First detection of the presence of naturally occurring grapevine downy mildew in the field by a fluorescence-based method†‡

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Early detection of fungal pathogen presence in the field would help to better time or avoid some of the fungicide treatments used to prevent crop production losses. We recently introduced a new phytoalexin-based method for a non-invasive detection of crop diseases using their fluorescence. The causal agent of grapevine downy mildew, Plasmopara viticola, induces the synthesis of stilbenoid phytoalexins by the host, Vitis vinifera, early upon infection. These stilbenoids emit violet-blue fluorescence under UV light. A hand-held solid-state UV-LED-based field fluorimeter, named Multiplex 330, was used to measure stilbenoid phytoalexins in a vineyard. It allowed us to non-destructively detect and monitor the naturally occurring downy mildew infections on leaves in the field.

Introduction

Viticulture and winemaking are both important economic activities and cultural issues in Europe. To protect their grapevines, European wine growers use 70 000 tons of pesticides each year that cost almost two billion euros. Most are fungicides, because fungal diseases can induce crop losses up to 70%. This is the motivation behind the European directive 128/2009/EC, whose aim is to implement a more sustainable approach to the use of plant protection products.

Fungicides aim to prevent two main diseases, powdery mildew and downy mildew, the latter being usually considered as the most damaging disease in viticulture. The downy mildew infectious agent is an oomycete named Plasmopara viticola (Berk & M.A. Curtis) Berl & de Toni. After one to two weeks of being present in the leaf it produces visible symptoms known as oil spots. One of the reactions of plants to both downy and powdery mildew is the synthesis of a variety of stilbenoid compounds. A useful characteristic of grapevine phytoalexins is that they produce a UV-induced violet-blue fluorescence (VBF). In vitro, the excitation maximum is at 320 nm and the fluorescence emission maximum is at 380 nm. In vivo, they are slightly shifted to longer wavelengths, with the excitation maximum at 330 nm and the emission maximum at around 400 nm.

This autofluorescent property of the stilbenoid phytoalexins, which is absent from healthy leaves, was exploited to detect the presence of downy mildew in greenhouse-grown plants, in outdoor-grown plants and in the field. Microscopic studies on live leaf pieces have shown that the fluorescence is mainly localised in epidermal cell walls close to the leaf surface.

The development of a portable fluorescence sensor, Multiplex 330 (FORCE-A, Orsay, France), hereafter Mx-330, allowed the application of this diagnostic method to leaves attached to the plant. In the greenhouse, infected leaves could be discriminated from control leaves from the first day post infection (DPI) on the abaxial side of leaves and the DPI 3 on the adaxial side. In the field, infected leaves could be discriminated from the control ones starting from DPI 6 on both sides. This is encouraging because there is a higher probability for leaves to be seen from the adaxial side by a vehicle-mounted sensor in the field. In addition, the adaxial side of the leaf displayed the same type of kinetics of VBF changes upon infection. This was the first demonstration of presymptomatic disease detection with real-time capacity for in field proximal sensing. None of the cited studies were done on naturally occurring infections.

The objective of this work was to follow naturally occurring infections from the local inoculum without knowing the time of infection. It was done on marked leaves in the field at various leaf levels in order to compare the Mx-330 sensing to visual assessments of the disease symptoms.
Material and methods

Experiment design
without other disease symptoms was compared to the VBF signal of leaves showing no symptoms at all. No differences could be found (Fig. S2 in the ESI†). In addition, it is not known whether black rot induces synthesis and accumulation of stilbenoids in grapevine leaves. Therefore, the presence of black rot was not taken into account for a further analysis of the VBF of leaves. On the other hand, the downy mildew infection led to a severe epidemic. For these reasons we considered *P. viticola* as the main cause of the changes in VBF.

The grapevine leaf VBF measured with the Mx-330 can be the result of additive contributions of several fluorophores. In healthy leaves it is mainly due to hydroxycinnamic acids. In *P. viticola* infected leaves the VBF of induced stilbenoids adds up to this autofluorescence. Moreover, the VBF of both healthy and infected grapevine leaves is always larger on the abaxial leaf side than on the adaxial one. This is why adaxial and abaxial VBF measurements need to be considered separately. Individual-leaf kinetics of VBF (Fig. 1) corresponded to the ones seen with artificial *P. viticola* infections in the greenhouse and in the field. Since the infections here occurred randomly from inoculum sources within the vineyard the date of appearance was different among leaves (Fig. 1). At the beginning of the measurement period, VBF levels measured by Mx-330 were around 60 mV and 95 mV, for the adaxial and the abaxial leaf sides, respectively, *i.e.* the usual level found in healthy leaves. It was followed by a significant transient increase in VBF with a highly variable VBF peak value. The VBF decreased thereafter with a general tendency to remain higher than in healthy leaves. The VBF signal is also dependent on the percentage diseased area of the measured leaf surface. Therefore, the epidemic development of the polycyclic pathogen complicates the kinetics of the VBF signal because a variable portion of the leaf surface area can be infected by a primary and a secondary infection on the same leaf (Fig. 1, leaf no. 1). Thus a global analysis of the VBF of a population of leaves was necessary to characterize the downy mildew infection at the plot level. For this global analysis we kept the six leaf categories separate: low, middle and high canopy heights at the east and the west row sides.

The VBF incidence kinetics for the six leaf categories were plotted separately for the adaxial (Fig. 2A) and the abaxial
(Fig. 2B) leaf side and compared to visual incidence measurements (Fig. 2C). Each category is represented by the mean of 20 marked leaves. The VBF incidence was earlier than visual incidence. At DOY 168, depending on the leaf category, 15 to 45% of leaves were classified as infected by abaxial VBF incidence compared to only 0 to 10% by visual assessment. This was also true for adaxial VBF incidence but with a lower value, 5–10%, and only for two categories. Visual incidence was already 5% for two categories since DOY 157, but it should be noted that this 5% corresponded to a single leaf.

VBF incidence showed a clear valley at DOY 174–176 before a subsequent large increase and at a time when visual incidence was sharply increasing (Fig. 2A and B). This was the direct consequence of the bell-shaped kinetic of VBF following *P. viticola* infection briefly described above (Fig. 1). When the VBF of the first infected leaves decreased after DOY 171 it decreased under the threshold, so these leaves were not counted as infected anymore while the leaves infected in the second phase had a VBF still below the threshold (Fig. 1). The large increase in VBF incidence was slowing down after DOY 182, and was even decreasing for two categories on the adaxial side. This multi-phase behaviour, which was also seen using visual incidence, especially with the plateau at DOY 171–182, was most probably related to the succession of primary, secondary and even higher-order infections.

The three leaf categories (low, middle and high) were well separated during the last infection phase (DOY 182–192) both in the visual incidence (Fig. 2C) and in the adaxial VBF incidence (Fig. 2A). The difference in kinetics of the three categories of leaves can be linked to the epidemiology of downy mildew. The primary inoculum is mainly found on the ground, closer to the low leaves contaminated by rain splashing.

The effect of the row side on leaf attributes, especially photosynthesis, is known for north/south oriented rows, but it seems to be too subtle to reflect on downy mildew incidence (Fig. 2) The east/west row-side dichotomy had no significant influence on incidence nor severity, independent of the assessment technique.

Fig. 3 confirms that VBF may be used to estimate disease severity. As expected, the correspondence was better when abaxial VBF severity was compared to visual severity. In fact, the adaxial VBF showed significant severity only after DOY 180. Abaxial VBF severity (Fig. 3A) followed the visual leaf severity kinetics (Fig. 3B) and even more the visual plot severity kinetics (Fig. 3C). This implies that the proportion of infected leaf area containing stilbenoids influenced the VBF signal more than the stilbenoid content per unit surface area.

Visual leaf severity showed a transitory peak at DOY 168 for the east-low and west-high leaf categories. The decrease after the peak is a consequence of the appearance of newly infected leaves with lower severity after DOY 168. These newly infected leaves contribute mathematically to the decrease in the mean. This coincided with the appearance of the first visually detected infected leaves in three other categories (Fig. 2C and 3C). This is another sign of the beginning of the second phase of infection.

**Proximal sensing of diseases**

The temporal and spatial dynamics of plant pathogens can be quantified by visually assessing disease intensity (incidence and severity). However, the accuracy and precision of visual disease assessments performed by different raters continues to be called into question. In addition, a sensitive automatic
mapping of diseases is needed for precision pest management. Indeed, until now the successful reflectance-based remote sensing of diseases was limited to the changes in green biomass due to defoliation. Fluorescence, although technically more demanding than reflectance, is a far more sensitive technique. Under practical agronomical conditions the difference is about a thousand fold. The theoretical sensing limit of fluorescence is a single molecule. Furthermore, fluorescence can reveal molecules that absorb UV light, like stilbenoids, that cannot be seen by reflectance. Previous attempts to use fluorescence sensing in the field concerned yellow rust in wheat using a xenon lamp-based imaging spectrograph. More often, the experiments were restricted to greenhouses using, for example, laser-induced detection of chlorosis in citrus or to the laboratory even when a UV lidar was used for wheat rust detection. As reviewed recently, crop disease sensing using fluorescence in the field is still in its infancy. The latest attempt investigated leaf diseases in barley using the Multiplex 3 fluorescence sensor. Thermal imagery is another interesting optical sensing technique. It was applied to downy mildew detection on grapevine, but only with artificial inoculation on individual leaves in the greenhouse. This restriction was also applied in the latest attempt to use variable chlorophyll fluorescence imaging on P. viticola infected leaves.

The present version of the sensor has a limited functioning distance to a few centimetres. This limits tractor-mounted sensing. However, an earlier version of the Multiplex was already mounted on a parallelogram frame (a ski) on a tractor in order to glide along the canopy and to allow continuous mapping of leaf characteristics. With the development of new more powerful LEDs, UV-based non-contact fluorosensing from a larger distance will be possible. This was already done with the Multiplex 3. We are currently working on the implementation of such a powerful UV source to a new version of the sensor meant to be mounted on tractors for continuous mapping.

The variability of diseases in the field can be temporal, due to the kinetics of the infection, and spatial, because of the spreading of the infection from the initial hot spots. Therefore, both temporal and spatial surveys of diseases are important for efficient prevention and treatment. Even if not specific for the downy mildew, the VBF has the advantage of detecting leaves with visible symptoms and can also detect asymptomatic early stage infections, even in the field, as shown in this work. The advantages of early and automatic detection of disease outbreaks will be twofold. First, it would help viticulturists to choose the right curative plant protection product, a group known to be more efficient in the early phases of infection. Second, it would provide objective information on the first primary infection that is needed as an input variable for forecast models based on meteorological data. The VBF-based method will allow early detection of suspicious hot spots or larger zones of the vineyard. The subsequent identification of the origin of the disease or of the abiotic stress can be done by other more specific sampling techniques. The automatic mapping will also be useful in order to comply with the European regulation for organic viticulture. This regulation (EC 834/2007) allows the application of authorised plant protection products only in case of an established threat to the crop. Mounted fluorescence sensors on tractors will allow these surveys while the grower is performing other viticultural practices: hedging, leafing, fertilisation or spraying. This time-sharing approach would be the most economic, without precluding specific survey services.

Conclusion & prospects

We showed that stilbenoid VBF is a valuable signal to detect and monitor naturally occurring downy mildew epidemic in vineyards. At the same time, we also showed that the Mx-330 is an adequate tool for this measurement on a leaf-to-leaf basis. The presence of this signal on the adaxial side of leaves makes it suitable for vehicle-mounted proximal sensing. Based on the Mx-330 VBF measurements we proposed two indices ‘VBF incidence’ and ‘VBF severity’. They were both linked to the downy mildew disease intensity when this disease was the only one present. They are comparable to the information given by visually assessed disease incidence and severity. This should be confirmed and refined on a larger scale and using repeated experiments. This approach should also be tested for the detection of powdery mildew, which was not present in the experimental plot in 2014.

This new approach using phytoalexin-based fluorescence can be generalised to other crops like resveratrol fluorescence in peanuts or coumarin fluorescence in sunflower, for example. We need to detect the disease in the field in order to achieve the goal of precision agriculture: put the right doses, to the right place, at the right time. This will decrease the pollution of the environment by pesticide treatments. It will also help to protect the grape growers and the produced wine from contamination.

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