Method Article

DNA-based detection of grapevine trunk-disease pathogens from environmental spore samples [☆],[☆☆]

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A B S T R A C T

In California vineyards, spore dispersal of fungi that cause grapevine trunk diseases Botryosphaeria dieback and Eutypa dieback occurs with winter rains. Spores infect through pruning wounds made to the woody structure of the vine in winter. Better timing of preventative practices that minimize infection may benefit from routine spore-trapping, which could pinpoint site-specific time frames of spore dispersal. To speed pathogen detection from environmental spore samples, we identified species-specific PCR primers and protocols. Then we compared the traditional culture-based method versus our new DNA-based method.

- PCR primers for Botryosphaeria-dieback pathogen *Neofusicoccum parvum* and Eutypa-dieback pathogen *Eutypa lata* were confirmed species-specific, through extensive testing of related species (in families Botryosphaeriaceae and Diatrypaceae, respectively), other trunk-disease pathogens, and saprophytic fungi that sporulate in vineyards.
- Consistent detection of *N. parvum* was achieved from spore suspensions used fresh or stored at -20°C, whereas consistent detection of *E. lata* was achieved only with a new spore-lysis method, using zirconia/silica beads in a FastPrep homogenizer (MP Biomedicals; Solon, Ohio, USA), and only from spore suspensions used fresh. Freezing *E. lata* spores at -20°C made detection inconsistent.
- From environmental samples, spores of *E. lata* were detected only via PCR, whereas spores of *N. parvum* were detected both via PCR and in culture.

Specifications table

Subject Area:	Agricultural and Biological Sciences
More specific subject area:	Plant Pathology
Method name:	DNA-based detection of spores of grapevine trunk-disease pathogens
Name and reference of original method:	No original method available
Resource availability:	Resources are included in the text and also in Supplementary Table 1.

Description of Protocol

Background

Trapping spores from the environment can detect dispersal of a fungal pathogen to a healthy crop [1]. At a time of year when the host is known to be susceptible to infection, such pathogen detections can inform disease-management decisions. However, spore traps indiscriminately catch spores of all species producing and dispersing spores at the same time, which can confound detection of the pathogen from an environmental sample. In the absence of a selective growth medium, growth of the pathogen spores in culture, relative to those of other fungi also trapped, impacts the accuracy of detecting the pathogen when all spores are washed off the trap and spread on fungal growth medium, for example for the slow-growing fungus *Phaeomoniella chlamydospora*, which is one of the causal agents of Esca of grapevine [2]. The species-specificity of methods for detecting pathogens from spore traps can be greatly improved by DNA-based methods, assuming there is a region of the pathogen genome that is unique, relative to related species and unrelated species that might be dispersed to spore traps at the same time.

We developed DNA-based methods to detect spores of Botryosphaeria-dieback pathogen *Neofusicoccum parvum* and Eutypa-dieback pathogen *Eutypa lata* in California vineyards. Spore traps placed in severely symptomatic vineyards during winter rains have been used to detect *N. parvum* [3]. Similarly, spore traps placed in severely symptomatic apricot orchards and vineyards have also been used to detect *E. lata* [4,5]. Given there are no non-destructive means of eradicating the chronic wood infections they cause, it is important to prevent infection. Preventative practices can minimize infection of pruning wounds, which are susceptible in winter (between leaf fall in December and budbreak in March) by spores of *N. parvum* [6] and *E. lata* [7,8]. However, it is difficult to know exactly when to time the practices, as there are no climate-based models to predict spore production, dispersal, germination, or infection. As such, spore trapping, coupled with a rapid method of spore detection, could aid in future development of climate-based models.

In the past, researchers have used distinct spore-trapping methods (i.e., passive versus active spore traps) and identification methods (e.g., culture-based versus DNA-based) when studying spore dispersal of *E. lata* and *N. parvum*. For example, researchers have used volumetric spore traps (Burkard Mfg. Co. Ltd., Rickmasworth, Herts, England) and identified *E. lata* sexual spores (ascospores) under the microscope, based on spore shape and dimensions [4,5]. Also, glass microscope slides coated in petroleum jelly were used to trap spores, and, after spores were washed off the slides and the suspensions plated on growth medium, *N. parvum* colonies were identified in culture [3]. These detection methods are lengthy procedures that require technical expertise. More recently, researchers developed a DNA-based method using qPCR to detect multiple species related to *N. parvum* in the fungal family Botryosphaeriaceae, for spores collected with a volumetric spore trap [9]. However, this DNA-based method was not species-specific, as it amplified ten species. In the present study, we identified species-specific PCR primers for *E. lata* and *N. parvum*. Next, we developed procedures that

Table 1

Primer sets screened for specificity to California isolates of *Eutypa lata* and *Neofusicoccum parvum*. Primers amplify microsatellite loci, hence the ranges of amplicon sizes.

Target species	Primer name	Sequence (5'–3')	Amplicon size (bp)	Reference
Eutypa lata	B03f	CGAATAACTAACTACGCCCTACC	201–234	Baumgartner et al. (2009)
	B03r	GGGAGAAGAATGCGAGAATG		
Neofusicoccum parvum	BOT17	GGCGCAATCTCGATTCGAGC	228–258	Slippers et al. (2004)
	BOT18	CCACGATGTCCGTTCATCG		

allowed PCR amplifications of specific fungal DNA from spore suspensions. Lastly, we tested detection of each pathogen from environmental spore samples using our new DNA-based method, compared to the traditional culture-based method.

Method Details

First, we confirmed species specificity of *E. lata* primers B03f and B03r [10] and *N. parvum* primers BOT17 and BOT18 [11], all of which were originally developed to amplify microsatellite markers (single-sequence repeats) for population genetics studies (Table 1). We screened a total of 61 species, represented by 96 isolates, from our culture collection of known species (Supplementary Table 1). The 96 isolates represented the following six groups:

1. *E. lata* (six isolates from California). *Eutypa lata* is the target species for primers B03f and B03r. Isolate identity was originally confirmed by sequencing the rDNA internal transcribed spacer region (ITS) [12] and the secondary locus β-tubulin (BTUB) [13,14], the latter of which has been shown to be informative for species delineation in the fungal family Diatrypaceae [15].
2. *N. parvum* (five isolates from California and Texas). *Neofusicoccum parvum* is the target species for primers BOT17 and BOT18. Isolate identity was originally confirmed by sequencing ITS and the secondary locus translational elongation factor 1-α (TEF) [16], the latter of which has been shown to be informative for species delineation in the fungal family Botryosphaeriaceae [15].
3. Diatrypaceae relatives (28 isolates of 17 species related to *E. lata*). These are not target species for primers B03f and B03r (nor for *N. parvum* primers BOT17 and BOT18). Isolate identity was originally confirmed by sequencing ITS and BTUB, except for type specimens from the Centraalbureau voor Schimmelcultures (CBS) collection (Westerdijk Fungal Biodiversity Institute, Royal Netherlands Academy of Arts and Sciences), which are presumed accurate.
4. Botryosphaeriaceae relatives (23 isolates of 13 species related to *N. parvum*). These are not target species for primers BOT17 and BOT18 (nor for *E. lata* primers B03f and B03r). Isolate identity was originally confirmed by sequencing ITS and TEF.
5. Other trunk-disease pathogens (21 isolates of 17 species). These are not target species for *E. lata* primers B03f and B03r, nor for *N. parvum* primers BOT17 and BOT18. This includes pathogenic fungi that cause other grapevine trunk diseases (e.g., Black foot, Esca, Phomopsis dieback). It also includes wood-infecting fungi of native trees in the vineyard landscape (e.g., *Salix*). These species may or may not sporulate in winter, but they colonize grapevines. Isolates were originally identified to the species level by sequencing ITS and is considered putative, except for cases in which TEF was also sequenced.
6. Saprophytic fungi and other fungi not known to cause trunk diseases (13 isolates of 12 species). These are not target species for *E. lata* primers B03f and B03r, nor for *N. parvum* primers BOT17 and BOT18. These fungi sporulate in the rainy season in vineyards, based on a preliminary spore-trapping experiment we conducted at the USDA Agricultural Research Service National Clonal Germplasm Repository's Experimental Vineyard (Winters, California, USA) in November 2013. Isolates were originally identified to the species level by sequencing ITS and is considered putative, except for cases in which BTUB or TEF were also sequenced.

PCR reactions with *E. lata* primers (B03f and B03r) and *N. parvum* primers (BOT17 and BOT18) consisted of the following reagents: 1 × PCR Go*Taq* Colorless Buffer (Promega; Madison, Wisconsin,

USA), 1 U Go*Taq* polymerase (Promega), 1 µ*M* of each primer (MWG-Biotech; Ebersberg, Germany), 2 m*M* MgCl$_2$, and 0.5 m*M* dNTPs. Cycling conditions for *N. parvum* primers were as follows: 1 cycle at 95°C for 10 min; 40 cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. For *E. lata* primers, a 'touchdown' version of the program for *N. parvum* primers was used, with an initial denaturation of 2 min, followed by annealing starting at 63°C, and dropping 0.1°C every cycle for a total of 40 cycles. PCR products were detected, as visualized on agarose gel.

Second, after confirming primer specificity with DNA extracted from pure cultures (Supplementary Table 1), we tested detection from serial dilutions of spore suspensions (with no DNA extraction) as template for PCR reactions. Asexual spores (conidia) of *N. parvum* isolate Napa816 were collected with a sterile probe from pycnidia on 2-month cultures on potato dextrose agar overlaid with a sterilized grape leaf (24 h of fluorescent light, at room temperature) and were suspended in sterile water. Sexual spores (ascospores) of *E. lata* were collected from perithecia embedded in field-collected stromata from *Nerium oleander* L., in Solano County, California, by soaking the stromata in distilled water for 1 hour, suspending upside down overnight in a Petri dish containing 1 ml water, and pipetting up the spore suspension.

For *N. parvum*, PCR products were detected, as visualized on agarose gel, from spore suspensions used fresh or stored at -20°C. In contrast, we did not detect *E. lata* consistently from spore suspensions stored at -20°C. We also did not detect *E. lata* after DNA extraction (MoBio PowerSoil DNA Isolate Kit; Qiagen) of spore suspensions used fresh or stored at -20°C. For *E. lata*, PCR products were consistently detected only from fresh spore suspensions, using the following spore-lysis procedure to help break down the cell wall of the spores:

1. Do not freeze *E. lata* spore suspension before using this procedure.
2. To 500 µl of spore suspension in a 2 ml screw-cap tube, add 1/50 volume of 50x TE buffer, pH 10, vortex, and add 1 ml 0.1 mm zirconia/silica beads (BioSpec Products; Bartlesville, Oklahoma, USA). Incubate at 65°C for 3 min.
3. Vent the 2 ml tube and bead-beat in a FastPrep homogenizer (MP Biomedicals; Solon, Ohio, USA) for four 1 min runs at top speed, allowing it to cool for at least 3 min. between runs. Homogenate can be frozen after this step, if necessary.
4. Use homogenate as template for PCR. To minimize inhibition from cell-wall compounds or anything that may have been present on the surface of the spores, the PCR mixture (as detailed above for use with DNA from pure cultures as template) was amended with 0.25% BSA (Roche; another brand we tried was inhibitory) and 0.5% PVP-40 (Sigma-Aldrich) [17]. Check for inhibition by including a reaction 'spiked' with a very low concentration of target DNA from pure culture, alongside the homogenate template.

Primers we did not use, due to lack of specificity, were the following:

1. Primers Pmo1f and Pmo2r for Esca pathogen *Phaeomoniella chlamydospora* [18], which amplified products from *Neofusicoccum nonquaesitum* isolate y1.BotN5t3, *Truncatella angustata* isolate Napa792, and *Alternaria* sp. sect. *Infectoriae* isolate y1.BotL4rt5 of the same size as that of *P. chlamydospora* isolate C42, albeit with different DNA sequences (see Supplementary Table 1 for sequence details and other information on isolates).
2. Primers Eut02 F2 and Eut02 R2 to detect *E. lata* [19], which amplified several species in the Diatrypaceae in addition to *E. lata* (*E. lata* var. *aceri* isolate CBS 217.87, *Cryptosphaeria ligniota* isolate Napa311; see Supplementary Table 1 for sequence details and other information on isolates).
3. Nested primer pairs EBdF and EBdR, and IBdF and IBdR for Botryosphaeria-dieback pathogen *Botryosphaeria dothidea* [20], which did not amplify any of our *B. dothidea* isolates (Napa815, Napa817, Napa830, Napa860, Wolf804; see Supplementary Table 1 for sequence details and other information on isolates).

Lastly, our new DNA-based method was compared to the traditional culture-based method. During year 4 of a four-year study, in which spore dispersal was compared in young, asymptomatic vineyards versus mature, symptomatic vineyards in wine-grape regions Lodi and Napa, in California [21], we used the DNA-based method for detection of *E. lata* and *N. parvum* (as described above).
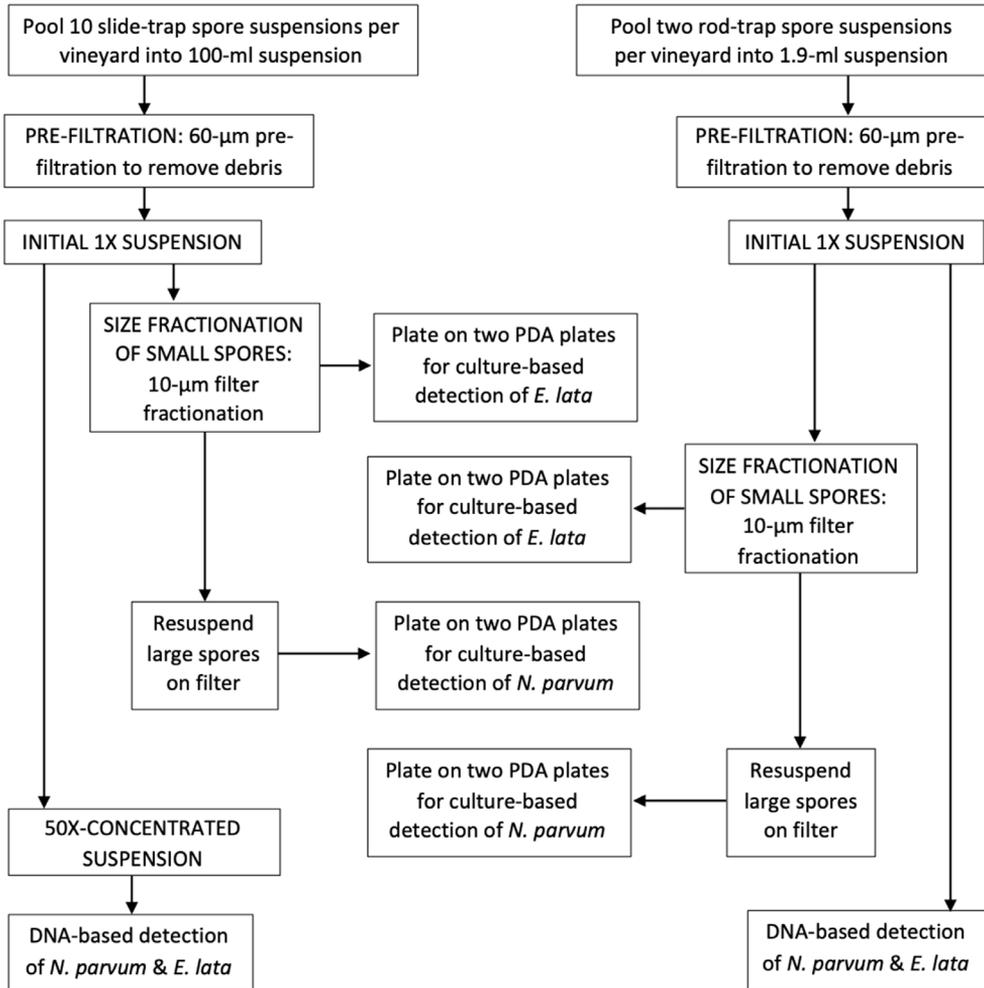
Fig. 1. Processing steps for environmental spore-trap samples from slide traps and rod traps, for culture and DNA-based detection of *Eutypa lata* and *Neofusicoccum parvum*. For culture-based detection, from each of 12 vineyards sites, at a total of nine time points, two different suspensions from each trap type were plated on eight total PDA plates. For DNA-based detection of *E. lata*, spore suspensions from both trap types were homogenized fresh (i.e., samples were not frozen before homogenization). For DNA-based detection of *N. parvum*, spore suspensions from slide traps were further concentrated, and suspensions from each trap type were used directly as template for PCR detection (i.e., no DNA extractions were done and samples were not frozen before use).

Environmental samples from two types of spore traps (slide traps and rod traps) were collected at nine time points from 12 sites [21]. To wash spores from slide traps, the 10 slides per site were placed out into individual 50 ml tubes and shaken with 10 ml 0.1% Tween20 wash solution (by hand or in a 168 rpm reciprocating shaker) for 60 s, and then the 10 suspensions per site were pooled (Fig. 1). To wash spores from rod traps, the two rods per site were vortexed in their 2 ml collection tube with 1 ml wash solution for 60 s at top speed. Tubes containing the rods were vortexed briefly with an additional 900 µl of wash solution that was pooled with the first 1 ml. To remove debris, spore suspensions from either trap type were first passed through a pre-filtration step with a 60 µm pore nylon, 25 mm-diameter nylon net filters held in Swinnex® holders (Millipore, Billerica, Massachusetts, USA), to give the initial 1X suspension. A portion of the initial 1X suspension from the slide traps

Table 2

Culture versus DNA-based detections of *E. lata* and *N. parvum* from environmental spore-trap samples. Each value is pooled across nine time points and three sites. Mature and young vineyard sites (three of each) were located in each of two California wine-grape regions (Lodi and Napa). For culture-based detection, a 'detection' is defined as the presence of a pathogen colony cultured on one or more plates (with one or more colonies per plate) per time point per site. For DNA-based detection, a 'detection' is defined as the presence of an amplicon of the correct size for either *E. lata* or *N. parvum* (Table 1) per time point per site.

Region	Age	Trap	*E. lata*		*N. parvum*	
			culture	DNA	culture	DNA
Lodi	Mature	Rod	0	0	0	0
Lodi	Mature	Slide	0	4	0	0
Lodi	Young	Rod	0	0	0	0
Lodi	Young	Slide	0	4	1	0
Napa	Mature	Rod	0	1	0	0
Napa	Mature	Slide	0	7	1	0
Napa	Young	Rod	0	2	0	1
Napa	Young	Slide	0	5	1	0

was serially concentrated to 50X by centrifuging it in a swinging-bucket centrifuge at 2,000 rpm for 5 min, removing the appropriate amount of supernatant to leave a tenfold-concentrated suspension, re-suspended, and then concentrated another five-fold (using the same procedure) to give the 50X-concentrated suspension. Rod-trap suspensions were not concentrated in the same way as those of the slide traps, as the active spinning of the former is thought to sample more air and, thus, catch more spores.

Size-fractionated portions of the 1X suspension, made by filtration with a 10 μm pore polycarbonate Isopore® filter (Millipore), were plated immediately on PDA amended with 1 ppm of each of the antibiotics ampicillin, neomycin, and tetracycline ('PDA plates'). For each time point per site, and including spore suspensions from both trap types, 200 μl per spore suspension was plated on each of eight total plates (Fig. 1): suspension with small spores from slides (two PDA plates), suspension with large spores from slides (two PDA plates), suspension with small spores from rods (two PDA plates), and suspension with large spores from rods (two PDA plates). Large-spored species included *N. parvum* and other Botryosphaeria-dieback pathogens (conidia are 19.0–26.0 × 8.5–12.5 μm [22]). Small-spored species included *E. lata* and other Eutypa-dieback pathogens (ascopores are 5.0–15.0 × 1.5–2.5 μm [23]). For each timepoint, site, and trap type, *E. lata* or *N. parvum* cultured from one or more PDA plates (with one or more colonies per plate) was recorded as a 'detection'. For DNA-based detection, a 'detection' was defined as the presence of an amplicon of the correct size for either *E. lata* or *N. parvum* (Table 1) per timepoint per site.

Eutypa lata was detected only via PCR and not in culture. The limit of detection was 10 to 30 spores per PCR reaction for the slides, based on amplification of standards with known spore counts. For the rods, only 50 μl of rod suspension was homogenized per site, and the limit of detection for this amount was 600 to > 1,950 spores. Indeed, there were more *E. lata* detections from slides than from rods (20 versus three detections, respectively). There was no significant association between region and vineyard age for *E. lata* detections ($\chi^2 = 0.02$, $p = 0.88$; Table 2). In other words, the patterns of observed detections were consistent, with more detections in Napa than in Lodi, for both mature and young sites. *Neofusicoccum parvum* was detected both via PCR and in culture, but very rarely. The limit of detection for the latter was 10 to 50 *N. parvum* spores per PCR reaction. However, with so few *N. parvum* detections, it is difficult to compare the methods.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101494.

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