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ORIGINAL ARTICLE

Influence of constitutive phenolic compounds on the response of grapevine (*Vitis vinifera* L.) leaves to infection by *Plasmopara viticola*

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Abstract Flavonols and hydroxycinnamic acids are known to contribute to plant resistance against pathogens, but there are few reports on the implication of flavonols in the resistance of grapevine against *Plasmopara viticola*, and none on the involvement of hydroxycinnamic acids. In order to analyze the effect of flavonols on *P. viticola* infection, variable amounts of flavonols were induced by different light conditions in otherwise phenologically identical leaves. Differences in content of leaf hydroxycinnamic acids were induced at the same time. A non-invasive monitoring of flavonols and hydroxycinnamic acids was performed with Dualex leaf-clip optical sensors. Whatever the light condition, there were no significant changes in flavonol or in hydroxycinnamic acid contents for control and inoculated leaves during the development of *P. viticola* until 6 days after inoculation. The violet-blue autofluorescence of stilbenes, the main phytoalexins of grapevine that accumulate in inoculated leaves, was used as an indicator of infection by *P. viticola*. The implication of leaf constitutive flavonols and hydroxycinnamic acids in the defence of *Vitis vinifera* against *P. viticola* could be investigated in vivo thanks to this indicator. The increase in stilbene violet-blue autofluorescence started earlier for leaves with low flavonol content than for leaves with higher content, suggesting that constitutive flavonols are able to slow down the infection by *P. viticola*. On the contrary, constitutive hydroxycinnamic acids did not seem to play a role in defence against *P. viticola*. The non-destructive nature of the methods used alleviates the major problem of destructive experiments: the large variability in leaf phenolic contents.

Keywords Defence against pathogens · Downy mildew · Flavonols · Hydroxycinnamic acids · Spectrofluorimetry · Stilbenes

Abbreviations

ANOVA	Analysis of variance
DAD	Diode array detector
DPI	Days post-inoculation
Dx-Flav	Dualex Flav
Dx-HCA	Dualex HCA
DW	Dry weight
Flav	Flavonols
HCA	Hydroxycinnamic acids
HPLC	High-performance liquid chromatography
PDA	Photodiode array detector
PAR	Photosynthetically active radiation
QSEU	Quinine sulfate equivalent unit
TQD	Tandem quadrupole mass spectrometry detector

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UPLC Ultra performance liquid chromatography
VBF Violet-blue fluorescence

Introduction

Downy mildew caused by *Plasmopara viticola* is one of the most destructive diseases in viticulture (Wong et al. 2001). It affects both the yield of grapes and the quality of wine produced. Grapevine resistance to downy mildew involves complex and not yet fully understood mechanisms (Allegre et al. 2007; Chong et al. 2009; Alonso-Villaverde et al. 2011; Gessler et al. 2011). Phenolic compounds, recognized for their antifungal activity (Nicholson and Hammerschmidt 1992; Hammerschmidt 2005), seem to have a primary role in the grapevine–*P. viticola* interactions (Dai et al. 1995a, b; Mondolot-Cosson et al. 1997). Of particular importance are the stilbenes, the main phytoalexins in grapevine (Jeandet et al. 2002). Stilbenes are not detectable in healthy leaves but their synthesis and accumulation are induced by *P. viticola* and also by other biotic or abiotic stresses (Langcake and Pryce 1976; Chong et al. 2009; Jeandet et al. 2010). The implication of flavonols (Flav) in grapevine defence against *P. viticola* has also been shown (Dai et al. 1995b; Agati et al. 2008). Lower resistance of plants grown under high nitrogen supply (Bavaresco and Eibach 1987) is also probably linked to a lower Flav leaf content. Flavonols are naturally synthesized and accumulated in grapevine leaves grown in vineyards with a primary role of protection against UV (Kolb et al. 2001; Kolb and Pfündel 2005). They are constitutive phenolic compounds whose accumulation depends on UV-light regime (Bidel et al. 2007; Morales et al. 2011). In the defence against *P. viticola*, they act as constitutive compounds (Agati et al. 2008), but Flav formation induced by downy mildew has also been reported (Dai et al. 1995a, b; Mondolot-Cosson et al. 1997).

Hydroxycinnamic acids (HCA) are also accumulated as constitutive phenolic compounds in grapevine leaves (Cerovic et al. 1999; Kolb et al. 2001; Kolb and Pfündel 2005; Pfündel et al. 2006; Agati et al. 2008) with a role as UV-screening epidermal compounds (Kolb et al. 2001; Kolb and Pfündel 2005). The relationship between light and HCA content in leaves is species-dependent (Bidel et al. 2007). In grapevine leaves, synthesis of HCA is stimulated by strong visible light but not by UV-light (Kolb et al. 2001). Hydroxycinnamic acids act as antifungal compounds in a number of plant-pathogen interactions (Morrissey and Osbourn 1999) and they play a major role in the defence of cereals against insects, fungi and bacteria (Niemeyer 1988). However, the HCA implication in the grapevine–*P. viticola* interaction is unknown.

Stilbenes are autofluorescent compounds (Hillis and Ishikura 1968), displaying a violet-blue fluorescence (VBF) under UV-light with excitation and emission maxima around 320 and 390 nm, respectively, in methanol as well as in leaves (Poutaraud et al. 2007). The main six stilbene compounds produced by grapevine leaves have very similar fluorescence spectra precluding their discrimination in vivo (Poutaraud et al. 2007; Bellow et al. 2012). Still, total stilbene content in grapevine leaf has been measured in vivo by UV-induced blue fluorescence (Poutaraud et al. 2007; Poutaraud et al. 2010).

Flavonols have a very low fluorescence yield but they can be assessed in vivo in the leaf by the chlorophyll fluorescence screening method (Bilger et al. 1997; Ounis et al. 2001; Cerovic et al. 2002).

Hydroxycinnamic acids are more fluorescent than flavonols especially when esterified to cell walls, emitting blue fluorescence under UV excitation (Cerovic et al. 1999). In the vacuoles, where a large part of HCA is located in grapevine (Kolb et al. 2001; Pfündel et al. 2006), they have a low fluorescence yield (Bellow et al. 2012). Thus, in this work the chlorophyll fluorescence screening method was also used for a non-invasive in vivo measurement of HCA content in leaves (see Bidel et al. 2007).

The main objective of this work was to clarify the implication of Flav and HCA in the defence of *Vitis vinifera* against downy mildew. Towards that goal, the grapevine leaf content in constitutive Flav and HCA was modulated by exposing greenhouse-grown plants to different UV-light conditions before inoculation by *P. viticola*. We used the in vivo stilbene VBF as an indicator of the infection by *P. viticola*. The leaf response to infection of the susceptible genotype *Vitis vinifera* L. cv. Cabernet Sauvignon was monitored on a daily basis. Two portable leaf-clips, both based on the chlorophyll fluorescence screening method, were used to follow daily the epidermal Flav content and the epidermal HCA content in parallel with the stilbene VBF.

Materials and methods

Plant material, experimental design and leaf sample preparation

Greenhouse-grown plants of *V. vinifera* L. cv. Cabernet Sauvignon were propagated in a greenhouse by cuttings. The fifth leaves counted from the apex of three and a half-month-old plants having 12–14 fully expanded leaves were used. Two experiments were performed, one in July and one in August. For the UV-light acclimation experiment (cf. Fig. 2) the greenhouse-grown plants were set outdoor next to the greenhouse in a never shaded place in Orsay, France (Lat. 48°70′ N, Long. 2°17′ E) in July (about 18 h of daylight). The plants were set in a north–south oriented row, and were not

moved for the daily Flav and HCA optical measurements. During the 10 days of this UV-light acclimation experiment, the mean photosynthetically active radiation (PAR) was 46 ± 14 mol photon m^{-2} day^{-1}. For the inoculation experiment (cf. Figs. 3, 4, 5, 6 and 7) four UV-light conditions were used to obtain leaf samples with different Flav content: (1) ''greenhouse'', plants were kept in the greenhouse; (2) ''outdoors'', plants were set outdoor under the conditions described for the UV-light acclimation experiment, but in August (about 16 h of daylight and a PAR of 26 ± 13 mol photon m^{-2} day^{-1}); (3) ''no-UV'', plants were set outdoor as described for condition 2, but with half of the leaf (along the central vein) completely protected from UV by a plastic-foil filter (Lee 226 UV, Lee Filters, Andover, UK) (see Kolb et al. 2001 for the transmittance spectrum) (see the Online Resource 1, picture); (4) ''UV'', the second leaf-halves were exposed to normal daylight (partners of the leaf-halves bearing an anti-UV filter of condition 3). For condition 3, the anti-UV filter chamber allowed a free air circulation around the leaf-half, especially on the abaxial side where stomata are located. Conditions 2 and 4 were similar in terms of light exposure (normal daylight with UV), but condition 4 gave the possibility to avoid the influence of leaf heterogeneity by comparing the two halves of the same leaf, as one half was submitted to condition 3 and the other half to condition 4. Comparison between samples of conditions 2 and 4 also allowed us to investigate the influence of leaf detachment (see below). The four UV-light conditions started 10 days before the inoculation by *P. viticola*.

Flavonol and HCA optical measurements were performed in the laboratory before applying the different UV-light conditions and 10 days later just before inoculation. For inoculation, which took place in the laboratory, the leaves were rinsed with demineralized water and then immersed in the inoculum solution (2.4×10^4 sporangia mL^{-1}) for 5 h in the dark. For conditions 1 (greenhouse) and 2 (outdoors), the leaves were detached just before inoculation and cut into two along the central vein. One leaf-half was inoculated and the other half was used as control and immersed in water instead of the inoculum solution. For conditions 3 (no-UV) and 4 (UV), leaves were kept attached to the plants and were totally immersed in the inoculum solution for inoculated leaves and in water for control leaves. After inoculation, all samples (detached leaves and leaves attached to the plant) were kept in the laboratory. They were illuminated by daylight through a large window completely filtering out UV-B.

Optical measurements of flavonols and hydroxycinnamic acids

Epidermal phenolic compounds, which are representative of total leaf phenolics (Kolb and Pfündel 2005; Barthod et al. 2007), were optically estimated in situ using a portable leaf-clip device, the Dualex® (Force-A, Orsay, France). Two versions of the Dualex were used: the Dx-Flav and the Dx-HCA. The Dx-Flav measures the epidermal absorbance at 375 nm, mainly due to Flav, by comparing the chlorophyll fluorescence signals at two different excitation wavelengths (375 and 650 nm) (Goulas et al. 2004; Cartelat et al. 2005). The Dx-Flav measurements are largely insensitive to the presence of HCA (Cerovic et al. 2005). This was verified in field-grown grapevine leaves (Agati et al. 2008). A very good correlation between Dx-Flav and the total Flav content expressed on an area basis was also found for grapevine leaves (Agati et al. 2008).

The Dx-HCA was designed to estimate the HCA content in leaves. It is similar to Dx-Flav except that it measures the epidermal absorbance at 322 nm, and thus compares the chlorophyll fluorescence signals excited at 322 and at 650 nm. Its 322 nm LED source, a UVTOP 310 LED (Sensor Electronic Technology Inc., Columbia, SC, USA) plus a DUG 11 filter (Schott AG, Mainz, Germany), was chosen close to the HCA absorption maximum and away from the Flav absorption maximum (Fig. 1). However, Dx-HCA measurements are influenced by the presence of Flav. At 322 nm the Flav molar extinction coefficient is about two-thirds that of HCA (Fig. 1). Thus, to obtain an estimation of the epidermal HCA content of the leaves, the Dx-HCA measurements were corrected for the contribution of the Flav to the absorbance at 322 nm. As mentioned before, the Dx-Flav is largely insensitive to HCA and their contribution to the 375 nm epidermal absorbance measured by the Dx-Flav was neglected here. Dx-Flav and Dx-HCA measurements were done successively on the same spots for each leaf and the Dx-Flav measurements were used for correction according to Eq. 1:

$$\text{Dx-HCA}_{\text{index}} = \text{Dx-HCA}_{\text{measurement}} - \left(\frac{\varepsilon_{322}}{\varepsilon_{375}}\text{Dx-Flav}_{\text{index}}\right) \quad (1)$$

where ε_{322} and ε_{375} are the molar extinction coefficients of quercitrin (quercetin 3-*O*-rhamnoside) at 322 and 375 nm, respectively (Fig. 1). Quercetin 3-glycosides, which all have the same absorption spectrum, accounted for almost all the Flav: quercetin 3-*O*-glucuronide is the main one present in Cabernet Sauvignon leaves (see below) as in Sangiovese (Agati et al. 2008).

For the UV-light acclimation experiment, Dx-Flav and Dx-HCA measurements were made outdoor every day at about the same time, starting the day when the plants were taken outdoor. The same four regions, equally distributed on each side of the main vein, were measured on both leaf sides every day. For each region, the sum of the corrected measurements for adaxial and abaxial side was used. For the inoculation experiment, measurements were made in

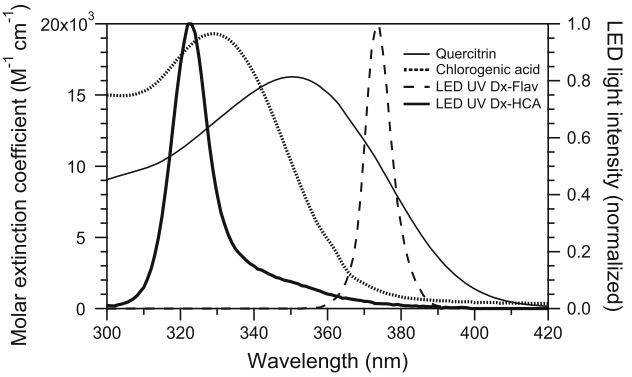

Fig. 1 Spectra of molar extinction coefficients of chlorogenic acid in methanol and quercitrin (quercetin 3-*O*-rhamnoside) in 50/50 methanol/water (v/v) compared to the normalized spectra of the UV-light sources of the Dx-Flav and of the Dx-HCA

the laboratory, the day when the plants were taken outdoor and every day from inoculation onwards. The measurements were made the same way as for the UV-light acclimation experiment, but with three regions for each leaf-half.

Measurements of spectra

Absorption spectra were recorded on the spectrophotometer HP8453 (Hewlett–Packard, Agilent Technologies, Massy, France), using quercetin 3-*O*-rhamnoside (Extrasynthèse, Lyon, France), chlorogenic acid (C3878, Sigma-Aldrich, Saint Quentin Fallavier, France), spectroscopic grade methanol and Milli-Q water. The spectra of the UV LED sources of the Dx-Flav and of the Dx-HCA were recorded using an optic-fiber spectrograph (HR4000CG-UV-NIR, Ocean Optics, Dunedin, FL, USA).

Spectrofluorimetry

Excitation and emission fluorescence spectra were acquired with a spectrofluorimeter (Cary Eclipse, Varian, Les Ulis, France) using a double-arm optical fiber bundle (C Technologies, Cedar Knolls, NJ) made of 147 randomized fibers. The two arms of the bundle were coupled to the excitation and emission part of the spectrofluorimeter 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 leaf samples by a proprietary leaf-clip. Under these conditions, every day at about the same time, the spectra of the same marked circular area (diameter 5.5 mm) of the abaxial surface of each leaf-half were recorded from just before inoculation (0 days post-inoculation, DPI) to 6 DPI. 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 (Louis et al. 2006). In addition, fluorescence intensity was expressed in quinine sulfate equivalent units (QSEU) (Cerovic et al. 1999): 1000 QSEU correspond to the fluorescence of 1 μM quinine sulfate dihydrate in 0.105 M perchloric acid for 1-cm light-path square cells or, 1 nmol cm^{-2} for flat samples, excited at 347.5 nm and emitted at 450 nm, under the identical conditions used to acquire the sample fluorescence spectrum.

Extraction and HPLC–DAD analysis

Chromatographic quantification of flavonols was performed on control leaves after 10 days of UV-light conditioning (at the time of inoculation) and on inoculated leaves at 6 DPI. All leaf-halves were frozen at −80 °C. For each sample, the equivalent of 20–60 mg dry weight (DW) was extracted with methanol at 60 °C for 45 min. The ratio of about 20 mg leaf DW per mL of methanol allowed good flavonol extraction and detection by HPLC–DAD. For the DW/fresh weight ratio estimation, one additional leaf piece of each sample was weighed before and after drying at 60 °C for 3 days. The HPLC system consisted of a 1100 quaternary pump (Hewlett–Packard) equipped with a 1100 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 × 4.6 mm, Merck, Lyon, France) with a slightly modified HPLC gradient according to Jean-Denis et al. (2006). The absorption was measured between 200 and 400 nm, and chromatograms were recorded at 307 nm. Retention times of Flav were determined thanks to a UPLC-PDA-TQD system (Acquity, Waters, Saint-Quentin-en-Yvelines, France) used in strictly the same conditions as for HPLC–DAD on some of the methanolic leaf extracts. The UPLC-PDA-TQD system, that was also used for HCA quantification, consisted of an UPLC instrument (Acquity, Waters) connected to a photodiode array detector (PDA) and to a tandem quadrupole mass spectrometry detector (TQD) equipped with an electrospray ion source. A quercetin 3-*O*-glucoside standard (17793, Sigma-Aldrich) was also used. Acetonitrile, methanol (Merck) and sterilized water for injections (Aquettant, Lyon, France) were of HPLC grade.

Statistical analyses

Statistical analyses were performed using the software Statistica 6.1 (StatSoft Inc., Maison-Alfort, France). The significance of differences between mean values was assessed by a one-way analysis of variance (ANOVA).

The normality assumption of the ANOVA was tested with a Shapiro–Wilk test. A Levene test and a Brown–Forsythe test were used to check the homoscedasticity assumption. In the cases where normality or homoscedasticity were violated the result obtained by the ANOVA was confirmed by three non-parametric tests: Mann–Whitney U test, Wald–Wolfowitz runs test and Kolmogorov–Smirnov test.

Results

Daylight-UV induced changes in flavonols and hydroxycinnamic acids

The transfer of greenhouse-grown grapevine leaves to full sunlight outdoor induced an increase in epidermal Flav content (Fig. 2a). The effect was observed on both sides of the leaves but was larger on the adaxial side (data not shown), which is more exposed. The kinetics of Flav synthesis in the leaves upon transfer from the greenhouse to outdoor were: (1) a small increase on day one, (2) a fast increase from day two to day six, and (3) a much smaller increase thereafter. In parallel with daily Dx-Flav measurements, Dx-HCA measurements of epidermal absorption at 322 nm were recorded. Because of the overlap of the absorption of HCA and Flav in the UV-B, Dx-HCA measurements were corrected for the Flav contribution (see "Materials and methods" for details). The epidermal HCA kinetics after the plants were transferred outdoor (Fig. 2b) showed an anti-parallel behaviour to Flav.

On particular days, Dx-HCA measurements were more variable. These days, variations were also larger than for indoor measurements (see Fig. 7). This was due to a contamination of the Dx-HCA leaf clip measurements by sunlight. Dx-HCA is more sensitive than Dx-Flav to direct sunlight and therefore when measuring outdoor, the leaf clip should be shaded, which was not systematically done here. The higher sensitivity of Dx-HCA to direct sunlight was due to the lower light intensity of the 322 nm LED compared to the 375 nm LED of the Dx-Flav, which implied a much higher gain of the detector (Force-A, unpublished results).

In order to analyze the effect of Flav on *P. viticola* infection, different amounts of Flav were induced by four different natural UV-light conditions in otherwise phenologically identical leaves. Figure 2 shows that greenhouse-grown Cabernet Sauvignon leaves transferred to full sunlight outdoor had reached, within 10 days, a content equivalent to that of vineyard-grown leaves (Agati et al. 2008). This 10-day acclimation was also used to compare the effect of UV-light on the same leaf by installing an anti-UV filter on one half of the leaves. We also compared the grapevine–*P. viticola* interactions in attached leaves and in detached leaves usually used in this type of experiment.

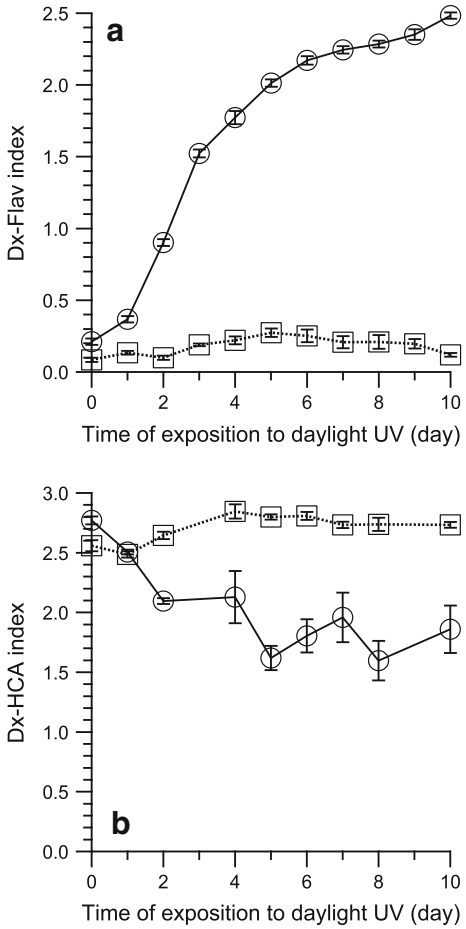

Fig. 2 Daily kinetics of Dx-Flav (**a**) and Dx-HCA (**b**) measurement of greenhouse-grown grapevine leaves after the transfer of the plants outdoor. Outdoor leaf data (*circles*, *full line*) are the mean of 12 Dualex measurements on three leaves (four measurements per leaf) while the control greenhouse leaf data (*squares*, *dotted line*) are the mean of four Dualex measurements on a single leaf. *Error bars* are the standard errors of the mean (often smaller than the markers). Dx indices are the sum of adaxial plus abaxial Dualex measurements and the Dx-HCA measurements were corrected for the absorbance of the Flav (see "Materials and methods" for details)

Four types of samples (produced by the 10-day UV-light conditioning) were inoculated: ''greenhouse'' and ''no-UV'' devoid of Flav and ''outdoors'' and ''UV'' in which Flav were induced. During the inoculation experiment, the Flav content of the leaves was monitored by Dx-Flav, but was also measured by HPLC–DAD after methanolic extraction on control samples at 0 DPI and on inoculated samples at 6 DPI (Fig. 3). The results of the HPLC quantification showed that greenhouse and no-UV leaves were devoid of Flav, and that outdoors leaves had a higher Flav content than UV leaves (Fig. 3). Only the three main Flav measured by HPLC in the methanol extract were presented in Fig. 3 because they accounted for 85 % of the Flav

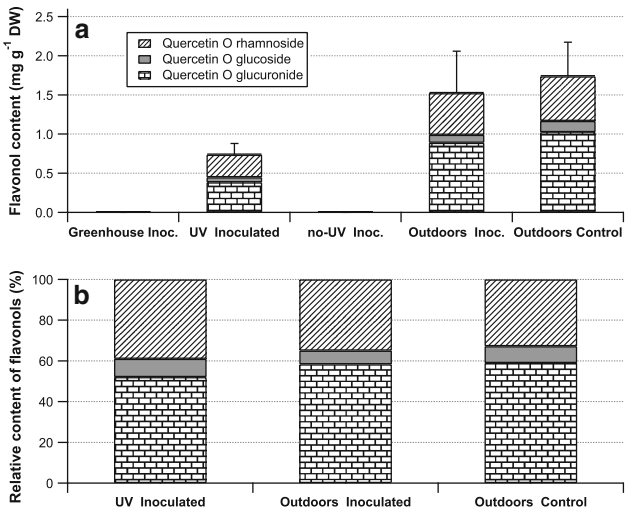

Fig. 3 Flavonol contents measured with HPLC–DAD of *V. vinifera* cv. Cabernet Sauvignon leaves after 10 days under different UV-light conditions. Leaf-halves without a UV-blocking filter (*UV*); leaf-halves with a UV-blocking filter (*no-UV*); whole leaf exposed to UV (*Outdoors*) and whole leaf not exposed to UV (*Greenhouse*). Samples were measured either before *P. viticola* inoculation after 10 days of exposition to full sunlight (*Outdoors Control*) or 6 days after inoculation (*Inoculated* or *Inoc.*). **a** Means of the three replicates are presented with standard errors calculated from the sum of the three flavonols assayed. **b** Relative content of the three flavonols assayed. Same symbols in **a** and **b**

(other Flav would not have been visible on the graph). In addition to the quercetin derivatives, small amounts of kaempferol and myricetin derivatives were also detected in the extracts. The main HCA detected in the extracts were, by descending order of content, caftaric acid, coutaric acid, chlorogenic acid and caffeic acid, in a much smaller amount. No significant differences were observed for the total leaf Flav content between outdoors inoculated samples at 6 DPI and outdoors control samples (Fig. 3a). The proportion of the three main Flav was also the same (Fig. 3b). All these indicate that infection by *P. viticola* had probably no effect on gross Flav synthesis and catabolism.

Kinetics of stilbenes upon infection by *P. viticola*

The in vivo blue-green autofluorescence excitation and emission spectra of the four different leaf samples were recorded on the very same region of the abaxial leaf side every day, from just before inoculation to 6 days after. The spectra of one typical no-UV inoculated sample are shown in Fig. 4. A three phase kinetics can be clearly observed: (1) from 0 to 2 DPI, no change in the spectra; (2) from 3 to 5 DPI, increase in fluorescence with changes of shape for both spectra due to the accumulation of stilbenes, as described by Poutaraud et al. (2007); (3) at 6 DPI, another change in the emission spectrum shape. The change of spectral shape observed for half of the greenhouse and no-UV inoculated samples at 6 DPI (Fig. 4) was attributed to a leaf dehydration induced by the pathogen, visible in the measured areas of the affected samples (Cerovic Z.G., unpublished). All control samples, during the whole 6-day experiment, had spectra identical to the spectra at 0 DPI presented in Fig. 4 (data not shown). All inoculated samples had similar spectra to those shown in Fig. 4, but with different kinetics for the different pre-inoculation UV-light conditions (spectra not shown). These kinetics are presented in Fig. 5 for a wavelength combination specific for stilbene VBF, excitation at 330 nm and emission at 400 nm. On one hand, the kinetics of the stilbene VBF of all samples with Flav (outdoors and UV) were very similar. On the other hand, the kinetics of the stilbene VBF of all samples devoid of Flav (greenhouse and no-UV) were very similar and clearly different from those of the samples with Flav. So we could group them (one group for samples with Flav and one group for samples without Flav) for a better statistical significance (Fig. 5). The fact that these two groupings could be made infers that there was no

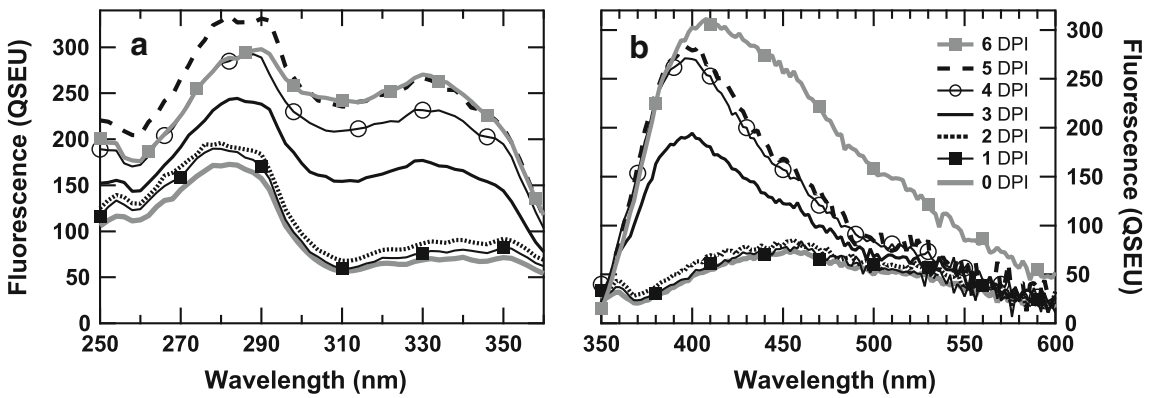

Fig. 4 Fluorescence spectra of the abaxial side of a no-UV leaf-half measured on the very same spot every day post-inoculation (*DPI*) by *P. viticola* during 6 days starting just before inoculation (0 DPI). **a** Excitation spectra (emission wavelength, 400 nm). **b** Emission spectra (excitation wavelength, 330 nm). Fluorescence is expressed in quinine sulfate equivalent units (QSEU)

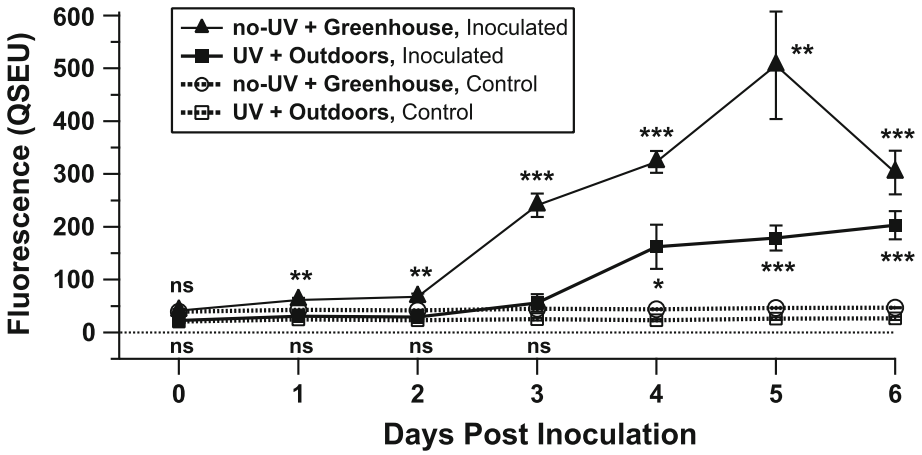

Fig. 5 Fluorescence (330 nm excitation, 400 nm emission) measured on the very same spot every day post-inoculation (DPI) by *P. viticola* during 6 days, starting just before inoculation (0 DPI) on inoculated or control leaves. Leaf-halves with Flav (*UV + Outdoors*) were grouped together as well as leaf-halves without Flav (*no-UV + Greenhouse*) for this analysis. Each point represents therefore the mean of five leaf-halves measured on the abaxial side, with the associated standard error of the mean (*error bars*). The results of ANOVA calculated every day between inoculated and control samples for each of the two groups, with Flav and without Flav, are shown above the markers for inoculated samples of *no-UV + Greenhouse*, and below the markers for inoculated samples of *UV + Outdoors*. *ns* not significant; * means are significantly different at $P < 0.05$; ** means are significantly different at $P < 0.01$; *** means are significantly different at $P < 0.001$. Fluorescence is expressed in quinine sulfate equivalent units (QSEU)

significant effect on the stilbene VBF of detaching the leaves during the 6 days of this experiment.

In Fig. 5 the different phases of the VBF described above can clearly be seen. Most importantly, the delay in stilbene VBF increase in the presence of Flav is striking. The difference between infected and control leaves became significant at 4 DPI, compared to 1 DPI in the absence of Flav. This suggests that the presence of constitutive Flav slowed down the infection by *P. viticola*.

Kinetics of Flav and HCA upon infection by *P. viticola*

The results of Flav and HCA Dualex measurements during the *P. viticola* infection are presented in Figs. 6 and 7, respectively. The lower epidermal Flav content in Fig. 6 than in Fig. 2, after the same time of exposition of the greenhouse leaves to outdoor sunlight, can be explained by the difference in PAR (cf. Agati et al. 2008). During the inoculation experiment the mean PAR was 26 mol photon m^{-2} day^{-1} compared to 46 mol photon m^{-2} day^{-1} during the UV-light acclimation experiment. For the four UV-light conditions, inoculation by *P. viticola* had no effect on Flav and HCA measurements (Figs. 6 and 7) during the first 6 days of the infection. Only the results for the UV and no-UV samples are shown because outdoors and greenhouse leaves gave similar results. We can conclude that there were no significant changes in Flav and HCA content during the development of the infection and so no synthesis or consumption of Flav and HCA was induced by *P. viticola* in the conditions of our experiments.

Leaf HCA content was higher in the samples devoid of Flav (greenhouse and no-UV) (Fig. 7) where in vivo

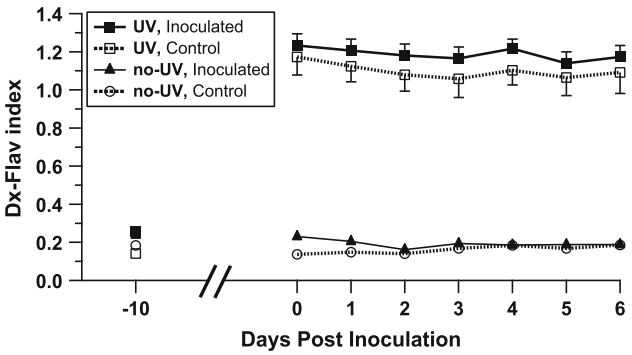

Fig. 6 Flavonols measured by Dx-Flav on *UV* and *no-UV* leaf-halves, inoculated (*Inoculated*) by *P. viticola* or not (*Control*). Measurements were done on the same spots before the UV-light conditioning (−10 days post-inoculation, DPI), just before inoculation (0 DPI) and every DPI during 6 days. Each point represents the mean of the total epidermal Flav content (adaxial plus abaxial) of nine measurements (three measurements for each of the three replicates) with the associated standard error of the mean (*error bars*). For *no-UV* leaf-halves the *error bars* were smaller than the size of the markers

stilbene VBF showed a faster *P. viticola* infection, an observation arguing against the role of HCA as constitutive compounds in the *V. vinifera* defence against *P. viticola*.

Discussion

As expected from previous experiments (Kolb et al. 2001; Kolb and Pfündel 2005; Bidel et al. 2007; Morales et al. 2010, 2011), the transfer of greenhouse-grown grapevine

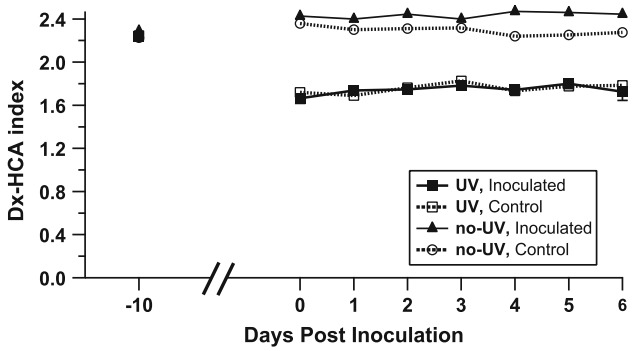

Fig. 7 Hydroxycinamic acid measurements by Dx-HCA on *UV* and *no-UV* leaf-halves, inoculated (*Inoculated*) by *P. viticola* or not (*Control*). Measurements were done on the same spots before the UV-light conditioning (−10 days post-inoculation, DPI), just before inoculation (0 DPI) and every DPI during 6 days. Each point represents the mean of the total epidermal HCA content (adaxial plus abaxial) of nine measurements (three measurements for each of the three replicates) with the associated standard error of the mean (*error bars*). For most of the data, *error bars* were smaller than the size of the markers. The Dx-HCA measurements were corrected for the influence of Flav (see "Materials and methods" for details)

leaves to full sunlight outdoor induced an increase in epidermal Flav content. This Flav synthesis was specifically induced by UV-B light (Kolb et al. 2001; Bidel et al. 2007) that was filtered out in the greenhouse. Indeed, UV-A were only partially filtered out in our greenhouse (Online Resource 2) and leaves were almost devoid of Flav (Fig. 2a). The synthesis of Flav is also positively correlated with the PAR (Agati et al. 2011a, b) and a contribution to Flav accumulation of the increase in PAR from greenhouse to outdoor (about 12 %, see Online Resource 2) was most probable. Still, the comparison between conditions 1 and 3 could not help to clarify this issue as the anti-UV filter also lowers the PAR by about 12 %. The kinetics of Flav synthesis in the leaves upon transfer from the greenhouse to outdoor were almost the same as those observed on *Ligustrum vulgare* L. upon transfer from 30 % daylight without UV to 85 % daylight (Agati et al. 2011b) and on *Morus nigra* L. upon transfer from the greenhouse to outdoor (Bidel L.P.R. and Cerovic Z.G., unpublished).

The anti-parallel behaviour of epidermal HCA kinetics to Flav kinetics after the plants were transferred outdoor has been observed previously, leading to the hypothesis that Flav may be synthesised at the expense of HCA since they are both derived from the same phenylalanine precursor (Bidel et al. 2007). In grapevine leaves, Kolb et al. (2001) observed, on the contrary, an increase in HCA upon transfer from the greenhouse to outdoor conditions, but this can be attributed to the large increase in visible light associated with the transfer. In their greenhouse, visible light was only 15 % of the outdoor intensity (Kolb and Pfündel 2005). Here the PAR was only about 12 % lower in the greenhouse than outdoor. Indeed, while the effect of light on HCA content depends on species (Bidel et al. 2007), it seems that in grapevine, HCA are stimulated by strong visible radiations (Kolb et al. 2001).

The phenolic composition found for Cabernet Sauvignon was qualitatively in accordance with previous analyses of leaf extracts for Silvaner (Kolb et al. 2001) and Sangiovese (Agati et al. 2008). The destructive HPLC–DAD analysis validated Dx-Flav measurements showing that no-UV leaves were devoid of Flav, even after *P. viticola* infection at 6 DPI. Indeed, the epidermal Flav content, measured by the Dx-Flav, is correlated to the total leaf Flav content (Kolb and Pfündel 2005; Barthod et al. 2007). These analyses showed a lower Flav content for UV samples than for outdoors samples. It was also seen on Dx-Flav data with a 15 % difference (data not shown). This 15 % difference is supposedly the consequence of a lower exposure of UV leaf-halves to sunlight. Indeed, the anti-UV filter present on the opposite half of the leaf, in addition to shading, added weight and changed the angle of the leaf (Online Resource 1).

Stilbenes are synthesized in response to the presence of *P. viticola*. They have a phytoalexin action on the pathogen that may be strong enough to stop the infection in some resistant genotypes (Chong et al. 2009) but not in a susceptible genotype like Cabernet Sauvignon. In susceptible genotypes, the pathogen spreads despite the increasing content of stilbenes (Poutaraud et al. 2010 and present study) as attested by the appearance of sporulation for inoculated leaves after a few days. In fact, the content of stilbene increases in parallel with the spreading of *P. viticola* in susceptible genotypes (Poutaraud et al. 2010). Based on these observations, the in vivo stilbene VBF was used as an indicator of the spreading of the infection by *P. viticola*. Although this seems legitimate, further experiments are needed to answer the question whether the intensity of the stilbene VBF may also be an indicator of the severity of the infection.

Flavonols are known to contribute to plant resistance against pathogens (Harborne and Williams 2000; Treutter 2005; Pourcel et al. 2007) but only a few reports show their implication in the resistance of grapevine against *P. viticola* (Dai et al. 1995a, b; Mondolot-Cosson et al. 1997; Agati et al. 2008). Indeed, greenhouse-grown leaves have been used for most studies on grapevine–*P. viticola* interactions, although these are devoid of Flav. This is one of the major differences with leaves grown in the field where Flav are present as constitutive compounds. Our results suggest that the presence of constitutive Flav slowed down the infection by *P. viticola* and, thus, confirms the findings of Agati et al. (2008) concerning the higher susceptibility to downy mildew of shaded leaves having less Flav.

A correlation between the *P. viticola* induced stilbene composition of grapevine leaves and the resistance level of

the genotype is now well established (Pezet et al. 2004; Chong et al. 2009; Alonso-Villaverde et al. 2011; Malacarne et al. 2011). Thus, it would be interesting to know if the presence of constitutive Flav has an influence on stilbene composition. Unfortunately, the present experiments did not allow us to give any conclusion about this: spectrofluorimetry in vivo is not able to discriminate between grapevine stilbenes (Poutaraud et al. 2007; Bellow et al. 2012).

Two reasons can be put forward to explain the decrease in stilbene VBF at 6 DPI for inoculated samples devoid of Flav (Fig. 5). First, stilbene synthesis might have decreased at 6 DPI due to a lower metabolism of these already damaged leaves (Malacarne et al. 2011). Second, it may be due to an increase in degradation of stilbenes by the pathogen. There is no data available on the degradation of stilbenes by *P. viticola*, but a laccase-mediated degradation of stilbenes (probably an oxidative detoxification) by *Botrytis cinerea* has been shown (Adrian and Jeandet 2006).

Both Dualex measurements of epidermal Flav (Fig. 6) and HPLC measurement of total Flav at 6 DPI (Fig. 3a) showed a total absence of Flav for greenhouse and no-UV leaves. This is in accordance with Dai et al. (1995b) who could not detect any Flav by histochemical microscopic observations on *V. vinifera* cv. Grenache greenhouse leaves infected by *P. viticola* during their 15 DPI investigation. While these authors could formulate the hypothesis that Flav formation plays an important role in the resistance for some other *Vitis* species, it appears not to be the case in *V. vinifera* where Flav seem to act only in the constitutive defence response against *P. viticola*.

Conclusions

In this study, the first use of two different Dualex devices in parallel and in connection with a deconvolution method allowed us to monitor both epidermal Flav and HCA non-destructively on the same Cabernet Sauvignon leaf region. From *P. viticola* inoculation to 6 days after, there were significant changes neither in Flav nor in HCA during this period of colonization of the leaves by the pathogen. In addition, the in vivo stilbene VBF was used as an indicator of *P. viticola* infection to investigate the implication of leaf constitutive Flav and HCA in the defence of *V. vinifera*. The accumulation of stilbenes, which took place for all inoculated leaves, started earlier for leaves with low flavonols content than for leaves with higher content, suggesting that constitutive flavonols are able to slow down the infection by *P. viticola*. On the contrary, constitutive HCA do not seem to play a role in the defence of Cabernet Sauvignon against *P. viticola*. The non-destructive nature of the methods used (in vivo stilbene VBF and Dualex) enabled us to follow the same leaf area before and after the inoculation by *P. viticola*. This alleviates the major problem encountered in destructive experiments: the large natural variability in leaf phenolic contents (Online Resource 3). Because of the non-invasiveness of these optical methods and the possibility to work on attached leaves, as demonstrated here, it would be very interesting to monitor *P. viticola* infection for much longer times with the prospects of using stilbene VBF for downy mildew diagnosis directly in the field. Another follow-up of this work would be the use of these techniques to investigate the role of Flav in the interaction of resistant grapevine genotypes with *P. viticola*.

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Conflict of interest ZGC declares a double link to the Force-A company: as one of the co-authors of the Dualex patent that the company exploits and as a part-time consultant to the company. Other authors have no competing interests.

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