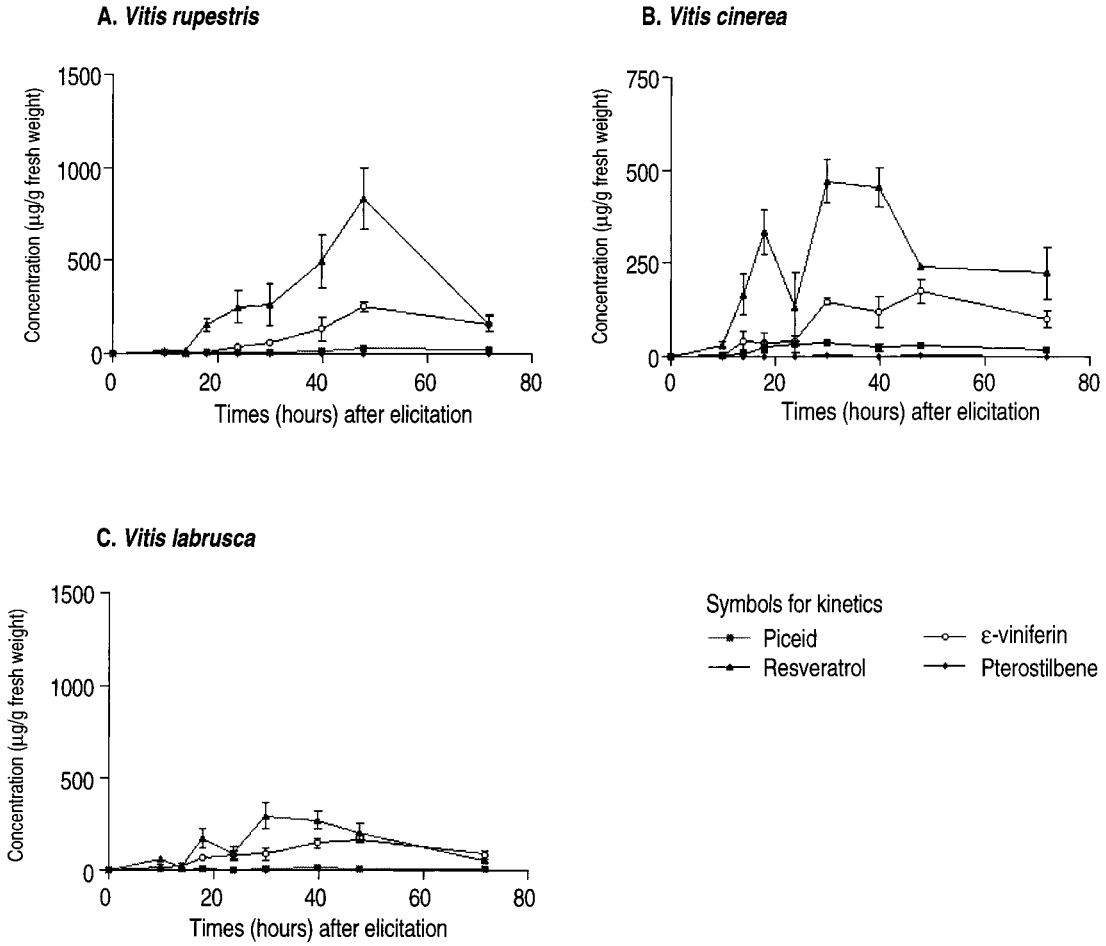

Figure 3. Time course changes in the production of resveratrol, piceid, ϵ-viniferin, and pterostilbene in three *Vitis* spp. following irradiation with short-wavelength radiations: (A) V. *rupestris*; (B) *V. cinerea*; (C) *V. labrusca*. Each point represents means of four leaves. Verticals bars represent standard errors of the four measurements.

to those of the corresponding 5 standards [see Materials and Methods and Jeandet et al. (1997)]; they cochromatographed with their standards, and their UV spectra were identical to those of the standards as shown by diode array detection. All UV spectra showed two bands corresponding to a high absorbance from 308 to 336 nm and from 281 to 313 nm. These two bands are characteristic of the *trans*-stilbenes (Jeandet et al., 1997).

Time course changes in the production of the four major stilbene phytoalexins of grapevines (resveratrol, piceid, ϵ-viniferin, and pterostilbene) are presented in Figures 3 and 4.

Resveratrol was the major stilbenic component that can be accumulated in grapevine tissues as induced by UV-C irradiation. This confirms numerous previously published results (Langcake and Pryce, 1977b; Langcake, 1981; Pool et al., 1981; Barlass et al., 1987; Dercks and Creasy, 1989; Sbaghi et al., 1995; Jeandet et al., 1997). Moreover, differences can be observed in the resveratrol production potential among the four *Vitis* spp. tested in response to UV-C irradiation. American species showed a higher capacity for resveratrol synthesis than the genetically susceptible species *V. vinifera*. This response was particularly high for *V. rupestris*, reaching up to 750 μg/g of fresh weight within 48 h after induction. These results are in good agreement with those of Dercks and Creasy (1989). However, the time course of production of resveratrol shown by *V. labrusca* was not significantly different from the ones shown by the three cultivars of *V. vinifera*. This is in accordance with our previous results (Sbaghi et al., 1995), which indicated that the resistance of *V. labrusca* to *B. cinerea* is coupled with a moderate ability to produce resveratrol.

Interestingly, analysis of the pattern of the time course changes of resveratrol production shows that all *Vitis* spp. have two peaks of resveratrol synthesis. The first peak appeared ~20 h following elicitation, whereas the second one was observed ~40 h after induction, depending on the species considered (see Figures 3 and 4). The first peak of resveratrol synthesis could correspond to a precocious defense response of the plant following elicitation, which enables the plant to rapidly counteract infection by a pathogen. After the second peak of synthesis, resveratrol production maintained a high level during 2 days, thus preventing pathogen development for a long time. The biosynthesis of resveratrol is controlled by stilbene synthase (Hain et al., 1993). Specifically, Melchior and Kindl (1990) have shown through the analysis of stilbene synthase mRNAs that this enzyme corresponds to a multigenic family. In accordance with our results, Wiese et al. (1994) have indicated that stilbene synthase mRNAs have two maxima of synthesis (corresponding to the two peaks of resveratrol synthesis) differing in time and corresponding to the expression of, at least, two different groups of genes. The differential expression of stilbene synthase genes was assumed to correspond to an

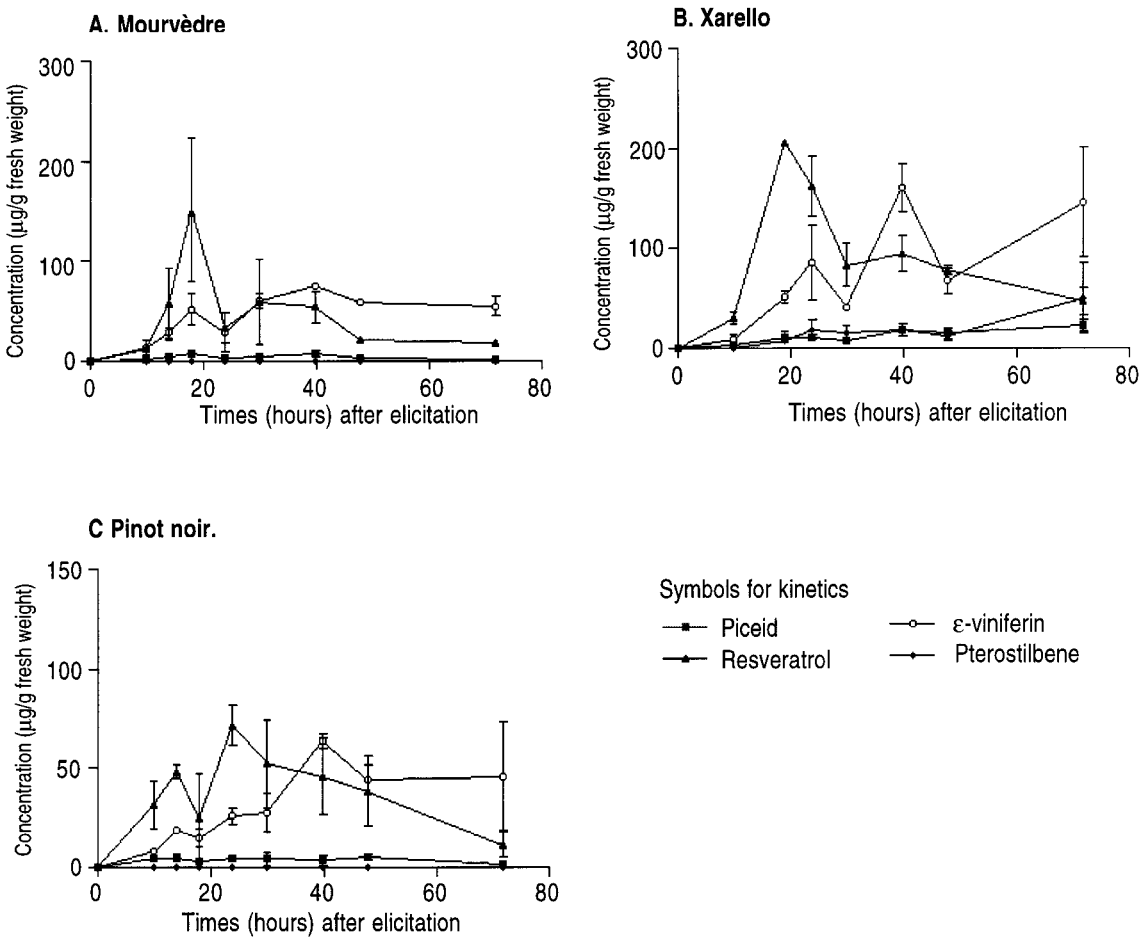

Figure 4. Time course changes in the production of resveratrol, piceid, ϵ-viniferin, and pterostilbene in three cultivars of *V. vinifera* following irradiation with short-wavelength radiations: (A) cv. Mourvèdre; (B) cv. Xarello; (C) cv. Pinot noir. Each point represents means of four leaves. Verticals bars represent standard errors of the four measurements.

adapted response of the plant upon infection or stress (Wiese et al., 1994; Adrian et al., 1997b).

Even though ϵ-viniferin was generally produced in lower amounts than its putative precursor resveratrol (Langcake and Pryce, 1977b), the time courses of production of resveratrol and ϵ-viniferin were very similar. Synthesis of ϵ-viniferin rose according to resveratrol synthesis, but the maximum peaks of synthesis of both compounds were shifted; the time course required to reach maximum ϵ-viniferin content was ∼40−50 h after induction (see, for example, *V. cinerea*, *V. labrusca*, and the three cultivars of *V. vinifera*). Since ϵ-viniferin was shown to be a resveratrol dehydrodimer formed during an oxidative dimerization process including peroxidase hydrogen peroxide (Langcake and Pryce, 1977c; Breuil et al., 1998), the maximum peak of ϵ-viniferin synthesis could thus correspond to a maximum in grapevine leaf peroxidase activities.

Generally, the relative proportions of resveratol and ϵ-viniferin in each species or cultivar are similar. Species or cultivars that produce high resveratrol quantities also have a high ϵ-viniferin content. These data confirm the results of Dercks and Creasy (1989). Here again a good correlation between the ability to synthesize ϵ-viniferin and grape disease resistance was found.

Piceid, the 3-*O*-β-D-glucoside of resveratrol, generally appeared 10 h after elicitation and maintained a low level up to 72 h. Contrary to expectation [see Jeandet et al. (1997)], the *cis* form of the resveratrol glucoside was never detected in the extracts. The constantly low levels of *trans*-piceid found in grape leaves could correspond to a hydrolysis of the glucoside moiety linked to the presence in the extracts of endogenous β-D-glucosidases (Ayran et al., 1978). It is likely that these enzymes remain active during the extraction process even when methanol/water (85:15) is used. The biological significance of the presence in the grapevine cell of a glycosylated form of resveratrol is at the present time not well understood. Piceid is likely a soluble form of resveratrol in the cell, owing to the fact that hydroxystilbenes such as resveratrol are scarcely soluble in water (Adrian et al., 1997). It has previously been suggested (Jeandet et al., 1997) that *trans*-resveratrol is synthesized in the free form and then rapidly glycosylated to *trans*-piceid.

With regard to pterostilbene synthesis, it is obvious from the results obtained that each *Vitis* species or cultivar may have its own characteristic response to induction. In *V. vinifera* cv. Pinot noir, pterostilbene was never detected during experiments. The sole plant in which pterostilbene was synthesized at a constantly high level was the Xarello variety. In that cultivar, the pterostilbene content rose from 20 µg/g of fresh weight, 20 h after elicitation, to 38 µg/g of fresh weight, 72 h after induction. No correlation could be observed between the pterostilbene content of grape leaves and that of its putative precursor, resveratrol. For example, in *V. rupestris*, the resveratrol content was ∼200 µg/g of fresh weight, 72 h after elicitation, while the pterostilbene concentration was at the same time only 5 µg/g of

fresh weight. Pterostilbene synthesis has already been reported to be very low in *Vitis* spp. (Langcake et al., 1979; Pezet et al., 1994; Adrian et al., 1997), depending on the intrinsic ability of each species or cultivar to convert resveratrol into its dimethylated derivative, pterostilbene. It should be mentioned here that there is now no direct evidence of the existence of a resveratrol methyl transferase capable of synthesizing pterostilbene from resveratrol. Owing to the results obtained, it is difficult to attribute to pterostilbene a major role in the defense mechanisms of grapevines against *B. cinerea* attacks as previously suggested (Pezet et al., 1988; Pezet and Pont, 1990), despite its high fungitoxicity. Unlike resveratrol or ϵ-viniferin, it was impossible to discriminate varieties differing in susceptibility to *B. cinerea* with regard to their ability to synthesize pterostilbene.

CONCLUSION

Simultaneous determination of ϵ-viniferin and pterostilbene together with that of resveratrol and its β-D-glucoside, piceid, allows us an excellent assessment of the phytoalexin production potential of grapevines. In contrast to other previously published methods, it was possible to characterize unambiguously the four major grapevine phytoalexins and to determine their respective roles in the defense mechanisms of that plant.

Resistance of *Vitis* genotypes to *B. cinerea* appears to be strongly correlated to the production of two major phytoalexins, that is, resveratrol and ϵ-viniferin. Resveratrol synthesis is especially high in the resistant genotypes of *Vitis* (i.e., *V. rupestris* and *V. cinerea*). Induction can lead to the accumulation of large quantities of resveratrol, which maintained for 2 days a high level (up to 750 μg/g of fresh weight in *V. rupestris* grape leaf extracts), quantities that can considerably exceed concentrations necessary to inhibit fungal growth. Obviously, resveratrol synthesis may largely be higher at the level of the infection sites (Dercks et al., 1989). This work confirmed the role played by resveratrol in the active defense mechanisms of grapevines.

Likewise, synthesis of ϵ-viniferin, a dehydrodimer of resveratrol with high fungitoxicity, seems to intervene in the defense mechanisms of *Vitis* resistant genotypes. ϵ-Viniferin was produced in relatively high amounts in all of the species or cultivars considered. Moerover, the relative proportions of ϵ-viniferin and resveratrol in each plant were very similar, confirming that both compounds are intimately connected biogenetically.

One question that remains for investigation concerns the role of piceid. This compound is likely a soluble form of resveratrol, but it is not clear from the results obtained here whether resveratrol is present in grapevine cells only in the form of its glucoside. Finally, it was shown that pterostilbene was generally present in very low amounts in grape leaf extracts, except for the variety Xarello.

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