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An infrared diagnostic system to detect causal agents of grapevine trunk diseases

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ABSTRACT

In most vineyards worldwide, agents of grapevine trunk diseases represent a real threat for viticulture and are responsible for significant economic loss to the wine industry. The conventional microbiological isolation technique used to diagnose this disease is tedious and frequently leads to false negatives. Thus, a dire need exists for an alternative method to detect this disease. One possible way involves infrared spectroscopy, which is a rapid, nondestructive analytical tool that is commonly used for quality control of feed stuffs. In the present work, a midinfrared spectrometer was tested as a fast tool for detecting agents of grapevine trunk disease. Midinfrared spectra were collected from 70 *Vitis vinifera* L. cv. Cabernet-Sauvignon one year old trunk-wood samples that were infected naturally in one viticulture nursery of the south of France. The samples underwent polymerase chain reaction and morphological identification, and the results were correlated to the midinfrared spectra by using multivariate analysis to discriminate between noninfected and infected samples. Based on comparison with some control samples, the highest percentage of correct identification of fungal contamination when using the midinfrared spectroscopy method is 80%.

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1. Introduction

The progression of grapevine trunk diseases (GTDs) that is currently occurring in most vineyards worldwide represents a major threat to viticulture and is responsible for significant economic loss to the wine industry. The decrease in productivity is due to externalized symptoms and the resulting early decline of plants in the field leads to a loss of wine typicality (Laveau et al., 2009).

The GTD Esca is caused by xylem-inhabiting *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* in association with several other fungi (Bertsch et al., 2013; Mugnai et al., 1999). In addition, a number of *Botrysphaeria* species have been identified as causal agents of Black Dead Arm (BDA) (Larignon et al., 2009). The more prevalent fungal species in France are *B. obtusa* and *B. parva*. Another fungus, *Eutypa lata*, causes Eutypa dieback (Rolshausen et al., 2006). Pathogens are thought to be present on the surface of canes and to infect plant material during soaking and stratification at the nursery and during vineyard management (Pouzoulet et al., 2013a). They attack perennial organs, leading to extensive inner necrosis in the trunk and arms (Mugnai et al., 1999; Rolshausen et al., 2006; Urbez-Torres et al., 2006). The symptoms of the disease appear several years after the first infection. GTD fungi have in common a slow growth and induce an extremely complex and variable symptom expression, making GTD difficult to diagnose in vineyards. Current methods to classify fungal species rely on morphological characteristics such as, in particular, reproductive structures. These methods are fastidious and frequently lead to false negatives because the pathogens are often overgrown by other microorganisms in semi-selective media (Aroca and Raposo, 2007).

Numerous studies have focused on molecular-based identification within a single fungal family (Aroca and Raposo, 2007) or detection of GTD fungi (Pouzoulet et al., 2013a). The cost and time required for such analytical methods limit their use for rapid screening of raw materials. Thus, a dire need exists for an alternative method, such as infrared spectroscopy, which would allow a fast screening of grapevine wood. This method would be similar to the use of infrared spectroscopy to analyze the quality of food and feed, which is now routine (Bertrand and Dufour, 2005). The use of infrared spectroscopy would also benefit from the studies that have identified the various fungi and yeasts responsible for different fungal diseases (Costa et al., 2007; Erukhimovitch et al., 2005; Fischer et al., 2006; Graeff et al., 2006; Huang et al., 2007; Levasseur-Garcia, 2012; Mariey et al., 2001; Peiris et al., 2010; Sankaran et al., 2012). For more information on the various infrared spectroscopic techniques for detecting plant diseases, please see the review by Sankaran et al. (Sankaran et al., 2010). To date, however, no study has used infrared spectroscopy to discriminate between GTD-infected and -noninfected grapevine trunks. Therefore, to address

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this need, the present study examines 70 naturally infected wood samples to investigate the potential of midinfrared (MIR) spectroscopy to detect GTD.

2. Materials and methods

2.1. Plant material

70 samples from one year-old canes of *Vitis vinifera* L. cv. Cabernet-Sauvignon clone 15 were used. The samples were collected from only one nursery, located in the Gaillac vineyard, (Midi-Pyrénées) in the south of France. The samples are grafted plants, grown open-air. After harvest, they were stored in the dark at 4 °C.

2.2. DNA extraction

The surface of the sample was cleaned by removing bark around the graft union and at the basal end of the rootstock, using a sterile scalpel in sterile conditions. Each sample is constituted by 5 wood sections of the same grafted plant, collected all along the plant to be representative of the whole plant. Each wood section size measures few millimeters of diameter and one millimeter on thickness. Wood sections were cut with clippers disinfected with 70% ethanol. Samples were lyophilized over 24 h and grinded at room temperature. Powder was incubated at 65 °C in a modified cetyltrimethyl ammonium bromide (CTAB) extraction buffer from Doyle and Doyle (Doyle and Doyle, 1987). 24:1 chloroform:isoamyl alcohol solution was added and the mixture was incubated on ice. Total DNA extraction was done by using a DNeasy plant mini Kit (Qiagen, USA) and following the manufacturer protocol. The final elution volume was 50 μl, and the samples were stored at −20 °C.

2.3. Primer design and sequence alignment

Primer sets were checked using Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/primer3/). Multiple sequence and primer alignments were performed with Multalin software (http://multalin.toulouse.inra.fr/multalin.html).

2.4. Qualitative PCR assays

PCR reactions were carried out in 25 μl reaction mixture containing: 12,5 μl of 2× Plexor™ Master Mix (Promega, Cat. No. A4031, Madison, USA), 0,2 μM of each primer, 10 ng of purified genomic DNA. Reaction conditions were: initial denaturation (94 °C – 5 min) followed by 35 cycles of denaturation (94 °C, 30 s), primer specific annealing temperature for 1 min and elongation (72 °C, 1 min), and a final elongation for 5 min at 72 °C. The amplified products were examined by polyacrylamide gel 4–12% (Invitrogen, Carlsbad) electrophoresis.

2.5. Quantitative real time PCR (rt-PCR) assays

Reactions were carried out in a final volume of 25 μl reaction mixture containing: 12,5 μl of 2× Plexor™ Master Mix (Promega, Cat. No. A4031, Madison, USA), 0,3 μM of each primer. Labelled Plexor primers (5′ Me-isodC) were synthesized by Eurogentec S. A (5′FAM labelled, ref.: PB-PP001–004; 5′TAMRA labelled, ref.: PB-PP100-004; 5′ROX labelled, ref.: PB-PPOO16-004; Liege science park, Seraing, Belgium) and were diluted in MOPS/EDTA buffer (Promega, Cat. No. Y510A, Madison, USA). Unlabelled primers were synthesized by Invitrogen (ref:10336-022, Fisher Bioblock Scientific, Illkirch, France) and diluted in nuclease/nucleic acid free water (Promega, Cat. No. P119A). A maximum of 2 μl of DNA template per reaction. Experiments were conducted with an ABI 7500 real-time PCR (rt-PCR) cycler using the software ABI SDS version1.4 with the default settings. The cycling program consisted of (i) an initial denaturation step at 95 °C for 5 min, (ii) forty 5 s cycles at 95 °C for denaturation, followed by 35 s at 65 °C for both annealing and extension, and (iii) an additional melting analysis of 40 min from 60 to 95 °C. The data were analyzed by using Plexor® Analysis software version 1.5.6.2 (Promega), and melt-curve thresholds were fixed to 20% of their respective 10^4 standards.

2.6. Conventional microbiological isolation

Five wood sections of the same grafted plants were collected all along the plant, on the neighborhood of the 5 wood previous sections for DNA extraction, to be representative of the whole plant. Each wood section size measures few millimeters of diameter and one millimeter on thickness. Wood sections were cut with clippers disinfected with one spray of 70% ethanol and plated at 26 °C in the dark in Petri dishes containing potato-dextrose-agar (Merck, Germany, Cat. No. 1-10130-0500). Fungi were detected and enumerated after 10 days. The fungal isolates were identified as Pch, Bob, Pal, or Bpv (referring to *Phaeomoniella chlamydospora, Botrysphaeria obtusa, Phaeoacremonium aleophilum* and *Botrysphaeria parva* respectively) on the basis of their morphological characteristics.

2.7. Fourier transform infrared spectroscopy measurements

Fourier transform infrared (FTIR) spectra of wood powder were measured by means of an Avatar FTIR spectrometer (Thermo Electron Corporation, USA). The spectral range spanned from 400 to 4000 cm^{-1} with a resolution of 2 cm^{-1} after 32 scans. Finely divided 1 mg samples of wood were ground and dispersed in a matrix of 150 mg KBr, followed by compression to form pellets.

2.8. Preprocessing of spectra

Spectra were preprocessed to remove the effects of light scattering and to compensate for baseline offset and bias. To obtain the best discrimination model, seven different types of spectral preprocessing were tested. These included no treatment (raw data), the first- and second-derivative Savitzky–Golay methods (D1 and D2, respectively), standard normal variate (SNV) ± detrending, and SNV ± D1 or D2. Derivatives were used to emphasize small bands and to resolve overlapping peaks. SNV removes the multiplicative interference due to scatter and particle size and corrects the variation in baseline shift (Sun, 2009). Spectral preprocessing was done by using the Unscrambler® Multivariate Data Analysis software (version X; CAMO A/S, Oslo, Norway).

2.9. Multivariate data analysis

Clustering analysis was used to determine the number of clusters that better define the wood dataset based on fungal contamination (percent contamination by Pch, Bob, Pal and Bpv). The K-means algorithm was used because of its simplicity (Tuffery, 2007): the only parameter that must be defined is the number of clusters to be located. Statistical analyses were done with XLSTAT version 2014.5.02 (Addinsoft, New York, USA).

After preprocessing, a principle components analysis (PCA) was done to compress the spectra into a set of linearly uncorrelated variables called principal components (PCs). The first PC accounts for the maximum variability in the MIR spectra, with subsequent PCs accounting for ever-decreasing variability (Tuffery, 2007). PCA was done with the Unscrambler® Multivariate Data Analysis software (version X; CAMO A/S, Oslo, Norway).

A chi-squared automatic interaction detector (CHAID) decision tree was used to build a discrimination model. The quality of the model was evaluated by calculating prediction accuracy and classification errors from confusion matrices (Visa et al., 2011). A confusion matrix was used. The *true positive* rate (TP) is the proportion of positive cases that were correctly identified; the *false positive* rate (FP) is the proportion

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