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Fast and Local Assessment of Stilbene Content in Grapevine Leaf by in Vivo Fluorometry

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Stilbenes are grapevine phytoalexins. These highly fluorescent molecules are generally analyzed by HPLC. This technique allows accurate assay of different stilbenes, but it is destructive, time-consuming, and neglects their spatial distribution. This is why we have tested a new method based on in vivo fluorescence using commercial spectrofluorometers that allowed fast and local assessment of stilbene content in grapevine leaves. Stilbene synthesis in grapevine *Vitis vinifera* var. Muscat Ottonel leaves was induced by *Plasmopara viticola* inoculation or UV-C irradiation. Fluorescence was measured both from the abaxial and adaxial sides of leaves, then stilbene content was analyzed by HPLC. It varied from 0 in control leaves to 15 mg g^{-1} dry weight in UV-treated leaves. Highly significant regressions were found between HPLC stilbene content and the corresponding leaf UV-induced blue fluorescence. Thus, in vivo fluorescence is a good tool for a rapid study of stilbenes synthesis in grapevine leaves that can potentially be extended to other fluorescent molecules.

KEYWORDS: Spectrofluorometry; HPLC-DAD; stilbenes; grapevine; pathogen resistance; fluorescence microscopy

INTRODUCTION

Phytoalexins are important factors in plant disease resistance. They belong to various groups of phytochemical low-molecular-mass products not detectable in healthy tissue but inducible under biotic or abiotic stresses (*1*). These antimicrobial metabolites inhibit the growth of pathogens in resistant interactions. Stilbenes are considered as the most important phytoalexin group in grapevine (*Vitis vinifera*) (*2*, *3*). They are known to contribute to the protection against various pathogens among which is downy mildew (*Plasmopara viticola*), the most destructive disease in viticulture (*4*, *5*). More generally, the content of these highly inducible stilbenes varied widely depending on factors such as genotype, climate, fungus infections, and UV exposure (*6*, *7*). The main stilbenes in grapevine are resveratrol (3,5,4′-trihydroxystilbene) and its derivatives: piceid (resveratrol 3-*O*-β-D-glucoside), pterostilbene (3,5-dimethoxy-4′-hydroxystilbene), ϵ-viniferin, and δ-viniferin (dimers of resveratrol).

Different analytical methods have been used to assay stilbenes: high-performance liquid chromatography (HPLC) in normal or inverse phase or gas chromatography direct or after derivatization (*8*). These quantitative methods imply extraction of molecules before the assay, and they need a certain amount of raw material to be accurate. So studies are generally performed on whole leaves or leaf segments of several square centimeters. Nevertheless, it is likely that resistance reactions against pathogens take place at a lower scale, i.e., at the cell or group-of-cells level.

Stilbenes are highly fluorescent molecules (*9*) displaying an intense blue fluorescence under UV light. Their detection has been based on fluorescence, UV absorption at a single wavelength, UV−vis absorption spectra with photodiode array detectors (DAD), or mass spectrometry (*10*, *11*). The fluorometric detection is about 20 times more sensitive and is more specific than UV absorbance detection (*12*−*14*).

Studies on the influence of stilbenes in the interaction between grapevine and downy mildew require simple and reliable tools allowing phytoalexins quantification at different scales of reaction. That is why we developed a new method allowing stilbene content assessment at large scale (square centimeters) and low scale (square millimeters) on grapevine leaves at the same time. By using leaf treated to produce different stilbene contents, we were able to correlate UV-induced blue fluorescence measured on the abaxial (lower) and the adaxial (upper) leaf surfaces and the stilbene content measured by HPLC-DAD in the extract of the corresponding leaf area.

MATERIALS AND METHODS

Plant Material and Leaf Sample Preparation. Greenhouse-grown plants of *V. vinifera* var. Muscat Ottonel (susceptible to *P. viticola*) were propagated in a greenhouse from cuttings. The sixth leaf, counted

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from the apex of 3.5 month old plants having 12−14 fully expanded leaves, was detached and then rinsed with demineralized water. Four modalities were performed to obtain leaf samples with different stilbene content: (a) control, no treatment; (b) inoculated leaves, abaxial leaf surfaces that were buoyed up in 50 mL of an inoculum of *P. viticola* at 2×10^4 sporangia mL^{-1} in 14 cm diameter Petri dishes during 5 h in darkness; (c) 7 min UV-C and (d) 14 min UV-C-treated leaves. Irradiation was performed by a UV-C tube (Osram, 30 W, 90 μW cm^{-2}, 254 nm, at 13 cm distance from the leaves). Three replicate leaves were set up for each of the four modalities. After treatments, the 12 leaves were put in 9 cm diameter Petri dishes with wet paper, the abaxial surface up. Petri dishes were placed in a culture chamber at 22 °C with 18 h of light (about 200 μmol photon m^{-2} s^{-1}) and 6 h of darkness. Five days after treatment each leaf was split in two parts for the two spectrofluorometric analyses, followed by methanolic extraction and HPLC-DAD analysis.

Standards and Solvents. *trans*-Resveratrol (R5010, Sigma-Aldrich, Saint Quentin Fallavier, France), quinine sulfate dihydrate for fluorescence 99.0% (22640 Fluka, Sigma-Aldrich, Saint Quentin Fallavier, France), *trans*-piceid, *trans*-ϵ-viniferin, *trans*-δ-viniferin, and *trans*-pterostilbene (kindly provided by R. Pezet, Changins, Switzerland) were used as control. Cis forms of stilbenes were obtained by photoisomerization under UV light of *trans*-stilbene standard solutions at 5 μg mL^{-1} (*9*). Dilutions were made in order to establish calibration curves in the range of 0.5−50 μg mL^{-1}. The reference solutions were maintained in the darkness at −20 °C.

Solvents used, acetonitrile, methanol (Merck, Darmstadt, Germany), and sterilized water for injections (Aquettant, Lyon, France), were of HPLC grade.

Spectrofluorometry. Excitation and emission fluorescence spectra were all acquired with the same spectrofluorometer (Cary Eclipse, Varian, Les Ulis, France) using different configurations adapted to the samples. For pure methanolic stilbene solutions (1 μM), a standard 90° configuration with 1 cm path length quartz cells (111-QS, Hellma, Paris, France) was used. For *P. viticola* aerial mycelium, which was very carefully collected with a scalpel from abaxial leaf surfaces under a binocular magnifying glass and placed in flat quartz cells (124-QS, Hellma), and for leaves the same setup based on a double-arm optical fiber bundle (C Technologies, Cedar Knolls, NJ) made of 147 randomized fibers was used. The two arms of the bundle were coupled to the excitation and emission part of the spectrofluorometer via a fiber-optic coupler accessory provided by Varian (part no. FA-VAR00-AP15). The common part of the fiber bundle was maintained at a fixed distance (5 mm) from the samples by a proprietary clip. Under these conditions the spectra of a circular area (diameter 5.5 mm) of the abaxial, and of the exactly corresponding adaxial, surface of leaves were recorded. The 5.5 mm diameter leaf discs were then cut, weighed, and placed in methanol for stilbene extraction. Excitation spectra were corrected with a calibrated photodiode (S1337-1010BQ, Hamamatsu, Massy, France), and emission spectra were corrected using a standard lamp with a known spectrum (LI-COR 1800-02) as described in detail previously (*15*). In addition, spectra were expressed in quinine sulfate equivalent units (QSEU) as previously suggested by Cerovic et al. (*16*): 1000 QSEU correspond to the fluorescence of 1 μM quinine sulfate dihydrate in 1.05 M perchloric acid for 1 cm light path square cells or, in general, 1 nmol cm^{-2} for short light path flat cells, excited at 347.5 nm and emitted at 450 nm, under the identical conditions used to acquire the sample fluorescence spectrum.

Fluorescence Macroscopic Imaging. Abaxial and adaxial leaf surfaces fluorescence was also scanned with a microplate fluorometer (Genios Pro Tecan, Lyon, France), used as microwell plate reader, equipped with specific filters: excitation at 330 nm (fwhm 10 nm) and emission at 389 nm (fwhm 20 nm). Leaf halves were put in a 96-well optical reaction plate (MicroAmp 96-well tray/retainer set 403081, Applied Biosystem, Courtaboeuf, France). Four wells were merged (**Figure 1**) to make one single well to allow the fluorescence acquisition on a surface of $10.4 \times 10.4 = 108$ mm^2, on which $8 \times 8 = 64$ measures were acquired. A pixel corresponded therefore to a surface of $1.3 \times 1.3 = 1.7$ mm^2. So, the surface corresponding to a standard well of a 24-well plate was measured with a 1536-well plate resolution. The plate with the leaf was just flipped over to make the two leaf sides measurements on the same area. The plate was adapted to be at the same focal point of the apparatus whatever the side measured. Two 108 mm^2 areas per leaf (both abaxial and adaxial) were analyzed. The two corresponding leaf discs were then cut, weighed, and their stilbenes extracted. Two filters, one green no. 244 and the other blue no. 385 (Chris James Lighting London, U.K.), were used as solid fluorescence standards. They were fixed in the plate in wells near the leaf sample (**Figure 1**). The linearity of the spectrofluorometer was tested using solutions of quinine sulfate from 1 to 250 μM.

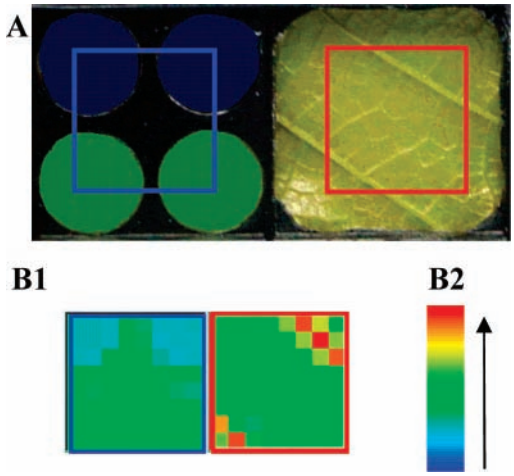

Figure 1. Illustration of data acquired with the microplate fluorometer (Genios Pro). (**A**) Photograph of the area corresponding to 8 wells of a 96-well microplate, Applied Biosystem; 4 wells were cut and merged in the right-side part. Blue and red squares show fluorescence acquisition areas. The upper part of the blue square corresponds to two wells with the blue filter and the lower part to two wells with the green filter. These filters are used as solid fluorescent internal standards. In the red square a *V. vinifera* cv. Muscat Ottonel leaf treated 7 min under UV-C, 5 days posttreatment, is seen. (**B1**) In each square the fluorometer acquired 8×8 pixels of fluorescence data: pixel size, 1.3×1.3 mm^2. (**B2**) Fluorescence color scale: in blue, low fluorescence (0 QSEU), and in red, high fluorescence (1470 QSEU in that case).

Fluorescence measurements at fixed wavelengths could be expressed in QSEU thanks to the knowledge of the quinine sulfate fluorescence excitation and emission spectrum.

Fluorescence Microscopy Imaging. After fluorescence spectra acquisition, small pieces of leaves were mounted in water with Tween (about 1%, v/v) and observed under an inverted epifluorescence microscope (Axiovert 200, Zeiss, Göttingen, Germany) equipped with a monochrome cooled CCD camera (AxioCam Mrm, Zeiss, Germany). The excitation light was provided by a 100 W mercury vapor lamp (HBO, Zeiss, Germany). The 365 nm mercury line was selected using an interference filter (330WB80, Omega, Brattleboro, U.S.A.). The fluorescence was transmitted to the CCD through a long-pass dichroic beam splitter (DCLP400, Omega, Brattleboro, U.S.A.) and a long-pass emission filter (LP400, Omega, Brattleboro, U.S.A.). The images of 1388×1040 pixel size corresponded to an area of 2.2 and 0.04 mm^2 acquired with the $\times 5$ and $\times 40$ magnification (Plan-Neofluar objectives, Zeiss), respectively. Experiments were automated using the software AxioVision (AxioVision 4.4, Zeiss, Germany), and images were processed using the software Image J (Image J 1.36b, National Institutes of Health, U.S.A.).

Extraction and Chromatographic Conditions for HPLC-DAD Analysis. A solid−liquid extraction was performed in darkness on fresh leaf discs with methanol at 60 °C for 45 min. The ratios between dry-weight leaf discs and methanol were comprised between 10 and 30 mg mL^{-1} that allowed good stilbene extraction and detection by HPLC-DAD. Two additional leaf discs were cut in each of the 12 leaves, weighed, then dried at 105 °C for 48 h and weighed again to be able to express the results on a dry-weight basis.

The HPLC system consisted of a 1100 quaternary pump (Hewlett-Packard, Agilent Technologies, Massy, France) equipped with a 1100

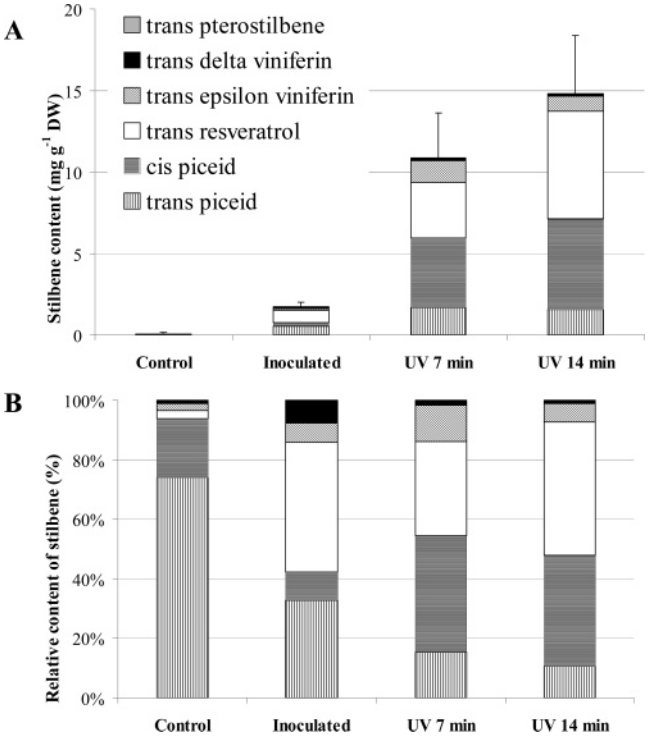

Figure 2. Stilbene contents measure with HPLC-DAD of *V. vinifera* cv. Muscat Ottonel leaves 5 days posttreatment (control, inoculated with *P. viticola*, treated under UV-C 7 or 14 min). Mean of the three replicates leaves (**A**). Standard errors were calculated on the sum of the six stilbenes assayed. Relative content of the six stilbenes assayed (**B**). The legend follows the order presented in the bars of the bar graph.

photodiode array multiwavelength detector (Hewlett-Packard), a 1100 vacuum degasser (Hewlett-Packard), and a 234 automatic injection module (Gilson, Villiers-le-Bel, France). The analyses were carried out at 20 °C on a Lichrospher end-capped RP-18 column (5 μm, 250 mm × 4.6 mm, Merck, Lyon, France) with a slightly modified HPLC gradient according to Jean-Denis et al. (*17*). The trans and cis isoforms of the five main stilbenes, piceid, resveratrol, ϵ-viniferin, δ-viniferin, and pterostilbene, were assayed. These five molecules and their isomers were used as external standards. The absorption was measured between 200 and 400 nm, and chromatograms were recorded at 307 nm. The stilbenes were identified by comparing their retention times and their UV absorption spectra with those of the reference standards.

Statistical Analyses. Statistical analyses were performed using the software StatBox Pro (version 2.5, Grimmer softwares, Paris, France).

RESULTS AND DISCUSSION

HPLC-DAD Analysis. The different treatments applied to the leaves were chosen to obtain highly differentiated leaf samples in term of stilbene content with the aim to validate our in vivo fluorometric assay method. According to the literature, UV-C, probably by causing oxidative stress, can also induce an accumulation of stilbene compounds. These compounds may be quantitatively different from phytoalexins induced by pathogens, but they are chemically similar (*18*−*20*). Soleas et al. (*10*) considered the reversed-phase gradient HPLC diode array detection as the most robust method to assay the stilbenes. The assay of the six main stilbenes present in our extracts was performed by this method. The contents of the cis form of resveratrol, viniferins, and pterostilbene were very low, so they were not taken into account. As expected, we obtained very different mean stilbene contents between modalities (**Figure 2A**) from nearly 0 for control leaves to 15 mg g^{-1} dry weight (DW)

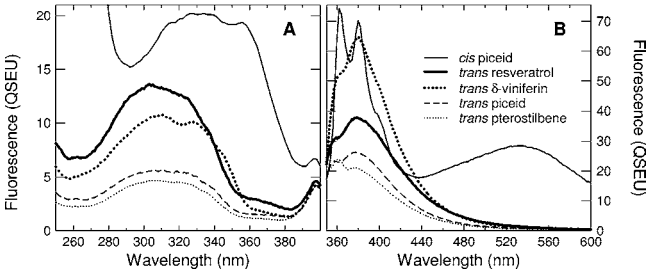

Figure 3. Fluorescence spectra of stilbene compounds in solution. Excitation (**A**) (emission wavelength, 450 nm) and emission (**B**) (excitation wavelength, 320 nm) fluorescence spectra of pure stilbenes (1 μM) in methanol measured on the spectrofluorometer (Cary Eclipse): *trans*-resveratrol, *trans*-δ-viniferin, *trans*-pterostilbene, *trans*- and *cis*-piceid. Fluorescence is expressed in quinine sulfate equivalent units (QSEU). See Materials and Methods for details. The 398 nm peak in the excitation spectra and the 356 nm peak or shoulder in the emission spectra are not fluorescence but Raman light scattering by the solvent (methanol).

for leaves treated by UV-C light during 14 min. Inoculation with *P. viticola* sporangia induced lower stilbene synthesis 5 days after infection (1.8 mg g^{-1} DW) than UV irradiation. The relative amount of the different stilbenes (**Figure 2B**) was not the same depending on the modality. For *cis*-piceid it was higher with UV treatment than in the control or the inoculated leaves. The relative amount of free resveratrol was the highest in that case. The occurrence of free resveratrol in the extracts could be linked to the presence of β-D-glucosidases which probably remain active during the extraction process. As already reported (*21*), the content and the relative amount of *trans*-pterostilbene were very low.

Excitation and Emission Fluorescence Spectra of Stilbenes and Leaves. Fluorescence spectra of pure *trans*-stilbenes in methanol (**Figure 3**) showed a broad excitation peak (290−330 nm) and an emission maximum around 380 nm, in accordance with previous studies (*12*, *22*). So, excitation and emission spectra for all *trans*-stilbenes studied were almost identical. By contrast, the cis form of piceid had a red-shifted excitation band (310−360 nm) compared to that of the trans form and an additional large peak at 260 nm (not shown on **Figure 3**). On the emission side, *cis*-piceid was also different. The emission below 400 nm had two maxima in accordance with the published *cis*-resveratrol emission spectrum (*22*) and an additional broad emission band centered at 535 nm of presently unknown origin. The trans−cis isomerization of piceid induced also a large increase in fluorescence in accordance with the higher fluorescence quantum yield (about 7 times) of *cis*-resveratrol (*22*).

Excitation spectra of pure stilbenes in solution were acquired with an emission wavelength of 450 nm, which is not the maximum for stilbenes. We chose this emission wavelength to keep the same wavelength conditions for all the samples and to be able to record the excitation spectra until 400 nm. Though, we checked that there was no difference in the general shape of the excitation spectra with emission wavelengths of 450 and 400 nm (data not shown).

Control Muscat Ottonel leaves, without induced stilbenes, had only a low basal autofluorescence (**Figure 4**). This blue-green autofluorescence peaking at 440−450 nm is due to the presence in plant tissues of intrinsic fluorescent molecules (*16*). Inoculation of leaves by *P. viticola* and UV treatments led to an important increase in fluorescence, as well as a modification of the shape of excitation and emission fluorescence spectra. For emission spectra, the change in shape was important with the

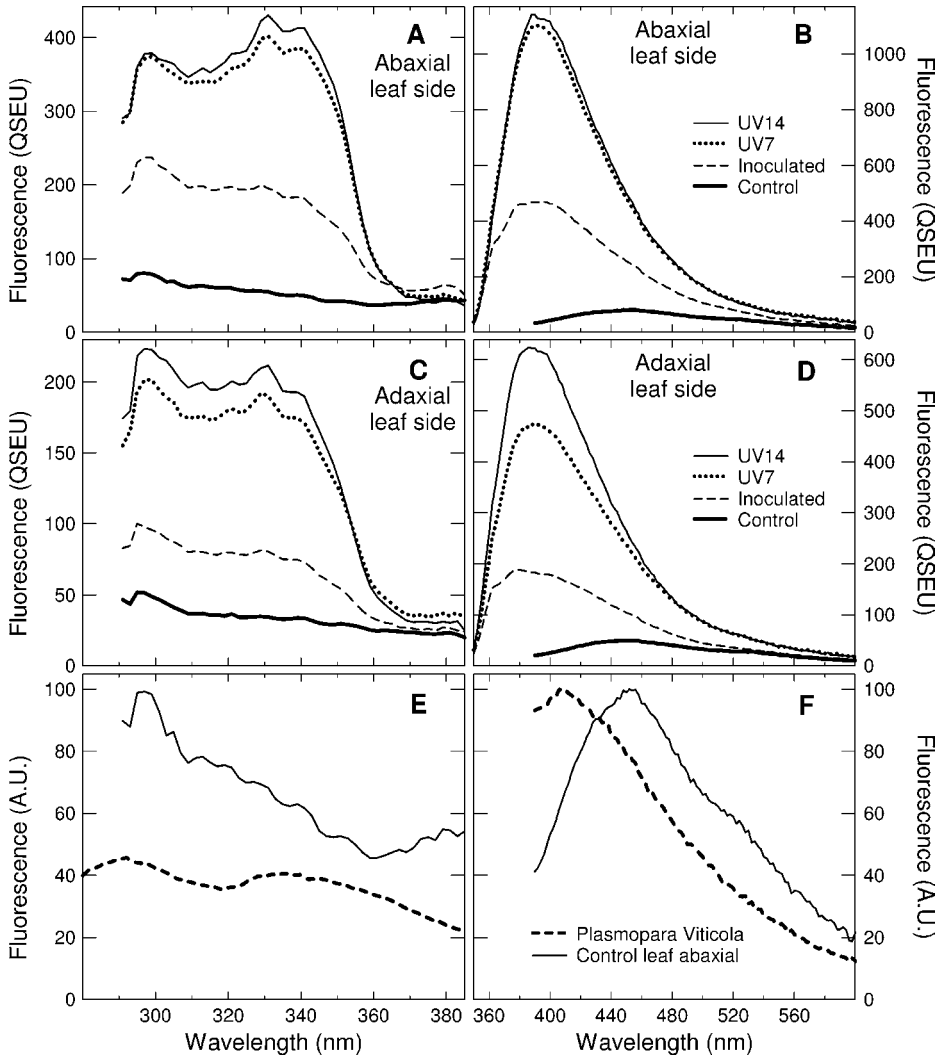

Figure 4. Fluorescence spectra of leaves and mycelium. Excitation (**A** and **C**) (emission wavelength, 450 nm) and emission (**B** and **D**) (excitation wavelength, 320 nm) fluorescence spectra acquired with the spectrofluorometer (Cary Eclipse). Each curve represents the mean of three replicates that were measured on the abaxial (**A** and **B**) and adaxial (**C** and **D**) surface of *V. vinifera* (cv. Muscat Ottonel) leaves, 5 days posttreatment. Control, without treatment; inoculated, inoculated with *P. viticola*; UV7, 7 min under UV-C light; UV14, 14 min under UV-C light. Fluorescence is expressed in quinine sulfate equivalent units (QSEU). See Materials and Methods for details. Excitation (**E**) (emission wavelength, 450 nm) and emission (**F**) (excitation wavelength, 320 nm) fluorescence spectra of *P. viticola* (aerial mycelium) carefully taken from the abaxial surface of a *V. vinifera* (cv. Muscat Ottonel) leaf and set in a flat (0.5 mm) quartz cell. Fluorescence is expressed in arbitrary units normalized to the emission maximum. Abaxial excitation and emission fluorescence spectra of control leaf also normalized to the emission maximum (arbitrary units) are shown again on graphs **E** and **F**, for comparison.

emergence of a 390 nm peak that was 4−14 times more intense than the 450 nm peak of control leaves, in accordance with the induction and accumulation of stilbenes. For excitation spectra, the fluorescence increase concerns a rather wide wavelength band (290−350 nm) again in accordance with the wide excitation peak of stilbenes (290−360 nm taking into account cis and trans forms, **Figure 3**). In the four modalities tested, the fluorescence of abaxial leaf surface was nearly 2 times higher than that of the adaxial (**Figure 4**). In control leaves this difference is explained by a larger diffusion of the abaxial side of bifacial leaves (*16*). But for induced leaves, this difference could also come from a higher synthesis of the stilbenes on the abaxial side. Still, the increase can be seen on both leaf sides. This is important for possible future in-the-field measurements.

The highest fluorescence (**Figure 4**) was obtained with the UV-treated leaves. The shape of the excitation spectrum of the abaxial side of these leaves was notably different from the shape of the excitation spectrum of the same side of inoculated leaves. These differences are much smaller on the adaxial side. The presence of *cis*-piceid in larger amounts in UV-induced leaves (**Figure 2**) can explain why these leaves have an increased proportion of fluorescence excited at 340 nm than inoculated leaves (cf., the shift in the excitation spectrum of *cis*-piceid **Figure 3**). The fluorescence of *P. viticola* itself could be another source of these differences. The analyses of the leaves were performed 5 days postinduction. Sporangiophores and sporangia were then present in important amounts on abaxial leaf surfaces of Muscat Ottonel (**Figure 5**), a variety highly susceptible to this pathogen. It has been shown previously on hyphae that *P. viticola* emits blue fluorescence under UV excitation (*23*, *24*). Here we show that sporangiophores and sporangia also emit blue fluorescence (**Figure 5**, parts **B** and **D**). We show also the excitation and emission fluorescence spectra of aerial mycelium of *P. viticola* recorded with the excitation−emission wavelength combination used for leaves (**Figure 4**). Aerial mycelium is usually not present with *P. viticola*, but in certain conditions it can appear on the abaxial surface of Muscat Ottonel leaves. From the spectra in **Figure 4** and literature data (*25*) we

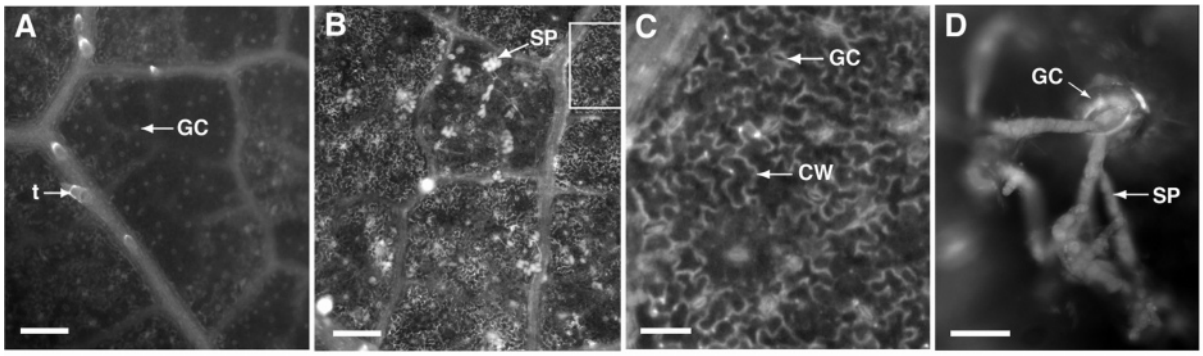

Figure 5. Microscopic images of blue autofluorescence under UV excitation of abaxial surfaces from control (**A**) and inoculated (**B**–**D**) leaves of *V. vinifera* cv. Muscat Ottonel (7 days posttreatment). Image **C** is a zoom on the upper-right part of image **B** (white rectangle). Images **A** and **B** were acquired under the same conditions and are shown here with the same fluorescence intensity gray-level scale. In images **A** and **B**, full black and full white pixels correspond to a relative fluorescence intensity of 0% and 100%, respectively. In image **C**, the scale was changed so that black and white pixels correspond to a relative fluorescence intensity of 0% and 80%, respectively. In image **D**, the image has been improved by a contrasting treatment. In control (**A**), blue autofluorescence was emitted by vein, trichomes (t), and to a lesser extent by guard cells (GC). In inoculated leaf (**B**–**D**), the blue autofluorescence was also emitted by these structures, but in addition, it emanated from epidermal cell walls (CW) and sporangiophores (SP). The bars correspond to 100 (**A**, **B**), 50 (**C**), and 20 μm (**D**).

hypothesize that the fluorophores responsible of the 290 and 340 nm excitation peaks were proteins (tryptophan) and NAD(P)H, respectively. The sporangiophores and sporangia probably have the same fluorophores like the aerial mycelium. Although being fluorescent in the blue, the contribution of *P. viticola* to the leaf fluorescence is small compared to that of induced stilbenes for two reasons. First, the shape of the fluorescence spectra of the adaxial and abaxial side of inoculated leaves was not significantly different. Second, from **Figure 5B** it can be calculated that sporangiophores and sporangia contribute less than 6% to the UV-induced blue fluorescence of the surface of image **B**.

Relation between Extract Absorption and Fluorescence of the Adaxial and Abaxial Leaf Surfaces. The same leaf, split in two equal parts, was use for the studies on the two fluorometers to avoid experimental bias linked to the level of organ development, the age of the leaf, or the concentration of the inoculum (*26*, *27*). Although strict experimental conditions were used, an important variation of the stilbene content was observed among UV treatments and inoculation modalities (**Figure 6**). Stilbene content measured with HPLC-DAD and adaxial and abaxial leaf surfaces fluorescence were performed on exactly the same area of the leaf. Highly significant correlations were found between total stilbene content and leaf fluorescence on the abaxial side ($R^2 = 0.80$ for the microplate fluorometer Genios Pro and 0.82 for the spectrofluorometer Cary Eclipse; $p < 0.001$) and the fluorescence on the adaxial side ($R^2 = 0.77$ for the microplate fluorometer and 0.69 for the spectrofluorometer; $p < 0.001$), respectively. A multiple regression aggregating the two sets of fluorescence data obtained on both leaf sides with the stilbene content measured by HPLC-DAD improved the coefficient of determination of the regression (stilbene HPLC-DAD = 0.008(abaxial fluorescence) + 0.016 (adaxial fluorescence) − 5.164, $R^2 = 0.90$ for the microplate fluorometer, and stilbene HPLC-DAD = 0.007(abaxial fluorescence) + 0.006(adaxial fluorescence) − 0.772, $R^2 = 0.85$ for the spectrofluorometer; $p < 0.001$) (**Figure 7**). This shows again that all the stilbenes were not located on the abaxial surfaces only. In both fluorometers, for two out of three inoculated leaves the stilbene content calculated with the models based on both adaxial and abaxial leaf fluorescence was higher than that measured by HPLC-DAD. It was probably due to the contribution of *P. viticola* fluorescence to the sample fluorescence.

The expression of the stilbene content per unit leaf area, rather than per dry weight, gives the same results (data not shown). The differences in fluorescence yields of stilbene derivatives present in the leaf (**Figure 3**) can explain partly the dispersion of points in the plots of macroscopic fluorescence measurements against HPLC quantification. Still, the correlation is very good thanks to the choice of the excitation and the emission wavelength and the presence of *trans*-resveratrol as the major compound. In any case, the proposed method does not intend to replace HPLC as an analytical technique but will certainly be useful for rapid screening and macroscopic imaging.

Heterogeneous Repartition of Stilbene on Abaxial and Adaxial Leaf Surfaces. Thanks to the highly significant relation found between leaf stilbene content and fluorescence, the latter could be potentially used down to the pixel level. So, the fluorescence of each pixel could allow the visualization of the stilbene repartition in the leaf **(Figure 8)**. The fluorescence of the control leaf was homogeneous; the coefficient of variation (CV) between the 64 pixels was about 5% for both abaxial and adaxial leaf surfaces. This is slightly higher than the variability seen on the two filters used as standards in the microplate fluorometer (CV on eight data around 2.5%, data not shown). For inoculated or UV-treated leaves, the CV was around 20% (**Figure 8**). Inoculation or UV treatments induced local reactions of size lower than the size of a pixel (1.7 mm^2). Although leaf treatments were performed homogenously, the reactions on the leaves were not. This was confirmed by fluorescence microscopy of a small abaxial area (**Figure 5**). On a microscopic scale, fluorescence was quite homogeneous for control leaves, with a higher fluorescence of veins and stomata, while it was clearly nonhomogeneous for inoculated leaves, with the appearance of increased fluorescence of the cell walls. As seen in **Figure 5B**, the fluorescence of sporangiophores and sporangia was also participating to the nonhomogeneity of the fluorescence of inoculated leaves.

In the case of induction with UV light, the highest fluorescence was always found near or on the veins on the abaxial (**Figures 1** and **8**) and adaxial surfaces of the leaves. This was not the case in inoculated leaves. On another hand, the fluorescence increase induced by inoculation was coming from small veins, which showed a fluorescence intensity slightly higher than control and mainly from the cell walls (**Figure 5**). The latter are hardly distinguishable on images of control leaves. These observations confirmed that phytoalexins are mainly

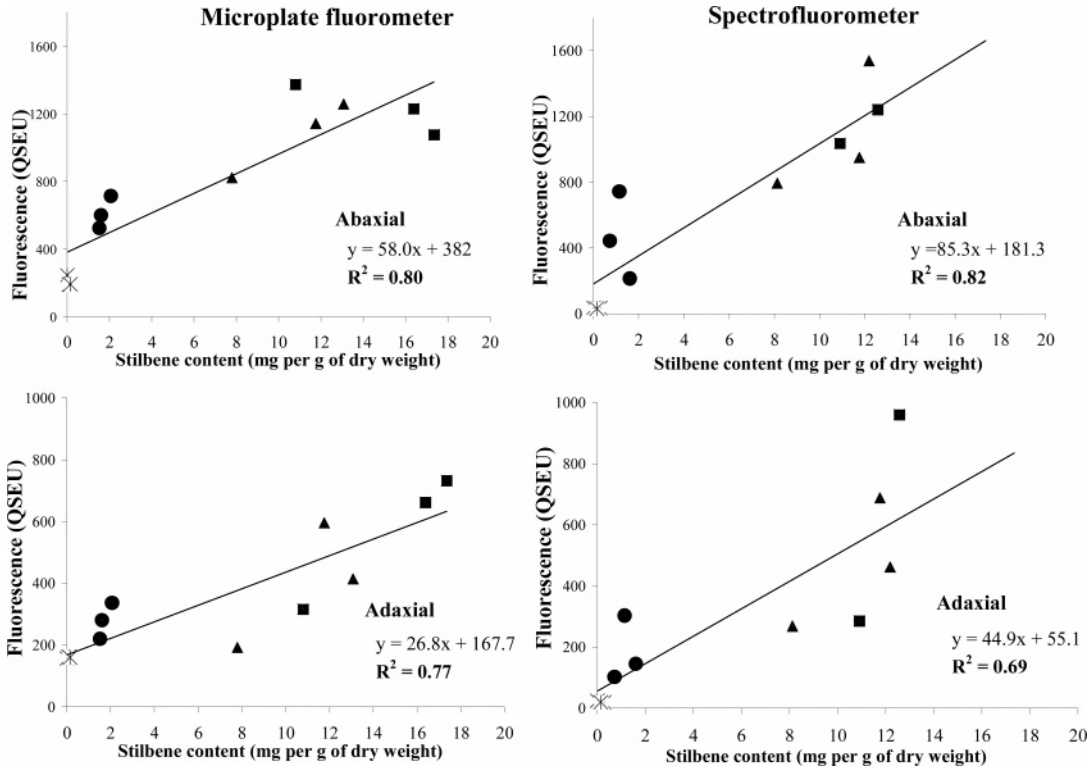

Figure 6. Relation between fluorescence acquired with the microplate fluorometer (Genios Pro) and the spectrofluorometer (Cary Eclipse) on *V. vinifera* cv. Muscat Ottonel abaxial and adaxial leaf sides and the HPLC-DAD analysis of the corresponding samples. The excitation/emission wavelength combination was 330/389 nm for the microplate fluorometer and 320/390 nm for the spectrofluorometer. Each point corresponds to the measurements on one leaf by the two methods. Because of the slightly different response of the two fluorometers, the two sets of data were not presented on the same figure. Only two measurements were performed with the spectrofluorometer on leaf treated with UV-C for 14 min. QSEU: quinine sulfate equivalent units. × control; ● inoculated; ▲ UV 7 min; ■ UV 14 min.

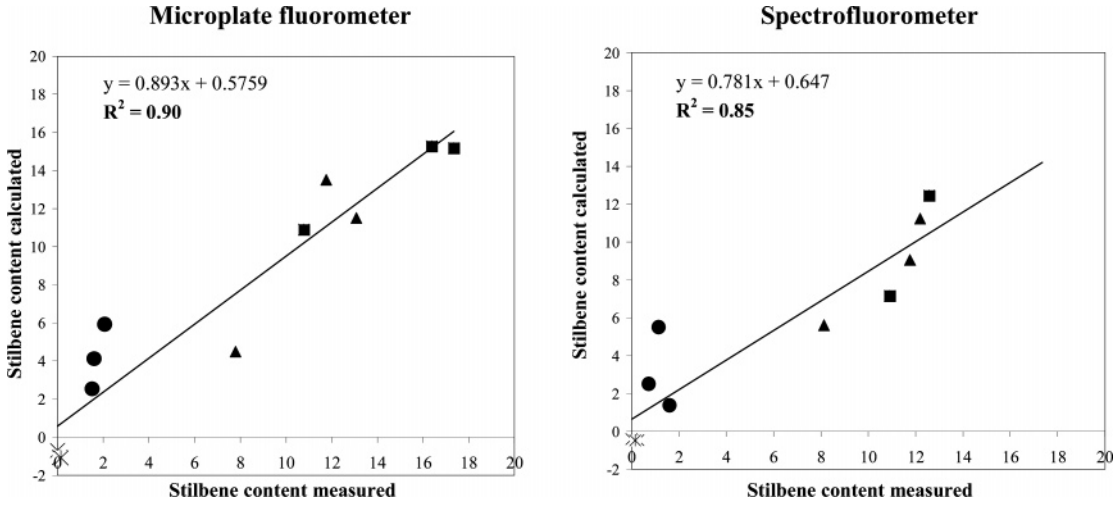

Figure 7. Relation between stilbene content calculated with a multiple regression model aggregating the abaxial and adaxial fluorescence data of *V. vinifera* cv. Muscat Ottonel leaves and the stilbene content (mg g^{-1} DW) measured with HPLC-DAD. Only two measurements were performed with the spectrofluorometer on leaves treated with UV-C during 14 min. × control; ● inoculated; ▲ UV 7 min; ■ UV 14 min.

accumulated in lignified tissues and veins, cell walls, and stomata (*19*, *27*) and that resveratrol could participate to the cell-wall reinforcement (*27*).

It would be important to see if the same behavior is maintained in outdoor-grown leaves. Constitutive flavonoids present in the epidermis might change the intensity of the response of the leaf to the pathogen, or at least the detectability of the accumulated stilbenes. Still, the described method is potentially applicable to other pathosystems of crops grown in the field or in the greenhouse.

We presented a rapid and efficient fluorometric approach to assess stilbene content on grapevine leaves: in vivo spectrofluorometry imaging using commercial microplate scanners constitutes a new tool to study the production of stilbene in leaves inducted by biotic or abiotic stresses at different levels, from leaf surfaces of a few square centimeters, to access the global stilbene content, to square millimeters (even square micrometers using new microspectrofluorometers), to determine very local reactions. The determination of the highest stilbene content at a local point could be important in the case of

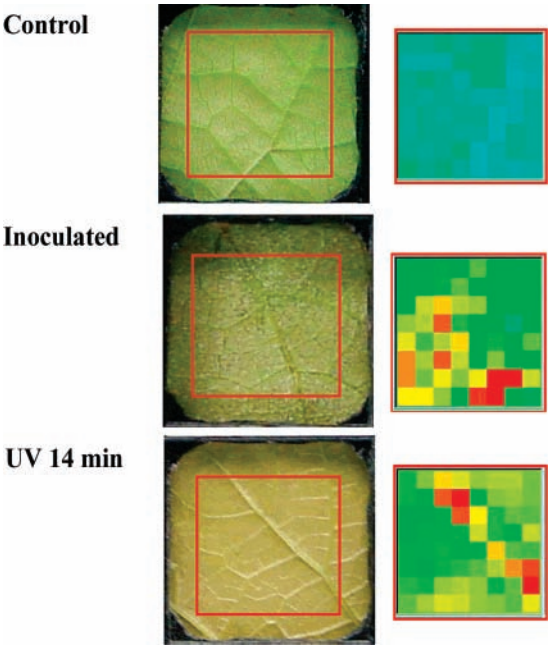

Figure 8. Examples of abaxial measurement of fluorescence acquired with the microplate fluorometer. The red squares on pictures of leaf samples (left side of the figure) correspond to the area of fluorescence acquisition presented on the right side. The color scale is the same as in Figure 1: blue for low and red for high fluorescence. The scale range was different for the three treatments going from 0 to 760 QSEU for the control, from 0 to 860 QSEU for the inoculated with *P. viticola*, and from zero to 1950 QSEU for the UV 14 min. The coefficients of variation calculated between the 64 fluorescence data were 5% for control, 20% for inoculated, and 17% for UV 14 min.

resistance study because it was hypothesized that the resistance is linked to the genotypes capacity to set up quickly and locally high stilbene content (*3*). HPLC analyses generally performed on square centimeters of leaf do not evaluate the heterogeneity of the repartition on the area studied but allow a good separation and an accurate assay of the extractable stilbenes. Fluorescence imaging could be, therefore, considered as a method of choice allowing the evaluation of the local content of stilbenes. This nondestructive method can be coupled with HPLC to determine the different molecules present in the studied sample. Furthermore, this approach can be extended to other kinds of molecules and organs. It constitutes a new means to study synthesis and localization of fluorescent secondary metabolites in plants.

ABBREVIATIONS USED

CV, coefficient of variation; DAD, photodiode array detector; DW, dry weight; fwhm, full width at half-maximum transmission; HPLC, high-performance liquid chromatography; QSEU, quinine sulfate equivalent units; UV, ultraviolet; NAD(P)H, nicotinamide adenine dinucleotide (phosphate), reduced form.

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