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Characterisation and detection of *Pythium* and *Phytophthora* species associated with grapevines in South Africa

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Abstract Replant and decline diseases of grapevines not only cause quantitative and qualitative yield losses, but also result in extra costs when vineyards have to be replanted. This study investigated the role of *Pythium* and *Phytophthora* in the decline syndrome in South Africa by determining (1) the species associated with nursery and established vines, and (2) pathogenicity of *Ph.* sp. *niederhauserii* and *P. vexans* relative to known grapevine pathogens. Quantitative real-time PCR (qPCR) assays were also developed for detection of the most prevalent oomycete groups. In total, 26 *Pythium* and three *Phytophthora* species were identified from grapevine nurseries and established vineyards. The most common infections in sampled nursery vines were caused by *P. vexans* (16.7%), followed by *P. ultimum* var. *ultimum* (15.0%) and *P. irregulare* (11.7%). In established vineyards, *P. irregulare* (18.0%) and *P. vexans* (6.2%) were also among the three most prevalent species, along with *P. heterothallicum* (7.3%). Three *Phytophthora* species were also identified from the sampled established vines, of which *Ph. cinnamomi* (5.1%) was predominant, followed by *Ph.* sp. *niederhauserii* (1.1%). In established vineyards a higher incidence and more diverse species composition was observed in spring and winter, than in summer. Pathogenicity studies showed that some *Ph.* sp. *niederhauserii* and *P. vexans* isolates were as aggressive as the known grapevine pathogens *Ph. cinnamomi* and *P. irregulare*. Sensitive qPCR assays were developed for the detection of *P. ultimum* var. *ultimum*, *P. irregulare*, *P. vexans* and the genus *Phytophthora*. These assays will be invaluable in limiting pathogen dispersal through screening of nursery material. This is especially important since pathogenic species were also isolated from healthy looking vines in nurseries.

Keywords Grapevine decline · Pathogenicity · *Phytophthora* sp. *niederhauserii* · *Pythium irregulare* · *Pythium vexans* · Real-time PCR

Abbreviations

RGP	Relative growth percentage
qPCR	Quantitative real-time PCR

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Introduction

Grapevine (*Vitis* spp.) replant and decline diseases cause financial losses not only due to a decrease in

harvest quality and quantity, but also due to costs involved in replanting dead and dying vines. A large amount of research on grapevine decline has focused on the role of fungi contributing to above ground grapevine trunk diseases, such as species in the Botryosphaeriaceae, Diatrypaceae, *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield and Mugnai) Crous and W. Gams and *Phaeoacremonium* spp. (Mugnai et al. 1999; Van Niekerk et al. 2004; Mostert et al. 2006; Trouillas et al. 2010). Although these pathogens can infect vines and cause disease on their own, severe symptom expression is often only triggered once the host is physiologically stressed (Gubler et al. 2004). Stress-causing microbial soilborne root pathogens may therefore be an important factor in the predisposition of vines to attack by trunk disease pathogens.

Several soilborne pathogens have been shown to adversely affect grapevine root systems world-wide, including fungal genera such as *Cylindrocarpon* Wollenw., *Fusarium* Link, *Verticillium* Hees and *Rhizoctonia* De Candolle and the oomycetes *Pythium* Pringsheim and *Phytophthora* de Bary (Bumbieris 1972; Marais 1979, 1980; Latorre et al. 1997; Gubler et al. 2004; Halleen et al. 2004; Van Coller 2004). In South Africa, Marais (1979, 1980) identified *Phytophthora* and *Pythium* species as the most common and widespread soilborne pathogens of grapevines, both in nurseries and established vineyards. Five *Phytophthora* species [*Ph. cinnamomi* Rands, *Ph. cryptogea* Pethybridge and Lafferty, *Ph. cactorum* (Lebert and Cohn) J. Schröter, *Ph. nicotianae* Breda de Haan (syn. *Ph. parasitica*) and *Ph. megasperma* Drechsler] and five *Pythium* species or complexes [*P. ultimum* Trow, *P. sylvaticum* complex Hendrix and Papa, *P. irregulare* complex Hendrix and Papa, *P. rostratum* complex Hendrix and Papa and *P. aphanidermatum* (Edson) Fitzpatrick] were identified in established vineyards and nurseries. Of these, *Ph. cinnamomi* was the most common and important root pathogen in established vineyards, while *P. ultimum* was more common in grapevine nurseries (Marais 1979, 1980). With the exception of *Ph. megasperma*, which was non-pathogenic, all these species caused crown and/or root rot on grapevines. *Phytophthora* and *Pythium* have also been associated with such symptoms on grapevines in other regions of the world, including Australia, Chile and the USA (Chiarappa 1959; Bumbieris 1972; Latorre et al. 1997; Gubler et al. 2004). In addition to those reported by Marais (1979, 1980) in South Africa, other species reported worldwide include *Ph. cambivora* (Petri) Buisman, *Ph. drechsleri* Tucker, *P. mamillatum* Meurs and *P. oligandrum* Drechsler (Williams and Hewitt 1948; Bumbieris 1972; Latorre et al. 1997; Gubler et al. 2004). Gubler et al. (2004) also reported an unidentified species of *Pythium* that was able to cause severe root and crown rot and even death of young vines.

Previous studies that have investigated the role of *Pythium* and *Phytophthora* species associated with grapevines used conventional culture based methods for the isolation of *Pythium* and *Phytophthora*, and morphological characteristics for identifying isolates to the species level. However, molecular techniques for both identification and detection of *Pythium* and *Phytophthora* species have several advantages over such conventional techniques, including higher accuracy and resolution and they are less labour-intensive (Okubara et al. 2005). Quantitative real-time PCR (qPCR) assays for pathogen detection have the additional advantages of being quantitative, less labour intensive, less prone to cross-contamination and having a higher throughput than conventional PCR assays (Hardegger et al. 2000).

In viticulture, changes in management strategies during the past 30 years that were aimed at increasing yields and quality of grapes may have altered the incidence and species composition of *Pythium* and *Phytophthora* populations from that previously reported (Marais 1979, 1980). Some evidence of this has been reported from the diagnostic clinic of the Agricultural Research council (ARC) at Stellenbosch in South Africa, where the frequency of isolation of *Pythium* species has increased steadily (F. Halleen, ARC-Nietvoorbij, Stellenbosch, South Africa, personal communication). Therefore the aims of this study were: (1) to determine the *Pythium* and *Phytophthora* species associated with nursery and established vines using molecular techniques; (2) to determine the pathogenicity of one *Phytophthora* species (*Ph.* sp. *niederhauserii*) and one *Pythium* species (*P. vexans* de Bary) that have not previously been reported as being associated with grapevines world-wide; and (3) to develop qPCR assays for the detection of the most common pathogenic species.

Materials and methods

Sampling in grapevine nurseries

Samples in nurseries consisted of entire nursery vines including roots and some rhizosphere soil. Three healthy and three diseased (weak growth, stunting, chlorotic foliage) vines were sampled in each of five nurseries representing four climatically different areas in the Western Cape region of South Africa [Clanwilliam (CW), Malmesbury (MB), Vredendal (VD) and Wellington (WL1 and WL2)] (Fig. 1). Samples were taken during February 2005 (summer) and again during October/November 2005 (spring), resulting in a total sample size of 12 vines per nursery over both seasons.

Sampling in established vineyards

Samples from established vineyards were taken from ten climatically different areas in South Africa [Ashton (AS), De Doorns (DD), Elgin (EL), Hermanus (HR), Malmesbury (MB), Marble Hall (MH), Mookgopong (MO), Stellenbosch (SB), Vredendal (VD) and Wellington (WL)] (Fig. 1), during summer (February/March 2005 and 2006), winter (July/August 2005) and spring (September/October 2005). With few exceptions, root samples were taken from one symptomatic (weak growth, stunting, chlorotic foliage and dieback) and one asymptomatic vine in each of three vineyard blocks at each location and during each season (i.e. three healthy and three diseased vines being sampled during each season at each site). The exceptions to this sampling strategy included: (1) MH, where six healthy vines were sampled in summer and no other samples were taken during winter or spring, (2) in MO where six diseased and six healthy vines were sampled in summer in addition to normal sampling in the spring and winter and (3) VD where four healthy and four diseased vines were sampled in summer and spring in addition to normal sampling in the winter. Considering the sampling in all three seasons and all ten regions, a total of 178 vines were sampled, which for the different seasons consisted of 37 healthy and 31 diseased vines for the summer sampling, 27 healthy and 27 diseased vines for the winter sampling, and 28 healthy and 28 diseased vines for the spring sampling.

Isolation

Roots of all samples were rinsed under running tap water to remove soil particles before surface steri-

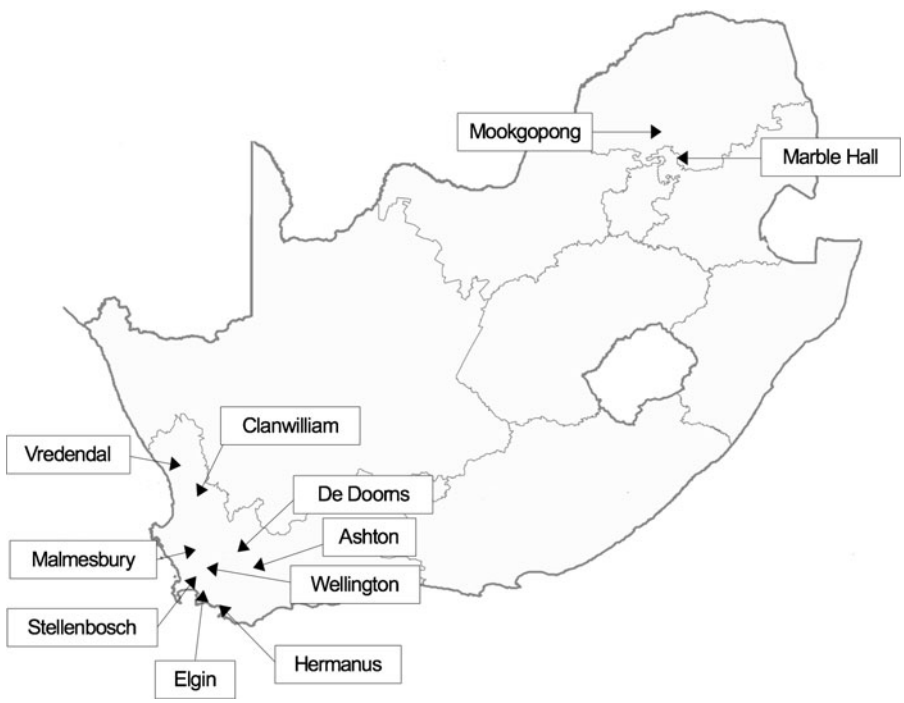

Fig. 1 Geographical location of nurseries and vineyards from which grapevines were sampled for the isolation of *Pythium* and *Phytophthora*. The five sampled nurseries were located at Clanwilliam, Malmesbury, Vredendal and Wellington in the Western Cape province of South Africa. The established vineyards that were sampled were located at Ashton, De Doorns, Elgin, Hermanus, Malmesbury, Stellenbosch, Vredendal and Wellington in the Western Cape province and in Mookgopong and Marble Hall in the Limpopo province

lisation by immersion in 70% ethanol for 2 to 3 s. From each vine, 20 root pieces of ca. 1 cm in length were then plated onto 90 mm petri-dishes containing *Pythium* and *Phytophthora* selective media, PARP and PARPH (Jeffers and Martin 1986), respectively. Additionally, for nursery samples the base of each vine was split open and four pieces of vascular tissue were also plated onto each of PARP and PARPH medium. Plates were incubated in the dark at ca 22°C and inspected daily for new growth for a period of up to 10 days. Hyphae emerging from the plated tissue were hyphal tipped onto corn meal agar (CMA, Sigma-Aldrich, St Louis, USA). These cultures were hyphal tipped again to ensure pure cultures were obtained.

Pythium and *Phytophthora* species identification

A subset of the isolates were selected for identification to the species level, with at least two isolates for each sampled vine, except in instances where only one isolate was obtained from a vine. Furthermore, in some cases where the two selected isolates per vine represented more than one species, more isolates from that vine were selected for species identification.

DNA extractions, amplification of the internal transcribed spacer regions (ITS1 and ITS2) and sequencing were performed as previously described (McLeod et al. 2009). The isolates were identified to the species level by conducting Basic Local Alignment Search Tool (BLAST) searches with the sequence data on GenBank. *Pythium* species were identified using only matches to sequence data submitted by Lévesque and De Cock (2004), except in a few instances where new species were described subsequent to this publication. *Phytophthora* species were identified mainly using sequences submitted by Cooke et al. (2000). In addition to molecular identification, representatives of molecular species were also identified using morphological characteristics as previously described (McLeod et al. 2009).

Pathogenicity trials

The pathogenicity of *P. vexans* and *Ph.* sp. *niederhauserii* was compared to that of *P. irregulare* and *Ph. cinnamomi* on the moderately resistant grapevine rootstock 101–14 Mgt. The trial was set up as a randomised block design with nine treatments (two isolates per species and one uninoculated control) and six replicates (blocks) per trial (i.e. 54 vines). The trial was repeated once. The isolates of *P. irregulare* and *P. vexans* that were used in the pathogenicity studies represented the single most common molecular group present in each of these species as previously identified, i.e. *P. irregulare sensu stricto* (*s.s.*) (Spies 2010) and *P. vexans* group A (*P. vexans s.s.*) (Spies et al. 2011).

Grapevine establishment and inoculation of planting media

Sand/bran inoculum of *Pythium* and *Phytophthora* were produced as previously described (Lamprecht 1986). The control consisted of uninoculated sand and wheat bran.

Dormant one-year-old cuttings of rootstock cultivar 101–14 Mgt were surface disinfested with Sporekill (120 g l^{-1} didecyl dimethyl ammonium chloride) [ICA International Chemicals (Pty) Ltd., Stellenbosch, South Africa] and treated with hot water (50°C for 30 min) to eradicate trunk disease pathogens (Fourie and Halleen 2004). Subsequently, the cuttings were rooted in water (ca 2 weeks) and transplanted to pasteurised Master Potting Mix (Master Organics, Cape Town, South Africa) in 2 l planting bags. Two capped plastic 7 ml test tubes were inserted at each of three positions around the rooted vines at planting to allow for subsequent sand/bran inoculations with minimal damage to the root systems. The vines were allowed to acclimatise for 3 weeks in a greenhouse at 28±7°C and ca 50% relative humidity prior to inoculation. The test tubes were removed and approximately 7 ml of inoculum was added to each hole (in total ca 1% vol/vol) that was then covered with soil. The shoot length at inoculation was recorded for each vine. For the first 3 weeks following inoculation, planting bags were kept in standing water in order to maintain high levels of soil moisture and encourage infection. For the remainder of the plant growth period, the vines were irrigated with a dripper irrigation system to the point of soil saturation once every 5 to 7 days. The vines were fertilized with Seagro [Premier Fishing (Pty.) Ltd., Cape Town, South Africa] every 2 to 3 weeks according to the manufacturer's instructions.

Evaluation of trial

After eight (trial 2) to nine (trial 1) months, the increase in shoot length was determined for each vine. The increase in shoot length of each vine was divided by the increase in shoot length of the control vine from the corresponding block. This value was expressed as a percentage, to reflect the percentage growth in relation to the control (i.e. control=100% growth) for each treatment, hereafter referred to as relative growth percentage (RGP).

Re-isolations were made from a total of six vines for each treatment, which consisted of three vines that were randomly selected from each of the two trials. The re-isolations were performed as described previously for the survey work on the established grapevines, except that only ten root pieces of each plant were plated onto PARP and PARPH media respectively. Growth on the PARP and PARPH dishes were confirmed as *Phytophthora* based on hyphal morphology or as *P. irregulare* or *P. vexans* based on colony morphology. Directly after re-isolation, the remainder of the roots of each plant were placed in a 50 ml BD Falcon™ tube (BD Biosciences, Erembodegem, Belgium) on ice and subsequently frozen at −84°C for DNA extractions.

Statistical analyses

The Shapiro-Wilk test was performed on the relative growth percentage (RGP) values to test for normality (Shapiro and Wilk 1965). The data, including the control (100%), were subjected to analysis of variance using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). Fisher's least significant difference (LSD) was calculated for each of these parameters at the 5% probability level (Ott 1998).

Development of qPCR detection assays

qPCR assays were developed for the detection of the most common and important soilborne oomycete pathogens of grapevines in South Africa, which included a genus-specific assay for *Phytophthora* spp., and species-specific assays for *P. irregulare*, *P. ultimum* var. *ultimum* and *P. vexans*. For the *Phytophthora* genus-specific assay, the conventional PCR detection assay developed by Schena et al. (2006) was adapted to a SYBR Green I qPCR assay. A SYBR Green I assay, based on the ITS region, was also developed for the *P. irregulare* species group, which included *P. irregulare* groups I and II *sensu* Matsumoto et al. (2000), *P. regulare* Masih and B. Paul, *P. cryptoirregulare* Garzón, Yánez and G.W. Moorman and *P. cylindrosporum* B. Paul. TaqMan assays were developed for *P. ultimum* var. *ultimum* and *P. vexans* using the ITS regions. The *P. vexans* assay was designed to detect at least three of the *P. vexans* groups (groups A, B and C) present within this diverse species group (Spies et al. 2011), as well as *P. cucurbitacearum* Takimoto and *P. indigoferae* Butler.

ITS sequences of the targeted *Pythium* species and closely related species were aligned with those of other species within the same *Pythium* clade *sensu* Lévesque and De Cock (2004), using MAFFT sequence alignment program version 6 (Katoh and Toh 2008). Potential primer and probe binding sites were identified using Geneious Pro v. 3.6.2 (Biomatters Ltd., Auckland, New Zealand). The primers and probes used in these studies are presented in Table 1.

Optimisation of qPCR conditions and validation of primers and probes

All qPCR reactions were performed on a RotorGene 6000 real-time rotary analyzer (Qiagen Inc., Valencia, CA, USA). All samples and controls were included in triplicate unless mentioned otherwise. Fluorescence was measured at the appropriate wavelength after each extension period. All SYBR Green I runs were followed by a melting curve analysis to confirm amplification of the target regions.

Optimal concentrations of reagents were determined by varying the concentrations of the primers (50, 300 and 900 nM in all possible combinations), probes (50 to 250 nM in increments of 50 nM) and $MgCl_2$ (3, 4 and 5 mM) in quadruplicate 40 µl reactions containing 1× Sensimix dT (containing $MgCl_2$ to a concentration of 3 mM) (Quantace Ltd., London, UK), 1 µl SYBR Green I (all assays, unless optimal probe concentration was being determined) and 30 ng of target DNA. Cycling conditions for optimisation of the *P. ultimum* var. *ultimum* and *P. vexans* assays consisted of initial denaturing at 95°C for 10 min followed by 40 cycles of denaturing at 95°C for 15 s and annealing/extension at 60°C for

Table 1 Primers and probes used for the quantitative real-time PCR (qPCR) detection of *Pythium ultimum* var. *ultimum*, *P. irregulare*, *P. vexans* and the genus *Phytophthora*

Target taxon	Primer or probe	Sequence (5′→3′)	Reference	Amplicon length
Phytophthora spp.	Yph1F	CGA CCA TKG GTG TGG ACT TT	Schena et al. (2006)	±450 bp
	Yph2R	ACG TTC TCM CAG GCG TAT CT	Schena et al. (2006)	
P. irregulare	PirF1	AGT GTG TGT GGC ACG TTG TC	This study	±120 bp[a]
	PirR3	GAT CAA CCC GGA GTA TAC AAA AC	This study	
P. ultimum var. *ultimum*	PulF2	GCA GGA CGA AGG TTG GTC TG	This study	102 bp
	PulR2	GTC CCC ACA GTA TAA ATC AGT ATT TAG GT	This study	
	PulP2	VIC-TGG ACT AGC TGA TGA ACT T-MGB	This study	
P. vexans	PvF1	TTT CCG TTT TGT GCT TGA TG	This study	147 bp
	PvR1	AGC GAA CAC ACC CAA TAA GC	This study	
	VexP1	HEX™-CCG TGT CTG CTG GCG GGT C-Iowa Black® FQ	This study	

[a] Due to intraspecific variation within the *P. irregulare* species complex (Spies 2010) the length of the amplified fragment varied between and within isolates

30 s. Optimisation for the *Phytophthora* and *P. irregulare* assays were performed as above with the exception that a three-step cycling program was used consisting of denaturing at 95°C for 10 s, annealing at 60°C for 15 s and extension at 72°C for 20 s. These conditions were also used for initial specificity tests for all assays, but modified conditions were necessary to attain specificity (see below). Concentrations with the lowest quantification cycle (C_q) values and largest increase in normalised fluorescence were chosen as optimal primer, probe and $MgCl_2$ concentrations.

The specificity of the qPCR assays was tested against a selection of *Pythium* and *Phytophthora* isolates as indicated in Table 2. All *Pythium* assays were tested against eight isolates representing seven of the clades of Lévesque and De Cock (2004) in addition to representatives of the clade the target taxon belongs to (*P. irregulare*-clade F, *P. ultimum* var. *ultimum*-clade I and *P. vexans*-clade K) and five isolates representing five *Phytophthora* species known as grapevine pathogens in South Africa. The *Phytophthora* assay was also tested against eight isolates representing *Pythium* clades B, D, E, F, H, I and J, eight isolates representing *Pythium* clade K (closely related to *Phytophthora*) and 11 isolates representing five *Phytophthora* species known as grapevine pathogens in South Africa. Specificity testing was done in 40 µl reactions containing 1× Sensimix dT (includes $MgCl_2$ at a concentration of 3 mM), 1 µl SYBR Green I (*P. irregulare* and *Phytophthora* spp. assays only), 30 ng genomic DNA and primers and probes (*P. ultimum* var. *ultimum* and *P. vexans* assays only) at optimal concentrations. Additional $MgCl_2$ was added to attain a final concentration of 5 mM in the *P. ultimum* var. *ultimum* and *P. vexans* assays. The optimal primer concentrations for the *P. ultimum* var. *ultimum* and *Phytophthora* spp. assays were 300 nM of each primer (PulF2, PulR2, Yph1F and Yph2R), while the optimal primer concentrations for the *P. irregulare* and *P. vexans* assays were 300 nM for primers PirF1 and PvR1 and 900 nM for primers PirR3 and PvF1. Optimal probe concentrations were 50 nM VexP and 150 nM PulP2 for the *P. vexans* and *P. ultimum* var. *ultimum* assays respectively. For the TaqMan assays (*P. ultimum* var. *ultimum* and *P. vexans*) 40 two-step cycles were used, whereas the SYBR Green I assays (*P. irregulare* and *Phytophthora* spp.) were subjected to 40 three-step cycles. Species-specific cycling conditions for the *P. ultimum* var. *ultimum* assay entailed 10 s denaturing at 95°C and annealing/extension at 60°C for 30 s. The same denaturing conditions with annealing/extension for 20 s at 62°C provided species-specific detection for the *P. vexans* assay. The *P. irregulare* assay was specific at cycling conditions of denaturing at 95°C for 10 s, annealing at 65°C for 5 s and extension for 20 s at 72°C. The *Phytophthora* assay was genus-specific using 10 s denaturing at 95°C, annealing at 62°C for 15 s and extension for 30 s at 72°C.

Table 2 Isolates used during validation of qPCR assays for the detection of *P. irregulare*, *P. ultimum* var. *ultimum*, *P. vexans* and the genus *Phytophthora*

Isolate	Species	Clade[a]	Assay[b]
PPRI 8759	*P. coloratum*	B	I, U, V, P
PPRI 8588	*P. torulosum*	B	I, U, V, P
PPRI 8597	*P. oligandrum*	D	I, U, V, P
PPRI 8601	*P. echinulatum*	E	I, U, V, P
PPRI 8636	*P. attrantheridium*	F	I
CBS 218.94	*P. cylindrosporum*	F	I
CBS 118731	*P. cryptoirregulare*	F	I
CBS 250.28	*P. irregulare*	F	I, U, V, P
CBS 493.86	*P. irregulare*	F	I
PPRI 8607	*P. irregulare*	F	I
STE-U 6752	*P. irregulare*	F	I
STE-U 6753	*P. irregulare*	F	I
STE-U 6771	*P. irregulare*	F	I
STE-U 6786	*P. irregulare*	F	I
PPRI 8605	*P. kunmingense*	F	I
PPRI 8609	*P. mamillatum*	F	I
PPRI 8611	*P. paroecandrum*	F	I
PPRI 8604	*P. spinosum*	F	I
STE-U 6800	*P. sylvaticum*	F	I
PPRI 8508	*P. helicandrum*	H	I, U, V, P
PPRI 8616	*P. heterothallicum*	I	U
PPRI 8620	*P. splendens*	I	U
PPRI 8621	*P. splendens*	I	U
PPRI 8615	*P. ultimum* var. *ultimum*	I	U
OW 1258	*P. ultimum* var. *ultimum*	I	U, V
OW 2152	*P. ultimum* var. *ultimum*	I	I, U
OW 2187	*P. ultimum* var. *ultimum*	I	U, P
OW 2211	*P. ultimum* var. *ultimum*	I	U
PPRI 8623	*P. perplexum*	J	I, U, V, P
PPRI 8625	*P. chamaehyphon*	K	V, P
OW 297	*P. helicoides*	K	V, P
STE-U 8629	*P. litorale*	K	V, P
STE-U 6127	*P. mercuriale*	K	V, P
STE-U 6748	*P. oedochilum*	K	V, P
CBS 119.80	*P. vexans*	K	V, P
PPRI 8632	*P. vexans*	K	V, P
STE-U 6708	*P. vexans*	K	U, V
STE-U 6720	*P. vexans*	K	V
STE-U 6730	*P. vexans*	K	V, P
STE-U 6735	*P. vexans*	K	I, V
STE-U 6738	*P. vexans*	K	V
STE-U 6745	*P. vexans*	K	V
ARG 512	*Ph. cactorum*	–	V, P
STE-U 7391	*Ph. cinnamomi*	–	U, P
STE-U 7392	*Ph. cinnamomi*	–	I, V, P
OW 1856	*Ph. cinnamomi*	–	P
OW 2300	*Ph. cinnamomi*	–	P
OW 2376	*Ph. cinnamomi*	–	P
OW 2004	*Ph. cryptogea*	–	V, P
AM 3	*Ph. nicotianae*	–	V, P
STE-U 6971	*Ph.* sp. *niederhauserii*	–	P
STE-U 6973	*Ph.* sp. *niederhauserii*	–	P
STE-U 6974	*Ph.* sp. *niederhauserii*		V, P

[a] *Pythium* clades designated according to Lévesque and De Cock (2004)

[b] The specificity of each assay was tested only against certain species. These are indicated by the letters I (*P. irregulare* assay), U (*P. ultimum* var. *ultimum* assay), V (*P. vexans* assay) and P (*Phytophthora* genus assay)

Validation of qPCR assays using grapevine root DNA from pathogenicity trials

Grapevine roots were frozen using liquid nitrogen and then ground to a fine powder for 30 s in a IKA A11 basic mill (IKA-Werke Gmbh & Co. KG, Staufen, Germany). DNA was extracted from triplicate subsamples (60 mg) of each root sample using the NucleoSpin® 96 Plant II kit (Macherey Nagel Gmbh & Co. KG, Düren, Germany) according to the manufacturer's instructions. DNA quality and quantity was estimated using a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). Initial qPCR runs with serial dilutions of grapevine root DNA spiked with DNA of pure culture target taxa indicated the presence of PCR inhibitors in the root DNA extract. Consequently all root DNA samples were diluted 1:10 using sterile distilled water, which alleviated most of the inhibition.

qPCR reaction conditions were as specified for the specificity assays, with the exception that 4 µl of root DNA was used per reaction. The quantitative ability and limit of detection for each assay was determined by constructing standard curves using seven serial dilutions of pure culture target taxon DNA (1 ng μl^{-1} to 1 fg μl^{-1}) in a 1:10 dilution of uninoculated grapevine root DNA extract. Initial runs confirmed the reproducibility of standard curves and subsequent

runs were quantified by including only one serial dilution per run and adjusting a previous standard curve to the new run based on the C_q value of the included serial dilution. qPCR products of selected positive samples from grapevine root DNA extracts were purified and sequenced as described above, in order to confirm amplification of the target taxa.

Results

Identification of *Pythium* and *Phytophthora* spp. From grapevine nurseries

More than 312 putative *Pythium* and *Phytophthora* isolates were obtained from nursery vines, of which 83 were identified to the species level. Molecular identification of isolates corresponding to known species was confirmed through morphological analyses of representative isolates of each species.

Pythium was isolated from all five nurseries, whereas *Phytophthora* was only isolated from one nursery. The Vredendal nursery had the lowest percentage of sampled vines infected by *Pythium* (16.7%). In the other four nurseries, 58.3% or more of the sampled vines were infected by *Pythium* (Table 3). With the exception of species recovered from single vines, all species were isolated from both healthy and diseased vines (Table 3). No clear trends were observed in the seasonal incidence of specific oomycete species.

In total 11 *Pythium* spp. and one *Phytophthora* species were isolated from nursery grapevine material. Based on the percentage of sampled vines infected across all five nurseries, the most common oomycete species were *P. vexans* (16.7%), *P. ultimum* var. *ultimum* (15.0%) and *P. irregulare* (11.7%). All three species were isolated from at least two nurseries, with *P. vexans* and *P. irregulare* being the most dominant species within certain nurseries. The only other species identified in samples from more than one nursery was *P. mercuriale.* The other oomycete species, including the only detected *Phytophthora* species, *Ph.* sp. *niederhauserii*, were each recovered from less than 7% of the sampled vines and only in individual nurseries. This also included isolate PPRI 8598 (GenBank accession FJ415916), which was previously suggested to represent a new species with

Table 3 Percentage of nursery grapevines infected by *Pythium* and *Phytophthora* species in five different nurseries

Species	Nurseries[a]					Vine health[b]		
	CW	MB	VD	WL1	WL2	Healthy	Diseased	Total[c]
P. sp. (aff. *acanthicum*)	8.3	0	0	0	0	0	3.3	1.7
P. chamaehyphon	0	0	0	8.3	0	3.3	0	1.7
P. coloratum	0	0	0	8.3	0	0	3.3	1.7
P. helicoides	0	**33.3**	0	0	0	3.3	3.3	3.3
P. irregulare	**41.7**	16.7	0	0	0	10.0	13.3	11.7
P. litorale	0	**33.3**	0	0	0	3.3	6.7	6.7
P. mamillatum	8.3	0	0	0	0	0	3.3	1.7
P. mercuriale	0	16.7	**16.7**	0	0	6.7	6.7	6.7
P. torulosum	0	0	0	8.3	0	0	0	1.7
P. ultimum var. ultimum	0	16.7	0	25.0	33.3	20.0	10.0	15.0
P. vexans	0	0	0	**33.0**	**50.0**	6.7	26.7	16.7
Ph. sp. *niederhauserii*	0	0	0	8.3	0	3.3	0	1.7
Total	58.3	75.0	16.7	75.0	66.7	56.7	60.0	58.3

[a] Nurseries were situated in Clanwilliam (CW), Malmesbury (MB), Vredendal (VD) and two locations in Wellington (WL1 and WL2). Values indicate the percentage of vines (from a total of 12 vines sampled at each nursery) infected by each of the oomycete species. The highest incidence(s) at each location is indicated in bold

[b] The percentage of apparently healthy or diseased vines (from a total of 30 vines each) infected by each of the oomycete species

[c] The percentage of vines (from a total of 60 vines) infected by each specific oomycete species

P. acanthicum Drechsler as its nearest neighbour in an ITS phylogeny (McLeod et al. 2009) (Table 3). Six of the *Pythium* species (*P. vexans*, *P. litorale*, *P. irregulare*, *P. mercuriale*, *P. torulosum* and *P. ultimum* var. *ultimum*) and *Ph.* sp. *niederhauserii* were recovered from the crowns of nursery vines.

Species composition of oomycete populations differed among the five nurseries. The number of species recovered at each site varied between one (VD) and six (WL1). *Pythium vexans* was the dominant species in both Wellington nurseries (33.3% sampled vines infected in WL1, 50% in WL2), while *P. irregulare* dominated in CW (41.7% sampled vines infected) and *P. litorale* and *P. helicoides* were the dominant species in MB (33.3% sampled vines infected by each) (Table 3).

Identification of isolates from established vineyards

A total of 449 putative *Pythium* and *Phytophthora* isolates were obtained from the roots of grapevines in established vineyards, of which 174 were identified to the species level. Molecular identifications of the known species were confirmed through morphological analyses of representative isolates of each species. Relative species recovery is presented as percentage sampled vines infected (Table 4). As in the nursery survey, most oomycete species (including the predominant species) were recovered from both healthy and diseased vines.

Pythium was isolated from all ten sampled locations, whereas *Phytophthora* was only identified in four of the locations. MB had the lowest percentage of sampled vines infected by *Pythium* and *Phytophthora* (27.8%), whereas the highest percentage sampled vines infected (88.9%) was at DD. Between 33.3% and 66.7% of the sampled vines at each of the remaining locations were infected by *Pythium* and *Phytophthora* (Table 4). Species diversity varied between sites ranging from two species obtained from MH to 13 species in HR (two *Phytophthora* spp. and 11 *Pythium* spp.).

In total, 22 different *Pythium* species were identified in established vineyards, with *P. irregulare* the most common (18.0% of sampled vines infected). *Pythium irregulare* also had the widest distribution, being present in nine out of ten locations, and was solely or jointly the predominant species at five of these sites (AS, DD, EL, MO and MB) (Table 4). *Pythium heterothallicum* (7.3% sampled vines infected) and *P. vexans* (6.2% sampled vines infected) were also fairly well distributed and were isolated in six and five locations respectively. The remainder of the *Pythium* species were each recovered from 2.3% or less of the sampled vines (Table 4).

In total, three *Phytophthora* species were isolated from established vines (Table 4). Among these *Ph. cinnamomi* was more frequently detected (5.1% sampled vines infected) than the other two species, but the distribution of *Ph. cinnamomi* was limited to only two regions (DD and VD). Although not widely distributed, this species was as prevalent at DD as *P. irregulare* (44.4% sampled vines infected). The other two *Phytophthora* species also co-occurred with *P. irregulare*. *Phytophthora* sp. *niederhauserii* (1.1% sampled vines infected), although not recovered from as many vines as *Ph. cinnamomi*, was also isolated from two locations (AS and HR).

The species diversities of *Pythium* and *Phytophthora* were higher in winter and spring than in summer (data not shown). Only four *Pythium* spp. (*P. irregulare*, *P. heterothallicum*, *P. vexans* and *P. helicoides*) and one *Phytophthora* sp. (*Ph. cinnamomi*) were isolated during summer. These five species were also isolated during the spring and winter, but the incidence of *P. irregulare* infected plants was much higher in spring and winter than in summer. Furthermore, some species such as *P. ultimum* var. *ultimum* and *Ph.* sp. *niederhauserii* were only isolated in spring and winter.

While one putative new *Pythium* species was identified from nursery vines, five putative new *Pythium* species were recovered from established vines. Each of these was only recovered from a single vine and was represented by either one or three isolates. The five putative species are referred to as *P.* sp. (aff. *violae*) (one isolate, GenBank accession FJ415937), *P.* sp. (aff. *canariense*) (one isolate, GenBank accession JF499669), *P.* sp. (aff. *monospermum*) (one isolate, GenBank accession JF431913), *P.* sp. (aff. *macrosporum*) (three isolates, GenBank accessions JF431917-9) and *P.* sp. (aff. *heterothallicum*) (three isolates, GenBank accessions JF431914-6), based on their closest match in ITS sequence BLAST analyses in GenBank. With the exception of *P.* sp. (aff. *violae*) and *P.* sp. (aff. *heterothallicum*), none of the putative new species had more than 95% ITS sequence similarity to any GenBank sequences. The *P.* sp. (aff. *violae*) isolate (PPRI 8614) had high ITS sequence

Table 4 Percentage of established grapevines infected by *Pythium* and *Phytophthora* spp. in vineyards from ten geographical regions

Species	Geographical region[a]										Vine health[b]		Total[c]
	AS	DD	EL	HR	MB	MH	MO	SB	VD	WL	Healthy	Diseased	
P. sp. (aff. *canariense*)	0	5.6	0	0	0	0	0	0	0	0	1.1	0	0.6
P. chamaehyphon	5.6	0	0	5.6	0	0	0	0	0	0	2.2	0	1.1
P. coloratum	0	0	0	0	5.6	0	0	0	4.6	0	0	2.3	1.1
P. echinulatum	0	0	0	0	0	0	0	11.1	0	0	1.1	1.2	1.1
P. helicoides	11.1	0	0	5.6	**16.7**	0	0	0	0	0	3.3	3.5	3.4
P. heterothallicum	0	0	5.6	0	0	**33.3**	8.3	**16.7**	**18.2**	5.6	9.8	4.7	7.3
P. sp. (aff. *heterothallicum*)	0	0	0	0	0	0	0	0	0	5.6	1.1	0	0.6
P. irregulare	**27.8**	**44.4**	**22.2**	22.2	**16.7**	0	**12.5**	5.6	9.1	11.1	18.5	17.4	18.0
P. kunmingense	0	0	0	0	0	0	0	0	0	5.6	0	1.2	0.6
P. litorale	5.6	0	0	11.1	0	0	0	0	0	0	3.3	0	1.7
P. sp. (aff. *macrosporum*)	0	0	0	5.6	0	0	0	0	0	0	1.1	0	0.6
P. sp. (aff. *monospermum*)	0	0	0	5.6	0	0	0	0	0	0	0	1.2	0.6
P. paroecandrum	0	0	0	**27.8**	0	0	0	5.6	0	5.6	5.4	2.3	3.9
P. perplexum	0	0	0	5.6	0	0	0	0	0	0	0	1.2	0.6
P. pyrilobum	5.6	0	0	0	0	0	0	0	0	0	1.1	0	0.6
P. recalcitrans	0	0	5.6	0	0	0	0	0	13.6	0	2.2	2.3	2.3
P. rostratifingens	0	5.6	0	0	0	0	0	0	0	5.6	1.1	1.2	1.1
P. spinosum	0	0	0	5.6	0	0	0	0	0	0	0	1.2	0.6
P. ultimum var. *ultimum*	5.6	0	0	0	0	0	8.3	0	4.6	0	1.1	3.5	2.3
P. vanterpoolii	0	0	0	0	0	0	0	0	0	**16.7**	1.1	2.3	1.7
P. vexans	0	0	16.7	5.6	0	**33.3**	8.3	0	9.1	0	6.5	5.8	6.2
P. sp. (aff. *violae*)	0	0	0	5.6	0	0	0	0	0	0	0	1.2	0.6
Ph. cinnamomi	0	**44.4**	0	0	0	0	0	0	4.6	0	3.3	7.0	5.1
Ph. cryptogea	0	0	0	5.6	0	0	0	0	0	0	0	1.2	0.6
Ph. sp. *niederhauserii*	5.6	0	0	5.6	0	0	0	0	0	0	2.2	0	1.1
Total	44.4	88.9	38.9	50.0	27.8	66.7	37.5	33.3	45.5	33.3	47.8	44.2	46.1

[a] Established vineyards were sampled in Ashton (AS), De Doorns (DD), Elgin (EL), Hermanus (HR), Malmesbury (MB), Marble Hall (MH), Mookgopong (MO), Stellenbosch (SB), Vredendal (VD) and Wellington (WL). Values represent the percentage of vines from each location infected by each of the oomycete species. In total 18 vines were analysed at each location, except for MO, MH and VD where 24, 6 and 22 vines were analysed respectively. The highest incidence(s) at each location is indicated in bold

[b] The percentage of apparently healthy and diseased vines (from a total of 92 and 86 vines respectively), which were infected by each oomycete species

[c] The percentage of vines from a total of 178 vines infected by each oomycete species

similarity (98%) to the CBS 132.37 isolate of *P. violae* and although related, it was shown in a previous study to be phylogenetically distinct from CBS 132.37, thus representing a putative new species (McLeod et al. 2009). The *P.* sp. (aff. *heterothallicum*) sequences had very high ITS sequence similarity (99%) to *P.* sp. P15703 (GU259268), an unpublished sequence of a putative new *Pythium* species from the World Phytophthora Collection (WPC).

Pathogenicity trials

Analysis of variance for RGP indicated no significant interaction between the treatments and the two trials (P=0.8376), consequently the data of the two trials were combined. The combined data revealed a significant effect of treatments (P=0.0307) on vine growth (Table 5) with five isolates (STE-U 6708, STE-U 6753, STE-U 6791, STE-U 7391 and STE-U

Table 5 Analysis of variance for the relative growth percentage (RGP) of 101–14 Mgt grapevines inoculated with *Phytophthora cinnamomi*, *Ph.* sp. *niederhauserii*, *Pythium irregulare* or *P. vexans* (two isolates each)

Source of variation	df	MS	P > F
Trial	1	11.30002	0.9479
Block	10	4961.71358	0.0600
Treatment	8	5983.29443	0.0307
Trial × Treatment	8	1368.32140	0.84

7392) causing significant growth reductions. *Phytophthora cinnamomi* was the only species of which both isolates caused significant stunting of 101–14 Mgt rootstock vines (Table 6). Of the remaining species, only one of the two isolates examined of *Ph.* sp. *niederhauserii* (STE-U 6791), *P. irregulare* (STE-U 6753) and *P. vexans* (STE-U 6708) caused significant growth reductions ($RGP_{STE\text{-}U6791}$=48.74%, $RGP_{STE\text{-}U6753}$=42.83%, $RGP_{STE\text{-}U6708}$=54.76%), relative to the uninoculated control. Therefore, only these isolates were designated as being pathogenic under the current assay conditions, with the remaining isolates being designated as non-pathogenic.

Grey to black necrotic lesions on larger roots and disintegration of fine rootlets were observed in some of the inoculated plants, but re-isolation of the different oomycete isolates from these symptomatic tissue was not always successful. This may have been due to the presence of secondary saprophytic invaders that eliminated the primary oomycete infections. *Phytophthora cinnamomi* was only recovered from 33% of the vines inoculated with STE-U 7391 and not from any vines inoculated with isolate STE-U 7392. *Phytophthora* sp. *niederhauserii* was only recovered from 17% of the vines inoculated with the non-pathogenic *Ph.* sp. *niederhauserii* isolate (STE-U 6794), whereas all vines inoculated with the pathogenic isolate of this species (STE-U 6791) yielded *Phytophthora*. Infection of vines with *P. irregulare* was confirmed for 50% and 83% of the vines inoculated with isolate STE-U 6771 (non-pathogenic) and STE-U 6753 (pathogenic), respectively. *Pythium vexans* was recovered from all vines inoculated with STE-U 6708 or STE-U 6724 (Table 6). No growth was observed on the PARP and PARPH dishes onto which roots of the uninoculated control vines had been plated.

Validation of qPCR assays in grapevine root DNA

Standard curves constructed from root DNA of the uninoculated control (diluted 1:10), which was spiked

Table 6 The effect of artificially inoculated *Phytophthora* and *Pythium* isolates on rooted cuttings of the grapevine rootstock 101–14 Mgt, and the percentage re-isolation and oomycete DNA concentrations in roots samples from the inoculated vines

Species	Isolate	RGP[a]	% Re-isolation[b]	Mean DNA concentration (fgμl^{-1})[c]
Ph. cinnamomi	STE-U 7391	42.60 c	33	8.82
	STE-U 7392	46.15 c	0	9.42
Ph. sp. *niederhauserii*	STE-U 6791	48.74 bc	100	29.70
	STE-U 6794	62.30 abc	17	5.91
P. irregulare	STE-U 6753	42.83 c	83	4.44
	STE-U 6771	90.50 ab	50	7.02
P. vexans	STE-U 6708	54.76 bc	100	1320.00
	STE-U 6724	82.93 abc	100	56.30
Control	–	100.00 a	0	0

[a] The effect of each isolate is expressed as the relative growth percentage (RGP) in relation to the uninoculated control (RGP=100%). $RGP = \left[(\text{increase in shoot length})_{\text{treatment}} \div (\text{increase in shoot length})_{\text{control}}\right] \times 100$. The trial was conducted twice, with six replicates per treatment in each trial. Values followed by different letters were significantly different according to the Student's *t*-test (P=0.0307, LSD=42.53)

[b] Percentage of vines from which the inoculated oomycete was recovered by re-isolation onto PARP and PARPH media

[c] Mean DNA concentrations of the oomycete species were determined using quantitative real-time PCR (qPCR). Values represent averages of 18 samples (six vines, three DNA samples per vine) tested in triplicate. Samples with C_q-values >40 were designated a DNA concentration of 0 fgμl^{-1}

with serial dilutions of the oomycete target DNA, had R^2 values of at least 0.99 and reaction efficiencies of 0.91 or higher. A degree of variability in quantification accuracy was observed when different isolates of the same species were used to construct the standard curve. Such variability was greatest among *P. vexans* isolates for which the amount of target DNA could be underestimated or overestimated by more than two orders of magnitude, when isolates from different groups (A to C) within this species (Spies et al. 2011) were used to generate the standard curve. For example, when serial dilutions of pure culture DNA from a *P. vexans* group A isolate was used for the standard curve, DNA of group B and C isolates at 10 ng μl^{-1} each would be estimated at ca. 1,600 and 3,000 ng μl^{-1} respectively (data not shown). For the assays of the other *Pythium* and *Phytophthora* species, such variation was generally below a single order of magnitude. Consequently, the detection limits presented here pertain specifically to the isolates used in the pathogenicity study. The *P. vexans* assay (group A isolates) was the most sensitive, being able to detect and quantify 6.9 fg μl^{-1} *P. vexans* DNA in a 1:10 dilution of grapevine root DNA spiked with pure culture DNA. The *P. ultimum* var. *ultimum* and *P. irregulare* assays had limits of 13 fg μl^{-1} and 10.6 fg μl^{-1} respectively, while the *Phytophthora* assay allowed detection of 12.7 fg μl^{-1} of target DNA.

qPCR using grapevine root DNA from the artificially inoculated vines generally indicated levels of target taxon DNA lower than the reliable quantitative range of the assays for the *Phytophthora* and *P. irregulare* assays, but not for *P. vexans*. The average *Phytophthora* DNA concentration in vines inoculated with *Ph. cinnamomi* isolates STE-U 7391 and 7392 were estimated at 8.82 and 9.42 fg μl^{-1} respectively (Table 6). In the *Ph.* sp. *niederhauserii* inoculated vines, on average the amount of target taxon DNA was within the quantitative range for isolate STE-U 6791 (29.7 fg μl^{-1}) that was pathogenic, but not for the non-pathogenic isolate STE-U 6794 (5.91 fg μl^{-1}). A similar low level of target DNA was observed for the *P. irregulare* treatments with 4.44 fg μl^{-1} and 7.02 fg μl^{-1} estimated for isolates STE-U 6753 (pathogenic) and STE-U 6771 (non-pathogenic) respectively. In contrast to the low levels of pathogen DNA observed in *Phytophthora* and *P. irregulare* inoculated vines, *P. vexans* DNA in vines from both *P. vexans* treatments were well within the quantitative range of the assay for this species. The pathogenic *P. vexans* isolate (STE-U 6708) had a higher DNA concentration (1.32 pg μl^{-1}) than the non-pathogenic isolate (STE-U 6724) (56.3 fg μl^{-1}) (Table 6). Thus, no clear relationship could be established between the degree of stunting, the amount of pathogen DNA in the roots and the degree to which the pathogens were recovered from inoculated material.

Discussion

The high oomycete species diversity identified during this study in grapevine nurseries (11 *Pythium* and one *Phytophthora* species) and vineyards (22 *Pythium* and three *Phytophthora* species), including six putative new *Pythium* species, differs remarkably from research conducted in the 1970s. Marais (1980) only identified five *Pythium* species complexes and five *Phytophthora* species in grapevine nurseries and in established vineyards only four *Pythium* and four *Phytophthora* species (Marais 1979). This relatively low species diversity is most likely due to (1) the higher accuracy and resolution of molecular protocols used in our study versus previously used morphological methods, and (2) the identification of *Pythium* species complexes by Marais (1979, 1980) as proposed by Hendrix and Papa (1974), which could have included several different species under the same name (i.e. the *P. irregulare* complex, *P. rostratum* complex and *P. sylvaticum* complex). Had this approach been used in the current study it would have resulted in only four fewer species being identified.

The current study and that of Marais (1979, 1980) also differed relative to the frequency and distribution of *Phytophthora* detected in vineyards. Marais recovered *Phytophthora* isolates (mainly *Ph. cinnamomi*) from all nine regions (including four regions sampled during the current study: MB, DD, SB and WL) where he sampled established grapevines (Marais 1979) and from ten of the 22 regions (including three regions sampled during the current study: MB, WL and VD) where he sampled grapevine nurseries (Marais 1980). The fact that our study indicates a lower incidence and distribution of *Phytophthora* suggests that changes in grapevine production systems over the past 20 years have suppressed the incidence and distribution of these pathogens. These

changes include: (1) a drastic increase in the use of fungicides with activity against *Phytophthora*, such as fosetyl-Al and metalaxyl for controlling the downy mildew oomycete pathogen, *Plasmopara viticola* (Berk. and M.A. Curtis) Berl. and De Toni, (Magarey et al. 1991; Fourie 2004); (2) the implementation of management practices such as the use of resistant rootstocks (Marais 1988) and hot water treatment of dormant nursery vines (Von Broembsen and Marais 1978); and (3) the planting of cover crops, such as wheat (a non-host of *Phytophthora*), between rows in some vineyards. These factors could have also contributed to changes in the *Pythium* species diversity. Despite the fact that the incidence of *Ph. cinnamomi* in South African vineyards and grapevine nurseries have declined since the 1970's, the high incidence and distribution of other pathogenic species, such as *P. irregulare* and *P. vexans*, which caused up to 43% or 55% growth reductions on vines under glasshouse conditions, indicates that soilborne oomycete pathogens may still have a significant negative impact on grapevine production through direct damage to the root system. Marais (1979, 1980) also observed severe stunting as well as death of vines that were inoculated with certain *Pythium* species under glasshouse conditions. Oomycetes can, furthermore, also negatively influence grapevine production indirectly by causing stress in the host, which can result in the aggravation of existing trunk disease pathogen infections. Future studies should use artificial inoculation studies with oomycetes in single inoculations, and when co-inoculated with trunk disease pathogens, such as *Phaeomoniella chlamydospora*, under glasshouse or field conditions to shed more light on the role of oomycetes in grapevine production under field conditions, and the symptoms they cause.

Six putative new *Pythium* species were also recovered, one from nursery vines and five from established vineyards. The majority of vineyards and nurseries sampled during this study are located in the Western Cape where the predominant natural vegetation type is fynbos, a highly diverse group of plants likely to harbour highly diverse soil microbial communities that have to date been poorly characterised. It is therefore possible that the putative new species recovered during this study infected grapevines where fynbos was cleared to establish new vineyards or the species immigrated from the native surrounding vegetation.

The most common *Pythium* species detected in nurseries were also among the most common species in established vineyards. The most common species in nurseries was *P. vexans*, followed by *P. ultimum* var. *ultimum* and *P. irregulare*. *Pythium irregulare* and *P. vexans* were also among the three most prevalent species in established vineyards, in addition to *P. heterothallicum*. These data support the proposal of Marais (1980) that *Pythium* spp., including pathogenic species, may be distributed to vineyard plantings in symptomless nursery material.

The seasonal changes in species diversity reported here confirm earlier reports of higher *Pythium* and *Phytophthora* incidences during winter and spring in grapevines (Marais 1988) and other cropping systems (Shearer and Shea 1987; Larkin et al. 1995; Ali-Shtayeh et al. 2003). Therefore, investigations into oomycete species associated with grapevines in South Africa should rather be conducted in spring and winter.

Several species from the current study have never been reported from grapevines in South Africa, most notably, *P. vexans* which was the most common *Pythium* species recovered. This species has only previously been reported in association with grapevines in Iran (Mostowfizadeh-Ghalamfarsa and Banihashemi 2005). Other frequently recovered *Pythium* species that are first reports for grapevines include *P. heterothallicum*, *P. paroecandrum* and *P. helicoides* (also isolated in one nursery). *Phytophthora* sp. *niederhauserii* is also a first report from grapevines. The pathogenicity of the aforementioned *Pythium* species is currently unknown and requires investigation in future studies since they might play a role as pathogens in vineyards as suggested by: (1) their site-specific dominance; and (2) reports of these species also causing disease in other perennial crops, including apple trees (*Malus* spp.; Mazzola et al. 2002), pecan seedlings [*Carya illinoinensis* (Wangenheim) K. Koch; Hendrix and Powell 1968], roses (*Rosa* spp.; Kageyama et al. 2002; Kageyama et al. 2003), and peach seedlings [*Prunus persica* (L.) Batsch; Mircetich and Fogle 1969]. *Pythium paroecandrum* has also been frequently isolated from citrus (*Citrus* spp.) orchards in South Africa (Thompson et al. 1995) and was weakly virulent when inoculated to trunks (Maseko and Coutinho 2002).

Due to the importance of most *Phytophthora* species as aggressive pathogens, and the widespread

occurrence of *P. vexans*, the pathogenicity of *Ph.* sp. *niederhauserii* and *P. vexans* was investigated using the moderately resistant rootstock 101–14 Mgt. The aggressiveness of these species was compared to that of *Ph. cinnamomi* and *P. irregulare* that are highly virulent on grapevines (Marais 1979, 1980). *Phytophthora cinnamomi* was the only species in assays for which both isolates examined were pathogenic; whereas for the other species, only one of the two isolates was pathogenic. Intraspecific variation in aggressiveness and/or pathogenicity is not uncommon in *Pythium* and *Phytophthora* and has been reported for species such as *Ph. cinnamomi*, *P. irregulare* and *P. sylvaticum* (Denman et al. 1995; Robin and Desprez-Loustau 1998; Mazzola et al. 2002).

Pythium vexans and *Ph.* sp. *niederhauserii* have been reported as pathogens of other hosts. On tree crops, *P. vexans* causes root rot and necrosis on durian (*Durio zibethinus* L.; Vawdrey et al. 2005), macadamia (*Macadamia integrifolia* Maiden and Betche; Serfontein et al. 2008) and apple trees (*Malus* spp.; Tewoldemedhin et al. 2011), seedling damping-off of wild cashew (*Anacardium excelsum* L.; Davidson et al. 2000), cankers on trunks of rubber trees (*Hevea brasiliensis* Müller Argoviensis; Zeng et al. 2005) and lesions on trunks of *Eucalyptus* spp. (Linde et al. 1994). The association of *P. vexans* with trunks and cankers and grapevine nursery crowns in our study, is interesting since *Pythium* species are generally perceived as pathogens of fine feeder roots (Hendrix and Campbell 1973; Cooke et al. 2000). However, it is important to note that *P. vexans* is not a typical *Pythium* species since it belongs to *Pythium* clade K *sensu* Lévesque and de Cock (2004), which may represent a distinct genus closely related to *Phytophthora* (Lévesque et al. 2008; Bala et al. 2010; Uzuhashi et al. 2010). *Phytophthora* sp. *niederhauserii*, although not yet officially described, was first identified in the USA from ivy (*Hedera helix* L.; Abad and Abad 2003) and has since been reported from several regions including Europe (Moralejo et al. 2009; Pérez-sierra et al. 2010) and Australia (Burgess et al. 2009) mainly from ornamental hosts, but also almond trees (*Prunus dulcis* Miller). On almond trees, symptoms included stem cankers and gummosis (Pérez-sierra et al. 2010). The recovery of *Ph.* sp. *niederhauserii* from the crowns of nursery vines in our study, also indicates the potential of this species to cause crown rot.

The qPCR assays that were developed for the most common and pathogenic oomycetes (*P. irregulare*, *P. vexans* and *P. ultimum* var. *ultimum*, and the genus *Phytophthora*) were efficient when evaluated on pure culture DNA, but certain problems were identified when the assays were validated on roots artificially infected with *P. irregulare*, *P. vexans* and *Phytophthora*. This may be due to the differential infection of tissue types (finer roots, coarser roots or crown) by these pathogens. For instance, *P. vexans*, which has the ability to infect coarser host tissues such as stems and trunks (Van der Plaats-Niterink 1981; Zeng et al. 2005; this study) could be easily detected from root systems, while *P. irregulare*, which is usually associated with fine, brittle rootlets that tend to disintegrate (Bumbieris 1972), was only detected at low levels. This general lack of correlation between the amount of pathogen DNA concentration in roots, and plant growth inhibition has also been reported for oomycete pathogens on apple (Tewoldemedhin et al. 2011). The low levels of DNA detected for *Ph. cinnamomi* in grapevine roots were unexpected since this species is able to infect coarser roots and crowns of grapevines (Latorre et al. 1997; Gubler et al. 2004). However, it is possible that the two *Ph. cinnamomi* isolates used in the pathogenicity trials were also mainly associated with finer rootlets that were lost during washing of the roots, since *Ph. cinnamomi* isolates may vary in their propensity to infect coarser root tissues (Robin and Desprez-Loustau 1998).

A complication with the use of qPCR assays for quantification of pathogenic species is that dissimilar DNA concentrations may be obtained when different isolates from the same species are used for quantification. This problem was identified for *P. vexans*, since standard curves constructed with isolates of *P. vexans* group A (Spies 2010), overestimated DNA concentrations of isolates of groups B and C by more than two orders of magnitude. This anomaly might be due to differences in the ITS copy number between isolates from the different groups or different amplification efficiencies of the target region in the different groups. This phenomenon was not observed for the other investigated *Pythium* species.

The findings of our study have improved our knowledge concerning the detection of oomycetes and their role in grapevine health in South Africa. The most common and known pathogenic *Pythium* (*P. vexans, P. ultimum* and *P. irregulare*) and *Phytoph-*

thora (*Ph. cinnamomi* and *Ph.* sp. *niederhauserii*) species that were identified may each function on its own in the grapevine decline syndrome, or when associated with other pathogens. These oomycetes could aggravate and induce symptom expression by existing infections of trunk disease pathogens such as *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., which are known as severe pathogens on stressed plants (Gubler et al. 2004). The possible involvement of asymptomatic nursery material in the distribution of pathogenic species in South Africa is important, and is likely due to vines only being examined visually. Therefore, our sensitive qPCR assays will be valuable for the screening of nursery material as a means to limit the dispersal of pathogens. The qPCR assays can also be invaluable in research applications such as the evaluation of management strategies aimed at minimising the contribution of *Pythium* and *Phytophthora* to grapevine decline. Future studies should assess the utility of rhizosphere soil as the sample unit to determine if a better correlation exists between pathogen DNA concentrations in this substrate and vine growth stunting, since some pathogenic species were only detected at low levels from root material.

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