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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 occuring downy mildew infections on leaves in the field.

Footnote
†Electronic supplementary information (ESI) available: Fig. S1. Temperature and precipitation for the surveyed vineyard in 2014; Fig. S2. Violet-blue fluorescence signals during the period before the onset of downy mildew infection compared to the black rot disease incidence; Fig. S3. Example of the variability of the violet-blue fluorescence signals. See DOI:

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[1]. Most are fungicides, because fungal diseases can induce crop losses up to 70%[2]. This is the motivation behind the European directive 128/2009/EC, whose aims 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[2]. 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[3, 4] and the fluorescence emission maximum at 380 nm[3, 4]. *In vivo*, they are slightly shifted to longer wavelengths, with the excitation maximum at 330 nm[3, 5] and the emission maximum at around 400 nm[5].

This autofluorescent property of the stilbenoid phytoalexins, which are absent from healthy leaves[5], was exploited to detect the presence of downy mildew in greenhouse-grown plants[4-6], in outdoors-grown plants[5, 6] and in the field[7]. Microscopic studies on live leaf pieces have shown that the fluorescence is mainly localised in epidermal cell walls close to the leaf surface[3, 4].

The development of a portable fluorescence sensor[5], 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[5]. In the field, infected leaves could be discriminated from control ones starting from DPI 6 on both sides[7]. 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[5, 7]. 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

Experiments were performed in an experimental vineyard near Blanquefort (Lat. 44.917° N, Long. 0.642° W) in the Bordeaux region (France) in 2014 on a north-south oriented row of *Vitis vinifera* cultivar Merlot Noir. Ten consecutive vine stocks were chosen and twelve leaves per stock were marked on May 23rd (BBCH 57, flowers separating), with six leaves per row side, both east and west. Leaves were selected at three canopy heights: low, middle and high, two leaves per height. This protocol produced six categories of leaves with 20 leaves per category and 120 marked leaves in total. Measurements started on May 23rd (day of the year (DOY) 143) and lasted until July 11th (DOY 192), with an overall frequency of two measurements per week. In the rare cases where a marked leaf was lost (accidentally detached), which happened six times during the whole experiment, it was replaced by a one nearby at the same height. No plant protection treatment was ever applied to this plot during the 2014 season. The weather data recorded during the survey are presented in Fig. S1 (see ESI†).

Visual disease assessment

Visual assessments of downy mildew, powdery mildew and black rot symptoms were made for every marked leaf in parallel to the Mx-330 measurements. The visual assessments only took into account the 6-cm central leaf area, which was measured using the Mx-330. The leaf severity was visually estimated independently for each disease. It was defined as the proportion of the leaf area with symptoms compared to the total 6-cm central leaf area. We used twelve classes for this estimation: 0 (no symptom), 1% (isolated spot), 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. The visual assessments were made without leaf side considerations.

For each leaf category we calculated: the ‘visual incidence’ as the percentage of infected leaves; the ‘visual leaf severity’ as the mean severity of infected leaves; and the ‘visual plot severity’ as the mean severity of all leaves (healthy leaf severity being zero). Although the measurements were performed on a single row, in this work we use the term

'plot severity' by convention[8]. It should also be recalled that numerically plot severity = incidence x leaf severity. Given the experiment design, the estimation of both disease incidence and disease severity had to be calculated on a leaf basis, not on shoot or plant basis as it is usually done[9].

Multiplex proximal sensing

We used the new Multiplex 330 (FORCE-A, Orsay, France) proximal sensor[5]. Mx-330 is a hand-held, multi-parametric fluorescence sensor based on LED excitation and filtered-photodiode detection that is designed to work in the field under daylight condition. It is based on the mechanical structure and electronics of the Multiplex 3[10], but specifically adapted to measure *in vivo* the stilbenoid VBF on grapevine leaves (335 nm excitation – 400 nm emission). The sensor illuminates a 6-cm-diameter area at a 4-cm distance from the sources and detectors. All marked leaves had a diameter exceeding 6 cm. The leaves were flattened as much as possible during the measurements by pressing them against the sensors with a hand covered by a black low-fluorescing glove. The Mx-330 measurements were performed on both leaf sides, the upper (adaxial) and the lower (abaxial). A UV-excited chlorophyll fluorescence (FRF_UV) of the leaf could be measured simultaneously with VBF thanks to the presence of an additional far-red (750 nm) detector in the Mx-330.

Measurement filtering and index calculations

We used the FRF_UV signal to remove abnormal VBF measurements by looking at complete individual leaf kinetics. Indeed, a non-destructive measurement on the same leaf allowed us to easily identify an abnormal measurement in a temporal series. The FRF_UV signal is independent of VBF fluorescence. It was rather constant during the whole survey (data not shown). However the FRF_UV signal was sensitive to the operators' diligence. A measurement triggered before the leaf was totally pressed against the sensor, a movement of the leaf during the measurement, direct strong sunlight entering the photodiode detector or an unknown effect can all produce an abnormal signal value. For each DOY and each leaf side, measurements with a FRF_UV value larger than two standard deviations from the mean were removed, because this signal was not influenced significantly by the presence or absence of the disease. This procedure was repeated once. In the end, for the whole survey only 8.7% of the measurements were discarded by this procedure.

The 120 marked leaves were organised into six leaf categories: low, middle and high canopy heights at the east and the west sides of the row. For each category and each measurement day, two indices were calculated based on the Mx-330 measurements of VBF.

First, the 'VBF incidence' defined as the number of leaves having a VBF above a fixed threshold divided by the total number of leaves. To choose the threshold for each leaf side, we calculated the mean and standard deviation of all VBF measurements of DOY 143, 146 and 148. Downy mildew symptoms were not observed on these dates. The threshold was defined as 2.7 standard deviations above the mean VBF. This value corresponds to the upper limit of a box plot and is close to the 99th percentile. With such a high threshold we were confident in selecting only infected leaves, i.e. to avoid false positives. Second, the 'VBF severity' index was calculated as the mean VBF value of all leaves of a given category.

Data were processed with the numerical/graphical software Igor 6 (WaveMetrics, Lake Oswego, Oregon).

Results and discussion

There was no powdery mildew infection during this particular survey. Black rot symptoms stayed low with a visual incidence below 15% and a maximum individual-leaf visual severity of 5%. The VBF signal of the leaves showing black rot symptoms 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[11]. In *P. viticola* infected leaves the VBF of induced stilbenoids adds up to this autofluorescence[3, 4]. 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[5] and in the field[7]. 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 n°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,B). This was the direct consequence of the bell-shaped kinetic of VBF following *P. viticola* infection [5, 7] 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[2]. The primary inoculum is mainly found on the ground, closer to the low leaves contaminated by rain splashing[12].

The effect of row side on leaf attributes[13], especially photosynthesis[14], is known for north/south oriented rows[15, 16], 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 Fig. 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[8]. In addition, a sensitive automatic mapping of diseases is needed for precision pest management[17]. Indeed, until now the successful reflectance-based remote sensing of diseases was limited to the changes in green biomass due to defoliation[8, 18]. 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[19]. 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[20, 21]. More often, the experiments were restricted to greenhouses using, for example, laser-induced detection of chlorosis in citrus[22] or to the laboratory even when a UV lidar was used for wheat rust detection [23]. As reviewed recently[17, 24, 25], 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[26]. Thermal imagery is another interesting optical sensing technique[27, 28]. It was applied to downy mildew detection on grapevine, but only with artificial inoculation on individual leaves in the greenhouse[28]. This restriction was also applied in the latest attempt to use variable chlorophyll fluorescence imaging on *P. viticola* infected leaves[29].

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[30]. 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 3.6[31, 32]. 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[33], 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[1]. 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[2, 34, 35]. The VBF-based method will allow an 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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Notes and References

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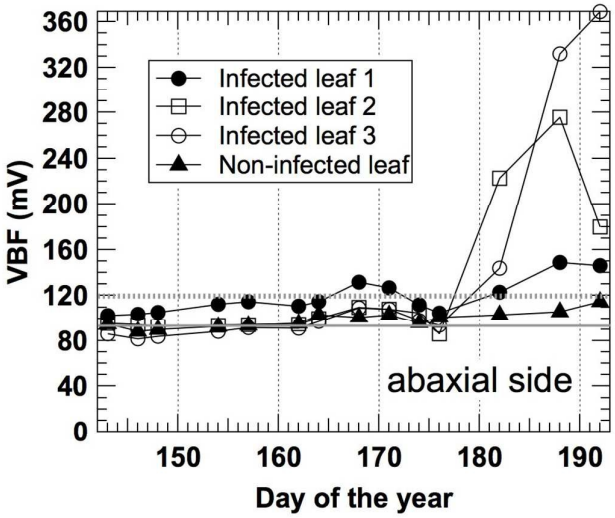
Figure captions

Figure 1. Examples of violet-blue fluorescence (VBF) kinetics of the abaxial side of three individual leaves naturally infected by downy mildew compared to a non-infected leaf. The non-infected leaf did not show any visual symptom until the end of the 50-days survey. The horizontal grey line and the grey dotted line are the mean VBF value for healthy leaves and the 2.7 standard deviations above the mean used as the threshold for incidence detection, respectively.

Figure 2. Adaxial (A) and abaxial (B) leaf sides violet-blue fluorescence (VBF) incidence compared to the visual incidence (C). Six categories of leaves with 20 marked leaves each were followed for 50 days during a naturally occurring downy mildew infection. Leaves were grouped in categories by their position in the canopy: low, middle and high, on the East or West side of a North/South oriented row.

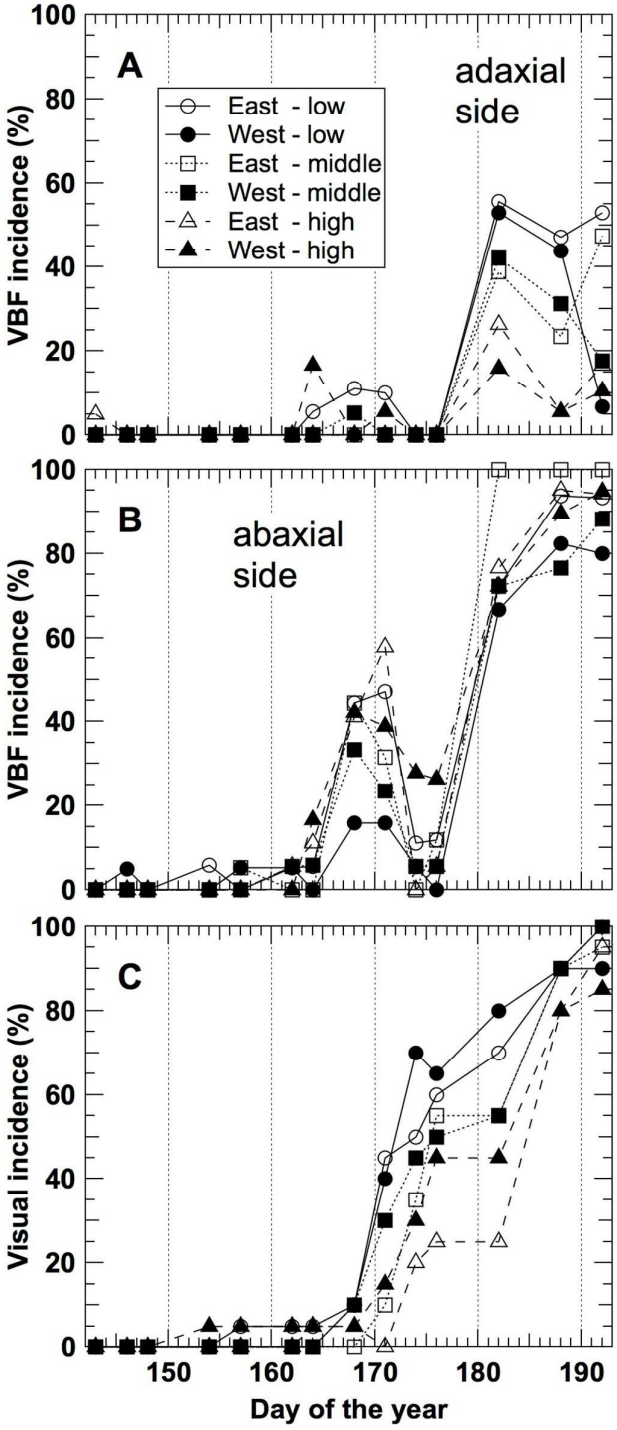
Figure 3. Adaxial and abaxial leaf side violet-blue fluorescence (VBF) severity (A) compared to the visual leaf severity (B) and visual plot severity (C). Six categories of leaves with 20 marked leaves each were followed for 50 days. Leaves were grouped in categories by their position in the canopy: low, middle and high, on the East or West side of a North/South oriented row. The two horizontal grey lines are the mean of the abaxial and the adaxial leaf side VBF measurements obtained from all healthy leaves before the *P. viticola* infection (DOY 143, 146 and 148).

409 **FIGURES**



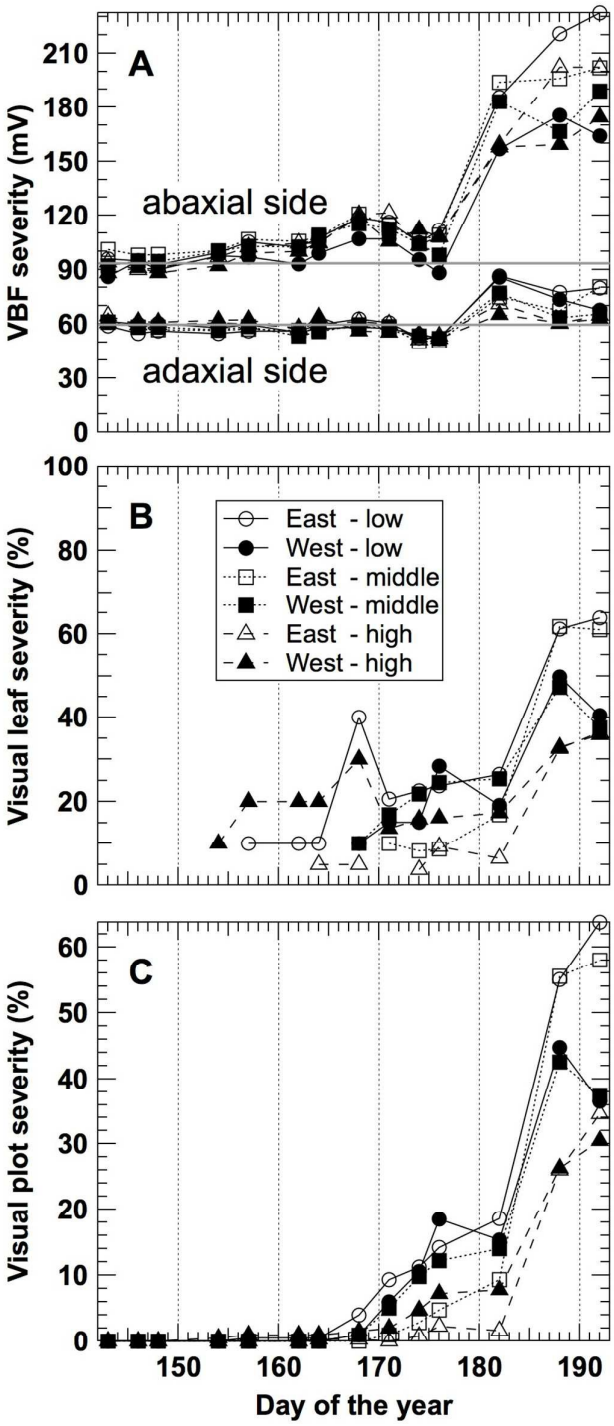
410

411 Figure 1.



412

413 Figure 2.



414

415 Figure 3.

The presence of a major grapevine disease was detected in the field by a new fluorescence proximal sensor. The approach based on UV-induced fluorescence of phytoalexins can be extended to vineyard mapping.

225x140mm (72 x 72 DPI)