MOLECULAR DETECTION OF *PHAEOMONIELLA CHLAMYDOSPORA* IN GRAPEVINE NURSERIES

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature:………………………… Date:…………………………

SUMMARY

MOLECULAR DETECTION OF *PHAEOMONIELLA CHLAMYDOSPORA* IN GRAPEVINE NURSERIES

Phaeomoniella chlamydospora is the main causal organism of Petri disease, which causes severe decline and dieback of young grapevines (1-7 years old) and also predisposes the wood for infection by other pathogens. Knowledge about the epidemiology and especially inoculum sources of this disease is imperative for subsequent development of management strategies. Through isolation studies it was shown that *Pa. chlamydospora* is mainly distributed through infected propagation material in South Africa. However, the infection pathways and inoculum sources in grapevine nurseries are still unclear. The only existing method to detect this pathogen in various media is by means of isolation onto artificial growth media. This has proven to be problematic since this fungus is extremely slow growing (up to 4 weeks from isolation to identification) and its cultures are often over-grown by co-isolated fungi and bacteria before it can be identified. The aim of this study was (i) to develop a protocol for the molecular detection of *Pa. chlamydospora* in grapevine wood, and (ii) to use this protocol along with others, to test different samples (water, soil, rootstock and scion cuttings and callusing medium) collected from nurseries in South Africa at different nursery stages for the presence of *Pa. chlamydospora*.

A protocol was developed and validated for the molecular detection of *Pa. chlamydospora* in grapevine wood. Firstly, several previously published protocols were used to develop a cost-effective and time-efficient DNA extraction method from rootstock pieces of potted grapevines. Subsequently, PCR amplification using species-specific primers (Pch1 and Pch2) was found to be sensitive enough to detect as little as 1 pg of *Pa. chlamydospora* genomic DNA from grapevine wood. The protocol was validated using various grapevine material from 3 different rootstock cultivars (101-14 Mgt, Ramsey and Richter 99) collected from each of 3 different nurseries, including grapevines that were subjected to hot water treatment. The basal end of the rootstock was parallel analysed for *Pa. chlamydospora* using isolations onto artificial medium and molecular detection. The identity of PCR products obtained from a subset of samples, that only tested positive for *Pa. chlamydospora* based on molecular detection, was confirmed to be *Pa. chlamydospora* specific through restriction digestion with *AatII*. Molecular detection was found to be considerably more sensitive than isolations, detecting *Pa.*

chlamydospora from samples with positive as well as negative isolations. On average, the molecular technique detected *Pa. chlamydospora* in 80.9% of the samples, whereas only 24.1% of the samples tested positive for *Pa. chlamydospora* by means of isolations. *Pa. chlamydospora* was not isolated from hot water treated samples. The results confirm the importance of hot water treatment for proactive management of Petri disease in grapevine nurseries. However, *Pa. chlamydospora* DNA was molecularly detected in hot water treated samples in frequencies similar to that detected in non-hot water treated samples. As expected, the DNA in hot water treated plants was not destroyed and could be detected by the developed molecular detection protocol. This is an important consideration when using molecular detection for disease diagnosis or pathogen detection and shows that these methods should be used in conjunction with other diagnostic tools. Most importantly, the DNA extraction protocol was shown to be 10 to 15 times cheaper than commercial DNA extraction kits.

Preliminary studies showed that the aforementioned molecular detection technique was not specific and sensitive enough for detection of *Pa. chlamydospora* in soil and water (unpublished data). Therefore, a one-tube nested-PCR technique was optimised for detecting *Pa. chlamydospora* in DNA extracted from soil, water, callusing medium and grapevine wood. Rootstock cane sections and soil samples were taking from the mother blocks from several nurseries. Water samples were collected from hydration and fungicide tanks during pre-storage and grafting. Scion and rootstock cuttings were also collected during grafting and soil were collected from the nursery beds prior to planting. The one-tube nested-PCR was sensitive enough to detect as little as 1 fg of *Pa. chlamydospora* genomic DNA from water and 10 fg from wood, callusing medium and soil. PCR analyses of the different nursery samples revealed the presence of several putative *Pa. chlamydospora* specific bands (360 bp). Subsequent sequence analyses and/or restriction enzyme digestions of all 360 bp PCR bands confirmed that all bands were *Pa. chlamydospora* specific, except for five bands obtained from callusing media and one band from water. Considering only *Pa. chlamydospora* specific PCR bands, the molecular detection technique revealed the presence of *Pa. chlamydospora* in 25% of rootstock cane sections and 17% of the soil samples collected from mother blocks, 42% of rootstock cuttings collected during grafting, 16% of scion cuttings, 40% of water samples collected after the 12-hour pre-storage hydration period, 67% of water samples collected during grafting and 8% of the callusing medium samples. These media should therefore be considered as potential inoculum sources or infection points of the pathogen during the nursery stages. The results furthermore confirmed previous findings that *Pa. chlamydospora* is mainly distributed through infected rootstock canes and cuttings. Infected scion cuttings were also shown to be potential carriers of

the pathogen. Management strategies should include wound protection of rootstock mother plants, eradicating this pathogen from rootstock-cuttings (e.g. hot water treatment), biological or chemical amendments in the hydration water and callusing medium and wound protection from soil borne infections.

OPSOMMING

MOLEKULÊRE OPSPORING VAN *PHAEOMONIELLA CHLAMYDOSPORA* IN WINGERD KWEKERYE

Phaeomoniella chlamydospora is die hoof veroorsakende organisme van Petri se siekte wat lei tot die agteruitgang en terugsterwing van jong wingerdplante (1-7 jaar oud) en veroorsaak verhoogde vatbaarheid van hout vir infeksie deur ander patogene. Kennis oor die epidemiologie en veral die inokulumbronne van die siekte is noodsaaklik vir die daaropvolgende ontwikkeling van beheerstrategieë. Isolasies het getoon dat *Pa. chlamydospora* meestal versprei deur middel van geïnfekteerde voortplantingsmateriaal in Suid-Afrika. Die infeksieweë en inokulumbronne in wingerdkwekerye is egter steeds onbekend. Die enigste bestaande metode vir die opsporing van die patogeen, in verskeie mediums, is deur middel van isolasie op kunsmatige groeimediums. Dit is egter gevind om problematies te wees aangesien die swam uiters stadig groei (dit vat tot 4 weke vanaf isolasie tot identifikasie) en die kulture is telkens oorgroei deur ander organismes voordat identifikasie kan plaasvind. Die doel van die studie was (i) om 'n protokol te ontwikkel vir die molekulêre opsporing van *Pa. chlamydospora* in wingerdhout, en (ii) om die protokol te gebruik, saam met ander, om verskillende monsters (water, grond, onderstok- en bostok-ente en kallusmedium) te toets, wat versamel is van kwekerye in Suid-Afrika, tydens verskillende kwekerystadiums, vir die teenwoordigheid van *Pa. chlamydospora*.

'n Protokol is ontwikkel en geverifieer vir die molekulêre opsporing van *Pa. chlamydospora* in wingerdhout. Eerstens is verskeie protokols wat voorheen gepubliseer is, is as grondslag gebruik vir die ontwikkeling van 'n ekonomiese en tydbesparende DNA ekstraksie protokol. Hierna is PKR (polimerase ketting reaksie) amplifikasie met spesie-spesifieke inleiers (Pch1 en Pch2) gevind om sensitief genoeg te wees om so min as 1 pg van *Pa. chlamydospora* genomiese DNA van wingerdhout op te spoor. Die protokol is geverifieer deur verskeie wingerdhoutmateriaal van 3 verskillende onderstokkultivars (101-14 Mgt, Ramsey en Richter 99) te gebruik, wat elk versamel is van 3 verskillende kwekerye. 'n Aantal van die wingerstokke is ook onderwerp aan warmwaterbehandeling. Die basale kant van die onderstok is parallel geanaliseer vir *Pa. chlamydospora* deur gebruik te maak van isolasies op kunsmatige groeimedium asook molekulêre opsporing. Die identiteit van 'n submonster van PKR produkte van verskeie monsters, wat slegs positief getoets het vir *Pa. chlamydospora* met die molekulêre

opsporing, is bevestig om *Pa. chlamydospora* spesifiek te wees. Dit is gedoen deur middel van restriksie ensiem analise met *AatII*. Molekulêre opsporing is gevind om aansienlik meer sensitief te wees as isolasies, deurdat *Pa. chlamydospora* opgespoor is van positiewe sowel as negatiewe isolasies. Die molekulêre tegniek het *Pa. chlamydospora* in 'n gemiddeld van 80.9% van die monsters opgespoor, terwyl slegs 'n gemiddeld van 24.1% van die monsters postief getoets het vir *Pa. chlamydospora*, deur middel van isolasies. *Pa. chlamydospora* is nie geïsoleer van die monsters wat warmwaterbehandeling ondergaan het nie. Die resultate bevestig hoe belangrik warmwaterbehandeling is vir die proaktiewe beheer van Petri se siekte in wingerdkwekerye. *Pa. chlamydospora* DNA is met die molekulêre tegniek opgespoor, in warmwaterbehandelde monsters, in getalle wat ooreenstemmend is met die van nie-warmwaterbehandelde monsters. Soos verwag, is DNA in warmwaterbehandelde plante nie vernietig nie en kon dit telke male opgespoor word deur die ontwikkelde molekulêre opsporing protokol. Dit is 'n belangrike feit wat in ag geneem moet word wanneer molekulêre opsporing gebruik word in siekte diagnose en opsporing van patogene en dit is 'n aanduiding dat die metodes gebruik moet word in samewerking met ander diagnostiese tegnieke. Die DNA ekstraksie protokol het getoon om tot en met 10 tot 15 kere goedkoper te wees as kommersiële DNA ekstraksie pakkette.

Voorlopige studies het getoon dat die bogenoemde molekulêre opsporings tegniek nie spesifiek en sensitief genoeg is vir die opsporing van *Pa. chlamydospora* uit grond en water nie (ongepubliseerde data). Daarom is 'n enkel-buis geneste-PKR tegniek geoptimiseer vir die opsporing van *Pa. chlamydospora* DNA wat geëkstraheer is vanaf grond, water, kallusmedium en wingerdhout. Dele van onderstokke en grond monsters is geneem vanaf moederblokke van verskeie kwekerye. Gedurende die voor-opberging en enting periodes is watermonsters versamel vanaf hidrasie en fungisied tenke. Bostok- en onderstokente is ook versamel gedurende enting en grond is versamel vanaf kwekerybeddens net voor uitplanting. Die enkel-buis geneste-PKR was sensitief genoeg om so min as 1 fg van *Pa. chlamydospora* genomiese DNA vanaf water en 10 fg vanaf hout, kallusmedium en grond op te spoor. PKR analise van die verskillende kwekerymonsters het getoon dat daar 'n teenwoordigheid is van verskeie putatiewe *Pa. chlamydospora* spesifieke bande (360 bp). Daaropvolgende analise deur middel van DNA volgordebepaling en restriksie ensiem analise het bevestig dat al die 360 bp PKR bande wel *Pa. chlamydospora* spesifiek is, behalwe vir vyf bande wat verkry is vanaf kallusmedium en een band verkry vanaf water. As slegs *Pa. chlamydospora* spesifieke bande in ag geneem word, is daar met molekulêre opsporing die teenwoordigheid van *Pa. chlamydospora* gevind in 25% van die onderstokke, 17 % van die grond versamel vanaf moederblokke, 42% van die onderstokente

versamel tydens enting, 16% van die bostokente, 40% van die watermonsters versamel voor die 12-uur hidrasie periode, 67% van die watermonsters versamel gedurende enting en 8% van die kallusmediummonsters. Hierdie mediums moet dus beskou word as potensiële inokulumbronne of infeksiepunte van die patogeen gedurende die kwekerystadiums. Die resultate bevestig ook verdere bevindinge wat aandui dat *Pa. chlamydospora* meestal versprei word deur geïnfekteerde onderstokke en ente. Geïnfekteerde bostokente is ook aangedui om potensiële draers van die patogeen te wees. Beheerstrategieë moet dus wondbeskerming van onderstok moederplante insluit, asook uitwissing van die patogeen vanaf onderstokente (bv. warmwaterbehandeling), toediening van biologiese of chemiese stowwe in die hidrasie water en kallusmedium en wondbeskerming teen grondgedraagde infeksies.

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1. MOLECULAR DETECTION OF *PHAEOMONIELLA CHLAMYDOSPORA* IN GRAPEVINES: A LITERATURE REVIEW

INTRODUCTION

Decline and dieback of young grapevines (1-7 years old) has been reported in several grape-growing countries such as the United States (California and Virginia), Italy, Australia, New-Zealand, Portugal, France and South Africa (Morton, 1995; Larignon & Dubos, 1997; Ferreira, 1998a; Scheck *et al*., 1998; Mugnai *et al*., 1999; Pascoe & Cottral, 2000). In South Africa, decline and dieback of young grapevines are most frequently attributed to Petri disease, or Black goo, as it was previously known (Fourie *et al*., 2000). Grape and wine production is very important in the agricultural industry and losses caused by the stunted growth and premature dieback is of great concern to the industry.

Petri disease is also a major component of the Esca disease complex of grapevines and the *Phaeoacremonium* disease complex (Crous *et al*., 1996). As the vine matures, the infection of *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams (2000) (main causal organism of Petri disease) will break down certain substances in the host that makes it more susceptible to the wood-rotting fungus, *Fomitiporia punctata* (Fr.) Murrill (1947) (Mugnai *et al.*, 1999). This leads to the development of esca-disease in older vines.

Management of Petri disease is therefore of great importance as it will not only affect young vines, but also predisposes wood of mature vines to infections that could lead to dieback. For the development of management strategies, knowledge of the etiology, symptoms and epidemiology of Petri disease is imperative.

PETRI DISEASE

Etiology

In 1912, the Italian plant pathologist Leonello Petri described the symptoms (internal brown wood streaks) associated with the decline of young vines for the first time. From these symptoms he isolated two fungi, which he named *Cephalosporium* sp. and *Acremonium* sp. (Petri, 1912).

Chiarappa (1959) came to the conclusion that the internal wood decay (black measles) and apoplexy of grapevines are both attributed to a *Cephalosporium* sp.

In 1974, Ajello *et al*. (1974) reported a human pathogenic fungus that was similar to the wood decay *Cephalosporium* spp. isolated by Chiarappa (1959). The human pathogenic fungus caused a subcutaneous infection in the kidney of a kidney-transplant patient. Ajello *et al*. (1974) found that this fungus was a new species of *Phialophora* and named it *Phialophora parasitica* Ajello, Georg & C.J.K. Wang (1974). It developed in the host's tissues in the form of dematiaceous mycelium. Hawksworth *et al*. (1976) reported later that *Phialophora parasitica* could also be associated with various woody hosts and that the *Vitis* isolate (CBS 239.74) that Chiarappa (1959) originally collected had some morphological differences.

Ferreira *et al*. (1994) conducted pathogenicity tests with *Phialophora parasitica* on *in vitro* plantlets and grafted plants in a glasshouse and also on graft unions. They found that *Phialophora parasitica* was consistently isolated where symptoms of slow dieback occurred. The symptoms observed by Ferreira *et al*. (1994) were discolouration of wood combined with extensive plugging of xylem tissue and callus inhibition of graft unions.

In 1996, Crous *et al.* (1996) described the fungal genus *Phaeoacremonium* for the first time and separated the genus *Phaeoacremonium* from *Phialophora*. *Phialophora* spp. have short, swollen and darkly pigmented phialides with a flaring collarette and *Phaeoacremonium* spp. have pigmented phialides with inconspicuous, non-flaring collarettes and aculeate conidiogenous cells. *Phaeoacremonium* resembles *Acremonium* but can be distinguished from *Acremonium* by its pigmented vegetative hyphae and conidiophores (Crous *et al*., 1996). Morphologically, the genus is therefore an intermediate between *Acremonium* and *Phialophora*. Yan *et al*. (1995) also published ITS sequence data that supported the separation of *Phaeoacremonium* from *Phialophora*. Crous *et al.* (1996) found that the new hyphomycete genus has five new species, *Pm. aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai (1996), *Pm. angustius* W. Gams, Crous, M.J. Wingf. & Mugnai (1996), *Pm. chlamydosporum* W. Gams, Crous, M.J. Wingf. & Mugnai (1996), *Pm. inflatipes* W. Gams, Crous, M.J. Wingf. & Mugnai (1996), *Pm. rubrigenum* W. Gams, Crous, M.J. Wingf. & Mugnai (1996), and the type species, *Pm. parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf. (1996), which was formerly accommodated in *Phialophora*. Based on the description by Petri (1912), Mugnai *et al*. (1999) placed the *Cephalosporium* strain and the *Acremonium* strain in *Pm. chlamydosporum* and *Pm. aleophilum*, respectively.

Dupont *et al*. (1998) presented ITS sequence data that demonstrated that *Pm. chlamydosporum* appeared to be more closely related to *Phialophora sensu stricto*, which is supposed to be an anamorph of the family Herpotrichiellaceae, than to the other species of *Phaeoacremonium*, which belongs to the family Magnaporthaceae. Crous and Gams (2000) also found several morphological differences and introduced a new genus, *Phaeomoniella* and typified that *Pm. chlamydosporum* should be called *Phaeomoniella chlamydospora*. In *Pa. chlamydospora*, the conidiophores are green-brown, with light green to almost hyaline conidiogenous cells and the conidia are not dimorphic and hyaline as in other species of *Phaeoacremonium*, but are pale brown and consistently straight, oblong-ellipsoidal to obovate (Crous & Gams, 2000). Groenewald *et al*. (2001) confirmed that *Phaeomoniella* was distinct from *Phaeoacremonium* on the bases of ITS and β-tubulin sequence data.

Several *Phaeoacremonium* spp. including *Pm. aleophilum*, *Pm. angustius*, *Pm. inflatipes*, *Pm. mortoniae* Crous & W. Gams (2001), *Pm. parasiticum*, *Pm. rubrigenum* and *Pm. viticola* J. Dupont (2000) have been found associated with decline symptoms and especially esca (Mostert *et al*., 2003). Other studies have also confirmed that *Pm. aleophilum* is a pathogen that causes Petri grapevine decline (Sheck *et al*., 1998; Adalat *et al*., 2000). However, data of the other species, and especially information indicating that these species are indeed causal organisms of Petri grapevine decline, remain unresolved. There is currently some confusion surrounding *Pm. inflatipes*. Some researchers report a lack of data indicating that this species is a pathogen of grapevine (Groenewald *et al*., 2001). Contrarily, other researchers (Scheck *et al*., 1998; Adalat *et al*., 2000; Eskalen *et al*., 2001) have found that *Pm. inflatipes* produces the same symptoms as *Pm. aleophilum* in grapevine. Some of the confusion surrounding the pathogenicity of *Pm. inflatipes* might have been due to incorrect classification since, Rooney-Latham *et al*. (2004) concluded through recent morphological and molecular tests that the isolates that were previously identified as *Pm. inflatipes* should now be classified as *Pm. aleophilum*.

Pa. chlamydospora was found to be a more aggressive coloniser of grapevine pruning wounds than *Pm. aleophilum* and *Pm. inflatipes*, which are both considered to be root pathogens (Adalat *et al*., 2000). Wallace *et al*. (2003) infected grapevine cuttings with *Pa. chlamydospora* and *Pm. aleophilum* and found that *Pa. chlamydospora* caused brown wood streaking in the rootstock cultivars, but not in the scion varieties. No visible internal symptoms were caused by *Pm. aleophilum*. They concluded that *Pa. chlamydospora* is the more virulent pathogen of the two. In New Zealand, the fungus most commonly isolated from diseased vines was *Pa. chlamydospora*

(Whiteman *et al*., 2003). Collectively, these results indicate that *Pa. chlamydospora* is the main causal organism of Petri disease.

Symptoms

Typical symptoms of Petri disease include stunted growth, shorter internodes, small leaves, smaller trunks and branches and a general decline of young vines resulting in plant death (Morton, 1995; Bertelli *et al*., 1998; Ferreira, 1998a; Fourie *et al.*, 2000; Sidoti *et al*., 2000; Whiteman *et al*., 2003). Leaves may also show some chlorosis, leaf roll and necrotic spotting (Morton, 1995; Ferreira, 1998a; Sidoti *et al*., 2000). Infected vines tend to be less vigorous and usually show poor blossoming the next season (Ferreira, 1998a). External trunk symptoms are not usually visible, but during severe infections some symptoms can be observed. Fissure and cracking of the trunk has been observed, and is usually associated with malformation of the round stem and small but deep pits (Morton, 2000).

In young vines, a black discolouration has been observed in the xylem vessels, but only in the rootstock and sometimes in the grafting union (Ferreira, 1998a; Eskalen *et al*. 2001; Whiteman *et al*., 2003). In some cases only a few of the xylem vessels show discolouration and in other cases it is a whole group of vessels that show a concentric pattern (Ferreira, 1998a). The discolouration is due to a black, tarry substance, which blocks the xylem vessels (Ferreira *et al*., 1994; Morton, 1995; Ferreira *et al*., 1999; Fourie *et al.*, 2000). The occlusions formed by the tarry substance leads to a reduction in water and mineral uptake, which leads to withering and dieback of plants (Ferreira *et al*., 1999). Parenchymatous growth has also been observed in the xylem vessels (Ferreira, 1998a).

Pascoe and Cottral (2000) found that *Pa. chlamydospora* did not distribute continuously through the length of an affected vessel, but was located at a specific point. In some cases, disease symptoms appeared at points remote from the actual infection (Pascoe & Cottral, 2000). The partial plugging of xylem vessels is probably causing the dieback and as more vessels become plugged, less growth can be sustained beyond the occlusion, which leads to plant death (Ferreira *et al*., 1994; Adalat *et al*., 2000).

Generally symptoms are not visible in wood younger than one year (Fourie *et al*., 2000). Morton (2000) did cross sections of different parts of wood that are older than one year in order to observe the symptoms caused by the decline fungus. In the rootstock trunk, black spots or a blackened sector within the oldest annual xylem ring was found surrounding the pith (Morton,

2000). Although these black dots extend out into other xylem rings, it was never found in the newest xylem tissue. In older vines, one might see necrotic heartwood. At the graft union there may be a black line around the graft itself with black goo dots just below it. The graft unions usually shows poor callusing or grafts may even fail in severe cases (Ferreira *et al*., 1994; Morton, 2000; Pascoe *et al*., 2000; Wallace *et al*., 2003). In the scion trunk, the black vascular streaking may not necessarily be in the center of the trunk, depending on where the fungus entered the plant. Morton (2000) also found that when *Pa. chlamydospora* enters through a pruning wound, the infection would move downward through vessels associated with the wound. In very young vines, the scion trunk above the union often appears normal, even if the rootstock below is severely infected (Morton, 2000). In roots, the symptoms are not always present, but when present it is in the center portion of the structural roots and not on or directly under the epidermis. Roots that are infected with the fungus appear to be normal from the outside, but they may be smaller than healthy uninfected roots (Morton, 2000). Adalat *et al*. (2000) found that the total number of roots was significantly reduced by fungal infection. This was probably due to the increased lesion size that led to the reduction of surface area from where roots could be produced.

Epidemiology

Pa. chlamydospora is considered as the major causal organism of Petri disease and is distributed throughout the world with its host, *Vitis vinifera* (Groenewald *et al*., 2001), but little is known about the epidemiology of Petri disease. Isolations from cuttings prior to planting demonstrated that the primary pathogens associated with Petri disease; such as *Pa. chlamydospora* and *Phaeoacremonium* spp. were already present in the apparently healthy rootstock propagation material as endophytes (Bertelli *et al*., 1998; Larignon, 1998; Halleen *et al*., 2003; Fourie & Halleen, 2002). Infected rootstock propagation material is therefore considered as a major inoculum source (Bertelli *et al.*, 1998; Edwards & Pascoe, 2002).

The importance of aerial dispersed spores has been shown by trapping spores of *Phaeoacremonium* spp. and *Pa. chlamydospora* in vineyards in California (Eskalen *et al*., 2003) and France (Larignon & Dubos, 2000). *Phaeoacremonium* spp. and *Pa. chlamydospora* produces conidia that can be dispersed aerially and usually penetrates the host through pruning wounds (Larignon & Dubos, 2000; Eskalen *et al*., 2003). The conidia of *Pa. chlamydospora*, that are most likely aerialy dispersed, has been isolated from berry surfaces but more commonly from the surface of spurs, cordons and trunks (Rooney *et al*., 2001; Eskalen *et al.*, 2003). *Phaeoacremonium* sp. has

also been isolated from the surfaces of spurs, but unlike *Pa. chlamydospora* it has been found on the surfaces of roots, leaves and soil clusters (Eskalen *et al*., 2003).

Edwards *et al*. (2001) found that *Pa. chlamydospora* sporulates abundantly in deep cracks and crevices on infected grapevines in the field. Pycnidia were observed among the sporulating hyphae of *Pa. chlamydospora* (Edwards & Pascoe, 2001). They ventured the reason for this was that cracks and crevices provide a protected humid environment, which is comparable to the moist incubation conditions provided in a laboratory. Edwards *et al*. (2001) also proposed the possibility that collembolans and mites might be responsible for dispersal of spores within cracks and crevices, especially because cracks are sheltered and provides limited scope for dispersal methods such as rain or wind.

Pa. chlamydospora is able to form chlamydospores that enable the fungus to survive for long periods in plant debris or soil. Chlamydospores are thought to form conidia that can penetrate vine roots in nurseries or vineyards (Bertelli *et al*., 1998). *Phaeoacremonium* spp. can penetrate uninjured roots (Feliciano & Gubler, 2001).

Pascoe and Cottral (2000) studied the colonisation of *Pa. chlamydospora* in the vine, after infection through a wound. The pathogen was initially only found in the xylem parenchyma adjacent to vessels in the inoculated area. The hyphae could be found intercellular and was densely packed in the interiors of the infected cells. The infected cells produced tyloses, and a hypha could frequently be found entering the vessel at the point of the intrusion of the tyloses. Later on, the hyphae spread further and could also be found in the parenchyma, cortical and pith cells of the stem. These authors have recorded no evidence of spore production inside the vessels.

On the contrary, Edwards *et al*. (2003) suspected that the infection might spread from the mother vines into canes via the spores that are carried in the sap flow. They found that *Pa. chlamydospora* and *Pm. aleophilum* were randomly spread along the full length of canes of infected Ramsey rootstock mother vines. Feliciano and Gubler (2001) inoculated shoots with *Pm. aleophilum* and observed through light microscopy that the fungus spreads through the intercellular spaces of the epidermis, cortex and pith. Spores were observed in the pith area as well as in the xylem. Hyphae were also observed in the epidermis, cortex, pith and vascular tissues, remote from the point of inoculation (Feliciano & Gubler, 2001). This confirmed the suspicion that spores or hyphae fragments cause the spreading of the infection inside the cane, rather than mycelium growth (Edwards *et al*., 2003).

Ferreira (1998a) and Ferreira *et al*. (1999) found that stress conditions resulting from planting, drought, poor drainage, nutrition deficiencies, soil compaction and/or infection by other root or trunk pathogens result in disease expression. The grafting process is also considered to be a stress factor. Since some grafting combinations are more compatible than others, grafting combination may also play a role in disease enhancement (Ferreira, 1998a).

Disease management

Vineyards. The use of poor quality or mishandled cuttings will increase the failure rate in material infected with *Pa. chlamydospora* (Morton, 2000). Since stress conditions induce disease expression, cultural practices must aim at reducing stress and keeping the vines as vigorous and healthy as possible (Ferreira, 1998a).

Pruning or grafting wounds can serve as entry portals for *Pa. chlamydospora*. Therefore, it is very important to treat wounds with fungicides, especially wounds made at the base of the plant (Larignon & Dubos, 2000; Morton, 2000). Larignon and Dubos (2000) found that infections were more serious with early pruning, and it is therefore very sensible to prune later in the winter when the wounds are not that susceptible. It is also important to use a wound sealant after pruning (Fourie *et al.*, 2000). Di Marco *et al*. (2000) found that pre-infection spraying of *Trichoderma* on fresh pruning wounds is effective in preventing Petri disease.

Systemic fungicides may inhibit internal spread of *Pa. chlamydospora* within vines, thereby reducing symptom development in vines affected by Petri disease (Jaspers, 2001). Groenewald *et al*. (2000b) and Jaspers (2001) found that systemic fungicides from the demethylation inhibitor, anilopyrimidine and benzimidazole classes were moderately effective in reducing *in vitro* mycelial growth and conidial germination. Laukart *et al*. (2001) found that the most effective fungicides for reducing the incidence of dark pith symptom in vines were DMI fungicides (prochloraz and fenarimol), benzimidazole (benomyl) and phosphonate (fosetyl-Al). Di Marco *et al*. (1999; 2000) also noted promising results if phosphonate is used as a foliar spray against esca on grapevines. The efficacy was attributed to a synergism that exists in mixtures of phosphorous acid and resveratrol, a common phytoalexin produced by grapevines (Di Marco *et al*., 1999).

The contact fungicides, thiram and chlorothalonil, also proved to be effective inhibitors of mycelial growth (Groenewald *et al*., 2000b). The disinfectant, hydroxyquinolene sulphate was also highly effective at reducing germination but less effective against mycelial growth (Jaspers, 2001).

These contact fungicides may be effective in reducing the inoculum found on berry surfaces, spurs, cordons and trunks, but further research needs to be conducted.

In California, different studies were done in order to find rootstocks that show resistance to *Pa. chlamydospora* and *Phaeoacremonium* spp. (Eskalen *et al.*, 2001). Inoculations revealed that rootstocks 3309, 420A, 110R, 5C, Schwarzmann, St. George and Salt Creek were least susceptible to *Pa. chlamydospora*. The rootstocks 16-16, 3309, AXR1, Salt Creek, 110R, 5C, Freedom and 140Ru were least susceptible to *Pm. inflatipes* and 1103, 420A, Harmony and Salt Creek were least susceptible to *Pm. aleophilum*. In South Africa, the rootstocks 101-14 Mgt, 99 Richter and Ramsey are known to be very susceptible to grapevine slow dieback and decline (P.H. Fourie, pers. comm.). Currently, there are no rootstocks that are known to be resistant to Petri disease in South Africa or any other grapevine cultivating country in the world.

Nurseries. Grapevine nurseries produce grafted vines by first producing rootstocks onto which scions are then grafted. In South Africa the rootstock cuttings are harvested and cut into pieces of 25-30 cm, during May and early June. The cuttings are drenched in a hydration tank for a period of 12 h and stored in a cold room at temperatures of 1°C to 4°C for a period of 2 to 3 months (Van der Westhuizen, 1981). During August and early September, the scion cuttings are harvested and cut into pieces and placed in a hydration tank (Van der Westhuizen, 1981). Before grafting, after cold storage, the rootstock cuttings are also placed in a hydration or fungicide tank. The scion is then grafted onto the rootstock by means of machine grafting (omega cut) or hand grafting. The graft-union is sealed with a wax layer and the grafted vines are placed in a callusing medium until the end of September or early October. The grafted vines are then planted in the nurseries, to induce root-production (Van der Westhuizen, 1981).

Whiteman *et al*. (2003) studied samples from all the stages in the propagation process. They found that there was a presence of *Pa. chlamydospora* at all stages and a very high presence in solutions where there was repeated exposure to plant material and especially in the hydration/fungicide tanks, pre- and post-storage. The percentage of positive samples was moderate from grafting tool washings and low from washings of callusing media (Whiteman *et al*., 2003). Wound protection and general hygiene is therefore very important during the nursery stages.

The use of fungicides in the nursery process might reduce the risk of spreading the disease from infected to uninfected plants, and perhaps lower the inoculum in infected plants (Morton, 2000). In some countries the cuttings can be treated with a fungicide (Chinosol or Captan) before

cold storage (Van der Westhuizen, 1981). Chemical strategies in South Africa mainly involve drenches of propagation material in a variety of broad–spectrum fungicides (captan, iprodione, 8–hidroxyquinoline sulphate) (Marias & van der Westhuizen, 1978) or quaternary ammonium sterilizing compounds. These fungicides were found to be moderately or poorly effective in reducing germination or mycelium growth of *Pa. chlamydospora* (Groenewald *et al*., 2000b; Jaspers, 2001). Fourie and Halleen (2004) treated rootstocks with benomyl and phosphorous acid prior to grafting and found these chemical treatments to cause a very low reduction in *Phaeomoniella* and *Phaeoacremonium* infection, especially when compared with hot water treatment.

Hot water treatment of propagation material has the potential to destroy *Pa. chlamydospora* in the propagation material. The grapevine cuttings, from the nursery, are drenched for 30 min in water with a temperature of 50˚C (Ferreira, 1998a). Ferreira (1998a, 1998b) also found that temperatures above 51˚C causes damage to the cutting and may reduce the viability of the cutting and temperatures below 49˚C would not be efficient to kill the pathogens. Hot water treatment was shown to be effective in eliminating or reducing pests and pathogens such as nematodes, phylloxera and Pierce's disease (Goheen *et al*., 1973). *Phytophthora cinnamomi* Rands (1922) was also effectively controlled by subjecting grapevine cuttings to hot water treatment (Von Broembsen & Marias, 1978). It was indicated in previous studies that hot water treatment is effective in eliminating the most well known fungal pathogens and endophytes from grapevine tissue (Crous *et al*., 2001). Varying results were achieved with hot water treatments to eliminate *Pa. chlamydospora*. Whiting *et al*. (2001) and Rooney and Gubler (2001) reported that hot water treatment was not effective in controlling *Pa. chlamydospora*. Contrarily, Fourie and Halleen (2004) observed a drastic reduction in the levels of *Pa. chlamydospora* after hot water treatment of naturally infected rootstock cuttings or uprooted nursery grapevines. Moreover, subsequent colonisation of treated rootstocks was also inhibited (Fourie & Halleen, 2004).

Biological strategies mainly consist of wound protection agents, soil drenches and rootstock drenches. According to Fourie *et al*. (2000), Messina (1999) found that the use of *Trichoderma* products, which contains a mixture of *T. harzianum* Rifai (1969) and *T. viride* Pers. (1794), in callusing boxes resulted in stronger graft unions and root systems in a shorter callusing period. On the contrary, Di Marco *et al*. (2004) found that the beneficial effect on the graft union was only in the first year. Fourie *et al*. (2001) also observed enhanced root development after *Trichoderma* soil amendments and Di Marco *et al*. (2004) confirmed this in later studies. Tolerance to stress would

also increase with enhanced root development, as water and nutrient uptake will improve (Harman, 2000). Fourie *et al*. (2001) also isolated a low percentage of Petri disease fungi from rootstocks treated with *Trichoderma*. In later studies, Fourie and Halleen (2004) further treated rootstocks with *T. harzianum* (Trichoflow), a mixture containing *Trichoderma* and *Gliocladium* and a bacterial biofertilizer (suspension of *Azospirillum brasilense*, *Pseudomonas fluorescence* and *Bacillus subtilis*) combined with sodiummolybdate/thiram. These treatments caused a very low reduction in *Phaeomoniella* and *Phaeoacremonium* infection, especially when compared with hot water treatment. On the contrary, post-callus application with *Trichoderma* significantly reduced the necrosis length caused by *Pa. chlamydospora* in the rootstock (Di Marco *et al*., 2000). Also, pruning wound application of grafted potted vines, prevented black goo and necrosis in the wood below the wound (Di Marco *et al*., 2004). Calderon *et al*. (1993) and De Meyer *et al*. (1998) also detected induced resistance in grapevines, against some diseases, following inoculation with *T. viride* and *T. harzianum*. Numerous factors are involved in the complex interaction between *Trichoderma*, the grapevine and the pathogen. For this reason, further studies are needed to improve the biocontrol of *Trichoderma* and move towards its practical implementation (Di Marco *et al*., 2004).

Molecular pathogen detection and diagnosis

Detection of harmful pathogens in plant material is essential to ensure safe and sustainable agriculture. Detection deals with establishing the presence of a particular organism within a sample. Timely detection of pathogens avoids planting of contaminated material. Detection may be by means of symptom identification or symptomless infections can be detected by isolation onto artificial growth medium.

Isolation of *Pa. chlamydospora* onto artificial media is problematic, since this fungus is extremely slow growing (up to 4 weeks from isolation to identification) and its cultures are often over-grown by co-isolated fungi before it can be identified (P.H. Fourie, pers. comm.). Furthermore, identification of *Pa. chlamydospora* based on morphology is problematic due to the comparative lack of diagnostic microscopic features and a high degree of morphological variability in cultures (Bindslev *et al*., 2002). A technique that could identify certain fungal species, without the need to obtain a pure culture would be very valuable. Another limitation of isolation techniques is that the pathogen has only been found at very low quantities (< 0.2%) (Fourie & Halleen, 2002). Fourie and Halleen (2002) therefore required many isolations. Consequently a sensitive technique, such as molecular detection (Schaad & Frederick, 2002) is needed, especially in asymptomatic propagative materials such as grapevine cuttings, for pathogen detection.

Until serological techniques were developed, the only reliable methods available for identification of fungi were isolation in culture and performing pathogenicity tests. Serological techniques were, however, not always that specific (Schaad & Frederick, 2002) and were very time consuming. Serological techniques were replaced by DNA dot-blotting techniques, which also exhibited some problems regarding sample contamination and was found to be time consuming and labour intensive (Holland *et al*., 1991; Schaad & Frederick, 2002). PCR (polymerase chain reaction) offers several advantages compared to more traditional methods of diagnosis. There is no need for organisms to be cultured prior to their detection and the technique possesses exquisite sensitivity (Henson & French, 1993). A single target molecule can also be detected in a complex mixture, without using labelled probes (Henson & French, 1993). Similar to serology, both narrow and broad selectivity is possible and, depending on the choice of primers, the method facilitates the detection of a single pathogen or many members of a group of related pathogens.

DNA extraction. Extraction of good quality nucleic acids from plants and fungi has been difficult in the past. Success of a DNA extraction is measured by its yield, condition (molecular weight and colour) and purity (Rogers & Bendich, 1994). Direct PCR (polymerase chain reaction) on fungal or plant material is not possible with fungal spores and therefore a DNA isolation technique is necessary, especially when working with woody propagation material (Schaad & Frederick, 2002). For the successful extraction of plant and fungal DNA, the cell walls must be broken in order to release the cellular constituents, the cell membranes must be disrupted to release the DNA and DNA must also be protected from endogenous nucleases (Rogers & Bendich, 1994). Grinding the tissue in dry ice or liquid nitrogen with a mortar and pestle breaks the cell walls and a detergent such as SDS (sodium dodecyl sulfate) or CTAB (cetyltrimethylammonium bromide) disrupts the cell membranes (Rogers & Bendich, 1994). EDTA (ethylenediaminetetraacetic acid) is a chelating agent that binds to magnesium ions and this detergent is added to protect the DNA from nucleases (Rogers & Bendich, 1994). Chloroform and phenol may also be added to separate proteins from DNA so that the tissue homogenate is emulsified (Rogers & Bendich, 1994).

Groenewald *et al*. (2000a) extracted *Pa. chlamydospora* genomic DNA from inoculated grapevine and tissue culture plants, using the modified CTAB method which was developed for extraction of fungal DNA out of small quantities of fresh leaf tissue (Doyle & Doyle, 1987). Lee and Taylor also extracted fungal DNA, using a SDS buffer, from single spores and fungal mycelium (Lee & Taylor, 1990). In the case of woody plants, such as grapevine, the presence of PCR inhibitors in DNA extractions is the chief limiting factor in using extracted DNA for PCR

amplifications (Minafra & Hadidi, 1992). Most extraction procedures do not remove contaminating plant polysaccharides or polyphenolic compounds that can have direct inhibitory effects on PCR amplifications (Demeke & Adams, 1992, Henson & French, 1993). Ridgway *et al*. (2002) developed an extraction protocol for the detection of *Pa. chlamydospora* from lignified wood. Their protocol requires the use of a CTAB buffer and the Dneasy Plant Mini kit (Qiagen, Germany)(Green & Thompson, 1999) for purification (Ridgway *et al*., 2002). Whiteman *et al*. (2002) developed a technique for isolating *Pa. chlamydospora* DNA from grapevine nursery soil using a SDS/phenol/chloroform DNA extraction method, which was processed in a Prep−A−Gene® DNA purification kit (Bio−Rad Laboratories Pty Ltd., New Zealand) to remove inhibitors (Whiteman *et al*., 2002). Another extraction protocol was developed for DNA extraction from soil using a SDS buffer and FastPrep® instrument (Bio101) and self−prepared PVPP (polyvinylpoly pyrrolidone) spin columns to remove inhibitors (Damm & Fourie, 2004).

PCR technique. The PCR technique was invented in 1984 by Mullis (Mullis, 1987). PCR is an *in vitro* method where specific DNA sequences are synthesised by enzymes, using two oligonucleotide primers (Erlich, 1989). Two primers each hybridise to the opposite strands of target DNA and leads to amplification of a specific region. Amplification of both DNA strands leads to a rapid exponential increase in target DNA copies. For the primers to anneal to template strands, the template strands must denaturate before extension of the annealed primer can follow (Erlich, 1989). Different temperatures are needed for denaturation, annealing and extension processes. Denaturation takes place between 94-96 °C and most DNA polymerase extend DNA at 72 °C (Bridge *et al*., 2004). The annealing temperature depends on the melting temperature (Tm) of primers. The primer extension products synthesised in one cycle can serve as a template in the next cycle and this causes the number of target DNA copies to double at every cycle. As the cycles are repeated, the quantity of amplicons rises exponentially (Bridge *et al*., 2004).

Initially the Klenow fragment of *E.coli* DNA polymerase I was used to extend the annealed primers, but it was found that this enzyme is inactivated by the high temperature that is required to separate the two DNA strands at the outset of each PCR cycle, so that fresh enzyme had to be added during every cycle (Erlich, 1989). The thermostable DNA polymerase (*Taq*) was isolated from *Thermus aquaticus* and enabled the addition of enzyme only once at the initiation of PCR cycles. Subsequently a thermal cycling device was developed that allowed automated PCR (Henson & French, 1993). A negative control (no template DNA) must be used to ensure that no contamination occurs during the PCR preparation and amplification. A standard positive control can be

incorporated as part of the test to help discriminate against false negatives and false positives and this will improve the overall confidence in the results achieved with the PCR reaction (Henson & French, 1993).

The specificity of PCR is typically analysed by means of gel electrophoresis (Erlich, 1989). The migration speed of the amplicons in the gel is dependent on their molecular mass, which is mostly determined by the number of nucleotides of the amplified DNA (Hartwell *et al*., 2000). The presence of the amplicons is checked by staining the agarose gel with ethidium bromide (BET), a molecule that intercalates between the stacked bases of DNA (Hartwell *et al*., 2000). UV illumination stains the DNA molecules so that it produces an orange fluorescence. The size of amplicons is verified by loading the PCR products next to a molecular weight marker, known as a DNA ladder (Henson & French, 1993).

Many factors affect the specificity and success of DNA amplification by PCR, therefore each reaction must be optimised (Henson & French, 1993). The correct primer, buffer salt and polymerase concentration must be used and the pH, annealing temperature and cycle periods must be optimised for each reaction (Bridge *et al*., 2004). This is especially important when genomic DNA extractions used for PCR amplification contain inhibitors. Therefore, many enhancers for the PCR reaction are available on the market. Examples of these are DMSO (dimethylsulfoxide), glycerol, BSA (Bovine Serum Albumin), formamide, PEG (polyethylene glycol), spermidine, Tris-HCl, KCl and gelatine (Innis & Gelfand, 1990). BSA increases the efficiency of a PCR reaction much more than both DMSO and glycerol and most of the other enhancers (Henegariu *et al*., 1997). The addition of albumin to tissue DNA samples increases the amount of DNA generated by neutralising many deleterious factors found in tissue samples, which inhibits the PCR reaction. Removal of PCR inhibitors from samples is also frequently accomplished by using polyvinyl pyrrolidone (PVP), which binds polyphenolic compounds (Henson & French, 1993).

The conventional PCR technique as introduced by Mullis in 1987, has been modified in various ways in the past decades. Modified PCR techniques include nested-PCR, co-op PCR and multiplex PCR to mention just a few (López *et al*., 2003). Nested-PCRs are increasingly being used, especially for plant pathogen detection due to its increased sensitivity compared to the conventional PCR (Bertolini *et al*., 2003). A nested-PCR uses two PCR primer pairs (external and internal primer pairs) for a single locus (Ma *et al*., 2003). The first pair (external pair) amplifies a fragment to which a second primer pair (internal nested primers) binds internally (Bridge *et al*., 2004). This prevents the wrong locus from being amplified since the probability is very low that a

second primer pair would also amplify it a second time. Nested-PCR increases pathogen detection in the order of ten to a hundred times, compared to conventional PCR.

One drawback of the nested-PCR is that cross-contamination of samples can occur, resulting in false positive sample testing. The increased risk of cross-contamination is due to the introduction of a second round of amplification that requires aliquoting of PCR products. Therefore, recent efforts have been focused on the development of a single tube nested-PCR that eliminates the need for aliquoting PCR samples for the second round of amplification, thus eliminating cross-contamination (Olmos *et al*., 1999).

Two single tube nested-PCR methods have recently been published. The first was published by Olmos *et al*. (1999). Their device consists of an Eppendorf tube containing two different PCR cocktails, which are physically separated by using the end of a standard 200 µl plastic pipette tip. The first PCR reaction mixture is placed in the Eppendorf tube and the PCR reaction mix for the second amplification is added into the pipette tip, where it remains due to capillarity (Olmos *et al*., 1999). After the first round of PCR, the Eppendorf tube is centrifuged so that the second PCR cocktail is mixed with the products of the first reaction. A second type of single tube nested-PCR has also been published by Tao *et al*., (2004), which involves a plastic film for the separation of the two reaction mixtures. The first round system is covered with mineral oil and the second round system is sequestered in the cap of the reaction tube by plastic film before the first round reaction. After the first round, the reaction tube is centrifuged so that plastic film breaks due to centrifugal force, resulting in the second round mixture being spun and mixed into the amplicons from the first round amplification.

Primer development and detection. Public databases harbour different nucleotide sequences that have been sequenced over the past several years. The largest nucleotide sequence database is present at the National Center for Biotechnology information (NCBI) in GenBank. Nucleotide sequence databases can be searched for the sequences of a particular organism that is unique to that organism, and might therefore be very useful as a potential target for development of organism specific PCR primers (Erlich, 1989). Some of these nucleotide sequences may encode for a specific product that is known to be unique to that specific organism. Genes that encode for a specific product unique to an organism are normally present as a single copy per cell (Erlich, 1989), which may be a disadvantage when designing PCR primers, because the sensitivity of the assay will be considerably lower. The sensitivity of the PCR primers increases when multicopy genes such as

16S rRNA are used (Pastrick & Maiss, 2000). The rDNA region of the fungal genome can be present in up to 200 copies (Russel *et al*., 1984), therefore the ribosomal repeat and internal transcribed spacer (ITS) regions were proven to be valuable for detection of many organisms (Sreenivasaaprasad *et al*., 1996). The sequence of ITS regions can be highly variable in fungi and this allows for differentiation between and within species (Böhm *et al*., 1999; Bonants *et al*., 1997; Niepold & Schober-Butin, 1997; O'Donnel, 1992; Ristaino *et al*., 1998; Schubert *et al*., 1999). However, although the ITS sequence of a large number of organisms are known, there are still millions of ITS sequences of culturable and non-culturable organisms that are unknown. Therefore, ITS primers, although sensitive, might not have high specificity. Consequently, when using ITS primers for pathogen detection in different environments for the first time, it is very important to ensure that they are specific. The specificity of PCR depends upon designing proper PCR primers that are unique to the target organism (Schaad & Frederick, 2002).

Two primer pairs have been developed for the detection of *Pa. chlamydospora*. Ridgway *et al*. (2002) performed a species-specific PCR, using the primers Pch1 and Pch2 (Tegli *et al*., 2000). The primers was synthesised, using the ITS regions of the nuclear ribosomal DNA, containing ITS1, ITS2 and the intervening 5.8 rRNA gene, as well as small portions of 18S and 28S rDNA. PCR amplification with these primers produced a 360bp fragment from isolates of *Pa*. *chlamydospora* (Tegli *et al*., 2000). This conventional PCR was found to be very sensitive and detected up to 5 pg of *Pa*. *chlamydospora* genomic DNA from woody grapevine tissue. Whiteman *et al*. (2002) used a nested-PCR approach with universal primers ITS4 and NS1 and was able to detect 50 fg of *Pa*. *chlamydospora* genomic DNA from artificially infested soil. Groenewald *et al*. (2000a) synthesised primers, PCL1 and PCL2 for *Pa. chlamydospora* from the internal transcribed spacers ITS1 and ITS2, including the 5,8S gene of the ribosomal DNA. The PCR amplification with these primers produced a 325bp fragment from isolates of *Pa. chlamydospora*. When grapevine tissue infected with *Pa. chlamydospora* was used as template for amplification with these primers (PCL1 and PCL2) a 325bp fragment was produced (Groenewald *et al*. 2000a).

CONCLUSION

Traditional methods for detection of *Pa*. *chlamydospora* through isolations onto artificial media cause problems due to the lack of a selective medium and difficulty in subsequent

identification of *Pa. chlamydospora*. Furthermore, it is not possible to isolate this fungus from water and soil samples, because there is no known selective medium to inhibit the numerous fast growing bacteria, yeast and other fungi present in soil and water. This may lead to false negatives, because the fungus is over-grown before it can be identified. Consequently, knowledge about the epidemiology and infection pathways of this pathogen is very limited. Molecular detection has been proven to be a fast and effective alternative for traditional detection methods and was shown to be effective in detecting *Pa. chlamydospora* from various media (wood and soil). However, due to the high costs involved, these techniques have not been widely used in epidemiological studies of *Pa. chlamydospora*.

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2. A PROTOCOL FOR MOLECULAR DETECTION OF *PHAEOMONIELLA CHLAMYDOSPORA* IN GRAPEVINE WOOD

ABSTRACT

Petri disease is a serious decline and dieback disease of young grapevines. The main causal organism, *Phaeomoniella chlamydospora*, is mainly distributed through infected propagation material. Pathogen detection and accurate diagnosis is currently based on fungal isolation onto artificial media. The fungus is, however, extremely slow-growing and cultures are often over-grown by co-isolated fungi before it can be identified. Therefore, a time-efficient and cost-effective protocol for the molecular detection of *Pa. chlamydospora* in grapevine wood was developed and validated. The developed molecular detection technique, using a species-specific PCR, detected as little as 1 pg genomic *Pa. chlamydospora* DNA. This protocol was validated with grafted grapevines from different nurseries, including grapevines that were subjected to hot water treatment. The basal end of the rootstock was parallel analysed for *Pa. chlamydospora* with isolations onto artificial medium and molecular detection. Molecular detection was found to be considerably more sensitive than isolations, detecting *Pa. chlamydospora* from samples with positive as well as negative isolations. The identity of PCR products obtained from a subset of samples that only tested positive for *Pa. chlamydospora*, based on molecular detection, was confirmed to be *Pa. chlamydospora* specific through restriction digestion with *AatII*. *Pa. chlamydospora* was not isolated from hot water treated samples. However, as expected *Pa. chlamydospora* DNA was detected in hot water treated samples in frequencies similar to that detected in non-hot water treated samples.

INTRODUCTION

Several trunk diseases can lead to a decline in the productivity and health of grapevines. Decline and premature dieback lead to considerable economic losses and are causing great concern in most grapevine growing countries. In South Africa, decline and dieback of young grapevine are most

frequently attributed to Petri grapevine decline, or Black goo, as it was previously known (Fourie & Halleen, 2001). The main causal organism of Petri grapevine decline is *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams (Crous *et al.*, 1996; Crous & Gams, 2000). The disease commonly occurs in grapevines of 1 to 5 years of age (Fourie & Halleen, 2001). As the plant matures, the fungus predisposes the wood to infection by various wood-rotting fungi, such as *Fomitiporia punctata* (Fr.) Murrill and can lead to the development of esca-disease (Mugnai *et al*., 1999).

Symptoms are generally not visible in one-year-old wood (Fourie & Halleen, 2001). Stress conditions such as severe pruning, drought, poor drainage, nutrition deficiencies and soil compaction are prerequisites for symptom expression (Ferreira, 1998; Ferreira *et al*., 1999). Typical symptoms of Petri disease include stunted growth, shorter internodes, small leaves, smaller trunks and branches and a general decline of young vines resulting in plant death (Morton, 1995; Bertelli *et al*., 1998; Ferreira, 1998; Fourie *et al.*, 2000; Sidoti *et al*., 2000; Whiteman *et al*., 2003). Internal wood symptoms include vascular streaking and blockage of the xylem vessels. It has been found that the fungus is present in apparently healthy propagation material in a latent or endophytic form. Infected rootstock propagation material is therefore considered as a major inoculum source (Bertelli *et al*., 1998; Edwards & Pascoe, 2002). It is suspected that cuttings might be infected from diseased mother plants (Crous *et al*., 1996; Mugnai *et al*., 1999; Edwards *et al.*, 2003). However, Fourie and Halleen (2002) did isolations from the basal ends of rootstocks and found the incidence of *Pa. chlamydospora* to be extremely low. Isolation of *Pa. chlamydospora* onto artificial growth media is problematic, since this fungus is extremely slow growing (up to 4 weeks from isolation to identification) and its cultures are often over-grown by co-isolated fungi before it can be identified and may therefore lead to false negative results. Therefore, a more sensitive detection technique is needed.

Molecular detection by means of DNA extraction and species-specific polymerase chain reaction (PCR) offers a fast alternative, which evade the above-mentioned problems (Ridgway *et al*., 2002). Protocols and methods for extraction and amplification of fungal DNA from pure cultures were developed (Lee & Taylor, 1990) and adapted for detection of *Pa. chlamydospora* from inoculated tissue culture plants (Groenewald *et al*. 2000). However, due to the presence of PCR inhibitors, these methods will not be suited for molecular detection from lignified wood. Therefore, Ridgway *et al*. (2002) developed an extraction protocol and species-specific PCR method for detection of this pathogen from lignified wood. Their protocol requires the use of a CTAB buffer and the

DNeasy Plant Mini kit (Qiagen, Germany)(Green & Thompson, 1999) for purification. Subsequently, a species-specific PCR is performed using the species-specific primers Pch1 and Pch2 (Ridgway *et al*., 2002). This PCR was found to be very sensitive, detecting <1 pg of *Pa. chlamydospora* genomic DNA. Although very time-efficient, the purification kit is very expensive for large-scale sampling experiments.

Hot water treatment at 50°C for 30 min was shown to be effective in eliminating or reducing pests and pathogens such as nematodes, phylloxera and Pierce's disease (Goheen *et al*., 1973). *Phytophthora cinnamomi* was also effectively controlled by subjecting grapevine cuttings to hot water treatment (Von Broembsen & Marias, 1978). It was indicated in previous studies that hot water treatment is effective in eliminating the most well-known fungal pathogens and endophytes from grapevine tissue (Crous *et al*., 2001). Varying results were achieved with hot water treatments to eliminate *Pa. chlamydospora*. Whiting *et al.* (2001) and Rooney and Gubler (2001) reported that hot water treatment was not effective in controlling *Pa. chlamydospora*. Contrarily, Fourie and Halleen (2004) observed a drastic reduction in the levels of *Pa. chlamydospora* after hot water treatment of naturally infected rootstock cuttings or uprooted nursery grapevines. Moreover, subsequent colonisation of treated rootstocks was also inhibited (Fourie & Halleen, 2004).

The aim of this study was to develop and validate a rapid and cheap DNA extraction protocol and a sensitive species-specific PCR for the detection of *Pa. chlamydospora* in symptomatic or asymptomatic grapevine wood. Molecular detection of the pathogen following hot water treatment of propagation material was also evaluated.

MATERIALS AND METHODS

Grapevine material

Potted grapevines that were naturally infected with the main causal organism of Petri disease (this was proved previously by isolation of the fungus; results not shown) were collected from ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa. The material was prepared for DNA isolation by removing the rooted basal end and cutting the rootstock into 2 cm pieces, as well as a small part of the scion (up to 6 cm above the graft union). The woody pieces were surface sterilised by submerging the

material in 70% ethanol for 30 s and in 3.5% sodium hypochlorite for 60 s and then again in 70% ethanol for 30 s. After the bark was removed, the 2 cm pieces were sterilised and stored at 4°C.

DNA extraction

Modifications of five previously published extraction protocols (Doyle & Doyle, 1987; Lee & Taylor, 1990; Groenewald *et al*., 2000; Ridgway *et al*., 2002) were tested on grapevine wood. DNA extracted by four of these protocols was successfully used to amplify DNA from *Pa. chlamydospora* cultures, but not from wood samples (results not shown). One of these methods, mostly based on the protocols of Lee and Taylor (1990) and Ridgway *et al*. (2002) showed promising results and was explored further. This protocol involved snap freezing with liquid nitrogen and grinding of the frozen sample to a powder by means of a pestle and mortar. After preparation, 0.5 g of the material, 550 µl of CTAB extraction buffer (1 M Tris, pH 7.5; 5 M NaCl; 500 mM EDTA, pH 8.0) was added to the grinded wood in a 2-ml Eppendorf tube and incubated at 65°C for 1 h. Thereafter, 400 µl chloroform:isoamylalcohol (24:1) was added followed by centrifugation at 1300 x g for 15 min. The watery supernatant was transferred to a new Eppendorf tube, 50 µl of 7.5 M ammonium acetate solution (pH 7.0) and 600 µl cold isopropanol were added and the samples were incubated at -20°C. After 1 h the samples were centrifuged at 15800 x g for 10 min. The supernatant was discarded and 1 ml cold 70% ethanol was added before incubation at -20°C for 30 min. After incubation, the samples were centrifuged at 15800 x g for 5 min and the supernatant discarded. The DNA pellet was dried on a bench, dissolved in 100 µl sterile ddH_20 and stored at 4°C.

PCR amplification

PCR was performed using the *Pa. chlamydospora*-specific primers Pch1 and Pch2, which were developed by Tegli *et al.* (2000). The reactions (total volume 25 µl) were performed using 0.65 units Biotaq (Bioline, London), 0.2 mM each of dATP, dTTP, dGTP and dCTP, 3 mM $MgCl_2$, 5 pmoles of each primer, 1x enzyme buffer, 1 mg/ml bovine serum albumin (BSA) and 5 µl of DNA solution. The amplification was performed on a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA) and the cycling conditions were: 5 min at 96°C, followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, 1 min and 30 s at 72°C and a 7 min extension step at 72°C to complete the reaction. DNA was isolated from a pure *Pa. chlamydospora* culture, using the method of Lee and Taylor (1990), and the 1:1000 dilution was used as a positive control in PCR reactions. Five

microlitres of each PCR product were separated by electrophoresis at 80 V for 1 h on a 1% agarose gel, stained with 0.5 µg/ml ethidium bromide, in a 0.5 × TAE buffer (0.04 M Tris, 0.02 M glacial acetic acid and 1.27 mM EDTA, pH 7.85) and visualised under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, United Kingdom). A negative control reaction (no template DNA) was also included during PCR preparation and amplification to ensure that no contamination was present.

To determine the sensitivity of the PCR reaction, known quantities of *Pa. chlamydospora* DNA was added to a DNA solution, which consisted of extractions from wood that had tested negative with the species-specific primers (Pch1 and Pch2). The different DNA concentrations of the purified DNA were determined with a fluorometer FL600™ (Bio-Tek, http://www.biotek.com) and added to the DNA wood extract to achieve final concentrations of 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, 100 fg/µl, 10 fg/µl and 1 fg/µl. Five microlitres from each of these spiked DNA extracts were used in PCR reactions and analysed with gel electrophoresis as described previously.

Validation

For validation of the protocol, one-year-old nursery-grown grapevines from 3 different rootstock cultivars collected from each of 3 different nurseries were randomly sampled after uprooting. The different rootstocks used were 101-14 Mgt, Ramsey and Richter 99 (18 grapevines per batch). The occurrence of *Pa. chlamydospora* in the basal end of each rootstock (1-3 cm from basal end) was determined according to the isolation methods used by Fourie and Halleen (2004). The rest of each 2 cm piece was subsequently used for DNA extraction and PCR amplification. Detection percentages (number of positive samples as a percentage of the total number of samples tested) were calculated and the data subjected to analysis of variance using SAS (SAS Institute Inc., SAS Campus Drive, Cary, North Carolina).

Hot water treatment

Forty-eight nursery grapevines Chardonnay/101-14 Mgt were sampled after uprooting. The grapevines were separated into six bunches of eight grapevines each. Three of these bunches were hot water treated (drenched in water at 50°C for 30 min) and the three untreated bunches were used as the control. The occurrence of *Pa. chlamydospora* in the hot water treated and untreated plants were determined by means of isolations and molecular detection as described previously.

Identification of putative *Pa. chlamydospora* PCR products obtained from wood

Enzyme restriction digestion was used to determine whether all 360 bp amplicons obtained from the wood samples were indeed *Pa. chlamydospora*. A subsample was randomly selected from the samples that tested positive with molecular detection, but negative with isolations. The samples were cut from agarose gels and purified using a QIAquick® Gel Extraction kit (Qiagen, Valencia, CA, USA) according to manufacturers instructions. Restriction enzyme digestion with *AatII* (Roche Diagnostics South Africa Pty Ltd, Randburg) was done according to manufacture's instructions. This restriction enzyme enables differentiation between *Pa. chlamydospora* and other closely related fungi (Whiteman *et al.*, 2002). The digested PCR products were separated by 1.5% agarose gel electrophoresis as described previously.

RESULTS

DNA extraction and PCR amplification

PCR amplification with *Pa. chlamydospora* primers (Pch1 and Pch2) was successful using DNA extracted with the modified method of Lee and Taylor (1990) and Ridgway *et al*. (2002) (Fig. 1). Furthermore, the PCR technique was sensitive enough to detect 1 pg of *Pa. chlamydospora* genomic DNA (Fig. 2).

Validation

Analysis of variance of the detection percentages revealed a significant difference between the molecular detection and isolation (P = 0.0003). On average, the molecular technique detected *Pa. chlamydospora* in 80.9% of the samples, whereas only 24.1% of the samples tested positive for *Pa. chlamydospora* by means of isolations. Depending on the incidence of *Pa. chlamydospora* in different batches, molecular detection was found to be substantially more sensitive than isolations (Fig. 3). DNA of *Pa. chlamydospora* was detected from 100% of the samples that tested positive with isolation.

Hot water treatment

Analysis of variance of detection percentages revealed a significant interaction ($P < 0.0001$) between treatment (hot water treated and untreated) and detection technique (molecular detection and isolations). This can largely be ascribed to the 100% detection of *Pa. chlamydospora* in hot water treated samples using molecular detection, whereas none of the hot water treated samples tested positive with isolations. In the untreated samples, *Pa. chlamydospora* was detected by means of PCR in 100% of the samples, and 91.7% by means of isolation.

Identification of putative *Pa. chlamydospora* PCR products obtained from wood

Restriction digestion of 360 bp PCR products, amplified with primers Pch1 and Pch2 from wood, with *AatII* yielded products of 127 bp and 233 bp (Fig. 4). These product sizes corresponded in size to those produced by digested genomic DNA from *Pa. chlamydospora*.

DISCUSSION

The developed detection protocol was proven to be robust, fast and highly sensitive. The preparation of the material might play a significant part in the success of the DNA extraction, especially snap freezing of the material with liquid nitrogen. Freezing the material enabled grinding of grapevine wood into a powdery form, which is important for releasing fungal DNA from woody tissue. This also breaks the fungal cell walls facilitating release of cellular constituents. By using this molecular detection technique, *Pa. chlamydospora* can be detected in a grapevine sample in less than 1 day, whereas diagnosis by means of isolations will take up to 4 weeks. Furthermore, a very low percentage of *Pa. chlamydospora* was detected from some of the rootstocks with isolations, this was due to the cultures being severely overgrown with bacteria, which led to false negatives.

Previous studies have shown that *Pa. chlamydospora* occur in rootstock cuttings, from mother plants, at very low incidences (Fourie & Halleen, 2002; Halleen *et al.*, 2003). On the contrary the molecular detection showed a very high presence of *Pa. chlamydospora* in most of the rootstock combinations, which indicates that the infection percentage increases while the grafted vines are growing in the field. Fourie and Halleen (2004) confirmed these results. This might be an indication

that *Pa. chlamydospora* is introduced into grafted vines during some nursery stages or that infection of young vines occurs in the field, with disease expression only being induced under stress conditions.

The molecular technique can detect as little as 1 pg of *Pa. chlamydospora* genomic DNA. Conventional PCR instead of nested-PCR makes the detection protocol faster and more cost effective. Furthermore, initial attempts to use nested-PCR resulted in many false positive samples, due to cross contamination of samples with PCR products during the second round of amplification (unpublished data). Most importantly, this extraction protocol was shown to be 10 to 15 times cheaper than commercial DNA extraction kits.

Pa. chlamydospora could not be isolated from the uprooted grapevines that were hot water treated. The results therefore confirm the importance of hot water treatment for proactive management of Petri disease in grapevine nurseries (Fourie & Halleen, 2004). However, as expected, the DNA in hot water treated plants was not destroyed and could be detected by the developed molecular detection protocol. This is an important consideration when using molecular detection for disease diagnosis or pathogen detection and shows that these methods should be used in conjunction with other diagnostic tools. Future molecular studies should be aimed at investigating the effect of hot water treatment at the RNA level. In theory, transcripts of RNA will only have a relatively short life span following pathogen death. RNA detection would therefore only be possible from living organisms (Klein & Juneja, 1997). However, this might be transcript dependent.

Molecular detection of *Pa. chlamydospora* must be used to identify potential inoculum sources and critical infection stages during the various stages of grapevine propagation. Knowledge of the disease cycle is essential for the development and evaluation of strategies for disease management.

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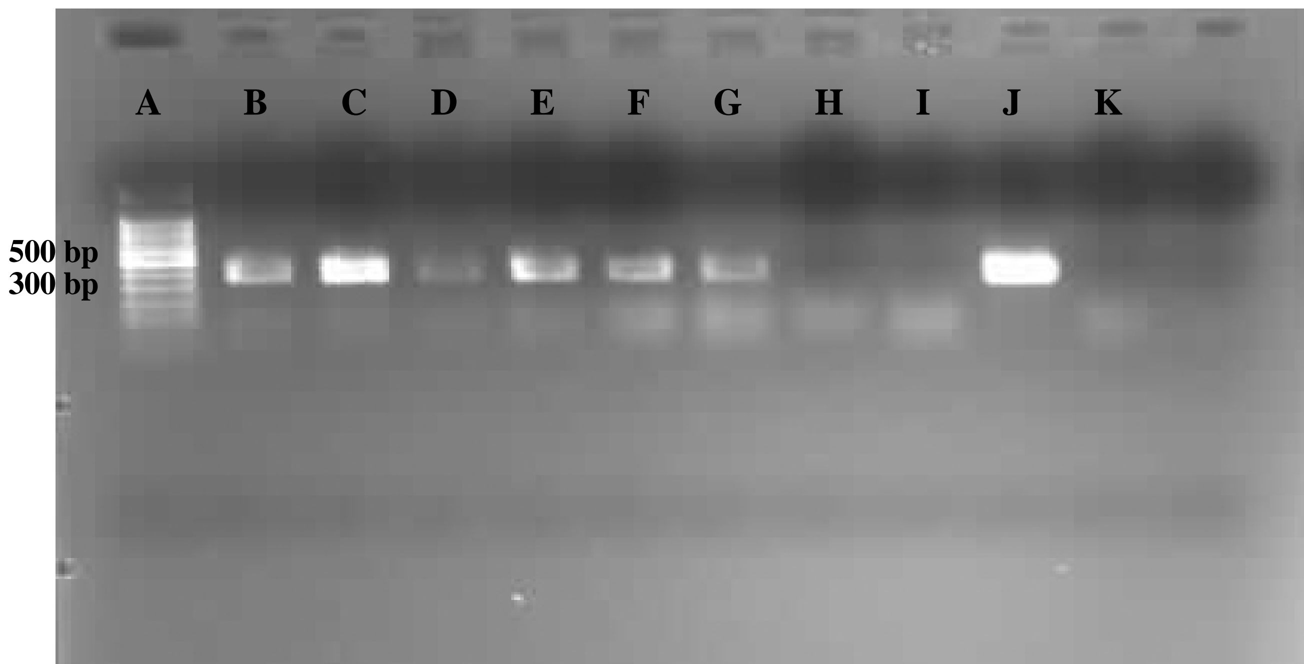

Fig. 1. Detection of *Pa. chlamydospora* in different parts of the grafting material using *Pa*. *chlamydospora* specific PCR primers: 3−5 cm (**B**), 5−7 cm (**C**), 9−11 cm (**D**), 11−13 cm (**E**) and 1−3 cm (**G**) from the basal end of the rootstock, 4-6 cm (**F**), 2−4 cm (**H**) and 0−2 cm (**I**) from the grafting union. **A** is the 100 bp DNA ladder, **J** positive control and **K** negative control.

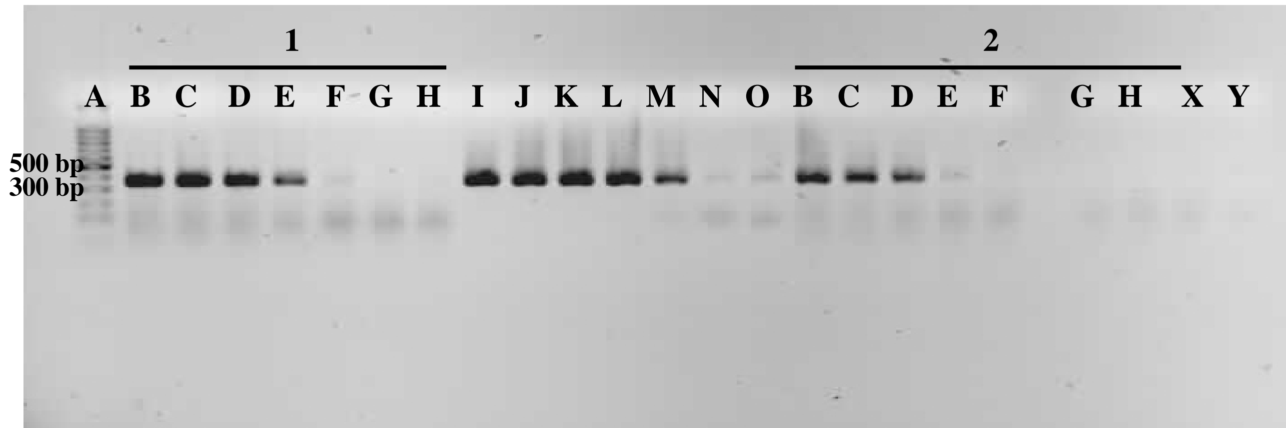

Fig. 2. Sensitivity of a *Pa. chlamydospora* species-specific PCR with primers Pch1 and Pch2. **(A)** 100 bp DNA ladder. **(B-H)** PCR amplification of DNA extracted from grapevine wood, that tested negative for *Pa. chlamydospora*, spiked with known quantities of *Pa. chlamydospora* genomic DNA (Isolates 1 & 2) (1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg). **(I-O)** seven positive controls of *Pa. chlamydospora* genomic DNA, suspended in water at different concentrations (1 ng -1 fg). **(X-Y)** two negative controls including DNA extracted from grapevine wood that tested negative for *Pa. chlamydospora* **(X)** and water **(Y)**.

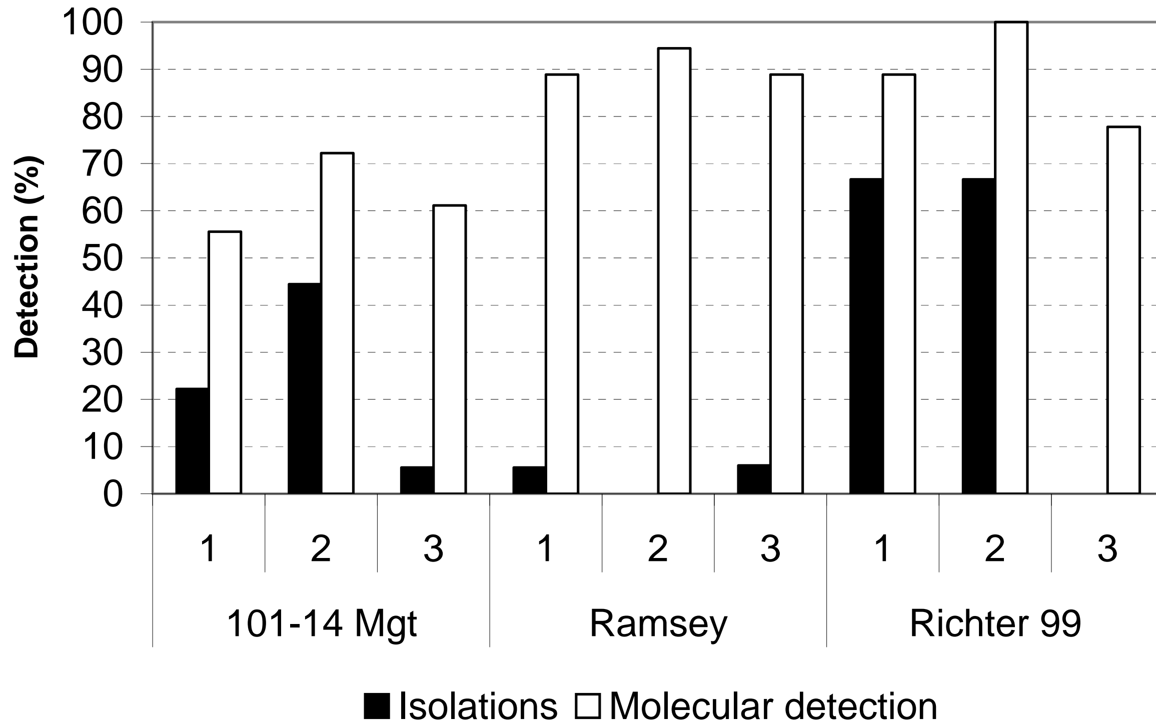

Fig. 3. Detection percentage of *Pa. chlamydospora* in 101-14 Mgt, Ramsey and Richter 99 rootstocks of grapevines from three nurseries (1,2,3) by means of isolations and molecular detection.

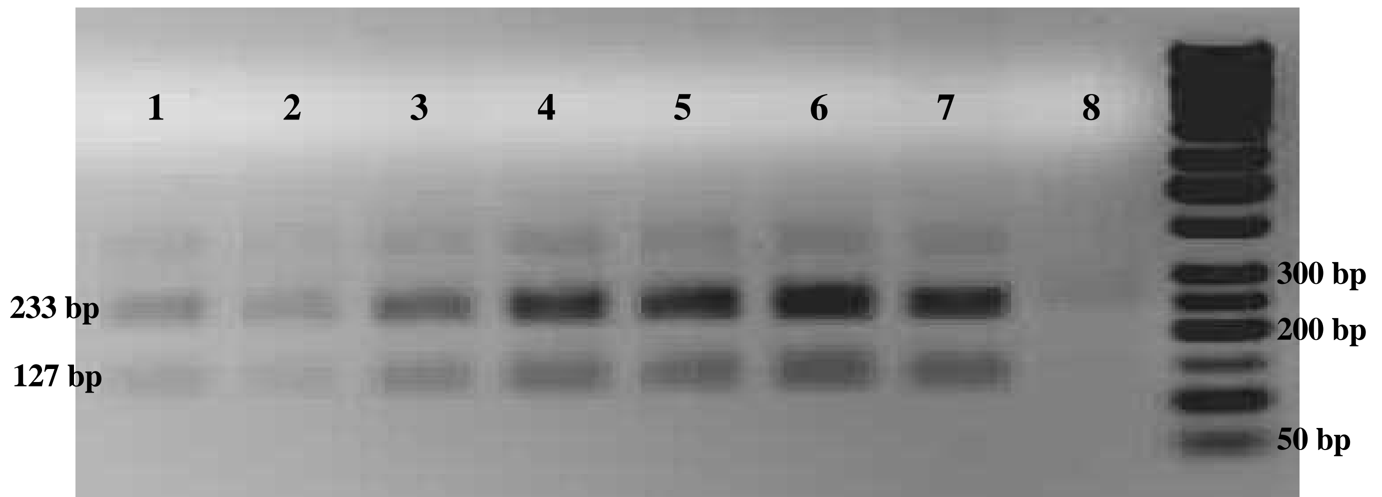

Fig. 4. DNA products resulting from restriction digestion of 360 bp PCR amplicons with *AatII*. The 360 bp PCR amplicons were PCR amplified from wood DNA with *Pa*. *chlamydospora* species-specific primers (Pch1 & Pch2). **Far right**: 50 bp ladder, **1-6**: wood samples, **7**: positive control, **8**: negative control.

3. POTENTIAL INOCULUM SOURCES OF *PHAEOMONIELLA CHLAMYDOSPORA* IN SOUTH AFRICAN NURSERIES

ABSTRACT

Petri disease of grapevine is primarily caused by *Phaeomoniella chlamydospora*. This pathogen affects mostly young grapevines, but is also implicated in esca disease of older grapevines. Little is known about the disease cycle of this fungus. Infected propagation material was identified as a major means of dissemination of the pathogen. Recently, the pathogen was also detected from soil in South Africa and airborne conidia have been found in vineyards. The aim of this study was to use a molecular detection technique to test different samples (water, soil, rootstock and scion cuttings and callusing medium) collected from nurseries in South Africa at different nursery stages for the presence of *Pa. chlamydospora*. A one-tube nested-PCR technique was optimised for detecting *Pa. chlamydospora* in DNA extracted from soil, water, callusing medium and grapevine wood. The one-tube nested-PCR was sensitive enough to detect as little as 1 fg of *Pa. chlamydospora* genomic DNA from water and 10 fg from wood, callusing medium and soil. PCR analyses of the different nursery samples revealed the presence of several putative 360 bp *Pa. chlamydospora* specific bands. Subsequent sequence analyses and/or restriction enzyme digestions of all 360 bp PCR bands confirmed that all bands were *Pa. chlamydospora* specific, except for five bands obtained from callusing media and one from water. Altogether the molecular detection technique revealed the presence of *Pa. chlamydospora* in 35% of rootstock cuttings, 16% of scion cuttings, 58% of hydration water, 17% of soil and 8% of callusing medium samples. These media can therefore be considered as possible inoculum sources of the pathogen during the nursery stages.

INTRODUCTION

Petri disease (previously known as “black goo” and Petri grapevine decline) causes a reduction in the survival rate of young grapevines (Mugnai *et al*., 1999). External symptoms of

Petri disease include stunted growth, shorter internodes, small leaves, interveinal chlorosis, smaller trunks and branches and a general decline of young vines resulting in plant death (Morton, 1995; Bertelli *et al*., 1998; Ferreira, 1998; Fourie *et al*., 2000; Sidoti *et al*., 2000; Whiteman *et al*., 2003). Internal symptoms include a black discolouration and tyloses formation in the xylem vessels (Ferreira, 1998). *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* have been isolated from grapevine material showing these symptoms and are considered to be the causal organisms of this disease. In New Zealand, the fungus that was most commonly isolated from diseased vines was *Pa. chlamydospora* (Whiteman *et al*., 2003) as was the case in South Africa (Fourie & Halleen, 2001). Wallace *et al*. (2003) infected grapevine cuttings with *Pa. chlamydospora* and *Pm. aleophilum* and found that *Pa. chlamydospora* caused brown wood streaking in the rootstock cultivars, but not in the scion varieties and that no visible internal symptoms was caused by *Pm. aleophilum*. They suggested *Pa. chlamydospora* to be the more virulent fungus of the two. Collectively, these results indicate that *Pa. chlamydospora* is the main causal organism of Petri disease.

There is not much known about the disease cycle of *Pa. chlamydospora*. However, a few sources of primary inoculum have been identified. Spores of *Phaeoacremonium* spp. and *Pa. chlamydospora* have been successfully trapped in vineyards in California and France (Larignon, 1998; Eskalen *et al*., 2003). The fungus produces conidia that can be aerially dispersed and usually penetrates the host through pruning wounds (Larignon, 1998; Eskalen et *al*., 2003). Airborne conidia are therefore considered to be a primary inoculum source.

Pa. chlamydospora is able to form chlamydospores in soil and plant debris enabling the fungus to survive for long periods. Chlamydospores are thought to form conidia that can penetrate the roots of vines in nurseries or vineyards (Bertelli *et al*., 1998). The roots of vines do not have to be injured to enable penetration by *Phaeoacremonium* spp. from soil (Feliciano & Gubler, 2001). Infested soil is considered as a potential inoculum source since *Pa. chlamydospora* was found in nursery and vineyard soil by means of conventional species-specific PCR (Damm *et al*., 2004.) and nested-PCR (Whiteman *et al*., 2002).

Isolations from cuttings prior to planting demonstrated that the primary pathogens associated with Petri disease were already present in apparently healthy rootstock propagation material as endophytes (Bertelli *et al*., 1998; Larignon, 1998; Halleen *et al*., 2003). Fourie and Halleen (2002)

also found *Pa. chlamydospora* to be present in canes from rootstock mother plants. Edwards *et al*. (2003) suspected that the infection might spread from mother vines into canes via spores that are carried in sap flow. Feliciano and Gubler (2001) and Edwards *et al*. (2003) found spores and hyphal fragments to be randomly spread along the full length of the cane. The mother plants can therefore be considered as a primary inoculum source.

During the propagation process of grapevines in nurseries, there are various stages where *Pa. chlamydospora* can potentially infect the host (Whiteman *et al*., 2003). One of the earliest stages where infection can occur is when rootstock canes are harvested and cut into 25-30 cm pieces and drenched in a hydration tank for a period of up to 12 h. In South Africa, this usually takes place in May and early June, where after rootstock cuttings are stored in a cold room at temperatures of 1°C to 4°C for a period of 2 to 3 months (Van der Westhuizen, 1981). Scion cuttings are usually harvested during August and early September and are also hydrated in hydration tanks (Van der Westhuizen, 1981) where infection can take place (Whiteman *et al*., 2003). Following cold storage, rootstock and scion cuttings are again placed in hydration or fungicide tanks during the grafting process (this can occur pre-grafting or pre-callus). Scion cuttings are grafted onto rootstocks by means of machine grafting (omega cut) or hand grafting. Graft unions are sealed with a wax layer and grafted vines are callused (until the end of September or early October) in callusing medium consisting of fresh pine sawdust that was drenched in a fungicide suspension. The callusing media may also serve as a potential inoculum source (Whiteman *et al*., 2003). The grafted and callused vines are then planted in nurseries (Van der Westhuizen, 1981). In New Zealand nurseries, it was found that *Pa. chlamydospora* was present at all stages, especially in solutions where there was repeated exposure to plant material and in pre and post storage hydration/fungicide tanks. The percentage of positive samples was moderate from grafting tool washings and low from washings of callusing media (Whiteman *et al*., 2003).

Pa. chlamydospora and *Pm. aleophilum* is difficult to detect using traditional isolation methods due to their slow growth and lack of suitable selective media. These pathogens have also been found in very low quantities (< 0.2%) in rootstock canes (Fourie & Halleen, 2002). Therefore, a sensitive molecular technique is required for accurate and sensitive pathogen detection. Previously, a conventional PCR detection method was developed for detection of *Pa. chlamydospora* in grapevine wood, detecting up to 1 pg *Pa. chlamydospora* DNA (Chapter 2). However, preliminary studies showed that this technique is not specific and sensitive enough for

detection of *Pa. chlamydospora* in soil and water (results not shown). Whiteman *et al.* (2002) recently published a more sensitive nested-PCR for detecting as little as 50 fg of *Pa. chlamydospora* genomic DNA from artificially infested soil. One drawback of nested-PCRs is the increased risk of contamination and false positive sample testing due to aliquoting of PCR products from the external primer pair reaction to a new PCR tube containing the internal PCR primer pair. Therefore, recently published one-tube nested-PCR techniques that do not receive aliquoting of PCR products, have greater potential for accurate pathogen detection (Olmos *et al*., 1999; Tao *et al*., 2004).

The first objective of this study was to develop a technique for extracting DNA from water and callusing medium. DNA extraction techniques from soil (Damm & Fourie, 2004) and wood (chapter 2) have previously been developed. Subsequently, a one-tube nested-PCR technique was optimised to detect *Pa. chlamydospora* in water, soil, wood and callusing medium. These molecular techniques were subsequently used to determine if *Pa. chlamydospora* is present in samples (water, soil, wood, callusing medium) collected from different nurseries, which would allow the identification of different inoculum sources of *Pa. chlamydospora* in different nursery stages in South Africa.

MATERIALS AND METHODS

Sample collection

Wood. Rootstock canes from the cultivars 101-14 Mgt, Ramsey, 99 Richter and 110 Richter were collected from six nurseries. Samples consisted of five third-internode sections from one-year-old canes, which were sub-sampled from five randomly selected rootstock mother plants in a randomly selected vineyard row in each nursery. Four samples were collected for each mother block.

Rootstock cuttings from the cultivars 101-14 Mgt and Ramsey (five cuttings of each cultivar) were collected during grafting from 16 nurseries. Scion cuttings (five per cultivar) from a variety of cultivars were also collected during the grafting process from 19 nurseries.

Soil. Soil samples (approximately 50 g each) were collected in sterile plastic bags from the area surrounding each rootstock mother plant from which cane samples were removed. Samples

were collected at a depth of 10–20 cm. The 20 samples (5 per row and 4 rows per block) from each mother block were mixed and analysed as one composite sample (24 samples in total). After grafting, soil samples were also collected in sterile plastic bags from the nursery beds of 18 nurseries. Four sampling sites were randomly selected, samples collected at a depth of 10–20 cm, mixed and analysed as one composite sample.

Water. During pre-storage hydration two water samples were collected in 250 ml sterile glass bottles from each of 15 nurseries. One sample consisted of water from hydration tanks in which rootstock cuttings has been drenched for a period of at least 12 h. The other sample consisted of water from the hydration tank before the 12-h drenching process. Water samples were also collected during the grafting process (either pre-grafting or pre-callusing) from 21 nurseries.

Callusing medium. During the grafting process, callusing medium consisting of fresh pine sawdust was sampled in sterile plastic bags from 12 nurseries. These samples were taken prior to the callusing stage, before the callusing medium were packed with grafted cuttings or drenched.

DNA extraction

Wood. Wood samples were prepared for DNA extraction by removing a half centimetre piece from the 1-3 cm section of the basal end of each wood sub-sample. After the bark was removed, woody pieces were surface sterilised (70% ethanol for 30 s and in 3.5% sodium hypochlorite for 60 s and then again in 70% ethanol for 30 s) and stored at 4°C. The pieces of each sample were snap frozen with liquid nitrogen and grinded to a powder using a Mixer Mill Type MM 301 (Retsch Gmbh & Co. KG, Germany). Following grinding, the four rootstock cane samples from each mother block were added together to represent 24 samples (each sample representing a mother block). Likewise, 32 samples were prepared from rootstock cuttings (Ramsey and 101-14 Mgt samples from 16 nurseries) and 19 samples from the scion cuttings (various cultivars, 19 mother blocks). DNA was extracted from 0.5 g grinded wood as previously described (Chapter 2).

Soil. DNA was extracted from soils using a SDS buffer, FastPrep® instrument (Bio101, Savant, Farmingdale, NY, USA) and self-prepared PVPP (polyvinylpoly pyrrolidone) spin columns to remove inhibitors (Damm & Fourie, 2004). The PVPP columns were prepared by making an opening (1 mm diameter) in the bottom of a 0.5-ml tube and covering it with glass wool. The lid of the tube was removed and the tube placed into a 2-ml tube. A mixture of PVPP and high salt TE

buffer (0.1 g/ml, buffer: 100 mM NaCl, 10mM Tris, 1 mM EDTA, pH 8) was filled into the 0.5-ml tube and centrifuged for 1 min at 720 x g. This was repeated until the tube remained full after centrifugation. Prior to use, the column was spun dry. Soil (0.5 g) was placed in a 2-ml screw cap together with 0.5 g acid washed sand and 0.5 g glass beads (2 mm diameter). Phosphate buffer (300 µl) (100 mM NaH_2PO_4, pH 8) and 300 µl SDS extraction buffer (100 mM NaCl, 500 mM Tris pH 8, 10% SDS) were added and the tube was subsequently inverted. Chloroform:isoamylalcolhol (24:1) (400 µl) was added and the tube shaken in a FastPrep instrument (Bio101, Savant, Farmingdale, NY, USA) for 40 s at 4.5 m/s. The mixture was then centrifuged and the supernatant transferred into a new tube. Cold ammonium acetate (final concentration 2.5 M) was added, the tube was vortexed and centrifuged. The supernatant was transferred into a new tube, cold isopropanol was added and the tube was incubated at room temperature. After incubation, it was centrifuged and the supernatant discarded. The DNA pellet was washed with 70% ethanol, where after the DNA pellet was dried and re-suspended in 120 µl ddH_2O. The DNA solution was loaded on the prepared PVPP column and spun through the column.

Water. DNA extraction was done from 90 ml water of each water sample by centrifugation in a Beckman J2–21 Centrifuge (Beckman Instruments, California). Thirty millilitres of water was centrifuged for 10 min at 6000 rpm (rotor JA-20) in an oakridge centrifuge tube and the supernatant discarded, this step was repeated twice in the same tube, enabling 90 ml of water to be processed for a single DNA extraction. The pellet was re-suspended in 1 ml of a CTAB extraction buffer (1 M Tris, pH 7.5; 5 M NaCl; 500 mM EDTA, pH 8.0) and the solution transferred to a 2-ml Eppendorf tube. Glass beads (0.5 g) were added and tubes were shaken for 5 min at a 30 1/s frequency using a Mixer Mill Type MM 301 (Retsch Gmbh & Co. KG, Germany), followed by incubation at 65°C for 1 h. After incubation, 400 µl chloroform:isoamylalcohol (24:1) was added, followed by centrifugation at 1300 x g for 15 min. The watery supernatant was transferred to a new Eppendorf tube and 50 µl of 7.5 M ammonium acetate solution (pH 7.0) and 600 µl cold isopropanol were added. The samples were incubated for 1 h at -20°C before centrifugation at 15800 x g for 10 min. The supernatant was discarded and 1 ml cold 70% ethanol was added before incubation at -20°C for 30 min. After incubation, the samples were centrifuged at 15800 x g for 5 min and the supernatant discarded. The DNA pellet was dried on a bench, dissolved in 100 µl sterile ddH_20 and stored at 4°C.

Callusing medium. DNA was extracted from callusing medium by combining 0.5 g callusing medium and 0.5 g glass beads (2 mm) in a 2-ml tube. The samples were then snap frozen in liquid nitrogen and shaken for 5 min at a 30 1/s frequency using a Mixer Mill Type MM 301 (Retsch Gmbh & Co. KG, Germany). CTAB buffer (500 µl) was added to the tubes and shaken for 5 min at a 30 1/s frequency using a Mixer Mill Type MM 301 (Retsch Gmbh & Co. KG, Germany), followed by incubation at 65°C for 1 h. Thereafter, the extraction protocol was followed as described above for the water samples.

One-tube nested-PCR analyses

Following DNA extraction, one-tube nested-PCR was performed using a 0.5-ml Eppendorf tube that was compartmentalised with the end of a standard 200 µl plastic pipette tip (Olmos *et al.*, 1999). The end of the pipette tip served as a small cone, which was inserted into the Eppendorf tube to physically separate the two PCR cocktails in the same tube. The PCR reaction containing the external primer pair (universal primers ITS4 and ITS6) was dispensed in the bottom of the tube, with the PCR reaction containing the internal primer pair (species-specific primers Pch1 and Pch2 (Tegli *et al.*, 2000)) being dispensed in the pipette tip cone (Fig. 1). The external primer PCR reaction (25 µl) consisted of 1x PCR buffer (Bioline, Luckenwalde, Germany), 3 mM $MgCl_2$, 0.4 mM dNTPs (each) (ABgene, Rochester,New York, USA), 0.2 µM ITS4 primer, 0.2 µM ITS6 primer, 0.65 units of *Taq* DNA polymerase (Bioline), 1.25 µl (20mg/ml) BSA Fraction V (Roche Diagnostics South Africa, Randburg) and 5 µl of DNA template. BSA (bovine serum albumin) was dissolved in a buffer consisting of 50 mM Tris-HCL (pH 8), 0.1 M NaCl, 0.25 mM EDTA (pH 8) and 50% glycerol. The internal primer PCR reaction consisted of 1x PCR buffer (Bioline), 4 µM Pch1 primer, 4 µM Pch2 primer, 0.75 µl (20mg/ml) BSA Fraction V (Roche) and 1 unit of *Taq* DNA polymerase (Bioline) in a total volume of 15 µl. The final $MgCl_2$ concentration for amplification with the internal species-specific primer pair was 1.875 mM. The nested-PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) and the cycling conditions for the external amplification were: 3 min at 94°C, followed by 32 cycles of 30 s at 94°C, 30 s at 50°C, 1 min at 72°C and a final extension cycle of 7 min at 72°C. Following completion of the first external primer pair PCR cycles, tubes were vortexed for 20 s and briefly centrifuged (6000 x g for 2 s). Cycling conditions for the internal amplification were 3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 57°C, 30 s at 72°C and a 7 min extension step at 72°C to complete the reaction. Twelve microlitres of each PCR product were separated by electrophoresis at 100 V for 1 h on a 1.5%

agarose gel, stained with 0.5 µg/ml ethidium bromide in a 1 × TAE buffer (0.04 M Tris, 0.02 M glacial acetic acid and 1.27 mM EDTA, pH 7.85) and visualised under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, United Kingdom).

Sensitivity of the one-tube nested-PCR was determined by adding known quantities of *Pa. chlamydospora* genomic DNA to DNA extractions from wood, soil, water and callusing medium that had tested negative with the one-tube nested-PCR. Approximately five to six different negative samples from each medium were pooled before adding known quantities of genomic DNA. DNA concentrations of the purified DNA were determined with a fluorometer FL600™ (Bio-Tek, http://www.biotek.com) and added to the DNA extract to achieve final concentrations of 1 pg/µl, 100 fg/µl, 10 fg/µl and 1 fg/µl. Spore suspensions of *Pa. chlamydospora* were added to water samples to achieve final concentrations of 10^1 and 10^2 conidia/90 ml water. DNA extraction was done as previously described. Five microlitres from each of these spiked DNA extracts and artificially infested water were used in PCR reactions and analysed with gel electrophoresis as described previously.

Identification of putative *Pa. chlamydospora* PCR products

Enzyme restriction digestion and/or sequencing analyses were used to determine whether all 360 bp amplicons amplified from DNA samples (wood, soil, callusing medium and water) with the one-tube nested-PCR were indeed *Pa. chlamydospora*. All 360 bp amplicons that were obtained from amplifications were cut from agarose gels and purified using a QIAquick® Gel Extraction kit (Qiagen, Valencia, CA, USA). Restriction enzyme digestions with *AatII* and *MluNI* (Roche Diagnostics South Africa Pty Ltd, Randburg) were done according to the manufacturer's instructions. These restriction enzymes enabled differentiation between *Pa. chlamydospora* and other closely related fungi (Whiteman *et al*., 2002). The digested PCR products were separated by 1.5% agarose gel electrophoresis as previously described. A sub-sample of 22 (13%) of the 360 bp amplicons from the different samples was also sequenced using the primer Pch3 (5'-GATAATGACGCTCGAACAGG-3'). The sequencing reaction and cycle conditions were carried out as recommended by the manufacturer with an ABI Prism Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, California) containing AmpliTaq DNA Polymerase. The resulting fragments were analysed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut). The identity of the sequences was

determined by BLAST analyses.

RESULTS

One-tube nested-PCR analyses

The nested-PCR was sensitive enough to detect 1 fg of *Pa*. *chlamydospora* genomic DNA from water and 10 fg from callusing medium, wood and soil. It was furthermore able to detect as few as 10 spores suspended in 90 ml of water (Fig. 2).

Several putative *Pa*. *chlamydospora* PCR products (360 bp amplicons) were obtained with the one-tube nested-PCR (Fig. 3): 25% of rootstock cane sections collected from mother blocks, 42% of rootstock cuttings collected during grafting, 16% of scion cuttings, 40% of water samples collected after the 12 h pre-storage hydration, 76% of water samples collected during grafting, 50% of the callusing medium samples and 17% of the soil samples collected from mother blocks. *Pa. chlamydospora* was detected from rootstock cuttings, scion cuttings, hydration/fungicide tanks, soil and callusing medium. Although no 360 bp amplicons were obtained from soil collected from nursery beds, DNA was successfully extracted and the absence of PCR inhibitors was shown by amplification with an ITS2/ITS5 PCR in all samples (Fig. 4).

Identification of putative *Pa. chlamydospora* PCR products

All PCR amplicons that were 360 bp in size were sequenced and/or restriction digested to confirm that the PCR amplicons were *Pa. chlamydospora* specific. Products resulting from restriction digest using enzymes *AatII* and *MluNI* corresponded in size to those produced by digested genomic DNA from *Pa. chlamydospora*. *MluNI* yielded products of 79 bp and 281 bp (Fig. 5a) and *AatII* yielded products of 127 bp and 233 bp (Fig. 5b). Analyses of the GenBank ITS sequence data of *Pa. chlamydospora* (AF197986) confirmed that the aforementioned fragment sizes should be obtained with restriction digestion using *AatII* and *MluNI*. A subset of the 360 bp PCR amplicons was also sequenced. BLAST analyses showed that the sequences of the majority of the bands were 100% identical to *Pa. chlamydospora* (*Phaeoacremonium chlamydosporum*) in GenBank (accession numbers: AF197986, AF266656, AF266653, AF197987, AF 197973, AF017652). The only samples where 360 bp PCR amplicons could not be identified as being *Pa.*

chlamydospora, either through sequence or restriction digests, were in five callusing medium and one water sample. BLAST analyses of the callusing medium and the water amplicons showed that these PCR amplicons had highest homology to *Diplotomma venustum* (Körb.) Lettau (1860) (E-value: 10^{-41}) *Botryosphaeria stevensii* Shoemaker (1964) (E-value: 10^{-41}) and *Botryosphaeria dothidea* (Moug.) Ces. & De Not. (1863) (E-value: 10^{-87}).

Considering all bands that were positively confirmed as being *Pa. chlamydospora*, 25% of rootstock cane sections collected from mother blocks, 42% of rootstock cuttings collected during grafting, 16% of scion cuttings, 40% of water samples collected after the 12 h pre-storage hydration, 67% of water samples collected during grafting, 8% of callusing medium samples and 17% of soil samples collected from mother blocks contained *Pa. chlamydospora* (Fig. 6). *Pa. chlamydospora* was most frequently detected from 101-14 Mgt (13%) and 99 Richter (8%) mother plants and from the 101-14 Mgt (50%) cuttings during grafting.

DISCUSSION

Conventional PCR might not always provide the sensitivity and specificity needed for pathogen detection in samples such as water, soil and wood where a variety of organisms are present. The conventional PCR could detect 1 pg of *Pa. chlamydospora* genomic DNA from grapevine wood (Chapter 2) whereas the one-tube nested-PCR analyses could detect as little as 10 fg of *Pa. chlamydospora* genomic DNA. The one-tube nested-PCR is therefore a lot more sensitive than the conventional PCR. The chance of cross-contamination of samples leading to false positives is also minimized when using the one-tube nested-PCR as opposed to a conventional nested-PCR technique.

The use of primers for pathogen detection that anneal to ITS regions can sometimes also amplify ITS regions of other organisms. In this study, it proved important to confirm that 360 bp amplicons were *Pa. chlamydospora* specific by restriction enzyme digests or sequencing. If this was not done, a six-fold overestimation of the incidence of *Pa. chlamydospora* in callusing media would have been made. Therefore in media that has a large number of diverse organisms, many of which the identities and sequences are unknown, confirmation of PCR bands are required. The

identity of the organisms amplified from water and callusing media with the *Pa. chlamydospora* primers are unknown since the BLAST analyses yielded low E-values.

A quarter of the rootstock cane samples from the mother plants tested positive for the presence of *Pa. chlamydospora*. Fourie and Halleen (2002) did isolations from rootstock canes and found the incidence of *Pa. chlamydospora* to be extremely low (<0.2%). The presence of the pathogen in rootstock canes, which was directly removed from the mother plant, confirms previous reports (Ridgway *et al*., 2002) that infected rootstock mother plants are one of the primary inoculum sources. The canes from the mother plants of the cultivar 101-14 Mgt showed the highest incidence of *Pa. chlamydospora*, which correlates with results found by Fourie and Halleen (2004b) through conventional isolation techniques. Furthermore, *Pa. chlamydospora* DNA was detected from soil sampled in rootstock mother blocks. *Pa. chlamydospora* might be present in soil as mycelium, conidia, chlamydospores and/or other fruiting structures originating from infected mother plants.

Water samples from the pre-storage and grafting hydration and fungicide tanks tested positive for the presence of *Pa. chlamydospora*. None of the water samples that were taken before the 12-h hydration period tested positive for this pathogen. This is an indication that the water source was not an inoculum source. However, after a period of hydration the water was contaminated, presumably from infected or contaminated cuttings. There might be mycelium and conidia present on the surfaces of cuttings that washes off into the water during hydration, or it might even ooze from xylem vessels into the water. This contaminated water can subsequently serve as an important inoculum source, since all cuttings are hydrated prior to cold storage. Whiteman *et al*., (2003) also found a very high percentage of positive *Pa. chlamydospora* samples from both pre-storage and pre-grafting rehydration and fungicide tanks in New Zealand commercial nurseries.

Rootstock cuttings that were sampled during grafting tested positive for the presence of *Pa. chlamydospora*. The incidence increased from 25% in the rootstock canes sampled in mother blocks to 42% of the number of cutting samples tested. A number (16%) of the scion cutting samples, which were also sampled during grafting, tested positive for *Pa. chlamydospora*. These cuttings might have been infected during the hydration period or possibly from infected mother plants.

A very small number (8%) of the callusing medium samples tested positive for *Pa. chlamydospora*. These results correlate with the results from Whiteman *et al*. (2003), who found a

very low percentage of positive *Pa. chlamydospora* samples from washings of callusing media in New Zealand nurseries. Callusing medium mainly consists of fresh pine sawdust. *Pa. chlamydospora* is not a known pathogen of pine trees, which indicates that the callusing medium was possibly contaminated during the nursery stages and most probably through contaminated water, equipment or floors.

None of the soil samples collected from the nursery beds tested positive for *Pa. chlamydospora*. During the year before the grafted vines are planted, these nursery beds are either laid fallow or planted with a cover crop, such as wheat. The fact that no *Pa. chlamydospora* could be detected from these soils might indicate that the pathogen cannot survive in soil for such a long period. Alternatively the pathogen levels were so low that it could not be detected.

Several potential inoculum sources (i.e. rootstock mother plants, rootstock canes and cuttings, scion cuttings, drench water (pre-storage, pre- and post-grafting), callusing media and soil) were identified in this study. By implementing several strategies to reduce or eradicate inoculum from these potential sources, Petri disease can be pro-actively managed in grapevine nurseries.

Primarily, control measures must concentrate on protecting pruning wounds of mother plants, because these open wounds can be infected from aerially dispersed spores or contaminated soil, as they are in most cases very close to the soil surface. Di Marco *et al*. (2004) found that pruning wound protection by *Trichoderma* spp. significantly reduced necrotic lesions caused by *Pa. chlamydospora* in potted vines. Pre-infection spraying of *Trichoderma* on fresh pruning wounds was effective in preventing Petri disease (Di Marco *et al*., 2000). Soil amendments of *Trichoderma* further enhanced root development (Fourie *et al*., 2001; Di Marco *et al*., 2004), which will increase tolerance to stress as well as water and nutrient uptake (Harman, 2000).

Rootstock and scion cuttings must be treated to eradicate or lower the incidence of the pathogen. This may be done by hot water treatment, chemical or biological strategies. Fourie and Halleen (2004a) tested and compared these strategies by treating rootstocks with a variety of products. They found that chemical and biological strategies reduced pathogen infections in rootstocks, but compared to the hot water treatment it showed a very low reduction. With hot water treatment, a drastic reduction in the levels of *Pa. chlamydospora* in naturally infected rootstock cuttings was observed. Moreover, subsequent colonisation of treated rootstocks was also inhibited (Fourie & Halleen, 2004a).

Unprotected wounds on cuttings provide ideal infection openings for *Pa. chlamydospora* and other trunk disease pathogens from contaminated water in hydration tanks. Hydration tanks must therefore be sterilised after every hydration period and the water treated with chemical and/or biological control agents. South African nurseries use fungicides such as captan, iprodione, quaternary ammonium compounds or hydrogen peroxide mixtures in their hydration/fungicide drench tanks. Captan and iprodione were found to be moderately or poorly effective in reducing germination or mycelium growth of *Pa. chlamydospora* (Groenewald *et al*., 2000; Jaspers, 2001). The efficacy of the sterilising agents, such as quaternary ammonium compounds or hydrogen peroxide mixtures, against *Pa. chlamydospora* has not been shown. Further research must be conducted to find chemical and biological control agents that should protect wounds or effectively eradicate the pathogen or reduce mycelium growth and conidia germination to an acceptable level.

To avoid the graft union from being infected by the contaminated drench water or callusing medium the graft unions must be thoroughly protected with a wax layer and a biological or chemical protective layer, may also be very useful. Messina (1999) found that the use of *Trichoderma* products, which contains a mixture of *T. harzianum* and *T. viride*, in callusing boxes resulted in stronger graft unions and root systems in a shorter callusing period. It may also be useful to use an effective fungicide during the pre-callus drench.

Nursery soils must be subjected to fallow periods or crop rotation with non-host crops such as wheat, canola or lupins. It might also be possible to select cover crops with bio-fumigation properties such as *Brassica* spp. (Brown & Morra, 1997). The absence of *Pa. chlamydospora* from nursery bed samples in this study, might indicate that the current 1-year fallow or crop rotation period is sufficient in reducing inoculum levels in soils to undetectable levels.

The detection of *Pa. chlamydospora* in this study was based on the presence of pathogen genomic DNA. However, it is important to consider that the mere presence of DNA does not indicate whether viable pathogen propagules are present. For example, after hot water treatment pathogen DNA is detectable, even though the pathogen is no longer viable (Chapter 2). Therefore, in future studies the use of RNA detection might be more suitable as it will only detect viable organisms.

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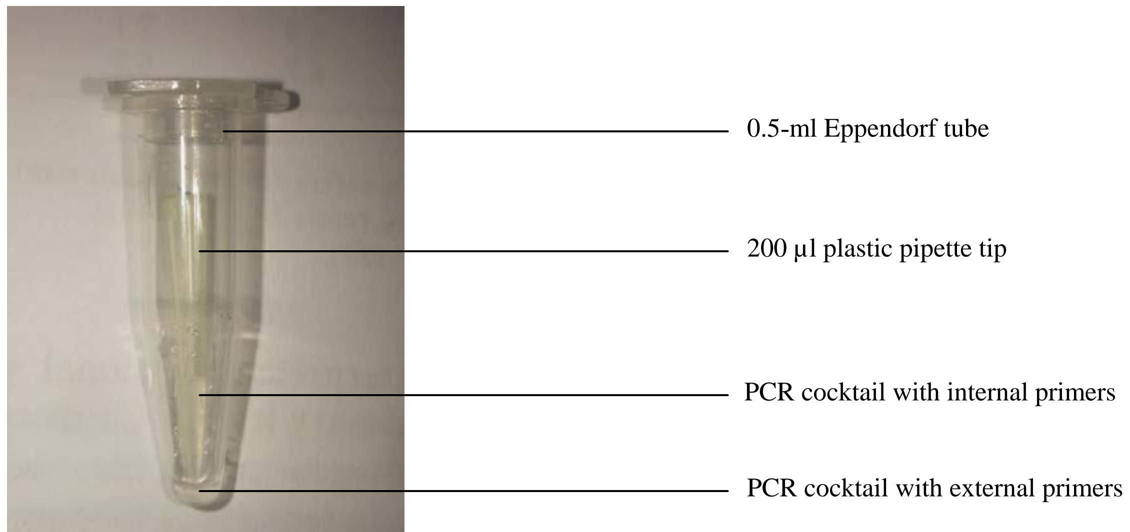

Fig. 1. One-tube nested-PCR using a 0.5-ml Eppendorf tube that was compartmentalised with the end of a standard 200 µl plastic pipette tip.

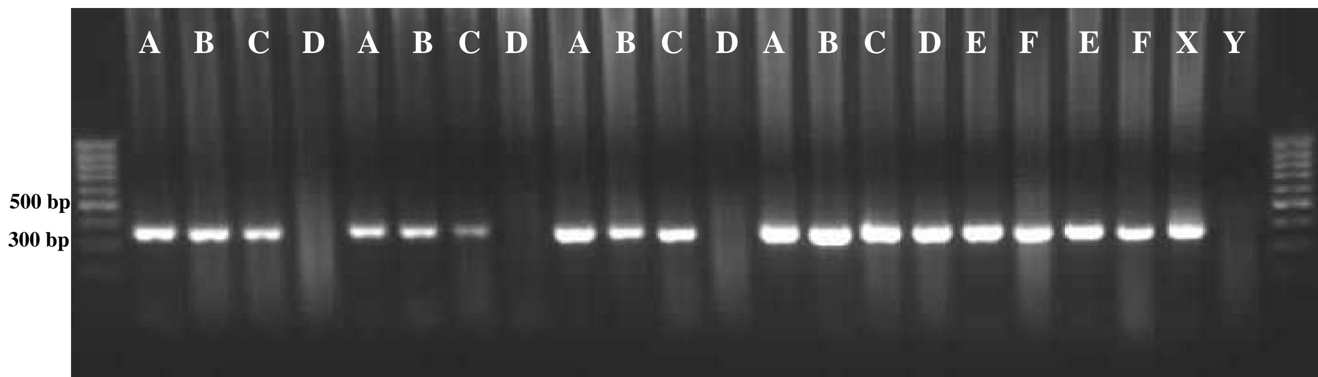

Fig. 2. Determination of the sensitivity of the one-tube nested-PCR with primers Pch1 and Pch2. DNA from grapevine wood (**1**), callusing medium (**2**), soil (**3**) and water (**4**), which tested negative for *Pa. chlamydospora* and were spiked with 1 pg (**A**), 100 fg (**B**), 10 fg (**C**) and 1 fg (**D**) of *Pa. chlamydospora* genomic DNA. The 90 ml water samples (**5**) were also spiked with 10^2 spores (**E**) and 10^1 spores (**F**). The 100 bp ladder is far left and right. A positive control **(X)** containing only genomic DNA and a negative control **(Y)** containing no template were included.

Fig. 3. Several putative *Pa. chlamydospora* specific bands (360 bp) generated in a nested-PCR with DNA extracted from wood and callusing medium. **Far left and right lane**: 100 bp DNA ladder, **1**: negative control, **2-4**: soil samples, **5-12**: wood samples, **13-18**: callusing medium samples, **19–21**: water samples **22**: positive control.

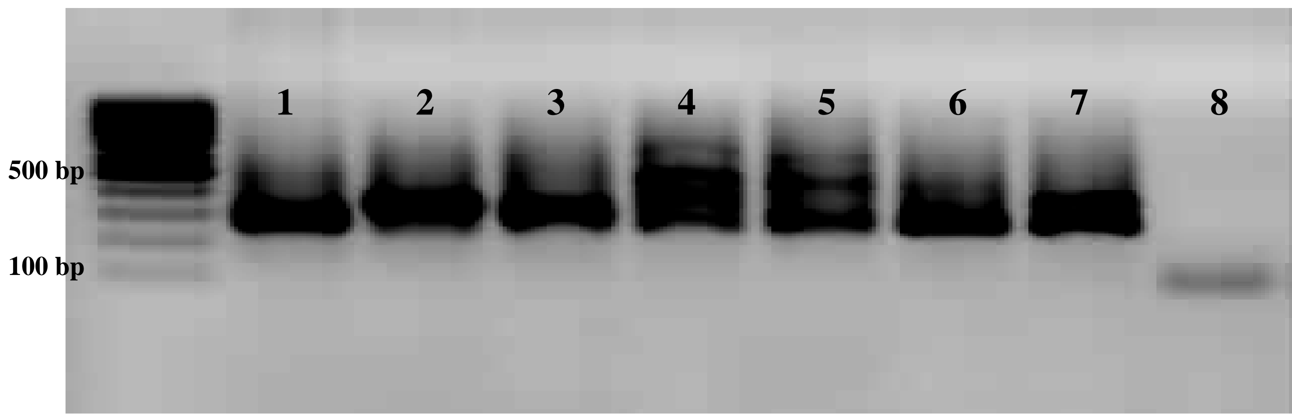

Fig. 4. PCR amplimers generated in a PCR using primers ITS2 and ITS5 on DNA extracted from grapevine nursery soil. Far left lane: 100 bp DNA ladder, soil samples 1-6, positive control (7), negative control (8).

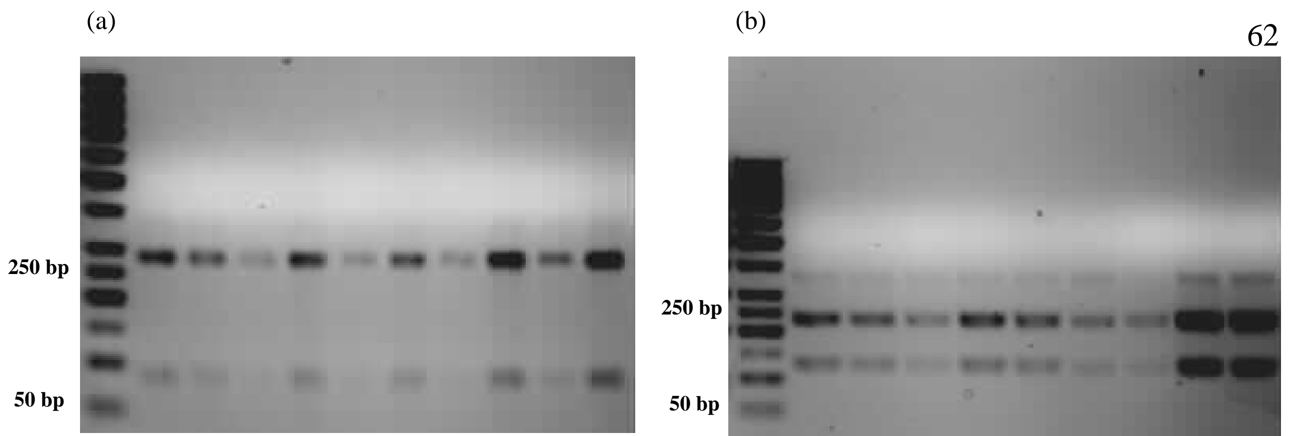

Fig. 5. DNA fragments resulting from digestion of putative *Pa. chlamydospora* PCR products (360 bp) with (a): *MluNI* yielding 79 bp and 281 bp fragments. The 50 bp DNA ladder is far left and the positive control far right and (b): *AatII* yielding 127 bp and 233 bp fragments. The 50 bp DNA ladder is far left and the positive control far right.

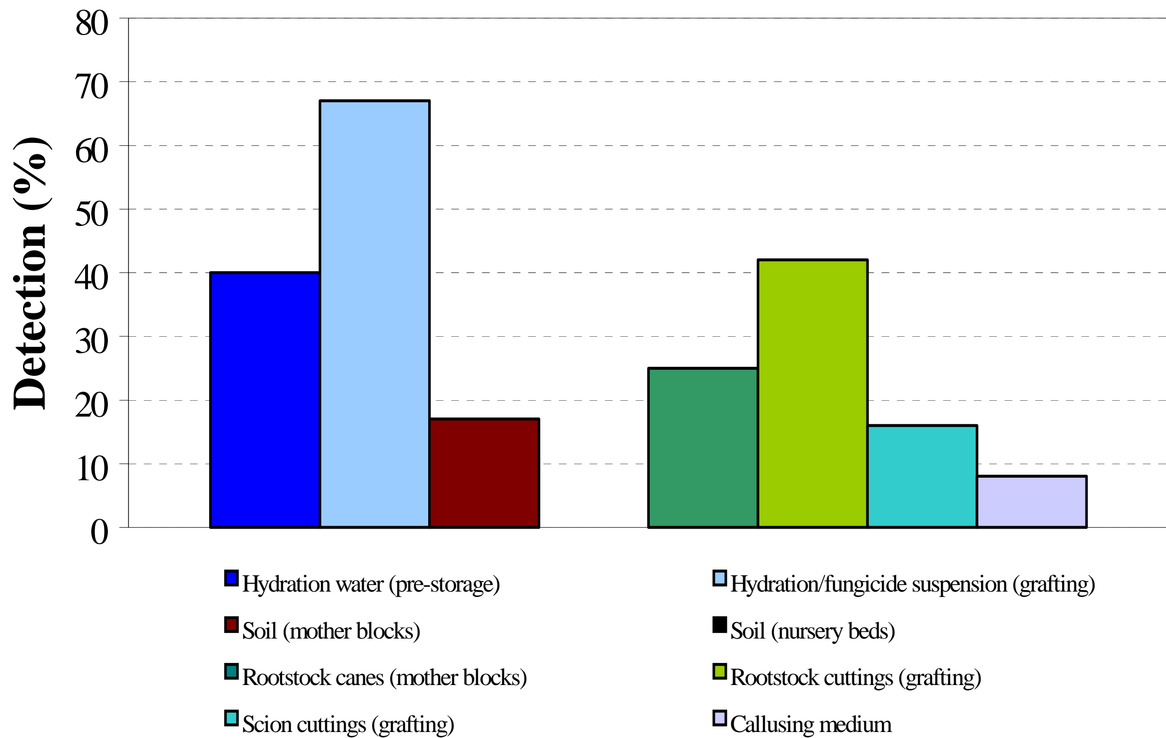

Fig. 6. Detection percentages of *Pa. chlamydospora* in wood, water, soil and callusing medium sampled at different stages during the nursery process. *Pa. chlamydospora* was detected using *Pa. chlamydospora* specific primers and a nested-PCR. The identity of all PCR amplicons were confirmed through restriction digestion or sequence analyses as being *Pa. chlamydospora* specific.