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Optically-assessed preformed flavonoids and susceptibility of grapevine to *Plasmopara viticola* under different light regimes

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Abstract. The role of flavonoids in the response of plants to *Plasmopara viticola*, the phytopathogen agent of downy mildew, was studied in the *Vitis vinifera* L. cultivar Sangiovese. Grapevines in the vineyard were exposed to two light regimes, 100% and 35% of full sunlight in order to induce differences in total leaf polyphenolic content. Epidermal leaf phenolic compounds were assessed optically, using the Dualex chlorophyll fluorescence-based portable leaf-clip. Dualex data were calibrated by means of HPLC analysis of extracts from the same measured leaves. Good correlations were obtained with total flavonoid contents, which consist mainly of quercetin 3-*O*-glucuronide. From the Dualex non-destructive measurements, we showed that full-sun exposed leaves contained 75% more flavonoids than shaded leaves. Inoculation of leaves with *P. viticola* sporangia resulted in a significantly lower infected leaf area in sun-lit leaves compared with shaded ones, as seen from subsequent analysis of the downy mildew severity. These results indicated an inverse relationship between preformed flavonoids and the susceptibility of grapevines to downy mildew. The rapid optical method for the non-destructive assessment of flavonoids presented here could be useful for large scale screening and predicting *V. vinifera* susceptibility to *P. viticola*.

Additional keywords: chlorophyll fluorescence, downy mildew, optical sensors, polyphenols, Sangiovese, *Vitis vinifera* L.

Introduction

Downy mildew (*Plasmopara viticola*) represents one of the most severe infections in grapevines (*Vitis vinifera* L.), as it affects both the yield and the quality of wine production (Agrios 1997).

The disease is usually prevented by repeated fungicide treatments of entire vineyards which cause a high economic and environmental impact. Therefore, there is great interest in enhancing the natural defences of grapevines against *P. viticola* and in developing new tools for detecting the most susceptible plants and early infection in order to limit the use of chemical treatments, as required in precision viticulture (West *et al*. 2003).

Grapevine resistance to downy mildew involves complex and not yet fully understood mechanisms (Aziz *et al*. 2003; Hamiduzzaman *et al*. 2005; Kortekamp 2006; Allègre *et al*. 2007). The production and accumulation of phenolic compounds, including flavonoids, in response to a pathogen attack seems to be of primary importance (Dai *et al*. 1995*b*; Dixon and Paiva 1995). Moreover, preformed constitutive flavonoids are also involved in several host-pathogen interactions (Treutter 2006) and their antifungal activity is widely recognised (Skadhauge *et al*. 1997; Harborne and Williams 2000). Recently, a low susceptibility to powdery mildew (*Erysiphe necator*) in grapevines was related to high constitutive leaf phenolic compounds, mainly flavonol glycosides (Keller *et al*. 2003). New indications of the role of flavonoid metabolism in resistance to downy mildew in other pathosystems are emerging, like for the monocot *Pennisetum glaucum* L. against *Sclerospora graminicola* (Geetha *et al*. 2005; Niranjan Raj *et al*. 2006). We wanted to know whether preformed flavonoids could confer resistance to *V. vinifera*, a dicot, against *P. viticola*.

The biosynthesis of flavonoids is greatly affected by the intensity and spectral composition of leaf irradiance (Rozema

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et al. 1997; Kolb *et al*. 2001). This aspect can explain some of the mechanisms by which light influences plant–pathogen interactions (Raviv and Antignus 2004; Roberts and Paul 2006). Numerous studies have shown that in several species under shade environments there was an increase in infection caused by different pathogens (Pennypacker 2000; Roberts and Paul 2006). Interestingly, there was also evidence of an inverse correlation between infection severity and the intensity of preinoculation light treatments (Shafia *et al*. 2001).

The role of non-phytoalexin flavonoids in the response of grapevine to *P. viticola* was inferred from studies on *in vitro* plantlets (Dai *et al*. 1995*a*). The aim of this work was to verify the role of preformed flavonoids as defence compounds against downy mildew on plants grown in the field, in which the flavonoid concentration is much larger than in greenhouse-grown plants. In addition, the advent of non-destructive means to follow leaf flavonoids *in vivo* allowed us to overcome the inherent variability of leaf-to-leaf flavonoid content and to follow the same leaf during the season. Because of the novelty of the technique for grapevine, we also performed destructive flavonoid analysis by HPLC to verify the specificity and robustness of the non-destructive method.

Materials and methods

Plant material and experimental conditions

The experiment was conducted during the 2006 summer in the Northern part of the Chianti region, Tuscany, Italy (43°47′N, 11°35′E) at an altitude of 180 m a.s.l.

Grapevines of *Vitis vinifera* (L.) cultivar Sangiovese were cordon-trained and spur-pruned (4–5 spurs with two buds per vine), the rows were oriented in a North–South direction, and the spacing was 1 m in the rows and 3 m between the rows. The canopy was trained in a single curtain between 90 and 210 cm from ground level.

Two light regimes (100% and 35% of solar radiation) were applied in the vineyard in order to induce differences in the total polyphenolic content in grapevine leaves, while maintaining similar canopy microclimatic conditions other than radiation intensity.

Three parcels of 18 × 5 m were covered by plastic shading nets perpendicular to the rows, 1.5 m above the canopies to ensure sufficient ventilation. Analogous parcels exposed to full solar radiation were used as control. The shading nets were installed on 12 May 2006 (day of year (DOY) 132). Each parcel included ∼30 vines and measurements were performed on the 12 central ones. Radiance at full sun-light and under the tunnels was measured using a double monochromator spectroradiometer (model SR9910-PC, Macam Photometric Ltd, Livingstone, Scotland). Daily cycles of radiance were recorded and the daily radiant exposure calculated. Under shade conditions, this was found to be ∼35% of direct solar radiation independently of the wavelength. Air temperature, relative humidity and leaf temperature were measured outside and under the tunnels in order to evaluate the potential effects of shading on the canopy microclimate. Air temperature and relative humidity were measured by means of Hobo thermometric microstations (Onset Computer Corporation, Bourne, MA, USA; thermistor resolution of 0.02°C and precision of 0.1°C). Leaf temperature was measured using a handheld infrared thermometer (model 100.3ZL, Everest Interscience Inc., Tucson, AZ, USA) on both sun-exposed and shaded leaves. Thirty leaves from 15 different plants were used for both light regimes and monitored during a sunny day from 0900 to 1700 hours.

Optical estimation of phenolic compounds

Epidermal polyphenols (EPhen), which are representative of total leaf phenols (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). The instrument determines the epidermal absorbance in the UV-A, mainly due to flavonoids, by comparing the chlorophyll fluorescence (ChlF) signals at two different excitation wavelengths (375 and 650 nm) (Goulas *et al*. 2004; Cartelat *et al*. 2005).

Two adaxial and two abaxial measurements were recorded in sequence from the middle part of the leaf avoiding the main veins. The EPhen content of single leaves was defined as the sum of the adaxial (AD) and abaxial (AB) values, and these were the average of the two measurements taken from each side.

For calibration, 30 leaves (shaded and sun-exposed) with total Dualex units in the 1.1–3.3 range were collected on 29 June 2006, measured for AD and AB again in the laboratory and four disks (12 mm in diameter) per measured area were punched out and frozen in liquid N, for successive extraction and HPLC analysis.

Polyphenol extraction and analysis

Freeze-dried leaf disks were ground in liquid N and extracted with 4 mL (50 : 50) of MeOH : H_2O (pH 2.0) + 100 µL of internal standard (kaempferol 1 mg/mL) while stirred overnight. After 5 min of centrifugation, the pellet was re-extracted twice, the first time using 2 mL and the second using 1 mL of the extracting solvent. The final three pooled supernatants were defatted twice with 3 mL of petrol ether, and the water–methanol phase was dry evaporated. Sample were recovered with 1.5 mL (50 : 50) of MeOH : H_2O (pH 2.0) and analysed using a HPLC/DAD apparatus. Authentic standards of quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside and kaempferol (internal standard) were purchased from Extrasynthèse SA (Lyon, Nord-Genay, France). Quercetin 3-*O*-rutinoside was from Fluka (Sigma-Aldrich, Milano, Italy), and chlorogenic acid was purchased from Roth (Karlsruhe, Germany). HPLC analyses were performed using a HP1100 liquid chromatograph equipped with a diode array detector (DAD) and an atmospheric pressure ionisation (API)-electrospray mass selective detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) operating in negative ionisation mode under the following conditions: gas temperature 350°C, nitrogen flow rate 10.0 L min^{-1}, nebulizer pressure 40 psi, quadrupole temperature 40°C, and capillary voltage 3500 V. Fragmentor operated in the range 80–180 eV. Each sample was filtered through a 0.45 µm filter before HPLC analysis. Polyphenols were separated using a 4.6 × 250 mm Polaris E RP_{18} (5 µm) column (Varian, Darmstadt, Germany) operating at 27 ± 0.5°C. The eluent was H_2O (adjusted to pH 3.2 by HCOOH)/CH_3CN. A four-step linear gradient solvent system was used, starting from 95% H_2O to 100% CH_3CN during a 53-min period, at the flow rate of 1.0 mL min^{-1}, as previously reported by Saracini *et al*. (2005). UV-VIS spectra

were recorded in the 190–600 nm range, and chromatograms were acquired at 350, 315, 280 and 254 nm. Identification of individual polyphenols was carried out using their retention times, UV-VIS and mass spectra. Quantification of single phenolic compounds was directly performed by HPLC–DAD using a four-point regression curves built with the available standards. The main compound, quercetin 3-*O*-glucuronide, and the other quercetin glycosides were calculated using the calibration curve of quercetin 3-*O*-rutinoside (rutin) at 350 nm. Analogously, kaempferol glycosides were calibrated by using kaempferol 3-*O*-glucoside at 350 nm. The tartaric esters of cynnamic acids (caffeoyl-tartaric acid, *p*-coumaroyl-tartaric acid and feruloyl tartaric acid) were quantified at 315 nm with the calibration curve of chlorogenic acid. All the curves with $r^2 > 0.9998$ were considered. All solvents were HPLC grade and were obtained from E. Merck, (Darmstadt, Germany). The quantitative data of phenolic compounds were expressed as nmol cm^{-2} of leaf area.

Extinction coefficients of polyphenol standards

The extinction coefficient spectra of 40 μM rutin and chlorogenic acid (CGA) solutions in a phosphate buffer (pH 6.8) with addition of 1% NaCl (w/v) were recorded using a diode array spectrophotometer (HP8453, Agilent, Les Ulis, France).

Microscopy

Cross sections (50 μm thick) of fresh leaf tissue, cut with a vibratory microtome (Vibratome 1000 Plus, Vibratome, St. Louis, MO, USA), were mounted in a phosphate buffer (pH 6.8) with the addition of 1% (w/v) NaCl. Sections were observed through the inverted epi-fluorescence microscope as described earlier (Agati *et al*. 2002) after *in situ* treatment with 0.5% (w/v) aqueous ammonia (Hutzler *et al*. 1998). Ultraviolet (365 nm) excitation and blue (470 nm) detection were used to selectively localise hydroxycinnamic acids, whereas blue excitation (436 nm) and yellow (546 nm) and red (680 nm) detection were used to image flavonoids and chlorophyll (Chl), respectively. Image spatial calibration using an oil-immersion × 40 Fluor (NA = 1.3) objective was 0.197 μm per pixel.

Monochrome images were also recombined after band-colour assignment in a single multicolour image with light-blue, yellow and red attributed to the 470, 546 and 680 nm fluorescence images, respectively. Profiles of fluorescence along the leaf cross sections were obtained by plotting the mean intensity of each row of pixels *v*. depth starting from the adaxial leaf surface.

Stomata density was evaluated, by means of the impression method previously reported (Palliotti *et al*. 2000), on the abaxial side of shaded and sun-exposed leaves (from the middle part of the shoots, between the 15th and 20th node from the base). Stomata from four 404 × 404 μm spots, corresponding to a leaf area of ~65 10^4 μm^2, were counted by using a ×10-objective and expressed as the mean ± s.d. for each light level ($n = 12$ different leaves).

Plasmopara viticola *inoculation*

Selected leaves, attached to the plants, located at the middle part of the shoots (between the 15th and 20th node from the base) were inoculated in the evening by spraying their abaxial surfaces with a sporangial suspension of *Plasmopara viticola* and enclosed in polyethylene bags overnight for 12 h.

Two inoculation experiments were performed on 7 July (DOY 188) and 20 July (DOY 201). In the first experiment (DOY 188), a suspension containing 10^6 sporangia mL^{-1}, measured by using a Burker counting chamber, was used to infect 48 (24 sun-exposed and 24 shaded) leaves, each from a different plant in the vineyard. Development of downy mildew infection was visually monitored and colour pictures of the adaxial side of all infected leaves were taken 10 days after inoculation (DAI), directly in the field, when oil spots were completely developed.

In the second experiment (DOY 201), a lower concentration (1.6×10^5 sporangia mL^{-1}) of the sporangia suspension was used to inoculate 72 (36 sun-exposed and 36 shaded) leaves. Colour pictures of the adaxial side of all inoculated leaves were taken in the field 12 DAI to be sure that oil spots were completely developed.

The severity of the downy mildew infection was assessed by an image elaboration of leaf pictures, using the ImageJ 1.34s public domain software (http://rsb.info.nih.gov/ij/, accessed 20 April 2004). The colour images were split into their RGB components. The red (R) monochrome component showed the greatest contrast between infected (light grey) and healthy (dark grey) tissues. A threshold in the grey levels was used to select the infected area. *P. viticola*-infected leaf area and total leaf area, manually selected, were then measured, with calculations being limited to and not limited to thresholded pixels, respectively. Since the grey level of leaf veins could not be distinguished from the infected tissue, vein area relative to the total leaf area was evaluated separately on healthy sun-exposed and shaded leaves using the same above method, and this was found to be ~6%. This amount was then subtracted from the evaluation of infected leaf areas. Severity was expressed as a percentage of infected area with respect to total leaf area.

Statistical evaluations

Results of measurements were subjected to a one-way analysis of variance to compare the mean values for the two light regimes. Curve fitting of experimental data and calculation of the 95% confidence limits were performed by using TableCurve 2D Windows v3.04 (Jandel Scientific Software, Corte Madera, CA, USA).

Results and discussion

Optical estimation of grapevine leaf polyphenols

The non-destructive optical measurement of leaf epidermal polyphenols performed by Dualex was calibrated by using HPLC analysis of extracts from the same leaf area.

The HPLC analysis of leaf polyphenols revealed the presence of the following compounds: quercetin 3-*O*-glucuronide, quercetin 3-*O*-glucoside, rutin, kaempferol 3-*O*-glucuronide, kaempferol 3-*O*-glucoside, and the tartaric esters of caffeic, *p*-coumaric and ferulic acid (caftaric, coutaric and fertaric acids). The main polyphenol compound present in Sangiovese was quercetin 3-*O*-glucuronide (Que 3-*O*-glc) which accounted for 60–70% of the total polyphenols. The other quercetin and

kaempferol glycosides were also found, in lesser amounts, in the leaf extracts. Among the hydroxycinnamates, caftaric acid was the most abundant molecule. The Sangiovese polyphenol composition found in this study was qualitatively in accordance with previous analyses of leaf extracts from the *V. vinifera* cultivar Silvaner (Kolb *et al*. 2001).

By plotting the Dualex values against polyphenol amounts measured by HPLC, we evaluated which class of compounds was best correlated with the Dualex optical signature. The best correlation was found between the total Dualex values, adaxial plus abaxial, and total flavonoids (Flav) expressed on an area basis ($r^2 = 0.938$) (Fig. 1*A*). This result was explained by the high absorbance of quercetin glycosides at 375 nm (Fig. 2), the detecting wavelength of the Dualex leaf-clip. On the contrary, Dualex data were not correlated at all with area-based total hydroxycinnamic acids (HCA) (Fig. 1*B*). A good relationship was also obtained between the Dualex values and the total phenolics (Phen) ($r^2 = 0.821$), probably because of the larger concentration of Flav with respect to HCA.

Since the Dualex detects the epidermal absorbance at 375 nm and HCA have a maximum absorbance at shorter wavelengths (Fig. 2), Dualex measurements were largely insensitive to the presence of HCA (Cerovic *et al*. 2005). This explains the extensive dispersion of data in Fig. 1*B*. Caffeoyl tartaric acid, the most represented HCA in Sangiovese leaves, would contribute significantly to Dualex measurements (Fig. 2) only if it were all located in the epidermis; however, it was not (cf. Fig. 3). Therefore, the most important aspect affecting detection of HCA by optical non-destructive methods was related to the compound localisation within the leaf tissue. This was evidenced by the multispectral fluorescence microscopy data that are reported in Fig. 3. Acquiring in sequence the fluorescence signal on a leaf cross section, treated with ammonia, at 470 (UV excitation) and 546 nm (blue excitation), we could selectively separate the distribution of HCA (Fig. 3*A*) from that of Flav (Fig. 3*B*). In Fig. 3*C*, the two above images were superimposed, along with the fluorescence image of Chl at 680 nm, in order to better visualise the co-localisation of compounds. The yellow fluorescence of Flav came mainly from vacuoles of the epidermal cells, whereas light-blue fluorescence of HCA was present in the walls of epidermal cells and mostly within the vacuoles of palisade cells. The fluorescence profiles from the adaxial cuticle towards the inside of the leaf (Fig. 3*D*) clearly evidenced the different distribution of Flav and HCA. Epidermal Flav, located within the first 20 µm were spatially separated from most of the Chl molecules, located deeper than 30 µm. This spatial separation was a fundamental prerequisite of the spectroscopic method

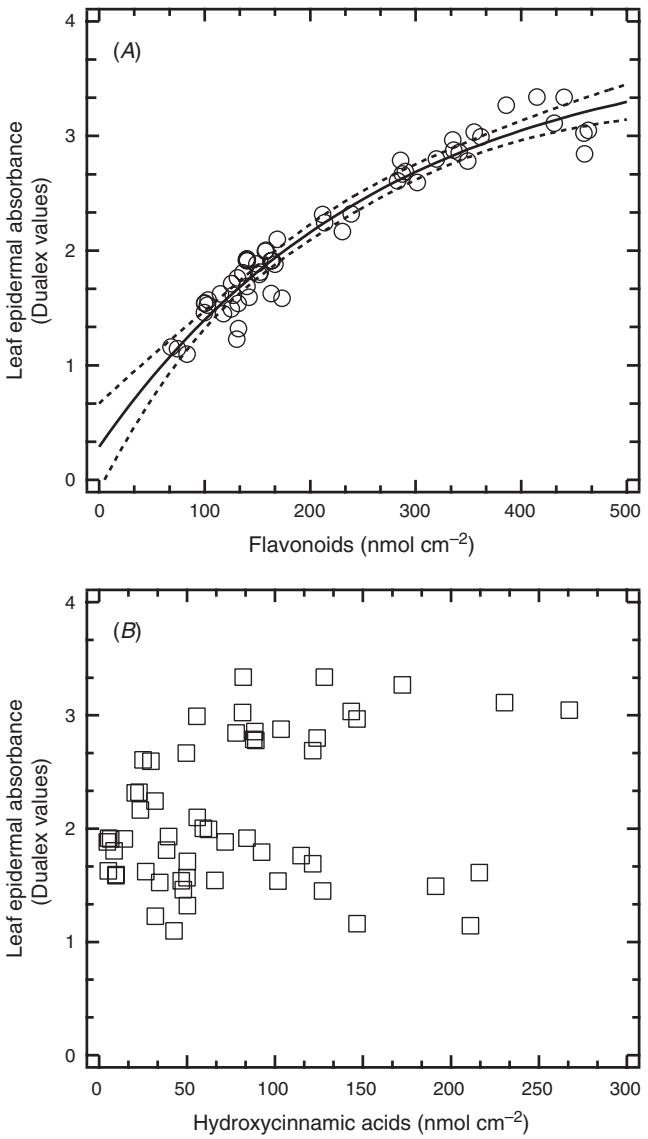

Fig. 1. Relationship between optically assessed leaf epidermal absorbance and leaf content of polyphenols quantified by HPLC of methanolic extracts in *Vitis vinifera*. (*A*) Dualex (adaxial plus abaxial) values *v*. flavonoids expressed on an area-basis; dashed line is the exponential fitting curve with $y = 3.85 - 3.56^{(-0.0037x)}$, $r^2 = 0.938$. Dotted lines indicate the 95% confidence limits. (*B*) Dualex (adaxial plus abaxial) values *v*. hydroxycinnamic acids expressed on an area-basis.

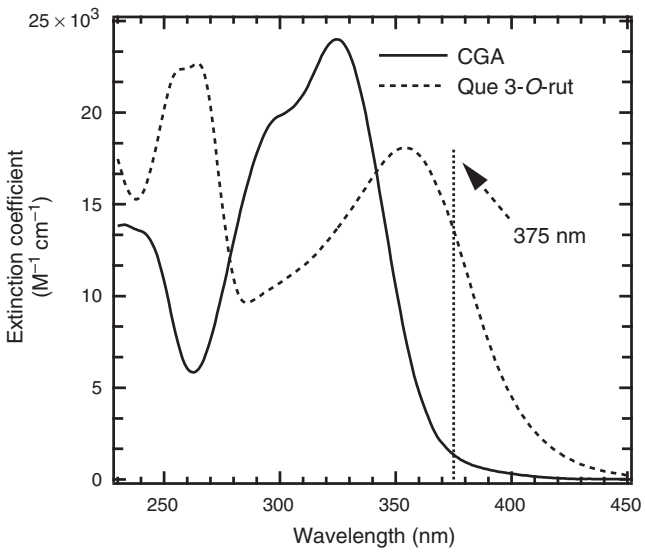

Fig. 2. Extinction coefficient spectra of chlorogenic acid (CGA) and rutin (quercetin 3-*O*-rutinoside (Que 3-*O*-rut)) in phosphate buffer (pH 6.8) with addition of 1% (w/v) NaCl.

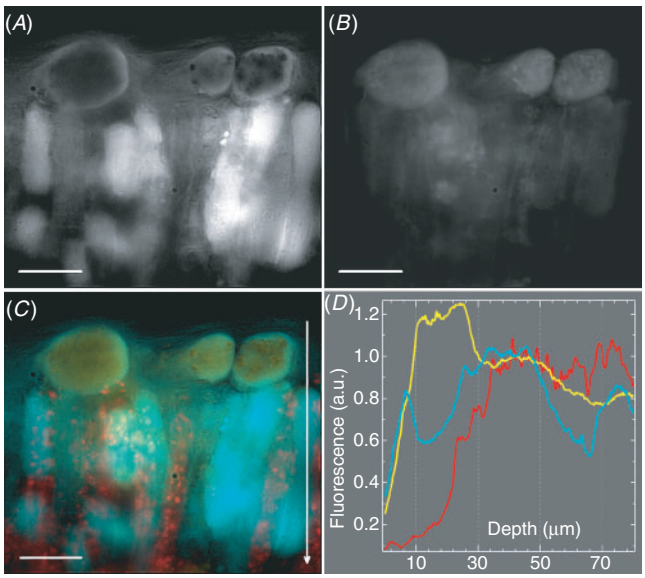

Fig. 3. Multispectral fluorescence microscopic analysis of a cross section of *Vitis vinifera* leaf treated with 0.5% (w/v) aqueous ammonia. (*A*) Fluorescence image excited at 365 nm and acquired at 470 nm showing hydroxycinnamic acids, (*B*) fluorescence image excited at 436 nm and acquired at 546 nm showing flavonoids. (*C*) Recombination of images in (*A*) and (*B*) coloured in light-blue and yellow, respectively, along with the chlorophyll fluorescence image acquired at 680 nm under blue excitation, coloured in red. Bars = 20 μm. (*D*) Fluorescence intensity profiles of the three fluorescence signals, hydroxycinnamic acids (light-blue), flavonoids (yellow) and chlorophyll (red), from the adaxial cuticle towards inside the leaf as indicated by the arrow in (*C*).

on which the Dualex device was based (Bilger *et al*. 1997). Accordingly, we found a good correlation between Dualex data and Flav leaf contents. In the case of HCA, they were mainly co-localised with Chl inside the palisade cells and therefore they cannot contribute to the epidermal absorbance assessed by the Dualex device. From the spectral and structural data presented above, it could be concluded that the Dualex leaf-clip almost exclusively detects flavonoids in grapevine leaves.

Evaluation of the severity of P. viticola *infection*

An example of sun-exposed and shaded leaves 10 days after inoculation is in Fig. 4*A* and *B*, respectively. Yellow 'oil spots' could clearly be seen on the adaxial surface of the shaded leaf. By splitting colour images into their RGB channels (Fig. 4*C*, *D* and *E*), we found that the red component (Fig. 4*C*) presented the highest contrast between infected and healthy leaf tissues. We then used the red channel image for further elaboration. The oil spots presented higher grey level values with respect to non-infected areas and could then be selectively identified by using a threshold, as reported in red in Fig. 4*F*. Measuring the leaf area of all pixels and those with intensity above the threshold made it possible to calculate the percentage of infected leaf tissue.

Relationship between leaf total flavonoids and leaf resistance to downy mildew

The evaluation of severity of *P. viticola* infection on grapevines is in Table 1 as the average percentage over all the sun-exposed

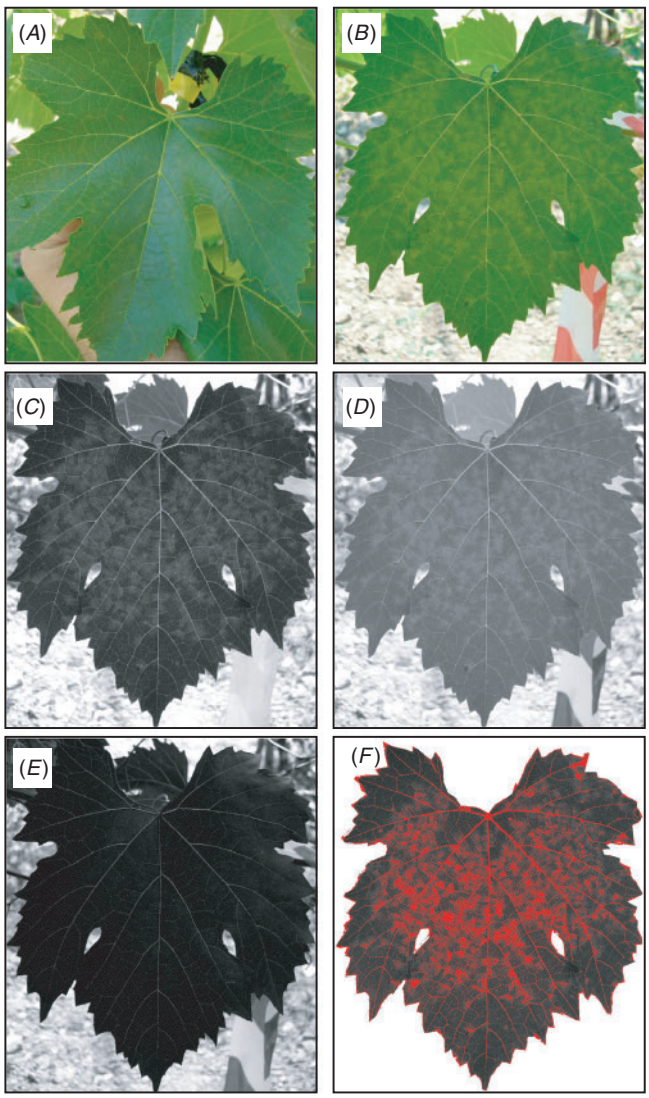

Fig. 4. Example of *Vitis vinifera* leaves showing downy mildew symptoms and image elaboration to assess severity of *P. viticola* attack. (*A*) Sun-exposed and (*B*) shaded leaves from inoculation experiment on day of the year (DOY) 188. (*C*), (*D*) and (*E*) RGB components, respectively, of image in (*B*). (*F*) infected area, in red, evidenced by thresholding.

and shaded infected leaves, along with the average content in Flav which was derived from the optical non-destructive measurement. Flav content was expressed in molar units of rutin-equivalents, as calculated from the Dualex-derived absorbance divided by the molar extinction coefficient of 13.5 μmol^{-1} cm^2 at 375 nm (Fig. 2).

For both inoculation experiments (DOY 188 and 201), the infected leaf area in sun-exposed leaves was significantly smaller than that of the shaded ones ($P < 0.0005$). The greatest severity of the disease observed following inoculation at DOY 188, as compared with DOY 201, was due to the greater sporangia concentration used. Furthermore, the less aggressive sporangia dose employed at DOY 201 induced a superior differentiation of resistance to the fungus between sun-exposed leaves and shaded leaves. In fact, the ratio of the average infected-leaf area of shaded to sun-exposed leaves was ~3 and 1.7, following the

Table 1. Flavonoid content and severity of *Plasmopara viticola* infection on grapevine leaves under two light regimes (100% and 35% solar irradiance)

One-way ANOVA resulted in a P value <0.0005 for all the sun/shade pairs. Data are mean ± s.d. $^{A}n = 22$, $^{B}n = 34$. DOY, day of the year

	Inoculation DOY 188[A]		Inoculation DOY 201[B]	
Sunlight irradiance (%)	Flavonoids (nmol cm^{-2})	Infected leaf area (%)	Flavonoids (nmol cm^{-2})	Infected leaf area (%)
100	191.3 ± 14.0	15.51 ± 9.24	180.6 ± 21.7	4.97 ± 3.58
35	110.2 ± 19.9	26.37 ± 7.55	101.6 ± 15.9	15.71 ± 8.40

DOY 201 and DOY 188 inoculations, respectively. The level of constitutive Flav in the grapevine leaves before inoculation was homogeneous for both experiments and was found to be 75% higher in sun-exposed *v*. shaded leaves ($P < 0.0005$). Therefore, our results suggested an inverse relationship between the leaf Flav content and the susceptibility to *P. viticola*. This link was better evidenced by averaging Flav values of leaves grouped within classes of infected areas (Fig. 5). For both inoculation experiments, it could be noted that a higher level of infected relative leaf area corresponded to a lower Flav content.

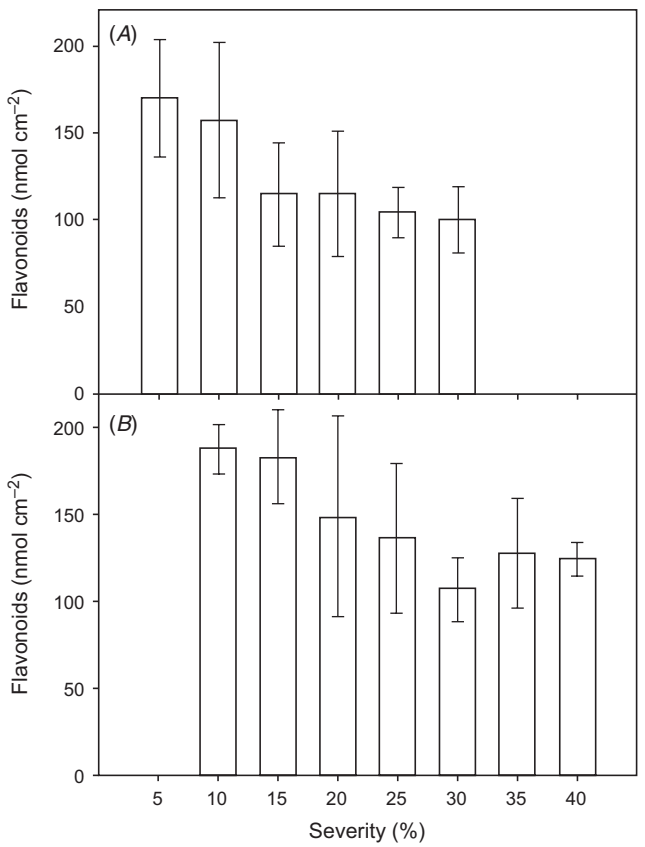

Fig. 5. Relationship between leaf flavonoids and severity, expressed as percentage of infected leaf area, of *Plasmopara viticola* from inoculation experiment on (*A*) day of the year (DOY) 201 and (*B*) DOY 188. Inoculated leaves of *Vitis vinifera* were divided in classes of severity and the corresponding total flavonoid (Flav) contents (mean ± s.d.) is reported in the ordinate axis.

The mean diurnal leaf temperature for sun-exposed and shaded leaves was 26.8 ± 0.4 and 27.8 ± 1.7 at 0900 hours ($P > 0.1$), respectively, and reached a maximum of 29.6 ± 0.5 and 30.8 ± 1.0 at 1500 hours ($P > 0.025$), respectively. Yet, no significant differences in air temperature and relative humidity between the sun-exposed and shaded sites were observed (Table 2). The similar canopy microclimatic conditions were assured by the quite tall (1.5 m above the canopies) shading structure that permitted high ventilation of the vineyard.

The quality of radiation was also unchanged by the filtering net, as shown by the ratio between UV and visible radiation (Table 2). Therefore, potential effects on the downy mildew diffusion due to different canopy microclimatic parameters other than light intensity could be ruled out.

It is known that light exposure has an inhibitory effect on the sporulation of *P. viticola*, as assessed by irradiating inoculated leaf samples under laboratory-controlled conditions (Brook 1979; Rumbolz *et al*. 2002). In our experiment, inoculation was performed in the field at night, therefore, all the treated leaves stayed in the dark during the first hours of the infection process. Moreover, the intercellular fungal colonisation seems not to be affected by light (Rumbolz *et al*. 2002).

Differences in leaf anatomy between sun-exposed leaves and shaded leaves may also play a role in the diffusion of the fungal disease. Stomata density on the abaxial leaf surface was 27% higher ($P < 0.01$) in sun-exposed leaves compared with shaded ones (249 ± 44 with respect to 197 ± 30 stomata × mm^{-2}), in agreement with data reported for the grapevine cultivars Cabernet Franc and Trebbiano Toscano (Palliotti *et al*. 2000). This would favour infection of the pathogen in sun-exposed leaves, in opposition to the observed results, and would even attenuate the observed relationship between Flav and severity. No significant differences were observed in the length of shoots and number of leaves per shoot between sun-exposed and shaded grapevine plants. On the other hand, sun-exposed leaves were thicker than shaded ones (+60%, as seen under microscopic view of leaf cross sections, data not shown).

Induced defence mechanisms, such as the production of phytoalexins, are known to contribute to the protection of grapevines against downy mildew (Hammerschmidt 1999). Synthesis and accumulation of stilbenes at the site of infection is a very fast response of the plant. A light dependency of the pathogen-induced production of stilbenes could explain the different extension of lesions in infected sun-exposed and shaded leaves. To our knowledge, no controlled studies on the combined effect of light and *P. viticola* elicitation of phytoalexins have been reported. It is known that the synthesis of stilbenes can

Table 2. Climatic parameters at the grapevine canopies under the two light regimes
One-way ANOVA for all the sun/shade pairs[A,B,C,D] resulted in P values >0.5

Vineyard site	Air temperature (°C)		Relative humidity (%)		Daily light exposure (MJ/m^2)	UV/Visible (%)
	Minimum[A]	Maximum[B]	Minimum[C]	Maximum[D]		
Sun-exposed	14.95 ± 3.08	29.11 ± 4.34	37.96 ± 8.50	97.58 ± 1.51	12.22	13.2
Shaded	15.26 ± 3.06	29.35 ± 4.42	37.55 ± 8.64	97.97 ± 1.62	4.52	12.7

be induced by UV-C radiation (Poutaraud *et al*. 2007; and references therein). Natural sunlight cannot directly induce the synthesis of resveratrol and related compounds because of its very low intensity below 300 nm at the earth level (Langcake and Pryce 1976). Accordingly, no significant amounts of stilbene derivatives were found in healthy *V. vinifera* leaves under natural conditions (Keller *et al*. 2003). On the other hand, different light intensities play a role in the regulation of phenylpropanoid biosynthetic pathway enzymes, and then have a potentially indirect effect on the stilbene accumulation. Under full sunlight, several phenylpropanoid pathway genes, such as those related to the synthesis of flavonoids, can be upregulated in a coordinated way (Jaakola *et al*. 2004). This is probably what happened in our grapevine experiment, according to the higher content in flavonoids found in the sun-exposed leaves compared with the shaded ones.

Previous studies on grape berries with a different content of anthocyanins have suggested a competition between chalcone synthase (CHS), the key enzyme in flavonoid biosynthesis, and stilbene synthase (StSy), the key enzyme of stilbene production (Jeandet *et al*. 1995). This competition hypothesis was questioned by the evidence that the induced synthesis of resveratrol at different developmental stages was similar in both red- and white-skinned grapes (Bais *et al*. 2000). Also, within the competition hypothesis, *P. viticola* would be expected to induce an enhanced synthesis of stilbenes in shaded rather than in sun-exposed leaves, with consequent minor leaf damage under lower light levels, but the opposite was found in our study.

Conclusions

Our results indicated that flavonoids can be significantly involved in the process responsible for the larger resistance to downy mildew in sun-exposed *v*. shaded grapevine leaves, although we cannot conclusively identify the main mechanism involved. The data were consistent with the antifungal activity possessed by flavonoid compounds (Picman *et al*. 1995; Skadhauge *et al*. 1997; Harborne and Williams 2000) and with their prompt accumulation in leaf tissues following *P. viticola* inoculation (Dai *et al*. 1995*a*). Localisation of flavonoids in the stomatal cells (Dai *et al*. 1995*b*), the site of pathogen entrance, and in nearby epidermis cells further support their antifungal action.

It is reasonable to expect that compounds already present in the plant at the time of attack would be more efficient in limiting pathogen diffusion than induced defence compounds. In this context, stimulation of flavonoid biosynthesis and accumulation in the leaves would be beneficial for the plant's protective mechanisms. The Protein Competition Model states that any stress factor experienced by the plant will induce a shift of carbon allocation towards secondary rather than primary metabolism (Jones and Hartley 1999). Accordingly, high-intensities of visible and UV radiation can enhance the production of phenolic compounds, especially flavonoids. The higher biosynthetic cost of flavonoids with respect to simpler polyphenols can be understood considering that they fulfil multi-functional roles such as the screening of UV radiation, scavenging of reactive oxygen species and also inhibition of pathogen's activity.

At present, what is the relative contribution of preformed flavonoids compared with induced stilbenes, and other defence reactions, to the higher resistance towards *P. viticola* of full-sun-exposed *V. vinifera* cannot be asserted. Studies have already been initiated to gain more insight on this important aspect (Poutaraud *et al*. 2007).

The present study has also shown that the level of leaf flavonoids in grapevines can be rapidly estimated by a non-destructive optical device. This approach can therefore be useful to predict susceptibility of grapevine to downy mildew, with the aim to reduce and localise fungicide treatments.

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