DEVELOPMENT OF RAPID DIRECT PCR ASSAYS TO IDENTIFY DOWNY AND POWDERY MILDEW AND GREY MOULD IN *VITIS VINIFERA* TISSUES

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Abstract

Aims: The development of a rapid and reliable direct PCR method to detect fungal propagules in grapevine tissues without prior DNA purification steps, and illustration of its potential use with different examples.

Methods and results: Different grapevine samples crushed in the presence of polyvinylpolypyrrolidone (PVPP) were used as templates for direct PCR amplification with primers specific for *Erysiphe necator*, *Plasmopara viticola*, *Botrytis cinerea* and *Vitis vinifera*. Sequencing of the PCR products confirmed the specificity of the amplifications. The sensitivity tested using conidia/sporangia dilution series was high, ranging from five sporangia for *P. viticola* to one conidium for *E. necator*. The potential of this technique is illustrated through the study of four epidemiological questions. Fungal propagules were observed in dormant buds using microscopy, but the responsible species could not be identified. Direct PCR revealed the presence of *E. necator* and *B. cinerea* in 29 % and 65 % of the buds, respectively. Downy mildew could be detected in asymptomatic leaves sampled in fields after potentially infectious events. In bunch, microscopic analysis of rachis sections showed the presence of hyphae growing in the green tissue. Direct PCR identified the presence of *P. viticola*.

Conclusion: A direct PCR method without DNA purification was demonstrated to be a simple and reliable method for the detection and identification of fungal pathogens in grapevine tissues. This method, together with microscopy, is a very interesting tool that can be used to study various epidemiological problems in the grapevine, including important unanswered questions such as the route of infection that leads to brown rot caused by downy mildew.

Significance and impact of the study: Direct PCR was shown to be a simple and versatile technique for the study of epidemiological questions in the grapevine. This technique could be extended to other pathosystems with minor adaptations.

Key words: *Plasmopara viticola*, *Erysiphe necator*, *Botrytis cinerea*, electron microscopy, direct PCR

Résumé

Objectifs: Développement d'une méthode simple et fiable d'amplification directe par PCR afin de détecter des propagules fongiques au sein de tissus de la vigne sans purification préalable de l'ADN et illustration du potentiel de cette méthode au travers de différents exemples.

Méthodes et résultats: Différents échantillons de vigne, broyés en présence de polyvinylpolypyrrolidone (PVPP), ont été utilisés directement comme matrice pour l'amplification par PCR avec des amorces spécifiques pour *Erysiphe necator*, *Plasmopara viticola*, *Botrytis cinerea* et *Vitis vinifera*. La spécificité des amplifications a été validée par le séquençage des produits de PCR. Des séries de dilution de conidies/sporanges ont permis de montrer la grande sensibilité des réactions, celle-ci variant de cinq sporanges pour *P. viticola* à une conidie pour *E. necator*. Le potentiel de cette technique est illustré au travers quatre questions épidémiologiques. La microscopie a permis de visualiser des propagules fongiques dans des bourgeons dormants sans toutefois permettre d'identifier les espèces en présence. *E. necator* et *B. cinerea* ont été détectés par PCR directe dans respectivement 29 % et 65 % des bourgeons dormants. Le mildiou a pu être détecté dans des feuilles asymptomatiques en champs après des épisodes potentiellement infectieux. Des observations microscopiques effectuées dans des sections de rafles ont permis de visualiser la présence d'hyphes en croissance dans les tissus verts et la méthode de PCR directe a permis d'identifier *P. viticola*.

Conclusion: La méthode de PCR directe sans purification de l'ADN se révèle être une méthode simple et fiable pour détecter et identifier la présence de pathogènes fongiques dans les tissus de la vigne. Cette méthode associée à la microscopie est un outil intéressant pour étudier différentes problématiques épidémiologiques chez la vigne, en particulier des questions ouvertes importantes comme la voie d'infection du mildiou causant la pourriture brune.

Signification et impact de l'étude: La PCR directe est une méthode simple et flexible pour l'étude de questions épidémiologiques chez la vigne. Cette technique pourrait être étendue à d'autres pathosystèmes, moyennant quelques adaptations.

Mots clé: *Plasmopara viticola*, *Erysiphe necator*, *Botrytis cinerea*, microscopie électronique, PCR directe

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INTRODUCTION

The study of complex relationships between organisms, such as microorganisms, and their substrates, environment or one another can currently be accomplished using methods that are completely independent of classical culture techniques. Methods such as polymerase chain reaction (PCR) (van Belkum *et al.*, 1998 ; Sachse, 2004), restriction fragment length polymorphism (RFLP) (Vaneechoutte, 1996), pyrosequencing (Petrosino *et al.*, 2009) and, more recently, metagenomics (Miller *et al.*, 2013 ; Ross-Davis *et al.*, 2013), metatranscriptomics (McGrath *et al.*, 2010) and other meta'omics methods (Lepage *et al.*, 2013) represent new ways to study complex ecosystems and their interactions (Segata *et al.*, 2013). The development of such methods has enabled the detection of specific organisms within a complex matrix.

PCR has become a widely used technique with applications in all biological and medical fields. However, in most cases, PCR involves prior purification of nucleic acids. A direct PCR method, without any DNA purification steps, was previously developed to detect latent *Botrytis cinerea* in young grapevine berries (Gindro *et al.*, 2005). This method, combined with microscopic analyses (Keller *et al.*, 2003), enabled the study of the infection at the bloom stage and during the following latency period. These data provided new insights into the epidemiology of grey mould and confirmed the importance of protecting the grapevine from *B. cinerea* infections during the bloom stage.

Among the approximately ten species of fungi that are considered to be major grapevine pathogens, three are responsible for most damage in vineyards : grey mould (*B. cinerea*), downy mildew (*Plasmopara viticola*) and powdery mildew (*Erysiphe necator*). A specific problem lies in the obligate biotrophic nature of *P. viticola* and *E. necator*, which prohibits their cultivation on artificial media, making their study much more complicated and time consuming. PCR amplification methods have been described for the identification or quantification of *B. cinerea* in air samples (Carisse *et al.*, 2009), *E. necator* in wine grapes and must (Stummer *et al.*, 2006) and air samples (Thiessen *et al.*, 2013), and *P. viticola* in grapevine leaves (Valsesia *et al.*, 2005). Several studies have successfully developed PCR reactions without prior DNA purification, relying upon lysis at the high temperature of amplification. For example, these techniques have been applied to whole bacterial cells (Gussow and Clackson, 1989), tobacco leaves and root pieces (Berthomieu and Meyer, 1991), fungal spores (Aufauvre-Brown *et al.*, 1993) and in direct diagnostics of bacteria and viruses in animal tissues (Olive, 1989). However, it is known that the amplification reaction can be inhibited by different parameters such as excessively high cell numbers, cell wall fragments, DNA-binding proteins, polysaccharides, phenols, detergents or insufficient cell lysis (Moreira, 1998 ; Dewey and Yohalem, 2004). Plant tissues are very rich in polysaccharides and polyphenols, including secondary metabolites as phytoalexins and lignin (Taiz and Zeiger, 2010), which can potentially inhibit PCR reactions, making purification steps of nucleic acids usually indispensable.

This paper describes a direct PCR method to detect fungal DNA in plants without prior DNA purification steps. The potential of this technique is illustrated through the study of four epidemiological questions concerning grapevines : the presence of *E. necator* in dormant buds, the occurrence of *B. cinerea* in dormant buds, the early detection of *P. viticola* in asymptomatic leaves in the field, and the detection of systemic development of *P. viticola* in the bunch.

MATERIALS AND METHODS

1. Plant material and artificial infections

Vitis vinifera L. cv. Chasselas grafted on 3309 were obtained from the Agroscope in Changins (Switzerland) and planted in experimental fields in 2005. They were cultivated for eight years until sampling. The experimental plot consisted of one row (distance between rows : 1.5 m ; between plants within a row : 0.75 m) conducted in "Guyot". Leaves, inflorescences, bunches and buds were all sampled from this experimental plot. For *P. viticola*, the leaves and grape clusters of three plants were artificially infected with an aqueous sporangia suspension at stage BBCH 57 (Hack *et al.*, 1992), namely when the inflorescences are fully developed, as previously described (Gindro *et al.*, 2003). In the case of *B. cinerea*, artificial infections were realised at the end of bloom (BBCH 69, all caps fallen) with an aqueous conidia suspension as described before (Viret *et al.*, 2004). In the case of *E. necator*, conidia were collected from infected leaves as described previously (Schnee *et al.*, 2008) and dormant buds were collected in February 2013 from plants in an untreated plot that were strongly infected in 2012. For the early detection of *P. viticola* infections on *V. vinifera* cv. Cabernet franc, entire leaves were randomly sampled in a vineyard in Bordeaux the day after potentially infectious conditions (rain or dew) and directly stored at -80°C until use.

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2. Sample preparation and PCR amplification

Plant samples, leaves (3 mg), flowers (40 mg), rachis sections (40 mg) and buds (20 mg) were crushed with a plastic pestle in an Eppendorf in 100 µl of water containing 1 % (w/v) polyvinylpolypyrrolidone (PVPP), except for buds, which received 500 µl of water. This crude extract was diluted 10- and 100-fold with nanopure water and used directly for PCR. Alternatively, bigger samples, such as entire leaves, were placed into an ELISA bag (Bioreba AG), frozen in liquid nitrogen and crushed with an homogeniser (Homex 6, Bioreba AG) into a fine powder and put on ice or stored at -20 °C until use. The powder was then suspended in 2 ml of 1x PBS containing Tween 20 (0.05 % v/v) and PVPP (2 % w/v). Aliquots of this solution were diluted 10- and 100-fold into water and used directly for PCR amplification. PCR reactions were performed in 25 µl volumes containing 2 units of Taq (Qiagen Taq DNA polymerase), 1x PCR buffer, 0.4 µM of each primer, 0.2 mM of each dNTP, 3 mM $MgCl_2$ and 18.3 µl of the diluted crude extract. The specific primers used in this analysis are described in Table 1. Amplifications were performed in a Biometra T3000 thermocycler with a first step at 97 °C for 15 min to lyse the cells, followed by 36 cycles at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 90 s and a final extension step at 72 °C for 10 min. Gel electrophoresis of PCR products was performed on 1 % agarose gels. Control amplifications were performed on fungal spores or sporangia as described previously (Gindro *et al.*, 2005). Spores and sporangia dilution series (1'000, 100, 10, 1 sporangia or conidia μl^{-1}) were performed to determine the sensitivity of the PCR reaction. Spore suspensions were counted under the microscope on standardised examination chambers (KOVA Glasstic Slide 10 with grids, Hycor Agilent Technologies, Amstelveen, The Netherlands) and the concentration adjusted to 1'000 spores μl^{-1}. PCR products were purified and sequenced to confirm the specificity of the reaction.

3. Transmission electron microscopy

Each sample, leaf fragment, rachis, dormant bud fragment and flower fragment was prepared according to Roland and Vian (1991), pre-fixed with a solution of 3 % glutaraldehyde-2 % paraformaldehyde in 0.07 M phosphate buffer at pH 7, embedded in 2 % agarose and post-fixed with a solution of 1 % OsO_4. The samples were then dehydrated in a graded series of ethanol solutions of 30-50-70-95-100 % (v/v) and embedded in LR White resin (14381-UC London Resin Company). After polymerisation (24 h at 60 °C), semi-thin (0.8 µm) and thin (0.08 µm) sections were cut and stained with a solution of 1 % methylene blue, sodium tetraborate and azure II for the semi-thin sections, or 2 % uranyl acetate followed by lead citrate according to Reynolds (1963) for the thin sections. Semi-thin sections were observed using a light microscope (Leica DMLB) equipped with a Leica DFC 490 FX camera. Thin sections were observed with a transmission electron microscope (Philips CM10) with a Mega View II camera.

RESULTS AND DISCUSSION

Direct PCR is a simple and rapid method for the detection and identification of a pathogen in non-symptomatic plant tissues. Figure 1 shows that the direct PCR methods described here are able to specifically detect the three major fungal pathogens of grapevine from a mix of symptomless infected green tissues. The different bands were purified from the gel and sequenced to confirm the specificity of the PCR amplification. Grapevine green tissues contain many phenolic and polyphenolic compounds as well as sugars, which are all potential inhibitors of

Table 1. Sequences of the different primers used in this study and amplicon length.

Organism	Primer name	Sequence	Amplicon (bp)
Plasmopara viticola	LSU 1+_PV	TAGTAACGGCGAGTGAAGCG	**698**
	LSU 2-_PV	GTTACGACTCGCATCAATCCA	
Erysiphe necator	Uc592+	AGTTAAAAGATGTCAACGCCGAAGA	**379**
	Uc946-	AGCGGCAAAAGATGAGTCAAAATTC	
Botrytis cinerea	C729+	AGCTCGAGAGAGATCTCTGA	**730**
	C729-	CTGCAATGTTCTGCGTGGAA	
Vitis vinifera	Vvin-F	CCTTCAGGTGGGTACAGTGG	**241**
	Vvin-R	CATTCCCAACTGCATCAGTCAC	

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PCR reactions. In the case of classical plant DNA extraction, all of these constituents are carefully removed by different purification steps. However, in direct PCR reactions, no prior purification steps are performed, and the inhibitors may still be present in the reaction mix. Some studies summarised the various compounds found in samples that inhibit or facilitate the enzymatic amplification reactions (Rossen *et al.*, 1992; Wilson, 1997). In the case of direct PCR on grapevine green tissue, PVPP, which chelates polyphenolic compounds, is added to the mix prior to the cell lysis step to avoid inhibition (95 °C 15 min). For the same purpose, the initial template mixture was diluted 10- to 100-fold. Because of this very rough purification step, it is very important to have a positive control to exclude the possibility that the absence of a band is due to the presence of inhibitors. Each sample was also PCR-amplified with *V. vinifera* primers as a positive control. The sensitivity of the direct PCR was tested with dilution series of conidia and sporangia in water. The results show a detection threshold of one conidium per microliter for the *B. cinerea* primers, which is consistent with previous results (Gindro *et al.*, 2005), one sporangium per microliter for *P. viticola*, and one conidium per microliter for *E. necator* (Figure 2).

The direct PCR method can be used to study different epidemiological questions such as the latency of these pathogens in apparently healthy tissues, or overwintering of pathogens, especially obligate biotrophs, in dormant buds. Microscopic studies are able to reveal the presence of fungal structures in plant tissues; however, the identification of the pathogens by microscopy is not possible in most cases due to the lack of identifiable species-specific structures. The primary infection of *E. necator* can have two different origins. The infection can be due to ascospores released from overwintering cleistothecia on the bark (Gadoury and Pearson, 1986; Gadoury and Pearson, 1987; Gadoury and Pearson, 1987; Gadoury *et al.*, 2012). According to climatic conditions and the *V. vinifera* cultivars,

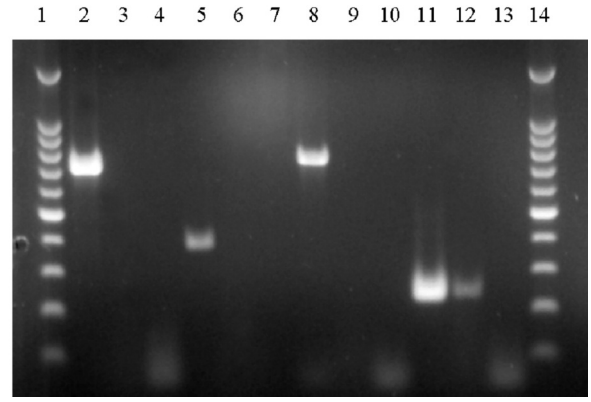


Figure 1. Direct PCR amplification of a mix of grapevine leaf pieces infected with *Plasmopara viticola* and *Erysiphe necator* and flowers infected with *Botrytis cinerea* (infected tissues).

Lanes 1 and 14: 1 kb DNA Ladder.
Lane 2: *P. viticola* primers with infected tissues,
lane 3: *P. viticola* primers with healthy tissues,
lane 5: *E. necator* primers with infected tissues,
lane 6: *E. necator* primers with healthy tissues,
lane 8: *B. cinerea* primers with infected tissues,
lane 9: *B. cinerea* primers with healthy tissues,
lane 11: *V. vinifera* primers with infected tissues,
lane 12: *V. vinifera* primers with healthy tissues,
lanes 4, 7, 10 and 13: negative control with water.
Amplicon sizes are: *P. viticola* 698 bp, *E. necator* 379 bp, *B. cinerea* 730 bp and *V. vinifera* 241 bp.

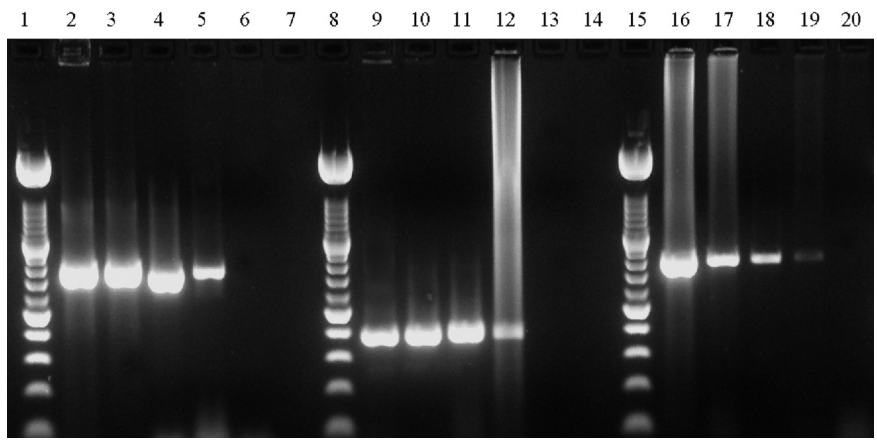


Figure 2. Direct PCR sensitivity tests by amplification of *Plasmopara viticola* sporangia, *Erysiphe necator* and *Botrytis cinerea* conidia dilution series.

Lanes 1, 8 and 15: 1 kb DNA Ladder. For *P. viticola*: Lanes 2 to 5: dilution series from 1'000, 100, 10 to 1 sporangia per microliter. Lane 6: negative control with water. Lane 7: empty. For *E. necator*: Lanes 9 to 12: dilution series from 1'000, 100, 10 to 1 conidia per microliter. Lane 13: negative control with water. Lane 14: empty. For *B. cinerea*: Lanes 16 to 19: dilution series from 1'000, 100, 10 to 1 conidia per microliter. Lane 20: negative control with water.

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overwintering mycelium in buds can also initiate the infection very early in the growing season leading to the formation of flag shoots (Corio-Costet, 2007). To set up the control strategy, it is important to determine if flag shoots are present or not. In Switzerland, cleistothecia, which can be abundant at the end of the growing season, are responsible for primary infections the next year. However, flag shoots are rarely observed and do not play a role in the epidemiology of powdery mildew in Swiss vineyards. We investigated if this correlated with the absence of mycelium in dormant buds of *V. vinifera* cv. Chasselas in Swiss conditions or if specific climatic conditions in spring or other factors are inhibiting the development of *E. necator* originating from the mycelium in buds. Dormant buds were collected in January in a non-treated plot of *V. vinifera* cv. Chasselas, which was naturally strongly infected with both powdery and downy mildew the year before. Microscopic analysis revealed the presence of numerous fungal structures including hyphae, spores and propagules in the fuzz of the bud (Figure 3). Even though fungal structures could be recognised, neither optical nor electronic transmission microscopy allowed for the identification of the fungi. Therefore, direct PCR was used to detect the presence of *E. necator*, as well as *P. viticola* and *B. cinerea*. From the 120 buds analysed, *E. necator* was detected by PCR in 35 buds (29 %), *B. cinerea* in 78 buds (65 %) and *P. viticola* could not be detected (0 %). *B. cinerea* and *E. necator* were present together in 20 buds (17 %). Despite the fact that flag shoots are not observed on Chasselas in Switzerland, one third of the buds in the non-treated studied plots are colonised by *E. necator*. These results are consistent with previous work (Rumbolz and Gubler, 2005) on *V. vinifera* cv. Carignane in California (USA), which reported 32.3 % of buds were infected. The presence of fungus in the dormant buds does not seem to play a role in the epidemiology of the fungus in the Swiss vineyard. It could be interesting to investigate whether *E. necator* is present at a similar level in buds of a standard treated plot. The direct PCR method is a precious tool to demonstrate that even if flag shoots are not observed in Switzerland, *E. necator* is present in dormant buds. The high incidence of *B. cinerea* was confirmed by traditional isolation on potato dextrose agar medium, showing that *B. cinerea* was isolated successfully from 40 out of 120 buds analysed (33 %), which represents approximately half of the incidence detected by direct PCR (65 %), but this fungus did not grow on artificial media. This result shows that direct PCR is highly sensitive, perhaps because non-viable fungal

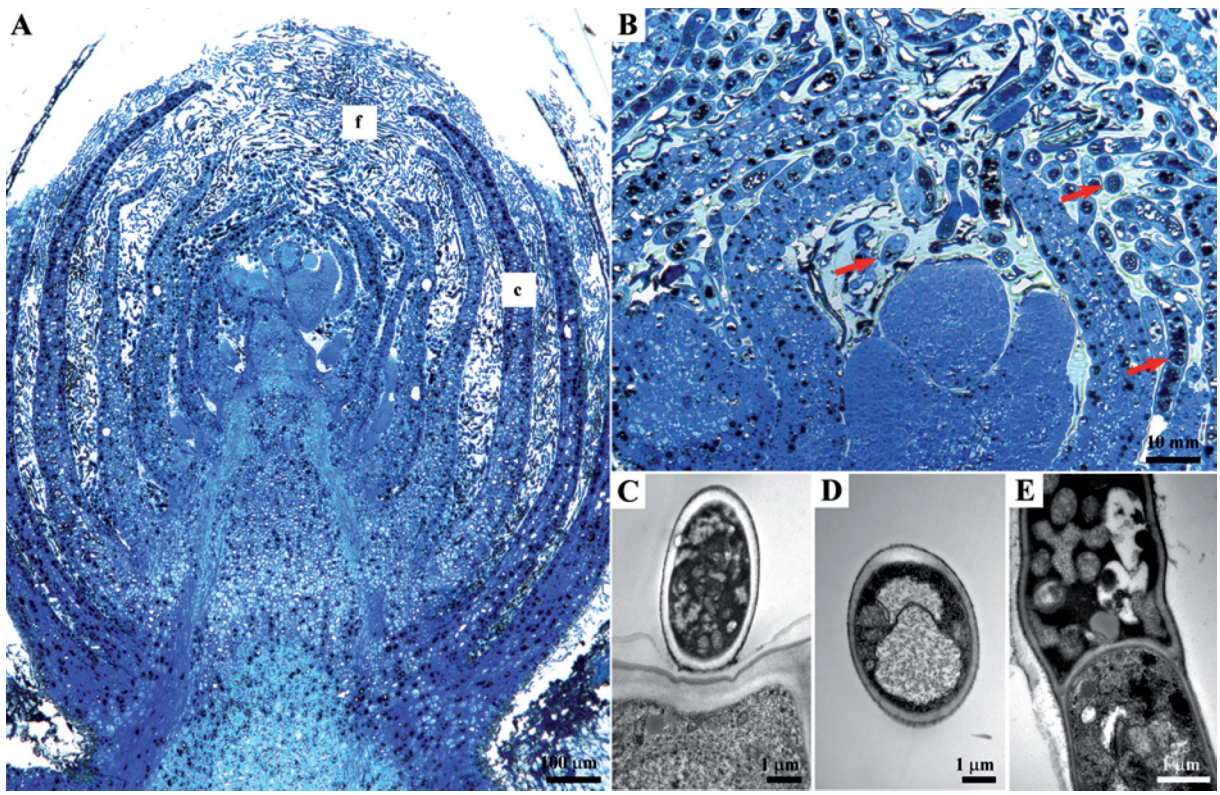


Figure 3. Ultrastructural observations of dormant buds of *Vitis vinifera* cv. Chasselas. A. Global view on a longitudinal semi-thin section. B. Detailed view of A showing fungal hyphae and propagules in the fuzz (red arrows). C and D. Fungal propagules observed on a thin section. E. Fungal hyphae observed on a thin section. c : cataphyll, f : fuzz.

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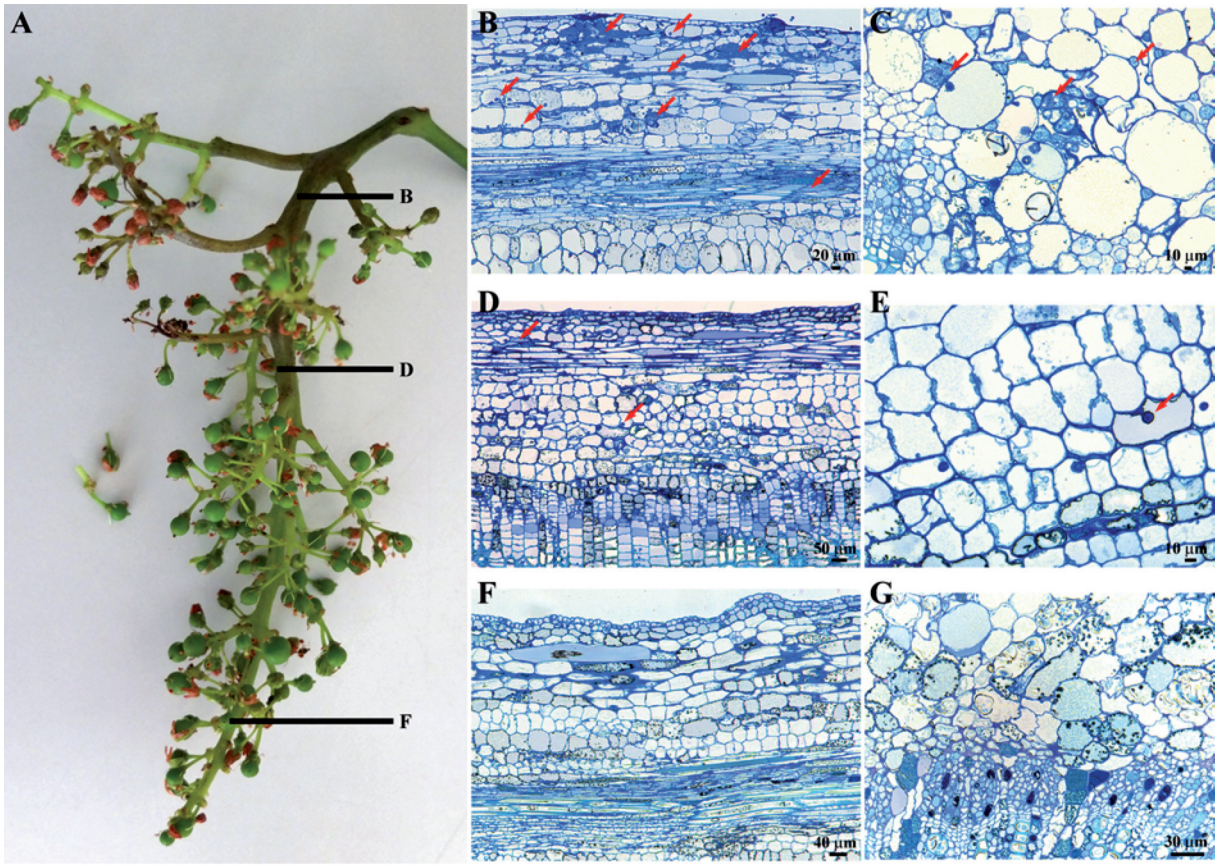


Figure 4. Microscopic analysis of semi-thin sections at three different points along a rachis of *Vitis vinifera* cv. Chasselas showing strong, weak and no symptoms of downy mildew infection. A. Grape cluster used for the study; brown rot symptoms can be observed along the upper part of the rachis. B. Semi-thin section strongly colonised by *P. viticola* with numerous hyphae and haustoria (red arrows). C. Detailed view of B. D. Semi-thin section with weak colonisation by *P. viticola* with only a few hyphae and haustoria (red arrows). E. Detailed view of D. F. Semi-thin section with no colonisation of *P. viticola*. G. Detailed view of F.

propagules or conidia are detected by direct PCR. As previously reported, *B. cinerea* is ubiquitous; thus, it is not surprising to detect it in buds. Even if *B. cinerea* is not infecting young shoots under natural conditions, this may be a source of inoculum for later infections, such as bloom infection and latency (Keller *et al.*, 2003). *P. viticola*, a highly specific biotrophic organism, is not able to colonise and survive in buds, which is consistent with previous reports on downy mildew epidemiology (Gessler *et al.*, 2011).

The direct PCR method was used for the early detection of downy mildew. Forecasting models are usually used to assess the right timing of fungicide treatments (Dubuis *et al.*, 2012). However, it is difficult to accurately evaluate the intensity and severity of forecasted infection episodes. Based on leaf wetness duration and temperature, forecasting models can be used to precisely evaluate if the weather conditions can lead to low, middle or strong infections. However, the real severity of the infection depends on the presence of inoculum, which can be difficult to evaluate. Direct PCR on randomly sampled whole leaves could be a simple method to evaluate the level of downy mildew infection before the appearance of oil spots. Such results could help growers make decisions about spraying a curative fungicide in the three days following the infection. For example, on the 41 leaves of *V. vinifera* cv. Cabernet franc sampled on 17 July 2013 after a few days with strong dew in the morning, eight leaves were positive (19.5 %). This is consistent with the percentage of 15 % of infected leaves monitored on 5 August 2013.

Another interesting epidemiological open question in which direct PCR technique can be used is the route of infection of *P. viticola* that leads to grey and brown rot in grape clusters. Previous works have shown that no artificial infections were possible after BBCH 69 in the conditions of the experiment

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(Gindro *et al.*, 2012). The absence of functional stomata on berries was described to be one possible cause. In the case of later downy mildew symptoms, one hypothesis discussed in this paper is that *P. viticola* develops systematically through the green tissues from infected leaves, tendrils, peduncles, rachis, pedicels or shoots, as previously postulated by Gregory (Gregory, 1915). Microscopic analysis of sections along a rachis, at three different points showing strong, weak and no macroscopic symptoms of downy mildew infection are shown in Figure 4. The upper part of the rachis is strongly colonised by *P. viticola*, and numerous hyphae and haustoria can be observed (Figure 4 B and C). In the middle part showing weak symptoms, *P. viticola* can be observed but with only few hyphae and haustoria (Figure 4 D and E). In the lower part of the rachis, no symptoms are observed, and *P. viticola* was not observed (Figure 4 F and G). Direct PCR confirmed the presence of downy mildew in the two symptomatic locations and its absence in the healthy area. This has confirmed that the hyphae observed are those of *P. viticola*. These observations also confirm that *P. viticola* is able to grow in the rachis at stage BBCH 73 and can be detected by direct PCR. These are only preliminary results, and the hypothesis that *P. viticola* grows systematically through the rachis to infect berries causing brown rot is under further investigation.

CONCLUSION

Direct PCR without DNA purification is a rapid method to detect and identify the presence of fungal pathogens in grapevine green and lignified tissues. This method can be used for various studies on fungal epidemiology, especially for obligate biotrophs and in field samples. The combination of microscopy and direct PCR enables the localisation and identification of the organisms present in symptomatic or non-symptomatic green tissues as well as in dormant buds. However, the method needs to be optimised for each use, especially in the removal of potential inhibitors of PCR reactions. Four examples of the usefulness of this technique in tackling different experimental hypotheses are presented. *E. necator* and *B. cinerea* are present in dormant buds, downy mildew could be detected in entire leaves shortly after rain or dew events, the presence of *P. viticola* in the rachis was detected and the progression of the disease could be followed during the season to better understand the appearance of brown rot. Moreover, the direct PCR method could be extended to study other pathosystems.

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