CHARACTERIZATION AND CONTROL OF *PHAEOMONIELLA CHLAMYDOSPORA* IN GRAPEVINES

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DECLARATION

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

SUMMARY

Petri grapevine decline, also known as black goo, slow die-back and Phaeoacremonium grapevine decline, causes significant losses of young vines world-wide. Species of *Phaeoacremonium*, *Phaeomoniella chlamydospora* and related genera are associated with this grapevine disease. This study investigates the *Phaeoacremonium*-complex and *Phaeomoniella chlamydospora*, focussing on the species isolated from grapevines. Fungicide sensitivity of *Pa. chlamydospora* and the possibility of employing molecular techniques for the detection of *Pa. chlamydospora* in grapevines were also investigated.

In an overview of the literature on Petri grapevine decline the disease history and the relatedness of Petri grapevine decline to esca is discussed. Petri grapvine decline occurs in propagation material or young vines. Infected material can appear asymptomatic and therefore the possibilities of molecular techniques for identification were also investigated in the literature.

In South Africa *Pa. chlamydospora* is the dominant organism causing Petri grapevine decline and therefore different fungicides were evaluated to control this fungus. Six isolates of *Pa. chlamydospora,* from Stellenbosch, Wellington, Somerset West and Malmesbury of Western Cape province, South Africa, were screened against twelve fungicides testing their effect on mycelial inhibition *in vitro*. These fungicides included benomyl, chlorothalonil, fenarimol, fosetyl-Al, iprodione, kresoxim-methyl, mancozeb, metalaxyl, prochloraz manganese chloride, quintozene, tebuconazole and thiram. Results provided the base-line sensitivity of South African isolates of *Pa. chlamydospora*. Benomyl, fenarimol, kresoxim-methyl, prochloraz manganese chloride and tebuconazole were the most effective (with EC_{50} values ranging from 0.01 to 0.05 µg/ml) for inhibiting mycelial growth of *Pa. chlamydospora in vitro*. This *in vitro* test gave a good indication of which fungicides could be selected for further studies in glasshouses and nurseries.

The molecular phylogeny of *Phaeoacremonium* and *Phaeomoniella* isolates from grapevines of South Africa, or isolates obtained from the Centraalbureau voor Schimmelcultures (CBS) in the Netherland, were investigated. Sequence data were created from the rDNA region and partial ß-tubulin gene of 33 of these isolates using the PCR technique. This sequence data were analysed with PAUP* version 4.0b2a. An analysis of the sequence data confirmed the genus *Phaeomoniella* to be distinct

from *Phaeoacremonium* (*Pm.*) based on DNA phylogeny. Although morphologically similar, the species status of *Pm. aleophilum* and *Pm. angustius* was confirmed with DNA phylogeny and cultural characteristics. *Pm. aleophilum* has an optimum growth rate at 30°C and the ability to grow at 35°C, where as *Pm. angustius* has an optimum growth rate at 25°C and cannot grow at 35°C. *Pm. viticola* was shown to be synonymous with *Pm. angustius*, and a new species, *Pm. mortoniae*, was newly described from grapevine occurring in California. Futhermore, *Pm. aleophilum* was newly reported from South Africa and grapevine isolates thought to be *Pm. inflatipes* were all re-identified as *Pm. aleophilum*. These findings therefore also shed some doubt on the possible role of *Pm. inflatipes* in Petri grapevine decline. It was confirmed that *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. angustius* are the species involved in Petri grapevine decline. *Pm. mortoniae* was isolated from grapevines, but its pathogenicity should still be confirmed and the role of *Pm. inflatipes* in Petri grapevine decline remains unclear.

Pa. chlamydospora has been routinely isolated from symptomless propagation and nursery material. Because the disease can take years to develop, it is crucial that healthy propagation material is used at planting. *Pa. chlamydospora* is a slow-growing fungus, and positive identification from symptomless grapevine tissue can take up to 4 wks. The possibility of employing molecular techniques for the detection of *Pa. chlamydospora* in apparently healthy grapevines was investigated. Species-specific primers (PCL1 and PCL2) based on the regions ITS1 and ITS2 were designed for *Pa. chlamydospora*. These primers were highly sensitive and amplification was achieved from genomic DNA of *Pa. chlamydospora* from as low as 16 pg. *Phaeoacremonium* spp., related genera and common fungal taxa from grapevines were tested with these primers, but positive amplification was achieved for *Pa. chlamydospora* only. The presence of *Pa. chlamydospora* in symptomless grapevine tissue culture plants was confirmed by PCR within 24 hours. These primers therefore allow rapid and accurate identification of *Pa. chlamydospora*. Testing on a larger scale with nursery material should be conducted to determine the feasibility of using these species-specific primers in the grapevine industry.

OPSOMMING

Petri-terugsterwing van jong wingerde, ook algemeen bekend as "black goo" en Phaeoacremonium-terugsterwing, veroorsaak wêreldwyd groot geldelike verliese in die wingerdbedryf. Spesies van *Phaeoacremonium*, *Phaeomoniella chlamydospora* en verwante genera word met hierdie wingerdsiekte geassosieer. In die tesis word 'n oorsig gegee van die geskiedenis van hierdie siekte, die verwantskap tussen Petri-terugsterwing en esca, en moontlike maniere van siektebestuur. Swamme wat by die siektekompleks betrokke is, kan in simptoomlose plantweefsel voorkom en daarom is die moontlikhede van die gebruik van molekulêre tegnieke vir swamidentifikasie in oënskou geneem.

In Suid-Afrika is *Pa. chlamydospora* die dominante swam wat met Petri-terugsterwing geassosieer word, gevolglik is verskillende fungisiedes vir die chemiese beheer van *Pa. chlamydospora* geëvalueer. Ses isolate van *Pa. chlamydospora*, versamel vanaf verskillende areas in die Wes-Kaap provinsie, is in dié studie gebruik. Benomyl, chlorothalonil, fenarimol, fosetyl-Al, iprodione, kresoxim-methyl, mancozeb, metalaxyl, prochloraz manganese chloride, quintozene, tebuconazole en thiram se effek op miselium inhibisie van *Pa. chlamydospora* is *in vitro* geëvalueer. Benomyl, fenarimol, kresoxim-methyl, prochloraz manganese chloride en tebuconazole was die mees effektiewe middels. Die effektiewe konsentrasie waarby 50% van die miselium groei geïnhibeer is (EK_{50}), was tussen 0.01 en 0.05 µg/ml vir die mees effektiewe groep middels. Benomyl, fenarimol, kresoxim-methyl, prochloraz manganese chloride en tebuconazole het *in vitro* goeie potensiaal getoon, en verder toetse moet *in vivo* uitgevoer word.

'n Molekulêre studie is van *Phaeoacremonium* en *Phaeomoniella* isolate; verkry uit Suid-Afrikaanse wingerde, of vanaf die "Centraalbureau voor Schimmelcultures" (CBS) van Nederland; gedoen. Deur van die PKR tegniek gebruik te maak, is die basispaaropeenvolgingsdata van 33 isolate, van die ITS1, 5.8S, ITS2 rDNA area en die gedeeltelike ß-tubulien geen verkry. Gekombineerde molekulêre data het die teorie ondersteun dat *Phaeomoniella* (Herpotrichiellaceae) gedistansieerd is van *Phaeoacremonium* (Magnaporthaceae). *Pm. aleophilum* en *Pm. angustius* was morfologies moeilik onderskeibaar, maar kon op grond van molekulêre data en kulturele eienskappe onderskei word. *Pm. aleophilum* se optimum groeitemperatuur was by 30°C en die swam besit die vermoë om by 35°C te groei.

Pm. angustius se optimum groeitemperatuur was by 25°C, maar het nie by 35°C gegroei nie. 'n Studie van molekulêre en kulturele eienskappe het getoon dat *Pm. angustius* en *Pm. viticola* sinoniem is. 'n Nuwe spesie, *Pm. mortoniae,* wat uit wingerde van Kalifornië geïsoleer is, is beskrywe. Verder is *Pm. aleophilum* die eerste keer in Suid-Afrikaanse wingerde aangetref en *Pm. inflatipes* isolate, wat vanuit wingerde geïsoleer is, is almal met molekulêre data gewys om *Pm. aleophilum* te wees. Hierdie bevindinge trek die rol van *Pm. inflatipes* in Petri-terugsterwing van wingerde in twyfel.

Phaeomoniella chlamydospora is in voortplantingsmateriaal en kwekerystokkies opgespoor. Omdat dit jare kan duur voordat siektesimptome ontwikkel, is dit belangrik om vroegtydig te weet of jong stokkies met *Pa. chlamydospora* geïnfekteer is. *Pa. chlamydospora* groei baie stadig en positiewe identifikasie van simptoomlose infeksies duur tot vier weke. Die toepassing van molekulêre tegnieke vir die vinnige identifikasie van *Pa. chlamydospora* in wingerde is dus ondersoek. Spesie-spesifieke oligonukleotiedes (PCL1 en PCL2) is vir *Pa. chlamydospora* ontwerp. Hierdie oligonukleotiedes is uiters sensitief en genomiese DNA van *Pa. chlamydospora* is van so laag as 16 pg geamplifiseer. *Phaeoacremonium* spp., verwante genera en algemene swamme vanuit wingerdmateriaal is met die oligonukleotiedes getoets, maar positiewe amplifikasie was slegs met *Pa. chlamydospora* moontlik. Die teenwoordigheid van *Pa. chlamydospora* is binne 24 uur in asimptomatiese wingerd weefselkultuurplantjies bevestig. Hierdie oligonukleotiedes identifiseer *Pa. chlamydospora* vinnig en akkuraat en toetsing op 'n groter skaal moet vervolgens met kwekerymateriaal onderneem word.

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CONTENTS

1. AN OVERVIEW OF PETRI GRAPEVINE DECLINE, AND THE POTENTIAL OF MOLECULAR TECHNIQUES FOR DISEASE DIAGNOSTICS

ABSTRACT

Petri grapevine decline causes devastating losses of young vines world-wide. This disease is also known as black goo, slow die-back or Phaeoacremonium grapevine decline. Petri grapevine decline is caused by *Phaeomoniella chlamydospora*, species of *Phaeoacremonium*, or related genera. External symptoms of diseased vines can be misleading, as slow die-back symptoms could be attributed to a variety of factors. However, internal symptoms like black vascular streaking is commonly associated with the disease. Although Petri grapevine decline occurs in propagation material and young vines, the latter may initially appear asymptotic. Molecular diagnostics, therefore, can play an important role in early detection and identification of these plant pathogens. This in turn can ensure clean propagation material. The PCR technique is therefore also discussed, underlining the important role that it can play in diagnostics of plant pathogens.

INTRODUCTION

In Italy Petri (1912) initially found vine decline problems to be correlated with phylloxera invasion after replanting with American rootstocks. Petri further observed wood alterations and trunk wounds to be a cause of vine decline. He also consistently isolated species of *Cephalosporium* and *Acremonium* from these diseased wood specimens.

Wood discoloration with brown or black streaks is a common internal symptom of esca-infected vines, and seems similar to the wood streaking of declining vines reported by Petri. Petri (1912) suggested that the brown wood streaking predisposed the vines to wood decay and could represent the first signs of esca. Viala (1926) considered *Stereum hirsutum* to be the primary pathogen of declining vines, and Bonnet (1926) suggested that esca and black measles in California is the same disease, caused by *Fomes ignarius* and *S. hirsutum*. Chiarappa (1959) established a firm correlation between wood decay and measles of grapevines. He isolated and

confirmed the pathogenicity of a *Cephalosporium* species, and also confirmed the wood rotting ability of *F. igniarius in vitro*, but failed to show the same for *S. hirsutum*.

The *Cephalosporium* sp. from declining vines was examined by Hawksworth *et al.* (1976), and reported to morphologically closely resemble *Phialophora parasitica*. It differed in having darker pigmented conidiophores, which were sharply delimited from the conidiogenous cells, as well as smaller, usually straight conidia. Hawksworth *et al.* (1976) speculated that this isolate represented an undescribed species, but in the absence of further material no conclusion was reached. Recently studies found this *Cephalosporium* sp. (CBS 239.79) to represent *Phaeomoniella chlamydospora* (Crous & Gams, 2000).

Larignon and Dubos (1988) suggested a sequence of pathogens in esca-affected grapevines, with the initial colonisation by a *Cephalosporium* sp. and *Eutypa lata*. Shortly afterwards a slow die-back of grapevines in the Western Cape province of South Africa was associated with *P. parasitica* (Ferreira *et al.*, 1994). These grapevines showed symptoms of unequal to stunted growth, eventually leading to die-back. In a molecular study conducted by Yan *et al.* (1995), *P. parasitica* was shown to be genetically variable, suggesting that it could represent more than one species. In the same year Morton (1995) discovered a disease of grapevines in California that she named black goo. The symptoms were, however, similar to those observed by Petri (1912). In a separate study, Crous *et al.* (1996) compared numerous phialophora-like isolates from human patients and various different woody hosts. A new genus, *Phaeoacremonium* (*Pm.*), was subsequently established for *P. parasitica* and other similar fungi, most of which were associated with slow die-back of woody hosts. The establishment of this genus, as well as the description of several species, implied that more than one taxon could be involved in the grapevine wilt disease. The *P. parasitica* isolate (STE-U 809) associated with slow die-back in South Africa, as well as the *Cephalosporium* sp. (CBS 239.79) isolated by Chiarappa (1959), were found to represent *Phaeoacremonium chlamydosporum*. In a recent study *Pm. chlamydosporum* was relocated to the genus *Phaeomoniella* (*Pa.*) and for the remainder of this review will therefore be referred to under its correct name, *Phaeomoniella chlamydospora*.

Young grapevine decline, or Phaeoacremonium grapevine decline, was observed in vines in California by Scheck *et al.* (1998). The disease was attributed to three species, namely *Pm. aleophilum*, *Pa. chlamydospora* and *Pm. inflatipes*. Recently Petri grapevine decline was proposed as the name to be used for the syndrome known under names like black goo, slow die-back and Phaeoacremonium grapevine decline (Mugnai *et al.*, 1999). It is now generally accepted that this disease is caused by *Pa. chlamydospora*, species of *Phaeoacremonium* or related genera (Graniti *et al.*, 2000). In most countries, however, *Pa. chlamydospora* appears to be the dominant organism causing Petri grapevine decline.

ESCA VERSUS PETRI GRAPEVINE DECLINE

Grapevine diseases such as esca, apoplexy and black measles with a broad range of symptoms have been reported from Europe (Viala, 1926; Chiarappa, 1959; Graniti, 1960, Baldacci *et al.*, 1962; Dubos & Larignon, 1988). Several fungi are repeatedly associated with esca, of which the most common are *Pm. aleophilum*, *Pa. chlamydospora*, *Eutypa lata*, *Acremonium berkeleyanum*, *Botryosphaeria obtusa*, *Phellinus punctatus*, and *Stereum hirsutum* (Mugnai *et al.*, 1996; Larignon & Dubos, 1997; Mugnai *et al.*, 1999). Among the fungi involved in esca, *Phellinus punctatus* and *S. hirsutum* are commonly considered as the cause of wood decay, whereas the *Phaeoacremonium* spp. and *Pa. chlamydospora* are seen as pioneer fungi in disease development (Larignon & Dubos, 1987).

Mugnai (1998) reported that it was becoming more common for young vines to display esca symptoms in Italy. These vines showed a limited amount of rotten tissue. In contrast, however, they showed black stripes, brown-red wood coloration and brown necrosis. Isolations were subsequently carried out on unplanted grafts of different rootstock combinations from nurseries in Italy and France. The presence of *Pa. chlamydospora* was confirmed in apparently healthy propagation material. Several cases of young vines (2-5-yr-old) were found exhibiting symptoms of stunted and weak growth, shorter internodes and leaves that appeared smaller, chlorotic and sometimes necrotic. The following year the vines showed stronger decline symptoms and eventually died (cited in Mugnai *et al.*, 1999).

From these observations, it was concluded that there could be two distinct syndromes. Firstly a classic esca with its typical range of internal and external symptoms, and secondly a disease caused by *Pa. chlamydospora* occurring in young grapevine plants (Mugnai, 1998).

Esca has long been considered as a complex disease. Larignon & Dubos (1987) suggested a model for esca, based on a succession of the fungi involved. Mugnai *et al.* (1999) suggested another model that portrayed esca as an association between two different diseases. The one causing symptoms of vascular discoloration (caused by *Phaeoacremonium* spp. and *Pa. chlamydospora*) and the other one causing wood decay (caused by *Fomitiporia punctata*). The two diseases could coexist in the same vine or could develop together over time. *Pa. chlamydospora* and *Pm. aleophilum* are seen as the pioneer fungi in esca (Mugnai, 1996). The question arises, therefore, whether Petri grapevine decline is a precursor of esca. Would vines infected with *Pa. chlamydospora* develop into esca diseased vines when suitable environmental circumstances and *F. punctata* were present?

Recently it was reported that five syndromes could be distinguished, depending on the origin of infections, the occurrence of the associated fungi and the environmental factors. (Mugnai *et al.*, 1999; Graniti *et al.*, 2000)

- The first syndrome is the brown wood streaking as reported by Petri (1912). Rooted cuttings, rootstocks, grafted and mother plants are affected and often no external symptoms can be observed. This is caused by *Pa. chlamydospora*, *Phaeoacremonium* spp. or related genera.

- The second syndrome, a decline of young grapevines, occurring when propagation material or young grapevines are infected, is again caused by *Pa. chlamydospora*, species of *Phaeoacremonium* or related genera. Petri grapevine decline is proposed for this syndrome that is locally known under names like black goo, slow die-back and Phaeoacremonium grapevine decline.

- Young esca, proposed as the name of the third syndrome, is what Petri thought would evolve in esca proper. It is characterized by black or brown wood-streaking and xylem gummosis in actively growing grapevines. External

symptoms can be present or absent. This is also caused by *Pa. chlamydospora*, *Phaeoacremonium* spp. or related genera.

- 'White rot', proposed as the name of the fourth syndrome, is caused solely or mainly by *Fomitiporia punctata,* or other wood-rotting basidiomycetes. Infection takes place through wounds. It is characterized by wood rot and can be accompanied by external symptoms of the leaves and fruits.

- This brings us to the final syndrome, Esca proper. White rot develops in the trunk of mature or old vines, after or at the same time as brown wood streaking. It is caused by the combined or successive action of *Pa. chlamydospora, Phaeoacremonium* spp. and *F. punctata.*

ESCA

History

Esca has been reported frequently in Italy (Baldacci *et al.*, 1962; Mugnai *et al.*, 1996), France (Viala, 1926; Dubos & Larignon, 1988) and California (Chiarappa, 1959). Recently it was also reported from Australia, although it is believed to have been present for many years (Pascoe *et al.*, 2000). Esca results in the withering of vines, probable due to wood degradation. This disease seems complex with several organisms playing a role in wood degradation.

In California Chiarappa (1959) reported that black measles indicated the same pathological condition of the vines described in Europe as esca or apoplexy. Chiarappa (1959) noted that esca and black measles differed slightly in symptoms, which could be due to cultural and variety differences. Esca occurs more frequently in warm, temperate zones. Larignon and Dubos (1997) reported that the disease is less important in France than in other Mediterranean countries, and that in France it could be controlled by applications of sodium arsenite during winter dormancy. However, the occurrence of esca increased in Europe in recent years, probable due to changes in cultural techniques and the recent abandonment of sodium arsenite treatment in some countries.

Symptoms

Symptoms of esca can be either mild and obscure, or severe (Chiarappa, 1959; Baldacci *et al.*, 1962; Dubos & Larignon, 1988; Larignon & Dubos, 1997). The severe form of esca is known as apoplexy, healthy-looking vines will wither and dry up completely within a few days. Chiarappa (1959) reported that infected vines in California frequently exhibited the severe form during the earlier part of the growing season (May-June). Such vines characteristically show a sudden apical die-back of one or more shoots, leaf dehiscence, and a shrivelling of berries.

Vines with mild esca are characterized by foliage deterioration. Leaf symptoms vary from discoloration, showing interveinal islands of chlorotic and yellowish tissue, to necrotic spots. Fruit symptoms include purple spots scattered over the epidermis of berries. Fruit and leaf symptoms can occur together or separately.

Classic esca includes typical external (as discussed above) and internal symptoms. Internal wood symptoms can be classified into three groups (Fig. 1), namely a typical white decay in the centre of the vine, shades of brown necrosis and coloration varying from brown-reddish to pinkish wood, and vascular tissue showing black streaks (Mugnai *et al.*, 1996).

Casual organisms

As stated before, fungi repeatedly associated with esca are *Pm. aleophilum*, *Pa. chlamydospora*, *E. lata*, *A. berkeleyanum*, *B. obtusa*, *P. punctatus*, and *S. hirsutum* (Mugnai *et al.*, 1996; Larignon & Dubos, 1997; Mugnai *et al.*, 1999). Results from earlier studies found *Phellinus igniarius* and a *Cephalosporium* sp. to be the primary pathogens (Chiarappa, 1959). The names of these organisms have respectively changed to *Phellinus punctatus* and *Pa. chlamydospora* (Crous & Gams, 2000). Fischer (1996) found the correct name for the species *P. punctatus* was *Fomitiporia punctata*. Results from further studies of a group of isolates from Italy, suggested that previous records of *F. igniarius* were misidentifications (Fischer & Mugnai, unpublished data). *F. punctata* is the basidiomycete commonly associated with decayed wood of esca-affected vines in France and Italy. Therefore, for the

remainder of this review, this pathogen will be referred to under its correct name, *F. punctata*.

Two basidiomycetes were found associated with the central wood decay, namely *F. punctata* and *S. hirsutum*. In Italy the majority of white rot samples were colonised by *F. punctata* (Mugnai *et al.*, 1997). In France *F. punctata* was also the dominant basidiomycete isolated, with *S. hirsutum* isolated at very low frequencies (Larignon & Dubos, 1997). *F. punctata* and *S. hirsutum* can cause the typical white decay in grape wood (Chiarappa, 1997), but their activity is usually accompanied by pioneer fungi, of which *Pa. chlamydospora* and *Pm. aleophilum* seem to be the most important (Mugnai *et al.*, 1996; Larignon & Dubos, 1997). *Pm. aleophilum* and *Pa. chlamydospora* were isolated from the discoloured wood and the zones bordering the central decayed wood. *E. lata* was the fungus chiefly isolated from the sectorial brown necrosis and the zones adjoining the decayed wood. *Pa. chlamydospora* was isolated from the initial stages of the disease, where it occurred in the black stripes and brown-red wood (Mugnai *et al.*, 1996; Larignon & Dubos, 1997)(Table 1; Mugnai, 1998).

Mugnai *et al.* (1997) tested the effect of some phenolic compounds on *F. punctata, S. hirsutum, A. berkeleyanum, Pm. aleophilium, Pa. chlamydospora*, and *E. lata*. Results from this study showed that the growth rates of *Pa. chlamydospora, Pm. aleophilum, A. berkeleyanum* and *E. lata* were less inhibited than *F. punctata* and *S. hirsutum* by the presence of phenols in artificial media. When medium containing gallic acid was pre-inoculated with a pioneer fungus, *F. punctata* grew better than in the non pre-inoculated control. From these results (Mugnai *et al.*, 1997) it was suggested that colonisation of grape wood by pioneer fungi could help the progression of *F. punctata* in the host. However, Sparapano *et al.* (2000a) reported that *F. punctata* is a primary pathogen, causing wood deterioration and spongy decay on both mature and young grapevines without the prior succession of *Phaeoacremonium* spp. or other wood-decaying fungi. Sparapano *et al.* (2000b) also showed competition and antagonism *in vitro* and *in vivo* for *Pm. chlamydospora*, *Pa. aleophilum* and *F. punctata*. These *in vitro* studies suggest that the growth of *F. punctata* is significantly limited by *Pa. aleophilum*, but not by *Pm. chlamydospora*. *Pm. chlamydospora* and *Pa. aleophilum* grew agonistically due to competition for primary resources, however not challenging each other directly. In woody tissue of grapevine the effect of *F.*

punctata was also significantly limited by *Pm. aleophilum*, but not *Pa. chlamydospora*.

Control

Management of esca relies mainly on cultural practises to prevent and reduce infection. Although these practises are recommended, they are insufficient to ensure effective disease control. Esca is still controlled by chemicals like sodium arsenite in France, Portugal and Spain where its use has not been banned. The trend to use more environmental friendly products, however, will result in the banning of this product in the near future. Di Marco (Mugnai *et al.*, 1999) states that chemicals like triazoles and several others were tested and found to be ineffective in controlling esca. However, a reduction in esca symptoms were observed in the field for vines treated with fosetyl-Al via trunk injections. These vineyards had a moderate incidence of the disease, and were not seriously affected by white rot (Di Marco *et al.*, 2000). Further investigation of antifungal activity of phosphorous acid (to which fosetyl-Al is degraded), resveratrol and pterostilbene are needed. Two-yr-old grafted vines were treated with fosetyl-Al and after two months inoculated with *Pm. aleophilum* and *Pa. chlamydospora*. Results of this experiment showed treated plants inoculated with *Pm. aleophilum* were statistically different compared to the untreated, inoculated control. No significant difference was observed for *Pa. chlamydospora*. Bisiach (Mugnai *et al.*, 1999) attempted biological control by applying a solution of three strains of *Trichoderma viride* onto fresh pruning wounds. However, no difference was found between treated and untreated vines.

Traditional cultural methods remain essential to prevent and treat esca, although these methods do not ensure complete control. These methods include the following:

- propagation material from nurseries or mother plants should have no wood darkening;
- vines showing foliar symptoms and white rot of the trunk, can be treated surgically by excising the rotten tissue and protecting the resulting wound;
- large pruning wounds should be avoided and dressed with a broad-spectrum fungicide;

- healthy-looking vines should be pruned before diseased vines;
- clean pruning tools should be used;
- pruning residues should be removed from vineyards or burned; and
- diseased and dead vines should be removed (Mugnai *et al.*, 1999).

Recently a new practice has been applied successfully in central Italy. A basal shoot for cutting is selected the first year chronic esca symptoms appear. The shoot is raised the following spring, which will replace the canopy in 2 to 3 years time. A traditional method used in ancient times is also still used in some Mediterranean regions. The diseased trunk is cut and kept open by inserting a stone. The rotten wood is left open, and this practice will delay the onset of foliar symptoms of esca for a certain period of time.

PETRI GRAPEVINE DECLINE

From 'Black goo' to 'Petri grapevine decline'

In 1995 Morton reported on a mysterious "new" disease that was observed in young vines in California. One symptom of the diseased vines was shiny black vascular tissue, which was associated with poor vine growth, and even the sudden collapse of the plant. The name black goo originates from the black gum produced by the plants in reaction to the disease (Fig. 2) (Morton, 1995). The wood coloration is due to a black compound that blocks the xylem. Electron microscopy showed that the blockage was due to tylosis, which in turn triggered the idea that it was due to a resistance reaction of the plant (Ferreira *et al.*, 1994).

Adalat *et al.* (2000) inoculated cuttings with *Pa. chlamydospora*, *Pm. aleophilum* and *Pm. inflatipes* and found that these fungi affected the ability of cuttings to callus. These three species also affected the plant height, number of internodes, total number of roots and leaf dry weight. They also found *Pa. chlamydospora* to be a more aggressive coloniser of grapevine pruning wounds than *Pm. aleophilum* and *Pm. inflatipes*. The fungus most commonly isolated from diseased vines in Italy is *Pa. chlamydospora* (Mugnai *et al.*, 1996), which also appears to be dominant in South Africa. Re-inoculation of this fungus into vines

resulted in dark venation and an accumulation of black gum in the vessels (Ferreira *et al.*, 1994; Mugnai *et al.*, 1997).

Petri grapevine decline emerged as a significant problem in vineyard establishment of South Africa. Although it remains unclear how widespread the problem is, the disease has been reported from young grapevines in Italy (Sidoti *et al.*, 2000), California (Scheck *et al.*, 1998), Australia (Pascoe, 1998) and South Africa (Ferreira, 1998).

Infection pathway

An appropriate approach towards disease management can be implemented if the infection pathway is known. Larignon and Dubos (2000) trapped spores of *Phaeoacremonium* spp. with spore catchers in vineyards. These data suggest that spores can be air-borne. Furthermore, it was also found that *Pa. chlamydospora* could penetrate vine canes through pruning wounds following rainfall. Bertelli *et al.* (1998) speculated that infection occurs in nurseries, or in the field through the root system.

The source of inoculum of *Pa. chlamydospora* is still unknown. Chlamydospores can persist in soil, and conidia can be produced in abundance during the saprobic phase on dead vine wood and other plant material (Mugnai *et al.*, 1999). This inoculum can infect pruning wounds or injured roots. Adalat *et al.* (2000) suggested that the source of infection in California could be through pruning wounds. *Pa. chlamydospora* has also recently been observed to produce a phoma-like synanamorph (Crous *et al.*, 2000; Pascoe *et al.*, 2000) *in vitro* on vine cuttings. Adalat *et al.* (2000) suggested the synanamorph could play an important role with pruning wound infections. Larignon *et al.* (2000) studied the microflora on pruning wounds, and suggested that *Pa. chlamydospora* use these as infection sites.

Phaeomoniella chlamydospora also occurs as a latent pathogen or endophyte in propagation material. Bertelli *et al.* (1998) isolated *Pa. chlamydospora* at high frequencies (16 to 55%) from rooted vine cuttings produced in Italy and France. Bertelli *et al.* (1998) further reported that *Pa. chlamydospora* was frequently isolated from the grafting area. They suggested that the pathogen came from mother vines already harbouring latent infections, or entered cuttings through cuts and wounds during grafting and callusing. *Pa. chlamydospora* has been reported to occur

endophytically in South African nursery material (S. Ferreira, pers. comm.). In Australia, Pascoe *et al.* (2000) reported that most rootstock mother vines of the Australian Vine Improvement Association was infected with *Pa. chlamydospora,* and nursery samples also tested positive. No apparent difference in varietal susceptibility was suspected, seeing that all common rootstocks tested positive for *Pa. chlamydospora* (Pascoe *et al.*, 2000). In South Africa this pathogen is isolated from diseased vines, from the roots upwards into the rootstock and sometimes in the graft, suggesting that it could be soil-borne. Pascoe *et al.* (2000) reported that the distribution of the infection is not continuous through the length of the affected vessels, but located at a particular point. The black goo symptoms can therefore appear at points remote from the actual infection. This could explain the occasional failure to isolate the fungus from typical black goo symptoms.

Petri grapevine decline are associated with young vines up to now. This suggests that the disease could originate in nurseries. Disease control measures in nurseries should thus ensure that clean material is provided to farmers. To achieve this goal, the possibility of a certification scheme should be considered.

Symptoms of Petri grapevine decline

Identification of Petri grapevine decline in vineyards is not an easy task. The diseased vine will appear stunted, and leaf symptoms will resemble those induced by water stress. In California, a "firing" of leaves was observed, which suggested that the plant had an insufficient water supply to its leaves. Whether the "firing of leaves" is of importance to the disease remains uncertain (W. D. Gubler, pers. comm.). Due to the various factors that can induce these symptoms, diagnosis of Petri grapevine decline should never be based solely on visual symptoms. Stunting or wilting can be due to a variety of reasons such as poor nutrition, poor soil fertility, lack of adequate irrigation, poor planting techniques, viruses, nematodes and poor quality planting stock.

When diseased vines are cut vertically along their length, black vascular streaking can be observed. The internal tissue appears silvery, which could be due to the drying out of epidermal tissue. Coloration and gumming are found in the lower parts of the rootstock. Vascular streaking, however, can also be due to a variety of other factors such as host response to injury, a disbudding scar, insect damage or other

fungal infections. However the black gummy streaks are quite typical when *Pa. chlamydospora* are present.

Control

Ferreira (1998) reported that systemic fungicides such as metalaxyl (Ridomil), fosetyl-Al (Aliette) and phosphoric acid (Phytex) depressed *Pa. chlamydospora* growth in laboratory and glasshouse tests. Ferreira (1998) suggested relieving stress conditions in vineyards, together with the use of Ridomil granules (40g/m^2), Phytex (400mL/10L) or Aliette (400g/L) for control of the disease in vineyards. However, to date Phytex has not yet been registered on vineyards in South Africa. The addition of leaf nutrients together with the application of chemicals is suggested for better results. Metalaxyl binds to soil particles, which makes uptake by the roots inefficient and thus unpractical for control (S. Ferreira, pers. comm.). The latter fungicides have yet to be tested *in vivo*. Di Marco *et al.* (2000) reported that phosphorous acid in combination with *Vitis* stilbenes (resveratrol and pterostilbene) showed control against *Phaeoacremonium* spp. (including *Pa. chlamydospora*) *in vitro*.

The use of a hot water treatment (50°C for 30 min) for the control of *Pa. chlamydospora* in propagation material was proposed by Ferreira (1998). Previously hot water treatments were used for controlling external pathogens and parasites such as nematodes and phylloxera. Longer hot water treatments (30 min) were recommended to nurseries with dormant vine cuttings to reduce the titre of Australian Grapevine Yellows and Crown Gall. However, reports of a high mortality rate using this technique, as well as the rapid expansion of the wine industry in Australia, led to reluctance in its implementation (Waite & May, 1999). Furthermore, it was reported that hot water treatment of 31°C for 30 min, was unsuccessful in reducing spore suspensions of *Pa. chlamydospora* and *Pm. inflatipes* in test tube experiments (W.D. Gubler, pers. comm.).

TAXONOMIC STATUS OF *PHAEOACREMONIUM* SPECIES AND *PHAEOMONIELLA CHLAMYDOSPORA*

Yan *et al.* (1995) characterized *Phialophora americana, Phialophora parasitica, Phialophora richardsiae* and *Phialophora verrucosa* with restriction enzyme mapping of the ribosomal DNA region containing 5.8S and the intergenic spacers. The 34

isolates of the *P. americana/P. verrucosa* complex were separated into eight phenotypic groups. Nine isolates of *P. parasitica* were divided into four groups and the six isolates of *P. richardsiae* remained within a single group. Sequence analysis of the same region was completed for 24 isolates, which represented 12 of the 13 restriction map groups. The gene regions were highly conserved, but the internal transcribed spacers (ITS1 and ITS2) showed variability among the species. *P. parasitica* was found to be genetically variable and *P. richardsiae* genetically homogeneous. This correlated with their morphological observations. The sequence data showed several regions within ITS1 and ITS2 suitable for species-specific PCR amplification.

In 1996 a new hyphomycete genus, *Phaeoacremonium*, was proposed for six new species, namely *Pm. aleophilum*, *Pm. angustius*, *Pm. chlamydosporum*, *Pm. inflatipes*, *Pm. rubrigenum* and *Pm. parasiticum* (Crous *et al.*, 1996). *Phaeoacremonium* is morphologically intermediate between *Acremonium* and *Phialophora*. It can be distinguished from *Acremonium* by its pigmented vegetative hyphae and conidiophores, and from *Phialophora* by its aculeate conidiogenous cells and inconspicuous collarettes.

Dupont *et al.* (1998) performed a morphological and molecular analysis of 25 *Phaeoacremonium* isolates that were obtained from diseased European and Californian grapevines. Dupont *et al.* (1998) found *Pm. chlamydosporum* to be more closely related to *P. verrucosa* than to the type species of *Phaeoacremonium*, *Pm. parasiticum*. They suggested that the taxonomic position of *Pm. chlamydosporum* should be reconsidered, as it appeared to be more closely related to *Phialophora verrucosa*, and thus also belonged to the *Herpotrichiellaceae*, rather than other species of *Phaeoacremonium*, which belong to the *Magnaporthaceae*. *Pm. angustius* (ex-type strain CBS 249.95) was also proposed as a synonym of *Pm. aleophilum* (ex-type strain CBS 246.91). Recently Dupont *et al.* (2000) concluded from sequence data (ITS and β-tubulin) of the ex-type strains, as well as RFLP patterns of additional strains, that these two species were conspecific. The ITS1 rDNA region showed a difference of only two nucleotides between these species, and the 5' end of the β-tubulin gene no differences. Growth patterns of these isolates were found to be similar, though the incubation period was significantly longer than that proposed in

previous studies (Crous *et al.* 1996; Crous & Gams 2000). Optimal growth temperatures for *Pm. aleophilum* and *Pm. angustius* were recorded as 30°C, and growth occurred at 35°C. A new species, *Pm. viticola,* was recently described based on molecular data and cultural characteristics (Dupont *et al.*, 2000). This species is closely related to *Pm. aleophilum*. *Pm. viticola* also produces a yellow pigment in culture, and has an optimum growth at 25°C.

Crous and Gams (2000) proposed *Phaeomoniella,* typified by *Pa. chlamydospora,* as a new hyphomycete genus to accommodate the causal organism of Petri grapevine decline. Morphologically the genus is similar to *Phaeoacremonium*. It is distinguished from the latter based on its cultural characteristics, conidiophore morphology, and its uniformly straight, and slightly pigmented conidia (Crous & Gams, 2000). In culture, fresh isolates of *Pa. chlamydospora* have a white, yeast-like growth, which later forms dark green colonies (Fig. 2), once again being distinct from species of *Phaeoacremonium*. Although yeast-like growth phases and pleomorphism have been noted for the *Phialophora*-complex (Wang, 1979), this has never been observed for species of *Phaeoacremonium sensu stricto*. Tegli *et al.* (2000) studied the genetic variation of isolates of *Pa. chlamydospora* and *Pm. aleophilum* by analysis of amplification profiles obtained in RAPD-and RAMS-PCR experiments. These data confirmed the absolute divergence between species in this complex.

PCR AS A DIAGNOSTIC TOOL

History of PCR

Polymerase chain reaction is described as a method where nucleic acid sequences can exponentially be amplified *in vitro*. It is necessary to have sufficient information of the ends of the sequence to synthesise oligonucleotides that will anneal to the target DNA. To be synthesised enzymatically, the target sequence does not have to be in a pure form. It could be a minor fraction of a complex mixture (Mullis & Faloona, 1987). This makes PCR a very powerful tool. DNA can be amplified from a single human hair, a drop of dried blood at a crime scene or from ancient mummy tissue.

Saiki and co-workers (1988) reported the use of a thermostable DNA polymerase in the polymerase chain reaction. This enzyme was isolated from

Thermus aquaticus (*Taq*) and is known to be an important improvement to the PCR technique. Amplification reactions could be performed at higher temperatures and the specificity, yield, sensitivity and length of products were improved. The use of higher temperatures for primer annealing and extension increased the specificity of the amplification reaction. From 10^{-6} µg starting DNA, a PCR product of between 0.5 µg to 1 µg of the target DNA, with a length up to 2 kb, could be obtained from 30-35 cycles of amplification (Sambrook *et al.*, 1989) showing the increase in sensitivity. Presently DNA polymerase of *Thermicus aquaticus* is produced by genetically engineered bacteria.

PCR as a technique

PCR exponentially amplifies specific DNA sequences by *in vitro* DNA synthesis. This technique consists of three important steps: melting of target DNA (denaturation), annealing of two oligonucleotide primers to denatured DNA strands and primer extension (elongation) by the thermostable DNA polymerase. This three-step procedure is repeated in cycles (Fig. 3). Each successive cycle doubles the amount of DNA synthesised in the previous cycle. The DNA fragment is exponentially accumulated where the specific target fragment is 2^n and n is the number of cycles.

Taq polymerase

Taq polymerase was isolated from *Thermus aquaticus*. *Thermus aquaticus* is a thermophillic, eukaryotic microorganism able to grow at temperatures of 70-75°C. The bacterium was isolated from Hot Springs in Yellowstone National Park, USA (Brock & Freeze, 1969). *Taq* DNA polymerase has a relative high optimum temperature for DNA synthesis; its optimum elongation temperature is between 70-75°C.

A great disadvantage of *Taq* is that it lacks editing functions and incorporates incorrect nucleotides at a rate of 20×10^{-4} nucleotides per cycle during amplification. The error frequency seems to increase in the presence of higher concentrations of dNTPs and Mg^{++}. This misincorporation rate was reduced to less than 10^{-5} nucleotides per cycle by changing PCR conditions like lowering the concentration of dNTP's and $MgCl_2$, higher annealing temperatures and shorter extension times (Eckert

& Kunkel, 1990). For sequencing, the population of amplification products is analysed and misincorporated nucleotides are therefore not detected. Under normal reaction conditions *Taq* polymerase becomes limiting after 25-30 cycles (Sambrook *et al.*, 1989).

Oligonucleotides (Primers)

Sommer and Tautz (1989) suggested two minimal requirements for PCR primers: primer length should be between 20-24 nucleotides, and the last three 3' nucleotides should match completely. Usually oligonucleotides are used at a concentration of 1 μM during amplification and it will last for at least 30 cycles (Sambrook *et al.*, 1989). At too low concentrations of primers, the polymerase chain reaction is extremely inefficient. Specific primers can be derived from sequences of amplified or cloned DNA or RNA from the target microorganism. Primer specificity for the target sequences is influenced by factors like primer length, annealing temperatures, magnesium concentration and secondary structures of the target DNA or primers (Riedel *et al.*, 1992; Shen & Hohn, 1992).

To design species-specific PCR primers enough sequence information should be known. If the conserved templates are used for primer design, highly variable sequences that lie between the conserved templates can be amplified. This feature is useful for comparing sequences for constructing phylogenetic relationships. Following is a general recommendation list on primer design provided by the sequencing service centre at Iowa State University, U.S.A.:

- Long runs of a single base should be avoided in primers. Especially avoid three or more G's or C's in a row;
- Primers should have melting temperatures of above 50°C. Primers with higher melting temperatures produce better results than primers with lower melting temperatures;
- The G/C content of primers should be between 40 and 60 percent. Preferably above 50%;
- The primers should be 'stickier' at the 5' ends than the 3' ends. An 'sticky' end is indicated by a high G/C content (three bindings comparing with the two between 'A' and 'G'). If the 3' end is 'sticky' it could potentially anneal at

multiple sites on the template DNA. But a 'G' or a 'C' is desirable at the 3' end, as long as the above rule applies;

- Primers should not contain palindromes within themselves, meaning that they should not form hairpins;
- The chosen pair of primers should not contain sequences that will allow them to anneal to themselves or to the other primer (primer dimer formation);
- A computer search (e.g. blast) should be run to see if the primer is unique and especially the 8-10 bases of the 3' end; and
- Do not request inosine in sequencing primers. They do not work or give poor cycle sequencing results, with exceptions.

Inhibition and problems of PCR

Polymerase chain reaction can generate millions of DNA copies from a template. Contamination of the amplification reaction with other products can therefore create problems in research and in diagnostic applications. Multiple negative controls (no template DNA added to reaction) can give an indication if contamination occurs and great care should be taken while the reaction is prepared. A false positive can result from contamination by aerosols, hair, skin and gloves. Contaminated reagents of PCR reactions can be sterilised by applying short wavelength ultraviolet irradiation to the reaction mix prior to amplification (Sakar & Sommer, 1990). The DNA polymerase and target DNA should be added after the irradiation-inactivation step.

An important aspect of PCR is that primers bind specific to produce the desired DNA band. Higher annealing temperatures and lower $MgCl_2$ concentration enhance specific amplifications. Non-target amplifications can be minimised if DNA polymerase is activated only after the reaction has reached a temperature of above 70°C. The essential reagents (DNA polymerase, magnesium chloride, primers) should manually be added to the reaction tube at elevated temperatures. This approach is called "hot start" and was also found to minimise the formation of "primer-dimer" (Erlich *et al.*, 1991). Hung *et al.* (1990) found that tetramethylammonium chloride (TMAC) can dramatically reduce non-specific binding and therefore enhance the specificity of the reaction. One way to enhance the success rate and specific amplification of environmental DNA is to dilute the sample

(1:10 to 1:200) after the first few cycles (Mullis & Faloona, 1987) and then perform an additional round of PCR. This process may effectively dilute potential inhibitors to allow successful amplification. The diluted DNA will contain high ratios of target sequences compared to the total, non-target, background DNA (Steffan & Atlas, 1991).

Target sequences are usually purified or treated to remove DNA polymerase inhibitors. Polysaccharides (Demeke & Adams, 1992), phenolic compounds or humic substances can inhibit the PCR reaction (Henson & French, 1993). The inhibitory effect of various plant polysaccharides on PCR was tested (Demeke & Adams, 1992). Neutral polysaccharides were found not to inhibit PCR amplification of spinach DNA, but two acidic polysaccharides, dextran sulfate and gum ghatti inhibited PCR amplification. Demeke *et al.* (1992) found that Tween 20, Dimethyl sulphoxide (DMSO) and PEG can be used to help amplifications of DNA from plant species.

Some sort of purification is necessary to achieve maximum sensitivity in detecting the target sequence in environmental samples. However, if the sample contains many copies of the target sequence, boiling the sample for a few minutes is found to be adequate (Henson & French, 1993). For example wheat roots or crowns infected with *Gaeumannomyces graminis* were boiled and enough target DNA was released to produce a visible amplified product on an agarose gel after PCR. O'Brien (1998) investigated the conditions for extractions of amplifiable DNA from soil. They found the inclusion of Polyvinylpyrrolidone (PVP) or Polyvinlylpolypyrrolidone (PVPP) in extraction buffer significantly reduced the degree of inhibition with PCR.

Tetramethylammonium chloride (TMAC)(Hung *et al.*, 1990), DMSO (Bookstein & Lai, 1990), non-ionic detergents (Bachmann *et al.*, 1990) and the alteration of magnesium concentrations (Krawetz *et al.*, 1989) have been reported to influence the efficiency and specificity of the PCR reaction. Bachman *et al.* (1990) found that NP-40 and Tween 20 presumably prevented secondary structure formation, and Riedel *et al.* (1992) found that TMAC enhanced the specificity of the amplification at different priming. However, they also found a slight inhibition of amplification in the presence of TMAC.

The base composition of the template can affect the PCR amplification. For example, secondary structures in template DNA can hinder the extension of the

primers by polymerase. Shen and Hohn (1992) reported that DMSO in the reaction buffer allowed amplification of DNA with complex secondary structure. They also demonstrated that DMSO could be a decisive factor for *Taq*-mediated PCR amplifications of DNA fragments with complex secondary structures. Bookstein *et al.* (1990) found that 10% DMSO was necessary for the generation of a PCR band of the retinoblasto gene. This genes 5' region is very CG-rich and the addition of 10% DMSO probably decreased inter-or intra-strand base pairing (secondary structures). In contrast, Riedel *et al.* (1992) found that the inclusion of DMSO decreased the specificity of the amplification. At an Mg^{2+} concentration of 6.5 mM, the addition of DMSO lowered the specificity of the amplification. High concentrations of DMSO (10%) were also found to inhibit *Taq* DNA polymerase (Gelfand & White, 1990). Therefore advantages and disadvantages of DMSO should be measured to determine when it would be useful for PCR.

Application of PCR

Early and accurate diagnosis of plant diseases is a crucial component of integrated crop management, and control measures should be introduced at an early stage of disease development. Current taxonomic identification of filamentous fungi is based on micro and macro morphological characteristics; cultural morphology including colony and colour characteristics, as well as the size, shape, and development of sexual and asexual spores and spore-forming structures. Recent developments in molecular biology and biotechnology provide rapid, specific, and sensitive tools for the detection of plant pathogens (Miller & Martin, 1988). Molecular techniques can be used to detect fungal DNA from a pool of environmental DNA that was isolated from the natural substrate or matrix. Chen *et al.* (1993) compared monoclonal antibodies, DNA probes and PCR for detection of the Grapevine Yellow Disease agent. PCR was found to be the most sensitive, detecting grapevine yellow mycoplasma-like organisms when only 10^{-2} pg of DNA from the same source was used as template.

Fungal DNA can be detected from a pool of environmental DNA from natural substrates by using specific DNA probes or by using specific primers (Liew *et al.*, 1998a). The use of specific DNA probes is less sensitive than specific primers, due to the sensitivity of PCR amplification. However, DNA probes have been used to detect

DNA molecules that are species-specific in soil and host tissue (Rollo *et al.*, 1987; Goodwin *et al.*, 1989; Goodwin *et al.*, 1990). Detection with specific primers involves the use of primers from a specific region of the genome, containing phylogenetic information.

The first diagnostic application of PCR was to enhance the sensitivity of the prenatal diagnosis of sickle cell anemia (Saiki *et al.*, 1985). This genetic disease was caused by a nucleotide substitution in the ß-globin gene. Nevertheless, most genetic diseases result from a variety of mutations, making practical diagnostic applications of these approaches difficult. Usually this diagnostic test took several weeks using Southern blotting and hybridisation, but with PCR it took less than one day. PCR has several advantages above the more traditional methods of diagnosis. The target organism does not need to be cultured and the PCR technique is very sensitive. Theoretically it is supposed to detect a single target molecule in a complex mixture. Both narrow and broad selections can be made, depending on the choice of primers.

In practice only short DNA sequences of 50-2000 base pairs from the entire fungal genome are used in detection and identification of fungal taxa (Liew *et al.*, 1998a). Glass *et al.* (1995) constructed nine sets of oligonucleotide primers that amplified segments of DNA that span over one or more introns in conserved genes. These introns contain valuable information, which could allow differentiation between genera and even species. From these degenerated primers, organisms can be scanned for a species-specific area. By using data obtain from PCR products of these specific areas species-specific primers could be designed. Five of these primer sets amplified a product only from DNA of the filamentous ascomycetes and deuteromycetes. No plant DNA was amplified. These five primer sets were constructed form the *Neurospora crassa* genes for histone 3, histone 4, ß-tubulin and the plasma membrane ATPase (Glass & Donaldson, 1995).

Sequences used for phylogenetic information must have the same function in all taxa, evolving at a consistent rate, and be present in the genome as a single copy or several copies that evolved together (Bruns *et al.*, 1991). Regions that fulfil the above criteria are the ribosomal RNA genes (rDNA) from nuclear and mitochondrial genomes, the cytochrome oxidase genes, the actin genes and certain ribosomal protein elongation factors (Bruns *et al.*, 1991). The ribosomal RNA genes have proven to be

a very useful region of DNA. This region's characteristics and usefulness have recently been reviewed (Bruns *et al.*, 1991; Hillis & Dixon, 1991).

The ribosomal genes and intergenic spacers provide attractive targets for molecular detection and phylogenetic studies. They occur in high numbers, consisting of conserved and variable sequences and can be amplified and sequenced with universal primers based on the conserved sequences (Bruns *et al.*, 1991; Stackebrandt *et al.*, 1992; Henson & French, 1993). The 5.8S, 18S and 28S of fungal nuclear ribosomal RNA genes are situated head-to-tail in tandem, with 60-200 copies per haploid genome (Bruns *et al.*, 1991). Multi-copy target sequences allow greater sensitivity than single or low-copy sequences. Greater sequence variation is found in the internal transcribed spacer (ITS) regions than the conserved areas (Henson & French, 1993). ITS1 and ITS2 are the flanking areas of the 5.8S gene (Fig. 4). Adequate sequence differences in the ITS regions of the wilt fungi *Verticillium dahliae* and *V. albo-atrum* were found, and primers could be designed to specifically amplifying the DNA of each species (Nazar *et al.*, 1991). The rDNA region has also been used for designing species-specific primers for diagnostic purposes, detecting fungal pathogens in plant material (Brown *et al.*, 1993; Screenivasaprasad *et al.*, 1996; Johanson *et al.*, 1998).

The absence of readily diagnosed symptoms is common in many soil-borne diseases. Current routine methods for the detection and identification of *Phytophthora* species involve baiting from soil or isolation from infected plant tissue followed by pure-culturing of the organism and identification, based on morphological characteristics. This procedure is time-consuming and involves handling of large samples. rDNA and other areas in the fungal genome possess characteristics suitable for the detection of pathogens at the species level. *Pa. chlamydospora* is a slow-growing pathogen and young grapevines can be infected before symptoms of slow die-back appear (Bertelli *et al.*, 1998). Isolation of *Pa. chlamydospora* from symptomless material is difficult and time-consuming. Here a rapid and accurate method of diagnosis is of importance. PCR is a powerful tool that can effectively detect fungal plant pathogens in soil and plant tissue by the use of specific primers in the rDNA region (Nazar *et al.*, 1991; Brown *et al.*, 1993; Tooley *et al.*, 1997; Liew *et al.*, 1998b). Because of PCR sensitivity and the readily available results, it plays an important role in diagnostics. PCR is a tool that will become more

essential in plant pathology as the need for a quick and easy detection method for various plant pathogens becomes a priority in agriculture.

REFERENCES

Adalat, K., Whiting, C., Rooney, S. & Gubler, W.D. 2000. Pathogenicity of three species of *Phaeoacremonium* spp. on grapevine in California. *Phytopathologia Mediterranea* **39**, 92-99.

Bachmann, B., Luke, W. & Hunsmann, G. 1990. Improvement of PCR amplified DNA sequencing with the aid of detergents. *Nucleic Acids Research* **18**, 1309.

Baldacci, E., Belli, G. & Fogliani, G. 1962. Osservazioni sulla sintomatologia e sull'epidemiologia della carie del legno di vite (Mal dell'esca) di *Phellinus* (*Fomes*) *igniarius* (L. ex Fr.) Patouillard. *Rivista di Patologia Vegetale*, Serie III, **2**, 165-184.

Bertelli, E., Mugnai, L. & Surico, G. 1998. Presence of *Phaeoacremonium chlamydosporum* in apparently healthy rooted grapevine cuttings. *Phytopathologia Mediterranea* **37**, 79-82.

Bookstein, R. & Lai, C. 1990. PCR-based detection of a polymorphic *BamHI* site in intron 1 of the human retinoblastoma (RB) gene. *Nucleic Acids Research* **18**, 1666.

Brock, T.D. & Freeze, H. 1969. *Thermus aquaticus* gen. n. and sp. n., a non-sporulating extreme thermophile. *Journal of Bacteriology* **98**, 289-297.

Brown, A.E., Muthumeenakshi, S., Sreenivasaprasad, S., Mills, P.R. & Swinburne, T.R. 1993. A PCR primer-specific to *Cylindrocarpon heteronema* for detection of the pathogen in apple wood. *FEMS Microbiology Letters* **108**, 117-120.

Bruns, T.D., White, T.J. & Taylor, J.W. 1991. Fungal molecular systematics. *Annual Review of Ecology and Systematics* **22**, 525-564.

Chen, K.H., Guo, J.R., Wu, X.Y., Loi, N., Carraro, L., Guo, Y.H., Chen, Y.D., Osler, R., Pearson, R. & Chen, T.A. 1993. Comparison of monoclonal antibodies, DNA probes, and PCR for detection of the grapevine yellows disease agent. *Phytopathology* **83**, 915-922.

Chiarappa, L. 1959. Wood decay of the grapevine and its relationship with black measles disease. *Phytopathology* **49**, 510-519.

Chiarappa, L. 1997. Phellinus igniarius: the cause of spongy decay of black measles ("esca") disease of grapevines. Phytopathologia Mediterranea **36**, 109-111.

Crous, P.W., Gams, W., Wingfield, M.J. & Van Wyk, P.S. 1996. *Phaeoacremonium* gen. nov. associated with wilt and decline disease of woody hosts and human infections. *Mycologia* **88**, 786-796.

Crous, P.W. & Gams, W. 2000. *Phaeomoniella chlamydospora* gen. et. comb. nov., the causal orgainsm of Petri grapevine decline. *Phytopathologia Mediterranea* **39**, 112-118.

Demeke, T. & Adams, R.P. 1992. The effects of plant polysaccharides and buffer additives on PCR. *Biotechniques* **12**, 332-334.

Di Marco, S., Mazzullo, A, Calzarano, F. & Cesari, A. 2000. The control of esca: status and perspectives. *Phytopathologia Mediterranea* **39**, 232-240.

Dubos B. & Larignon, P. 1988. Esca and black measles. In *Compendium of Grape Diseases* (ed. R.C. Pearson & A.C. Goheen), pp 34-35. APS Press: St. Paul, Minnesota, U.S.A.

Dupont, J., Laloui, W. & Roquebert, M.F. 1998. Partial ribosomal DNA sequences show an important divergence between *Phaeoacremonium* species isolated from *Vitis vinifera*. *Mycological Research* **102**, 631-637.

Dupont, J., Laloui, W., Magnin, S., Larignon, P. & Roquebert, M. 2000. *Phaeoacremonium viticola*, a new species associated with esca disease of grapevine in France. *Mycologia* **92**, 499-504.

Eckert, K.A. & Kunkel, T.A. 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Nucleic Acids Research* **18**, 3739-3743.

Erlich, H.A., Gelfand, D. & Sninsky, J.J. 1991. Recent advances in the polymerase chain reaction. *Science* **252**, 1643-1650.

Ferreira, J.H.S. 1998. *Phialophora* terugsterwing - 'n Algemene probleem by jong wingerde. *Wynboer* **March**, T6-T8.

Ferreira, J.H.S., Van Wyk, P.S. & Venter, E. 1994. Slow dieback of grapevine: Association of *Phialophora parasitica* with slow dieback of grapevines. *South African Journal of Enology and Viticulture* **15**, 9-11.

Fischer, M. 1996. On the species complexes within *Phellinus*: *Fomitiporia* revisited. *Mycological Research* **100**, 1459-1467.

Gelfand, D.H. & White, T.J. 1990. Thermostable DNA polymerases. In *PCR protocols: A guide to methods and applications* (ed. M. Innis, D. Gelfand, D. Sninsky & T. White), pp 129-141. Academic: New York, U.S.A.

Glass, N.L. & Donaldson, G.C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**, 1323-1330.

Goodwin, P.H., English, J.T., Neher, D.A., Duniway, J.M. & Kirkpatrick, B.C. 1990. Detection of *Phytophthora parasitica* from soil and host tissue with species-specific DNA probe. *Phytopathology* **80**, 277-281.

Goodwin, P.H., Kirkpatrick, B.C. & Duniway, J.M. 1989. Cloned DNA probes for identification of *Phytophthora parasitica*. *Phytopathology* **79**, 716-721.

Graniti, A., Surico, G., & Mugnai, L. 2000. Esca of grapevine: a disease complex or a complex of diseases? *Phytopathologia Mediterranea* **39**, 16-20.

Hawksworth, D.L., Gibson, A.S. & Gams, W. 1976. *Phialophora parasitica* associated with disease conditions in various trees. *Transactions of the British Mycological Society* **66**, 427-431.

Henson, J.M. & French, R. 1993. The polymerase chain reaction and plant disease diagnosis. *Annual Review of Phytopathology* **31**, 81-109.

Hillis, D.M. & Dixon, M.T. 1991. Ribosomal DNA: Molecular evolution and phylogenetic inference. *Quarterly Review of Biology* **66**, 411-426.

Hung, T., Mal, K. & Fong, K. 1990. A specificity enhancer for polymerase chain reaction. *Nucleic Acids Research* **18**, 4953.

Johanson, A., Turner, H.C., McKay, G.J. & Brown, A.E. 1998. A PCR-based method to distinguish fungi of the rice sheath-blight complex, *Rhizoctonia solani*, *R. oryzae* and *R. oryzae-sativae*. *FEMS Microbiology Letters* **162**, 298-294.

Krawetz, S.A., Pon, R.T. & Dixon, G.H. 1989. Increased efficiency of the Taq polymerase catalyzed polymerase chain reaction. *Nucleic Acids Research* **17**, 819-820.

Larignon, P. & Dubos, B. 1987. Les séquences parasitaires impliquéesdans le syndrome del'esca. Symposium sur la lutte integrée en viticulture, Logrono, 3-5 mars 1987.

Larignon, P. & Dubos, B. 1997. Fungi associated with esca disease in grapevine. *European Journal of Plant Pathology* **103**, 147-157.

Larignon, P. & Dubos, B. 2000. Preliminary studies on the biology of *Phaeoacremonium*. *Phytopathologia Mediterranea* **39**, 184-189.

Liew, E.C.Y., Guo, L.D., Ranghoo, V.M., Goh, T.K. & Hyde, K.D. 1998a. Molecular approaches to assesing fungal diversity in the natural environment. *Fungal Diversity* **1**, 1-17.

Liew, E.C.Y., Maclean, D.J. & Irwin, J.A.G. 1998b. Specific PCR based detection of *Phytophthora medicaginis* using intergenic spacer region of the ribosomal DNA. *Mycological Research* **102**, 73-80.

Miller, S.A. & Martin, R.R. 1988. Molecular diagnosis of plant disease. *Annual Review of Phytopathology* **26**, 409-432.

Morton, L. 1995. Mystery diseases hit young vines. *Wines and Vines* **November**, 46-47.

Mugnai, L., Surico, G. & Esposito, A. 1996. Microflora associata al mal dell'esca della vite in Toscana (Fungal microflora associated with 'esca' grapevine disease in Tuscany). *Informatore Fitopatologico* **46**, 49-55.

Mugnai. L., Bertelli, E., Surico, G. & Esposito, A. 1997. Observations on the aetiology of 'esca' disease of grapevine. In *Proceedings10th Congress of the Mediterranean Phytopathological Union*, pp 269-272. Montpeiller, France.

Mugnai, L. 1998. A threat to young vineyards: *Phaeoacremonium chlamydosporum* in Italy. In *Proceedings of the 1998 Seminar and Workshop on Black goo: Symptoms and occurrence of grape decline* (ed. L. Morton), pp 35-42. Fort Valley, U.S.A.

Mugnai, L., Graniti, A. & Surico, G. 1999. Esca (Black measles) and brown wood-streaking: Two old and elusive diseases of grapevines. *Plant Disease* **83**, 404-418.

Mullis, K.B. & Faloona, F.A. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods in Enzymology* **155**, 335-350.

Nazar, R.N., Hu, X., Schmidt, J., Culham, D. & Robb, J. 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of verticillium wilt pathogens. *Physiological and Molecular Plant Pathology* **39**, 1-11.

O'Brien, P.A., Farbey, M.D. & Nowak, K.J. 1998. Detection of fungal phytopathogens by PCR. In *7th International Congress of Plant Pathology,* Section 3.3.15. Edinburgh, Scotland.

Pascoe, I. 1998. Grapevine Trunk Diseases in Australia: Diagnostics and taxonomy. In *Proceedings of the 1998 Seminar and Workshop on Black goo: Symptoms and occurrence of grape decline* (ed. L. Morton) pp 56-77. Fort Valley, U.S.A.

Pascoe, I. & Cottral, E. 2000. Developments in grapevine trunk diseases research in Australia. *Phytopathologia Mediterranea* **39**, 68-75.

Petri, L. 1912. Osservazioni sopra le alterazioni del legno della vite in seguito a ferite. *Le Stazioni Sperimentali Agrarie Italiane* **45**, 501-547.

Riedel, K.H.L., Wingfield, B.D. & Britz, T.J. 1992. Combined influence of magnesium concentration and polymerase chain reaction specificity enhancers. *FEMS Microbiol Letters* **92**, 69-72.

Rollo, F.R., Amici, A., Foresi, F. & di Silvestro, I. 1987. Construction and characterization of a cloned probe for the detection of *Phoma tracheiphila* in plant tissues. *Applied Microbiology and Biotechnology* **26**, 352-357.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. & Arnheim, N. 1985. Enzymatic amplification of β-globin genomic sequences and

restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**, 1350-1354.

Sakar, G. & Sommer, S.S. 1990. Shedding light on PCR contamination. *Nature* **343**, 27.

Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989. Molecular cloning: A Laboratory Manual. Laboratory Press: Cold Spring Harbor.

Scheck, H.J., Vasquez, S.J. & Gubler, W.D. 1998. First report of three *Phaeoacremonium* spp. causing young grapevine decline in California. *Plant Disease* **82**, 590.

Screenivasaprasad, S., Sharada, K., Brown, A.E. & Mills, P.R. 1996. PCR-based detection of *Colletotrichum acutatum* on strawberry. *Plant Pathology* **45**, 650-655.

Shen, W. & Hohn, B. 1992. DMSO improves PCR amplification of DNA with complex secondary structures. *Trends in Genetics* **8**, 227.

Sidoti, A., Buonocore, E., Serges, T. & Mugnai, L. 2000. Decline of young grapevines associated with *Phaeoacremonium chlamydosporum* in Sicily (Italy). *Phytopathologia Mediterranea* **39**, 87-91.

Sommer, R. & Tautz, D. 1989. Minimal homology requirements for PCR primers. *Nucleic Acids Research* **17**, 6749.

Sparapano, L., Bruno, G., Ciccarone, C. & Graniti, A. 2000a. Infection of grapevines by some fungi associated with esca. I. *Fomitiporia punctata* as a wood-rot inducer. *Phytopathologia Mediterranea* **39**, 46-52.

Sparapano, L., Bruno, G., Ciccarone, C. & Graniti, A. 2000b. Infection of grapevines by some fungi associated with esca. II. Interaction among *Phaeoacremonium chlamydosporum*, *P. aleophilum* and *Fomitiporia punctata*. *Phytopathologia Mediterranea* **39**, 53-58.

Stackebrandt, E., Liesack, W. & Witt, D. 1992. Ribosomal RNA and rDNA sequence analyses. *Gene* **115**, 255-260.

Steffan, R.J. & Atlas, R.M. 1991. Polymerase chain reaction: Applications in environmental microbiology. *Annual Review of Microbiology* **45**, 137-161.

Tooley, P.W., Bunyard, B.A., Carras, M.M. & Hatziloukas, E. 1997. Development of PCR primers from internal transcribed spacer region 2 for detection of *Phytophthora* species infecting potatoes. *Applied and Environmental Microbiology* **63**, 1467-1475.

Viala, P. 1926. Recherches sur les maladies de la vigne: Esca. *Annales des Epiphyties* **12**, 1-108.

Wang, C.J.K. 1979. Pleomorphic fungi imperfecti. In *The Whole Fungus, the sexual–asexual synthesis* (ed. B. Kendrick), pp 81-91. Kananaskis Foundation: National Museums of Canada, Canada.

Waite, H. & May, P. 1998. Trials and commercial application of long duration hot water treatment in Australia. In *Proceedings of the 1998 Seminar and Workshop on Black goo: Symptoms and occurrence of grape decline* (ed. L. Morton), pp 98-103. Fort Valley, U.S.A.

Yan, Z.H., Rogers, S.O. & Wang, C.J.K. 1995. Assessment of *Phialophora* species based on ribosomal DNA internal transcribed spacers and morphology. *Mycologia* **87**, 72-833.

Table 1. Presence of the commonly isolated fungi in esca-affected grapevines sampled in Italian regions (Mugnai, 1998)

Fungal species	% Black stripes	% Brown-red wood	% Brown-necrosis	% White decay
Eutypa lata	0.0%	3.5%	20.7%	3.6%
Pm. aleophilum	6.0%	7.2%	8.7%	3.1%
Pa. chlamydospora	58.5%	61.4%	22.7%	10.1%
Phellinus igniarius	0.3%	8.5%	2.6%	65.7%

Fig. 1. Internal wood symptoms of esca disease.

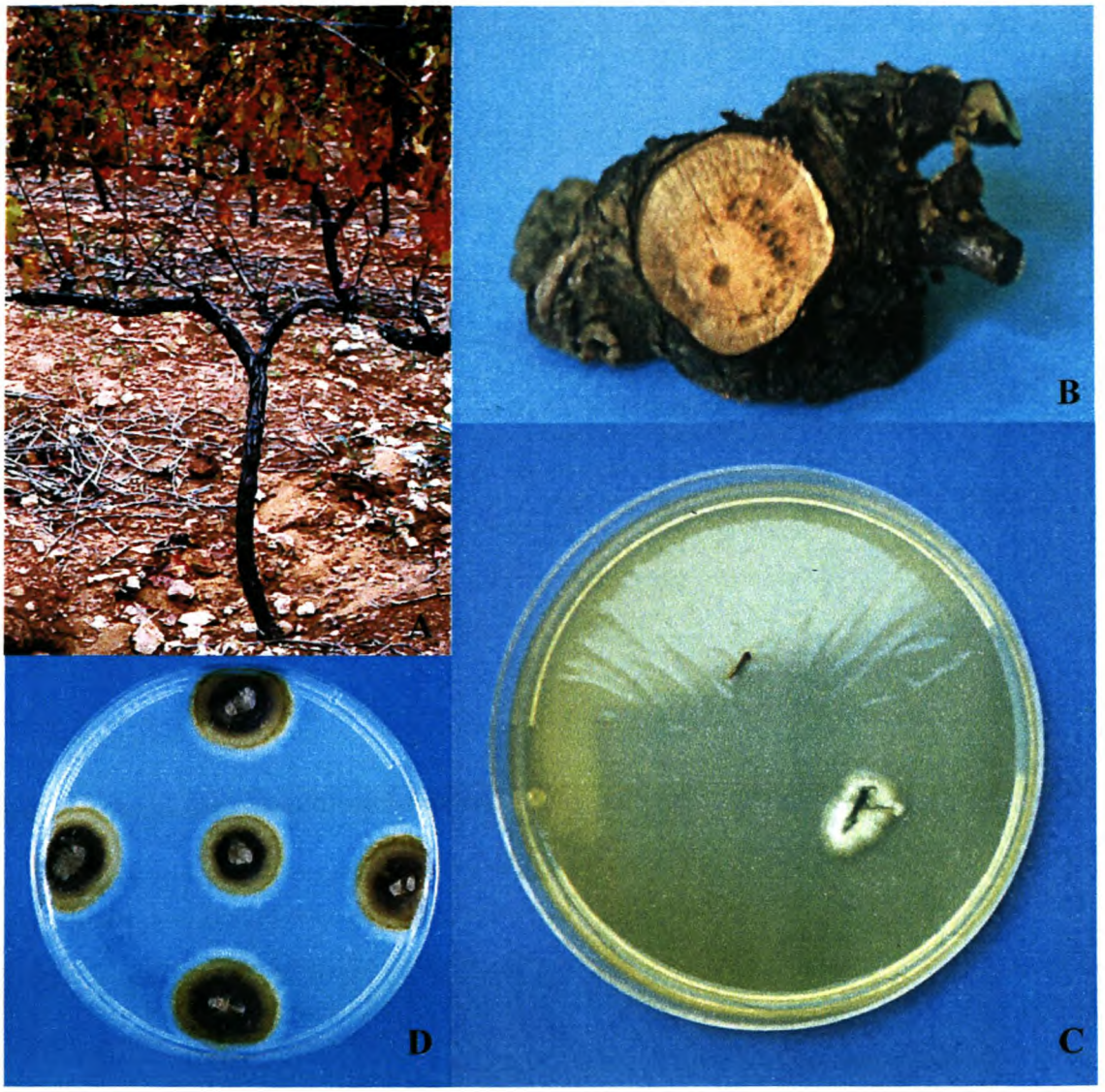


Fig. 2. *Phaeomoniella chlamydospora*, the dominant organism causing Petri grapevine decline in South Africa. A. External symptoms of Petri grapevine decline. B. A transverse section through an infected grapevine showing typical black spots. C. Young colonies of *Pa. chlamydospora* have a white yeast-like growth on MEA. D. Mature colonies of *Pa. chlamydospora* on MEA.

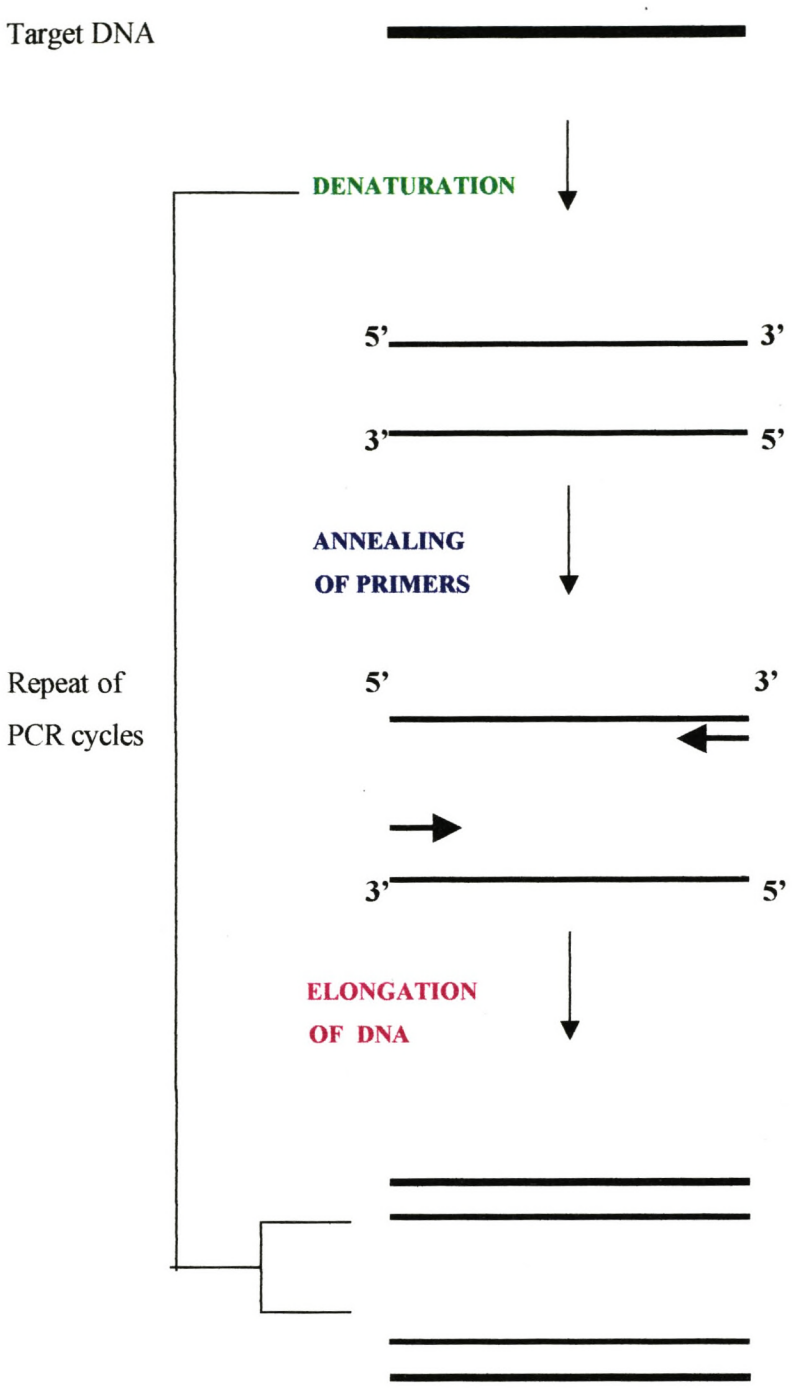


Fig. 3. The PCR technique consists of three important steps: **denaturation**, **annealing** and **elongation**.

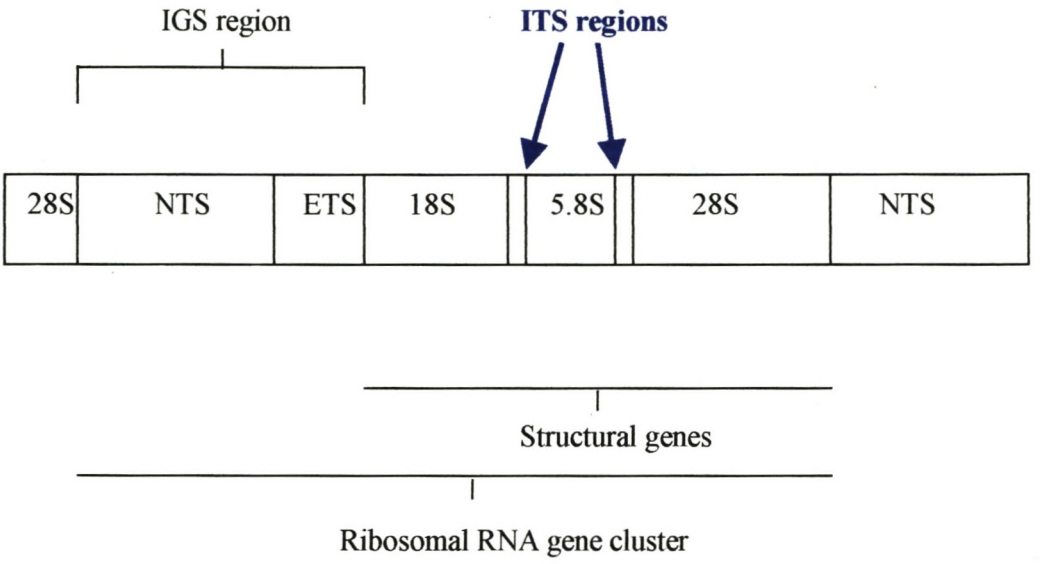


Fig. 4. The ribosomal RNA genes and the intergenic spacers.

2. FUNGICIDE SENSITIVITY OF *PHAEOMONIELLA CHLAMYDOSPORA*, THE CAUSAL ORGANISM OF PETRI GRAPEVINE DECLINE

ABSTRACT

Twelve fungicides, benomyl, chlorothalonil, fenarimol, fosetyl-Al, iprodione, kresoxim-methyl, mancozeb, metalaxyl, prochloraz manganese chloride, quintozene, tebuconazole and thiram were screened *in vitro* for mycelial inhibition of *Pa. chlamydospora* the causal organism of Petri grapevine decline. The isolates of *Pa. chlamydospora* were obtained from different geographical areas in the Western Cape province. The effective concentration at which 50% of mycelial growth was inhibited (EC_{50}) was calculated for each fungicide. Benomyl, fenarimol, kresoxim-methyl, prochloraz manganese chloride and tebuconazole were the most effective in inhibiting mycelial growth of *Pa. chlamydospora* with EC_{50} values ranging from 0.01 to 0.05 µg/ml. Data obtained in this study furthermore represent the base-line sensitivity of local isolates towards these fungicides, which is important for monitoring the development of pathogen resistance to fungicides.

INTRODUCTION

Grape production is an important agricultural industry in South Africa that earned an estimated R 1,4 billion in 1998 (KWV report, 1998). Losses caused by pests or diseases are thus of great concern to the industry. It has only recently been proven that the condition in which young grapevines show stunted growth and slow die-back is a disease caused by fungal pathogens (Morton, 1995; Ferreira, 1998; Scheck *et al.*, 1998; Mugnai, *et al.*, 1999). This disease is widespread and causes serious losses in South African vineyards.

Several fungi have been associated with slow die-back of vines worldwide, but the organism most commonly isolated from affected vines in South Africa was identified as *Phialophora parasitica* (Ferreira *et al.*, 1994). However, after an extensive study, Crous *et al.* (1996) established the genus *Phaeoacremonium* for a group of fungal pathogens associated with die-back diseases of woody hosts. Although several species of *Phaeoacremonium* have been associated with the disease, namely *Pm. aleophilum*, *Pm. chlamydosporum* and *Pm. inflatipes* (Morton, 1995;

Larignon & Dubos, 1997; Scheck *et al.*, 1998; Mugnai *et al.*, 1999), *Pm. chlamydosporum* is found to be the dominant fungus isolated from symptomatic young grapevines in South Africa (Groenewald *et al.*, unpublished data) and Italy (Mugnai *et al.*, 1996), respectively. A re-examination of numerous *Pm. chlamydosporum* isolates from diverse geographical regions showed several prominent morphological differences to be present between this species and others accommodated in *Phaeoacremonium*. Based on molecular (Dupont *et al.*, 1998; Groenewald *et al.*, in prep), morphological and pathological differences, a new genus *Phaeomoniella* Crous & W. Gams was therefore introduced, typified by *Phaeomoniella chlamydospora* (=*Phaeoacremonium chlamydosporum*) (Crous & Gams, 2000).

Much confusion has surrounded the name of the disease. Morton (1995) reported a mysterious disease that hit young vines in California in which diseased vines had shiny black streaks in their vascular vessels, showed poor vine growth and sometimes a sudden collapse of the vine. In response to the disease plants produced black gum and thus the name 'black goo' originated (Morton, 1995). In older vines the disease has been referred to as esca, apoplexy and black measles, but in young vines it is called Petri grapevine decline (Mugnai *et al.*, 1999).

In order to effectively implement integrated control of *Pa. chlamydospora*, it is important to know how infection becomes established. It is possible that *Pa. chlamydospora* could infect vines in nurseries or fields through the root system as a soil-borne pathogen. The pathogen could, however, also be introduced into grape rootstocks during the callusing stage in the nursery. Recently it was discovered that *Pa. chlamydospora* was present in apparently healthy propagation material (cuttings and rootstock) in a latent or endophytic form (Bertelli *et al.*, 1998; J.H.S. Ferreira, pers. comm.). This evidence supports the belief that the disease could have its origin in propagation material. Preplant treatment of nursery material to eradicate the pathogen would thus be of practical use to the industry.

One possibility of eradicating the pathogen from cuttings is to soak them in a solution of systemic fungicides so that the chemicals can be taken up and transported through the vascular tissues of the cuttings. Similarly, fungicide drenches could be taken up by the roots of rooted cuttings and transported through the plant and this may also offer good control. The purpose of this experiment was to screen a number of

fungicides against *Pa. chlamydospora* isolates, determining the effect of the different fungicides on mycelial inhibition. Results of this screening test also provide base-line sensitivities of South African isolates of *Pa. chlamydospora*. Data obtained in these *in vitro* tests should thus lead to further testing in pot and field trials.

MATERIALS AND METHODS

Selection of fungicides

Twelve fungicides representing contact and systemic products were used in the screening tests (Table 1). Chlorothalonil, iprodione, mancozeb, quintozene and thiram were selected as contact fungicides and benomyl, fenarimol, fosetyl-Al, kresoxim-methyl, metalaxyl, prochloraz manganese chloride and tebuconazole as systemic fungicides.

In vitro tests on mycelial inhibition

The screenings were carried out in a block repetition conducted in December 1998 and January 1999. All twelve fungicides were tested at the following concentrations: 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 5 µg a.i./ml. Chlorothalonil, thiram and mancozeb were also tested at 10, 50 and 100 µg a.i./ml. One litre stock solution was prepared for each fungicide. To attain the correct fungicide concentrations, dilutions from the stock solutions were made and added to 1000 ml 20% malt extract agar (MEA, Biolab) (50°C). Only MEA was present in the control medium. Plates were inoculated within 24-hours after they had been poured with a 5 mm diam. mycelial disc from the cultured *Pa. chlamydospora* isolates. Two mycelial discs were placed at an equal distance from each other on each plate. Six isolates of *Pa. chlamydospora* from different geographical regions (STE-U 2863-2868) were used for the tests. These isolates are maintained at the Department of Plant Pathology, University of Stellenbosch (STE-U). Linear mycelial growth was recorded after 14 days incubation at 22°C in the dark measuring the perpendicular diameters.

Statistical analysis was carried out on the data and the effective concentration at which 50% of the mycelial growth was inhibited (EC_{50}) was calculated where the % inhibition of each colony was calculated as a proportion of the control colonies. The % inhibition was plotted against the concentration for each fungicide/isolate

combination. The most suitable regression was fitted to each data set and the EC_{50} values calculated. An analysis of variance was conducted on the EC_{50} values to determine significant differences between the inhibitory effects of the various fungicides.

RESULTS

The tests on *Pa. chlamydospora* were performed in December 1998 and repeated in January 1999. No significant differences (P = 0.2538, Table 2) in the data obtained at the different test times (1998 and 1999) were found. Thus the pooled data over two years could be used for calculation of the EC_{50} values. Since there were no significant differences among isolates (P = 0.3354, Table 2) in response to the fungicides, the isolates could be used as replicates in the statistical analysis. Interaction between the isolates and fungicides (P = 0.6110, Table 2) was not significant. However, the effects of the various fungicides on mycelial growth were significantly different (P = 0.0001, Table 2).

The data analysis showed four types of responses of *Pa. chlamydospora* isolates to the fungicides. Firstly, no EC_{50} value could be calculated for fosetyl-Al, due to the lack of inhibition of mycelial growth. Fosetyl-Al was thus discarded from the statistical analysis. Secondly, inhibition of mycelial growth occurred, but only at high concentrations. Thirdly, there was effective mycelial inhibition of *Pa. chlamydospora* isolates at relatively low concentrations, and finally the mycelial growth was inhibited at extremely low concentrations, showing that these fungicides were very effective in inhibiting mycelial growth of *Pa. chlamydospora*. Mancozeb, metalaxyl and quintozene fell into the second group with EC_{50} values ranging between 10.891 and 13.590 (Group A, Fig. 1). The third group contained chlorothalonil, iprodione and thiram, with EC_{50} values between 1.379 and 5.151 (Group B, Fig. 1). The last group showed the lowest EC_{50} values ranging from 0.015 to 0.457. This group contained benomyl, fenarimol, kresoxim-methyl, prochloraz manganese chloride and tebuconazole (Group C, Fig. 1). Fungicides of groups B and C showed some degree of overlap (Fig. 1).

DISCUSSION

Until the epidemiology of the disease is understood, it will be difficult to achieve optimum integrated control. However, current information and recent observations suggest that the inoculum can be air-borne (Larignon, 1998) or that natural infection of planted vineyards probably occur through the roots. However, infected cuttings from nurseries are also planted in the field (Bertelli *et al.*, 1998), implicating nursery material in the spread of this pathogen.

An important management aspect of vascular diseases is prevention rather than the cure of the disease. The use of *Phaeomoniella*-free cuttings is of primary importance. Only healthy pathogen-free propagation material should be planted in pathogen-free soil. However, it is difficult to ensure pathogen-free nursery stock, because the fungus could be latent or endophytic in rootstock cuttings (Bertelli *et al.*, 1998). Therefore, preplant treatments with fungicides might be useful in eradicating the pathogen from cuttings that will be rooted. This experiment was performed to indicate which fungicides inhibit mycelial growth of *Pa. chlamydospora* so that these fungicides could be used for further testing on nursery material.

Our results showed that benomyl, fenarimol, kresoxim-methyl, prochloraz manganese chloride and tebuconazole gave effective inhibition of *Pa. chlamydospora* at low concentrations (0.01-0.5 ppm). Benomyl has been reported to control systemic pathogens like *Verticillium, Fusarium* and *Ceratocystis* under controlled conditions (Delp, 1995), and could thus be used if cuttings are soaked in a fungicide solution (cutting soak treatment: when cuttings are soaked in fungicides, before grafting). If benomyl is applied as a soil drench there may be problems with microbial degradation of the product. Delp (1995) suggested that soil drenching in field conditions do not offer effective disease control of certain systemic pathogens, and this will thus have to be confirmed for *Pa. chlamydospora*.

Fenarimol has been used primarily for protective and curative control of scab and powdery mildew on apples (Brown & Hall in Kuck *et al.*, 1995). However, Buchenauer and Rohner (in Kuck *et al.*, 1995) reported root uptake of fenarimol, but found it insufficient in providing disease control under field conditions. It is possible that fenarimol might be successful in the soaking treatment envisaged for vine

cuttings. The other DMI fungicides prochloraz manganese chloride and tebuconazole also gave good *in vitro* results and should thus be tested further.

Kresoxim-methyl represents a new group of fungicides, the strobilurins. Although little work has been done on soil treatment with kresoxim-methyl, it has been reported to break down rapidly to a biologically inactive acid in soil (Ypema & Gold, 1999). Thus this fungicide will not be able to be applied as a soil drench in practice, but the excellent *in vitro* results suggest that it should be tested for the cutting soak treatment. This treatment is explained above, but further research is of importance to determine the practical application of this treatment in nurseries.

Chlorothalonil is a contact fungicide with a broad spectrum of activity. For several foliar grapevine diseases it has a good effect if applied preventatively. No information on root uptake of chlorothalonil is available. It will be difficult for a contact fungicide to reach the xylem, because of its inability to move through plant tissue. Similarly the fungicide thiram, registered as a soil fungicide and seed treatment, will not be taken up by the vascular system and therefore have no effect on existing endophytic or latent infections and vascular wilt diseases. Dicarboximides such as iprodione are known as contact fungicides, which are generally applied prophylactically, but research has shown that there is some degree of systemic translocation of these fungicides (Cayle & Hide, 1980 in Pommer and Lorenz, 1995). Research should therefore be conducted on the application of these three fungicides as protective agents by dipping cuttings into the fungicide before planting or while they are stored during the callusing stage. Neither quintozene nor mancozeb inhibited mycelial growth of *Pa. chlamydospora,* and can therefore be excluded from any further testing.

Ferreira (1998) reported that fosetyl-Al and metalaxyl depressed *Phaeoacremonium* growth in the laboratory and in glasshouse tests. Our results showed no inhibition of mycelial growth at effective concentrations by fosetyl-Al or metalaxyl. Fosetyl-Al is reported to have weak curative action and is known to stimulate host resistance (Schwinn & Staub, 1995) rather than being fungicidal. Since it has a broad spectrum of applications ranging from foliar sprays to trunk injections and dip treatment (Schwinn & Staub, 1995), and is also known to move symplastically in plant tissue, it may be able to stimulate host defence mechanisms which would prevent the spread of the pathogen. Although fosetyl-Al was found to

be unsuccessful for the control of the esca disease complex (Mugnai *et al.*, 1999), Ferreira (1998) reported success in glasshouse trials. Di Marco *et al.* (2000) reported that phosphorous acid in combination with *Vitis* stilbenes (resveratreol and pterostilbene) showed effective control of *Phaeoacremonium* spp. (including *Pa. chlamydospora*) *in vitro*. Further testing to determine the exact effect of this chemical on disease control should be carried out.

Metalaxyl is known to target some protoctistan fungi, specifically the Oomycetes (Kerkernaar & Kaars Sijpesteijn, 1981). It has been effectively used to control *Pythium* and *Phytophthora* in soil (Schwinn & Staub, 1995). Ferreira (1998), however, reported effective disease control of *Pa. chlamydospora* with metalaxyl in laboratory and glasshouse tests. Results from our study showed that metalaxyl had no inhibiting effect on the *in vitro* mycelial growth of this fungus. The unexpected effective control of *Pa. chlamydospora* by metalaxyl, as reported by Ferreira (1998), is therefore unexplained by this study.

I suggest that dipping the base of the rootstock of nursery material in a fungicide solution before planting in nursery fields should also be investigated. Farmers can even use this technique before transplanting nursery material into fields on their farms. However, pot trials to test for efficacy of the various fungicides in disease control and to assess phytotoxic effects, should be conducted before field tests are performed, seeing that results of *in vitro* tests do not always correspond with field tests. Various factors, such as wind, sun (UV-radiation) and rain influence the efficacy of fungicides under field conditions. This *in vitro* test gives a good indication of which fungicides can be selected for further studies in glasshouses and nurseries. These data could therefore contribute to integrating an effective fungicide application programme. When used in combination with sanitation and stress relief, healthy, productive grapevines are an achievable goal.

REFERENCES

Bertelli, E., Mugnai, L. & Surico, G. 1998. Presence of *Phaeoacremonium chlamydosporum* in apparently healthy rooted grapevine cuttings. *Phytopathologia Mediterranea* **37,** 79-82.

Crous, P. W., Gams, W., Wingfield, M. J. & van Wyk, P. S. 1996. *Phaeoacremonium* gen. nov. associated with wilt and decline disease of woody hosts and human infections. *Mycologia* **88,** 786-796.

Crous, P.W. & Gams, W. 2000. *Phaeomoniella chlamydospora* gen. et comb. nov., a causal organism of Petri grapevine decline. *Phytopathology Mediterranea* **39**, 112-118.

Delp, C.J. 1995. Benzimidazole and related fungicides. In *Modern selective fungicides* (ed. H. Lyrs), pp. 291-303. Gustav Fischer Verlag: Germany.

Dupont, J., Laloui, W. & Roquebert, M.F. 1998. Partial ribosomal DNA sequences show an important divergence between *Phaeoacremonium* species isolated from *Vitis vinifera*. *Mycological Research* **102,** 631-637.

Ferreira, J.H.S., van Wyk, P.S. & Venter, E. 1994. Slow dieback of grapevine: Association of *Phialophora parasitica* with slow dieback of grapevines. *South African Journal of Enology and Viticulture* **15,** 9-11.

Ferreira, J.H.S. 1998. *Phialophora* terugsterwing - 'n Algemene probleem by jong wingerde. *Wynboer* **March,** T6-T8.

Kuck, K.H., Scheinpflug, H. & Pontzen, R. 1995. DMI fungicides. In *Modern selective fungicides* (ed. H. Lyrs), pp. 205-258. Gustav Fischer Verlag: Germany.

Kerkernaar, A. & Kaarssijpesteijn, A. 1981. Antifungal activity of metalaxyl and furalaxyl. *Pesticide Biochemistry and Physiology* **15,** 71-78.

Larignon, P. & Dubos, B. 1997. Fungi associated with esca disease in grapevine. *European Journal of Plant Pathology* **103,** 147-157.

Larignon, P., 1998. Esca disease from an European perspective. In *Proceedings of the 1998 Seminar and Workshop on Black goo: Symptoms and occurrence of grape decline* (ed. L. Morton), pp. 43-55. Fort Valley, U.S.A.

Morton, L. 1995. Mystery diseases hit young vines. *Wines & Vines* **November,** 46-47.

Mugnai, L., Surico, G. & Esposito, A. 1996. Microflora associata al mal dell'esca della vite in Toscana (Fungal microflora associated with 'esca' grapevine disease in Tuscany). *Informatore Fitopatologico* **46,** 49-55.

Mugnai, L., Graniti, A. & Surico G. 1999. Esca (Black measles) and brown wood-streaking: Two old and elusive diseases of grapevines. *Plant Disease* **83,** 404-418.

Nel, A., Krause, M., Ramautar, N., Van Zyl, K. 1999. A guide for control of plant diseases. National Department of Agriculture: Pretoria, South Africa.

Pommer, E.H. & Lorenz, G. 1995. Dicarboximide fungicides. In *Modern, Selective Fungicides* (ed. H. Lyrs), pp. 99-118. Gustav Fischer Verlag: Germany.

Scheck, H.J. Vasquez, S.J. & Gubler, W.D. 1998. First report of three *Phaeoacremonium* spp. causing young grapevine decline in California. *Plant Disease* **82,** 590.

Schwinn, F. & Staub, T. 1995. Oomycetes fungicides. In *Modern selective fungicides* (ed. H. LYRS), pp. 323-346. Gustav Fischer Verlag: Germany.

Ypema, H.C & Gold, R.E. 1999. Kresoxim-methyl. Modification of a naturally occurring compound to produce a new fungicide. *Plant Disease* **83,** 4-19.

Table 1. Fungicides screened against *Phaeomoniella chlamydospora*

Active ingredient	Trade name	Fungicide group	Type	Registered on grapevines in South Africa[a]
Benomyl	Benlate	Benzimidazoles	systemic	+
Chlorothalonil	Bravo	miscellaneous	contact	-
Fenarimol	Rubigan	DMI-pyrimidine	systemic	+
Fosetyl-Al	Aliette	Organic compounds	systemic	+
Iprodione	Rovral Flo	Dicarboximide	contact	+
Kresoxim-methyl	Stroby	Strobilurin	locally-systemic	+
Mancozeb	Dithane	Dithiocarbamates	contact	+
Metalaxyl	Ridomil	Acylanalines	systemic	+
Prochloraz manganese chloride	Octave	DMI-imidiazole	systemic	-
Quintozene	PCNB	Aromatic compounds	contact	-
Tebuconazole	Folicur	DMI-triazoles	systemic	+
Thiram	Thiram	Dithiocarbamates	contact	+

[a]According to Nel *et al.* (1999).

Table 2. Analysis of variance of EC_{50} values for significant differences amongst blocks, isolates of *Phaeomoniella chlamydospora* and fungicides

Source	[a]DF	Mean square	[b]*F* value	[c]*P* value
Block	1	23.7664362	1.34	0.2538 NS
Isolate	5	20.9396380	1.18	0.3354 NS
Fungicide	10	276.4873360	15.56	0.0001 S
Isolate:Fungicide	50	16.3818709	0.92	0.6110 NS

[a]DF: degrees of freedom.

[b]*F*-value: the ratio of variance associated with the particular effect measured to the natural variance.

[c]*P*-value: the probability (or confidence level) of the effects occurring.

NS: not significant.

S: significant.

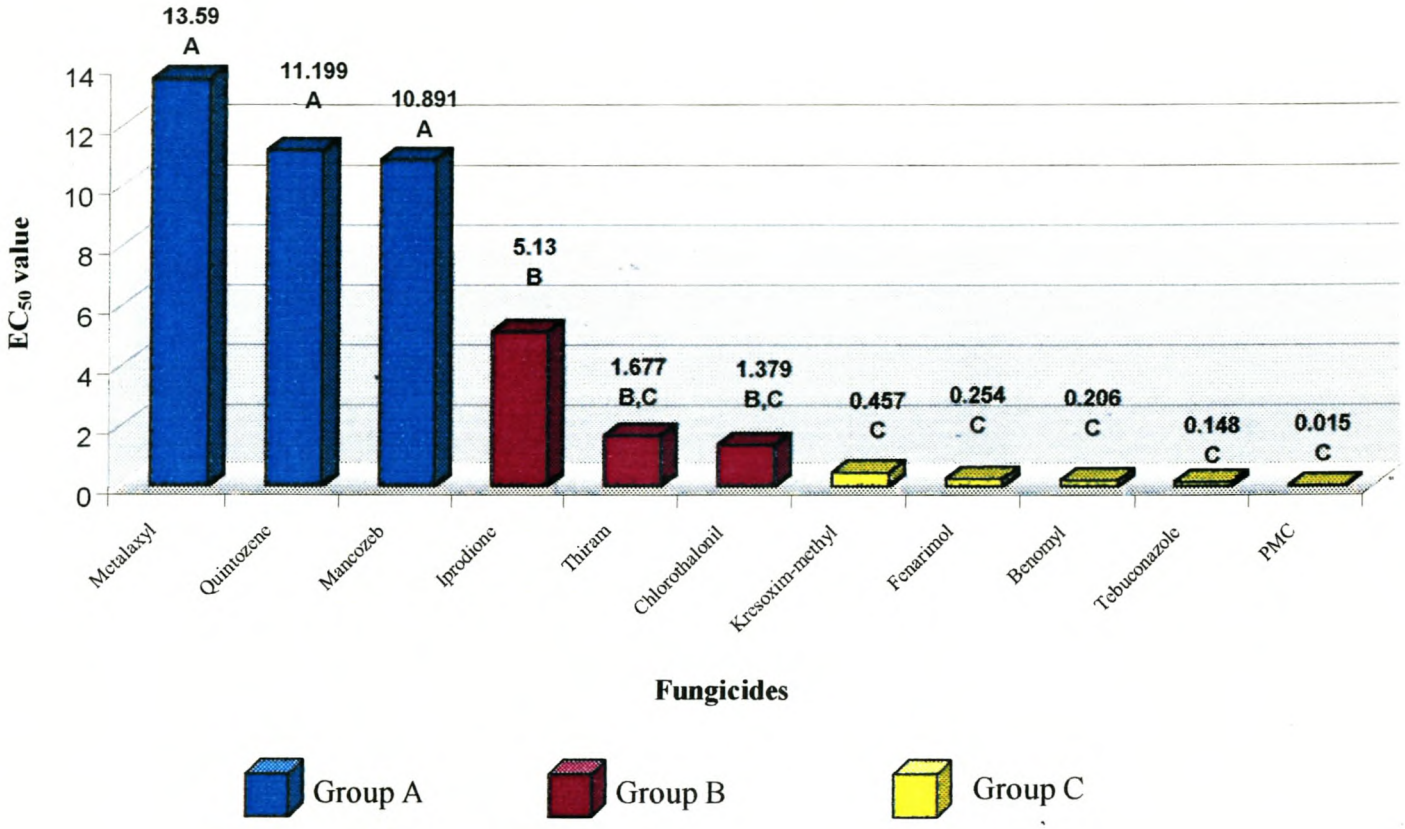


Fig. 1. Comparison of EC_{50} values calculated for fungicides from *in vitro* mycelial inhibition of *Phaeomoniella chlamydospora*.

3. ITS AND β-TUBULIN PHYLOGENY OF *PHAEOACREMONIUM* AND *PHAEOMONIELLA*

ABSTRACT

Based on ITS and β-tubulin sequence data of 33 isolates, the newly introduced genus, *Phaeomoniella* (*Pa.*), was confirmed as being distinct from *Phaeoaremonium* (*Pm.*). DNA phylogeny and cultural characteristics also confirmed the species status of *Pm. aleophilum* and *Pm. angustius*, which were recently reduced to synonymy. *Pm. aleophilum* has an optimum growth rate at 30°C and the ability to grow at 35°C, whereas *Pm. angustius* has an optimum growth rate at 25°C, and does not grow at 35°C. Furthermore, ITS and β-tubulin sequence data showed *Pm. viticola* to be indistinguishable from *Pm. angustius*, while a new species, *Pm. mortoniae*, could be distinguished from this complex.

INTRODUCTION

Petri grapevine decline, also known as slow die-back and black goo decline, is a well-known disease of grapevines worldwide (Mugnai *et al.*, 1999). Although the complex etiology and epidemiology of this disease has not yet been completely resolved, one of the major causal organisms is *Phaeomoniella chlamydospora*, a fungus that is well distributed throughout the world with its host, *Vitis vinifera*. In addition, Petri grapevine decline is a major component of the Esca disease complex of grapevines (primarily *Fomitiporia punctata* and *Stereum hirsutum*), and the Phaeoacremonium disease complex (primarily *Phaeoacremonium* spp.) (Crous & Gams, 2000).

As far as could be established, Petri (1912) was the first to report the disease from grapevines with brown and black wood streaks, with which he associated species of *Cephalosporium* and *Acremonium*. Chiarappa (1959) subsequently isolated and confirmed the pathogenicity of a *Cephalosporium* species from grapevine. Crous *et al.* (1996) compared morphologically similar isolates originating from human patients and various woody hosts, and subsequently established the genus *Phaeoacremonium* for these taxa. The genus contained six species, namely *Pm. aleophilum*, *Pm. angustius*, *Pm. chlamydosporum*, *Pm. parasiticum*, *Pm. inflatipes* and *Pm. rubrigenum*. The *Cephalosporium* species isolated by Chiarappa (1959) was found to

be representative of *Pm. chalamydospora* (Crous *et al.* 1996). In a later molecular study, however, Dupont *et al.* (1998) reported that *Pm. chlamydosporum* appeared to be unrelated to the other species of the genus in the Magnaporthaceae and closer to *Phialophora sensu stricto* (Herpotrichiellaceae). Based on these molecular, as well as additional morphological and pathological differences, a new genus, *Phaeomoniella* (*Pa.*), was therefore introduced, typified by *Pa. chlamydospora* (Crous & Gams 2000).

Other aspects that needed clarification were the pathogenicity of species in this complex (Scheck *et al.*, 1998), as well as their delimitation. Dupont *et al.* (1998) synonymized two taxa, and described one as new (Dupont *et al.* 2000a). *Pa. chlamydospora* is seen as the dominant organism causing Petri grapevine decline (Crous & Gams 2000). However, as stated previously, other *Phaeoacremonium* spp. are also involved in this disease complex. Accordingly, the aims of this study were to investigate the phylogeny of *Phaeomoniella* and *Phaeoacremonium* species using sequence analysis of two genomic areas, and to compare these data to cultural characteristics and morphology.

MATERIALS AND METHODS

Isolates and cultural characteristics

Isolates of *Phaeoacremonium* spp. were either obtained from symptomatic vines or from the Centraalbureau voor Schimmelcultures (CBS). These isolates (Table 1) are maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa (STE-U), as well as at CBS.

Single-conidial cultures were established for all isolates studied. Isolates were plated onto 2% malt extract agar (MEA; Biolab, Midrand, Johannesburg, South Africa), and incubated at 25°C under near-ultraviolet light to promote sporulation. Slide preparations were made in lactic acid and 30 examples of each structure measured. The 95% confidence interval was also determined for conidial dimensions, extremes in conidium length and width are given in parentheses in the description of taxa. To determine their cardinal temperature requirements for growth, isolates were plated on MEA and incubated for 8 days in the dark at seven different temperatures ranging from 10-40°C in 5°C intervals. Linear mycelial growth was measured by calculating the mean of four perpendicular colony radial measurements of three

repeats for every isolate at each temperature studied. The experiment was repeated once. Colony colour (reverse) was determined after 8 days at 25-30°C in the dark using the colour designations of Rayner (1970).

DNA isolation and amplification

Single-conidial isolates were grown on MEA plates and incubated at 25°C for 2-4 wks. DNA was extracted from fresh mycelium using the Promega Kit for isolation of genomic DNA from plant tissue (Promega Corporation, Madison, Wisconsin). The Nuclei lysis solution was substituted with SDS extraction buffer (20% SDS, 2 M Tris-HCl (pH 8), 1 M NaCl, 0.5 M EDTA).

Regions of two genes were amplified. The 5.8S nuclear ribosomal RNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) were amplified using primers ITS1 and ITS4 (White *et al.*, 1990). A 600 base pair fragment of the 5' end of the β-tubulin gene was amplified with primers T1 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass *et al.*, 1995). PCR reactions (total volume of 25 µL) comprised of 1.5 units Biotaq (Bioline, London, UK), 1 mM deoxynucleoside triphosphates, 4mM $MgCl_2$, 0.5 µM primer oligonucleotide and approximately 10 to 30 ng of fungal genomic DNA. Reactions were performed on a Perkin Elmer (Gene Amp PCR System 2400). PCR reactions consisted of the following: an initial denaturation for 4 min at 95°C, followed by 30 cycles of 1 min at 96°C, 30 s at 50°C and 1 min 30 s at 72°C. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen Inc., Valencia, California). Both strands of the ITS and β-tubulin PCR products were sequenced using the ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut). A Dye Terminator Cycle Sequencing Ready Reaction Kit containing an AmpliTaq DNA Polymerase (Perkin-Elmer) was used for the sequencing reactions. DNA samples were purified using Centri-Sep Spin columns (Princeton Separations, Adelphia, New Jersey) and loaded on the sequencing gel. A consensus sequence was created on Sequence Navigator for each isolate.

Phylogenetic analysis

Sequences obtained from this study and GenBank were aligned using the DNA and Protein Sequence Alignment (DAPSA) programme developed by Harley (1998). Alignment gaps were coded as missing data in the analysis. The sequences of

Fusarium oxysporum were used as outgroup for the joint data of ITS and β-tubulin (GenBank: AF132800 & U34424). *Amphisphaeria umbrina* (GenBank: AF009805) was used as outgroup for the analysis of ITS data. Fifty-four ambiguous characters from site 48 to 101 in the alignment were excluded from the analysis. Phylogenetic analyses were performed with PAUP* version 4.0b2a (Swofford, 1999). Maximum parsimony analysis was conducted using branch-and-bound search or heuristic search option using 1000 random addition sequence replicates. Bootstrap support (Felsenstein 1985) for internal branches was evaluated from 1000 heuristic searches and decay indices calculated using AutoDecay (Eriksson 1998) to further test the robustness of branches. Other measures including tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were also calculated. Congruence between the ITS and β-tubulin sequence data sets of 18 taxa was measured using the partition homogeneity test in PAUP*.

results

DNA phylogeny

Maximum parsimony analysis of the ITS1 data generated 15 equally most parsimonious trees (MPTs) with 61 parsimony informative characters in the alignment. The majority consensus tree of the 15 MPTs was evaluated with 1000 bootstrap replications and decay indices for clade stability. Maximum parsimony analysis of the combined data sets of ITS and the partial β-tubulin gene resulted in three trees with 588 parsimony informative characters in the alignment. The phylogenetic tree topology was evaluated with 1000 bootstrap replications and decay indices for the clade stability. The result of the partition homogeneity test ($P = 1.00$, where $P \geq 0.05$ was significantly incongruent) indicated that the two data sets are congruent. The final phylogenetic trees (Figs. 1 & 2) were compatible with accepted morphological delimitation of taxa in the *Phaeoacremonium*-complex.

In the ITS data set (Fig. 1), clade 1 represents *Pm. rubrigenum*, *Pm. parasiticum* and *Pm. inflatipes*. This clade consists mainly of human isolates. Clade 2 represents isolates of *Pm. aleophilum* and *Pm. angustius*, and with the exception of four isolates, all isolates were obtained from *Vitis vinifera*. This data set alone,

however, was insufficient to distinguish between all the morphological species acknowledged in *Phaeoacremonium*.

Aligned sequences of primers T1 and Bt2b showed 100% similarity between isolates CBS 101737-101739 (*Pm. viticola*) and CBS 100947 (*Pm. angustius*). The ex-type strain of *Pm. angustius* (CBS 249.95) differed from these isolates with 9 informative positions (1.7%). The ITS data showed 100% similarity between CBS 101737, 101739, 100947, and the type of *Pm. angustius*. Five deletions and one transversion (G to C) were observed for CBS 101738 when compared to the ITS sequencing data of other isolates of *Pm. angustius* (Table 2). Interspecies variation for *Pm. aleophilum* and *Pm. angustius* is shown in Table 2. More intraspecific variation was observed among isolates of *Pm. aleophilum* in the β-tubulin data set than in the ITS data set.

The combined data set (Fig. 2) supported the separation of *Pa. chlamydospora* from the genus *Phaeoacremonium*. Furthermore, the separation of *Pm. angustius* from *Pm. aleophilum* was strongly supported by bootstrap (99%) and a decay index (16) (Fig. 2), while isolates CBS 101737, 101738 and 101739 previously identified as *Pm. viticola* (Dupont *et al.* 2000a) were shown to be indistinguishable from *Pm. angustius*. Two similar isolates obtained from *V. vinifera* (CBS 101585 and 211.97) were found to be distinct from the presently known species based on morphology, cultural characteristics and phylogeny (Fig. 2), and are described as a new species of *Phaeoacremonium* below.

Taxonomic part

Phaeoacremonium mortoniae Crous & W. Gams, sp. nov. Figs. 3 & 4

Etym.: Named in honour of Dr Lucie Morton, for her contribution towards the understanding of black goo disease of grapevines.

Mycelium consisting of branched, septate hyphae occurring singly or in strands of up to 15, tuberculate (warts to 0.5 µm) to finely verruculose, pale brown to medium red-brown walls with darker septa, becoming lighter towards the conidiogenous region, 2–4 µm wide. *Chlamydospore*-like structures not observed. *Conidiophores* micronematous to macronematous, arising from aerial or submerged hyphae, erect, simple, cylindrical, pale brown, smooth to finely verruculose, straight to flexuous, 0–

3-septate, variable in length, 3–80 µm tall, 1–3 µm wide at base, generally not constricted at septa. *Conidiogenous cells* solitary, terminal or lateral, mostly monophialidic, pale brown to subhyaline, smooth to finely verruculose, elongate-ampulliform to lageniform or subcylindrical, not to prominently constricted at base, 3–20 µm long, 1–4 µm wide at the swollen part, 1.0–1.5 µm wide at the apex, with a terminal, narrowly funnel-shaped collarette, 1–2 µm long and wide. *Conidia* becoming aggregated in slimy heads at apices of conidiogenous cells, hyaline, dimorphic, partly subcylindrical, straight to allantoid, (3–)4–7 x 1.0–1.5(–2.0) µm, and partly shorter and ellipsoidal, 3–4(–7) × 1.5–2.0 µm.

Type. U.S.A. California, Sonoma County vineyard, stem of 13-yr-old Chardonnay vine, 20 Jul. 1998, L. Morton & L. van der Water, 12RS2-72098, (PREM 57084, dried holotype specimen, ex-type culture CBS 101585).

Cultural characteristics. Colonies on MEA (reverse) fuscous black (7""k), or alternating rings of fuscous black and greyish sepia (15""i), outer ring of growth greyish sepia to honey (21"b); surface pale mouse grey to mouse grey (15""d – 15""i), uniform in colour, or with a slightly lighter outer ring of smoke grey (21""f) mycelium, with or without a brown pigment that can diffuse up to 3 cm from the colony into the agar; colony margins smooth, surface forming radiating ridges in the agar with sparse aerial hyphae that tend to form hyphal strands, giving colonies a slightly woolly appearance in the middle; colonies reaching a radial growth of 10–11 mm at 30°C in the dark after 8 d. Cardinal temperatures for growth: min above 10°C, opt 30°C, max below 35°C.

Host. Fraxinus excelsior, Vitis vinifera.

Distribution. Sweden, U.S.A. (California)

Additional culture examined. SWEDEN. Stem wound in *Fraxinus excelsior*, under stripped bark, Dec. 1996, J. Stenlid, CBS 211.97.

Phaeoacremonium mortoniae can be distinguished from other species in the genus by its unique cultural characteristics. Of the species that have an optimal growth rate at 30°C (*Pm. rubrigenum* and *Pm. inflatipes*; Crous *et al.*, 1996), it is distinguished by having darker, fuscous black to greyish sepia colonies (reverse), as well as a diffuse brown pigment that can also form in the agar. These cultural

differences are also supported by its distinct phylogeny based on ITS and β-tubulin sequence data. Based on morphology alone, however, it would be difficult to distinguish these species, which once again stresses the importance of integrating cultural characteristics and molecular data for the identification of these taxa.

Although regarded as similar (Dupont *et al.*, 1998, 2000a, 2000b), isolates of *Pm. aleophilum* and *Pm. angustius* could be distinguished in the present study based on DNA phylogeny (Fig. 2) and cultural characteristics. All 12 isolates of *Pm. aleophilum* tested, obtained optimum growth on MEA at 30°C after 8 days, and were able to grow at 35°C. In contrast, however, isolates of *Pm. angustius* and *Pm. viticola* obtained optimum growth at 25°C, and were unable to grow at 35°C. A diffuse yellow pigment was observed in the agar for some isolates of *Pm. aleophilum* when incubated as described above. A similar yellow diffused pigment was also observed for isolates of *Pm. angustius*, while others again became red-purple in reverse, similar to that observed for isolates of *Pm. viticola*. Isolates of *Pm. mortoniae* again produced a more brownish diffused pigment. These pigments were more pronounced in older cultures, but generally disappeared with successive subculturing.

DISCUSSION

The molecular data obtained in this study confirm those of previous studies (Dupont *et al.*, 1998; Groenewald *et al.*, 2000), showing that *Pm. chlamydosporum* was more closely related to *Phialophora sensu stricto* (Herpotrichiellaceae), than other species of *Phaeoacremonium* (Magnaporthaceae). These data, as well as the morphological differences discussed by Crous & Gams (2000), therefore support the new genus *Phaeomoniella* as being distinct from *Phaeoacremonium*.

Dupont *et al.* (1998, 2000a, 2000b) also found insufficient evidence to distinguish *Pm. aleophilum* and *Pm. angustius*. Using sequence data (ITS and β-tubulin) of the type strains, as well as RFLP patterns of additional strains, they concluded that these two species were conspecific, with the ITS1 rDNA region showing only two nucleotide differences between these species, and the 5' end of the β-tubulin gene showing no differences (Dupont *et al.*, 2000a). Furthermore, they also found growth patterns to be similar, though the incubation period was significantly longer than that proposed in previous studies (Crous *et al.*, 1996; Crous & Gams,

2000). In the present study we were able to distinguish these two species by their ability to grow on MEA at 35°C (*Pm. aleophilum*) or below (*Pm. angustius*) after an incubation period of 8 d in the dark. Furthermore, although similar, we observed more genotypic divergence between these two species than that initially reported by Dupont *et al.* (1998, 2000a, 2000b).

Sequences of the partial β-tubulin gene of the ex-type strains of *Pm. aleophilum* (CBS 246.91) and *Pm. angustius* (CBS 249.95) showed distances of 15.5% from each other and for the rDNA data set differences of 4% were observed. Three isolates representing *Pm. viticola* (Dupont *et al.*, 2000a) (CBS 101739, 101737, 101738) were also found to be the same as *Pm. angustius* (CBS 249.95), with a 100% sequence similarity in the rDNA region. Phylogenetic analysis of the combined data set supported the separation of *Pm. angustius* from *Pm. aleophilum* with a strong bootstrap value and decay index. A practical and easy method for distinguishing these species seems to remain their cardinal temperatures for growth. *Pm. aleophilum* has an optimum growth rate at 30°C, and can grow at 35°C, whereas *Pm. angustius* has an optimum growth at 25°C and does not grow at 35°C. Although morphologically similar, the separation of *Pm. angustius* and *Pm. aleophilum* is therefore supported based on the DNA phylogeny and cultural growth characteristics.

This study has further shown that *Pm. aleophilum* is a prominent organism in grapevine decline with a wide geographic distribution, and that isolates thought to be *Pm. inflatipes* were in fact *Pm. aleophilum*. In fact, no records of *Pm. inflatipes* could be confirmed from grapevine, and its occurrence in this host remains to be proven.

Two prominent clades were resolved within *Phaeoacremonium* (Fig. 1). At present, however, we are not aware of any morphological characteristics that can be used to further divide the genus. Clade 1 consists of *Pm. rubrigenum*, *Pm. inflatipes* and *Pm. parasiticum*. This clade predominantly represents strains obtained from human patients. Although *Pm. parasiticum* is morphologically more similar to *Pm. inflatipes* (predominently warty hyphae), *Pm. rubrigenum* is again distinct in having more finely verruculose hyphae. Isolates of *Pm. rubrigenum* have thus far been mostly isolated from human hosts, and thus strain STE-U 3092, obtained from grapevines in South Africa, needs further clarification.

The second clade represents species obtained from plants (chiefly *Vitis vinifera*), namely *Pm. aleophilum*, *Pm. mortoniae* and *Pm. angustius*. These species are morphologically more uniform, and if evidence were ever presented to divide the genus further, these three taxa would clearly form one group that has paler to medium brown, finely verruculose to verruculose hyphae.

In conclusion, the *Phaeoacremonium* complex in *Vitis vinifera* is extremely difficult to identify based on morphological characteristics alone. It is therefore not surprising that in this study we found many *Phaeoacremonium* isolates to be misidentified. More isolates need to be included in future studies to resolve questions relating to what species occur in grapevines, and which of these play a role in decline disease of this host, as well as other plants and human disorders. Molecular techniques will play an increasingly important role in identification of relevant isolates. Here diagnostic testing with specific primers (Groenewald *et al.*, 2000; Tegli *et al.*, 2000) will also be important.

REFERENCES

Chiarappa, L. 1959. Wood decay of the grapevine and its relationship with black measles disease. *Phytopathology* **49**, 510-519.

Crous, P.W., Gams, W., Wingfield, M.J. & Van Wyk, P.S. 1996. *Phaeoacremonium* gen. nov. associated with wilt and decline disease of woody hosts and human infections. *Mycologia* **88**, 786-796.

Crous, P.W. & Gams, W. 2000. *Phaeomoniella chlamydospora* gen. et comb. nov., a causal organism of Petri grapevine decline and esca. *Phytopathologia Mediterranea* **39**, 112-118.

Dupont, J., Laloui, W. & Roquebert, M.F. 1998. Partial ribosomal DNA sequences show an important divergence between *Phaeoacremonium* species isolated from *Vitis vinifera*. *Mycological Research* **102**, 631-637.

Dupont, J., Laloui, W., Magnin, S., Larignon, P. & Roquebert, M.F. 2000a. *Phaeoacremonium viticola*, a new species associated with Esca disease of grapevine in France. *Mycologia* **92**, 499-504.

Dupont, J., Magnin, S., Paronnaud, J. & Roquebert, M.F. 2000b. The genus *Phaeoacremonium* from a molecular point of view. *Phytopathologia Mediterranea* **39**, 119-124.

Eriksson, T. 1998. AutoDecay Ver. 4.0 (programme distributed by the author). Department of Botany, Stockholm University, Stockholm.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using bootstrap. *Evolution* **39**, 783–791.

Glass, N.L. & Donaldson, G.C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**, 1323-1330.

Groenewald, M., Bellstedt, D.U. & Crous, P.W. 2000. A PCR-based method for the detection of *Phaeomoniella chlamydospora* in grapevines. *South African Journal of Science* **96**, 43-46.

Harley, E.H. 1998. Software Programme, DNA and Protein Sequence Alignment (DAPSA). Department of Chemical Pathology, University of Cape Town, Medical School, Observatory, South Africa.

Mugnai, L., Graniti, A. & Surico, G. 1999. Esca (Black measles) and brown wood-streaking: Two old and elusive diseases of grapevines. *Plant Disease* **83**, 404-418.

O'Donnell, K., & Cigelnik, E. 1997. Two different intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics Evolution* **7**, 103-116.

Petri, L. 1912. Osservazioni sopra le alterazioni del legno della vite in seguito a ferite. *Stazioni Sperimentali Agrarie Italiane* **45**, 501-547.

Rayner, R.W. 1970. *A mycological colour chart.* Commonwealth Mycological Institute. Kew, Surrey.

Scheck, H.J., Vasquez, S.J., & Gubler, W.D. 1998. First report of three *Phaeoacremonium* spp. causing young grapevine decline in California. *Plant Disease* **82**, 590.

Swofford, D.L. 1999. PAUP*. Phylogenetic analysis using parsimony (*and other methods), 4.0b1. Computer programme. Sunderland, Massachusetts, U.S.A.

Tegli, S., Bertelli, E. & Surico, G. 2000. Sequence analysis of ITS ribosomal DNA in five *Phaeoacremonium* species and development of a PCR-based assay for the detection of *P. chlamydosporum* and *P. aleophilum* in grapevine tissue. *Phytopathologia Mediterranea* **39**, 134-149.

White, T.J., Burns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. In *A Guide to Molecular Methods and Application* (ed. M.A. Innis, D.H. Gelfand, J. Shinsky & T.J. White), pp. 315-322. Academic Press: San Diego, U.S.A.

Table 1. *Phaeoacremonium* and *Phaeomoniella* isolates studied

Species	Culture no.	GenBank accession no.		Host and location
		ß-tubulin	ITS	
Phaeoacremonium aleophilum	CBS 246.91*	AF246811	AF017651	*Vitis vinifera*, Yugoslavia
	CBS 100397	AF246806	AF197981	*Vitis vinifera*, Italy
	CBS 100399		AF197991	*Vitis vinifera*, Italy
	CBS 100400	AF246807	AF197992	*Vitis vinifera*, Italy
	CBS 101358	AF246808	AF197993	*Actinidia chinensis*, Italy
	CBS 100401		AF197982	*Vitis vinifera*, Italy
	CBS 100402		AF197994	*Vitis vinifera*, Italy
	CBS 100548		AF197983	*Olea europaea*, Italy
	CBS 101568		AF197984	*Vitis vinifera*, California
	STE-U 3094 (MT78)	AF246812	AF197996	*Vitis vinifera*, South Africa
	STE-U 3093 (MT79)	AF246813	AF197985	*Vitis vinifera*, South Africa
	STE-U 3095 (MT80)		AF197995	*Vitis vinifera*, South Africa
	CBS 101006			*Actinidia chinensis*, Italy
	CBS 101008			*Actinidia chinensis*, Italy
Phaeoacremonium angustius	CBS 249.95*	AF246814	AF197974	*Vitis vinifera*, California
	CBS 101739	AF246816	AF197977	*Vitis vinifera*, France
	CBS 101738	AF192391	AF118137	*Vitis vinifera*, France
	CBS 101737	AF246817	AF197976	*Vitis vinifera*, France
	CBS 100947	AF246815	AF197975	*Olea europaea*, Italy
Phaeoacremonium parasiticum	CBS 860.73*	AF246803	U31841	Human, California, USA
	CBS 101007	AF246804	AF197980	*Actinidia chinensis*, Italy
	CBS 513.82		U31842	Human, New York, USA
Phaeoacremonium rubrigenum	CBS 498.94*	AF246802	AF197988	Human, USA
	STE-U 3092 (MT11)	AF246800	AF197978	*Vitis vinifera*, South Africa
	CBS 566.97	AF246801	AF197979	Human, Japan
	CBS 729.97		AF197989	Human, South Carolina, USA
Phaeoacremonium inflatipes	CBS 391.71*	AF246805	AF197990	*Quercus virginiana*, Texas, USA
	CBS 166.75		U31843	*Nectandra* sp., Cosra Rica
Phaeoacremonium mortoniae	CBS 211.97	AF246810	AF295329	*Fraxinus excelsior*, Sweden
	CBS 101585*	AF246809	AF295328	*Vitis vinifera*, California
Phaeomoniella chlamydospora	CBS 229.95*	AF253968	AF197973	*Vitis vinifera*, Italy
	STE-U 3066	AF253969	AF197986	*Vitis vinifera*, South Africa
	STE-U 3067		AF197987	*Vitis vinifera*, South Africa

* Ex-type culture.

Table 2. Intra- and interspecific variation within ITS1-ITS2 and partial β-tubulin gene DNA sequence of selected isolates of *Phaeoacremonium angustius* and *Phaeoacremonium aleophilum*.

Species	ITS1-ITS2				β-tubulin			
	No. of strains	Length	Differences (bp)	Variation (%)	No. of strains	Length	Differences (bp)	Variation (%)
Intraspecies								
Pm. angustius[a]	5	510	0-6	0.0-1.2	5	529	0-9	0.0-1.7
Pm. aleophilum[a]	14	482-490	0-3	0.0-0.6	6	499-528	0-32	0.0-6.4
Interspecies								
Pm. angustius - *Pm. aleophilum* (CBS 246.91, 249.95)[b]	2	510-514	25	4.9	2	500-502	86	17.1-17.2

[a] Strains listed in Table 1.

[b] Ex-type cultures.

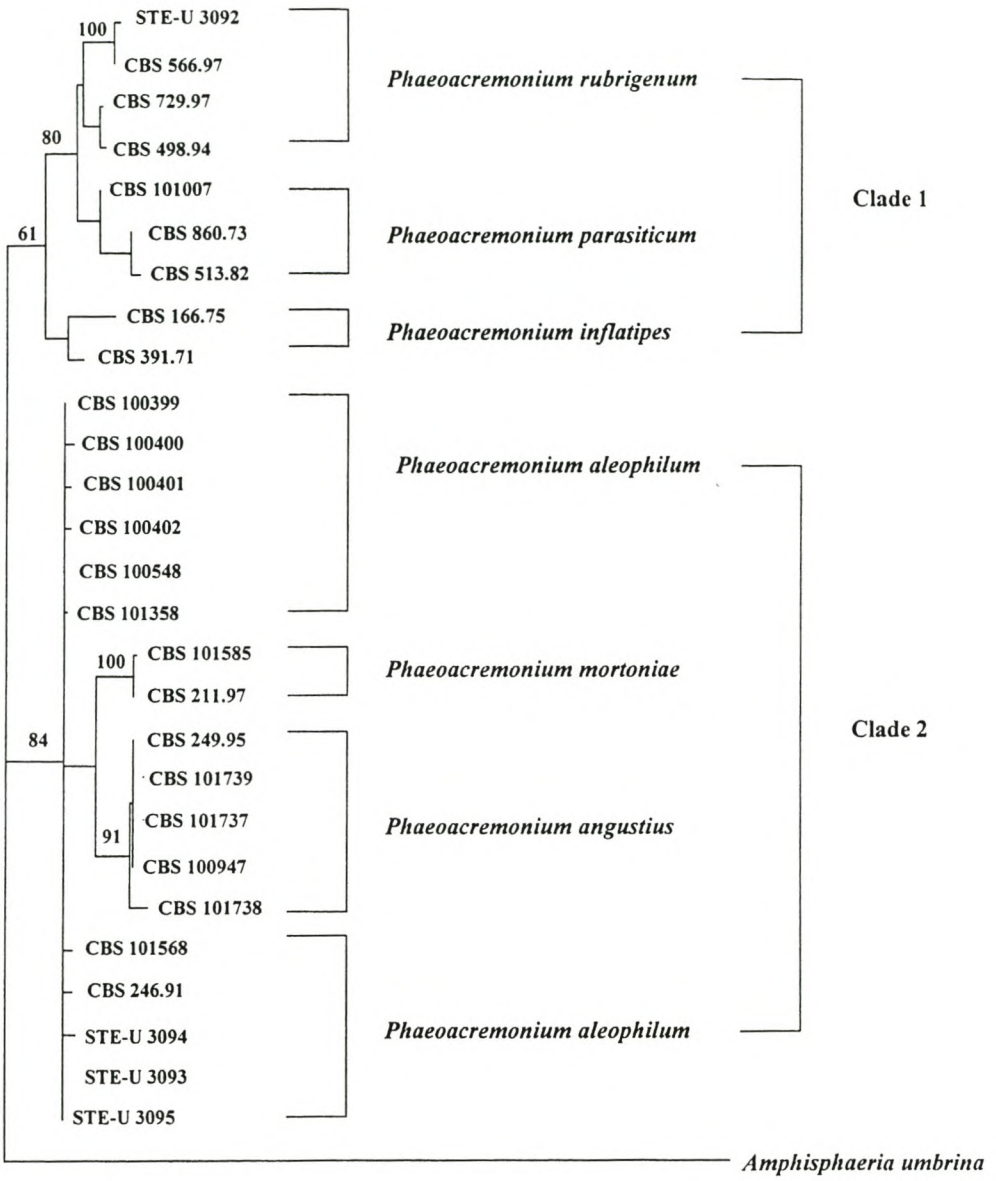


Fig. 1. One of fifteen most parsimonious trees generated with PAUP* 4.0b2a from aligned sequences of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions (tree length 396, CI = 0.826, RI = 0.833, RC = 0.688, HI = 0.174). A sequence of *Amphisphaeria umbrina* (AF009805) was used as outgroup.

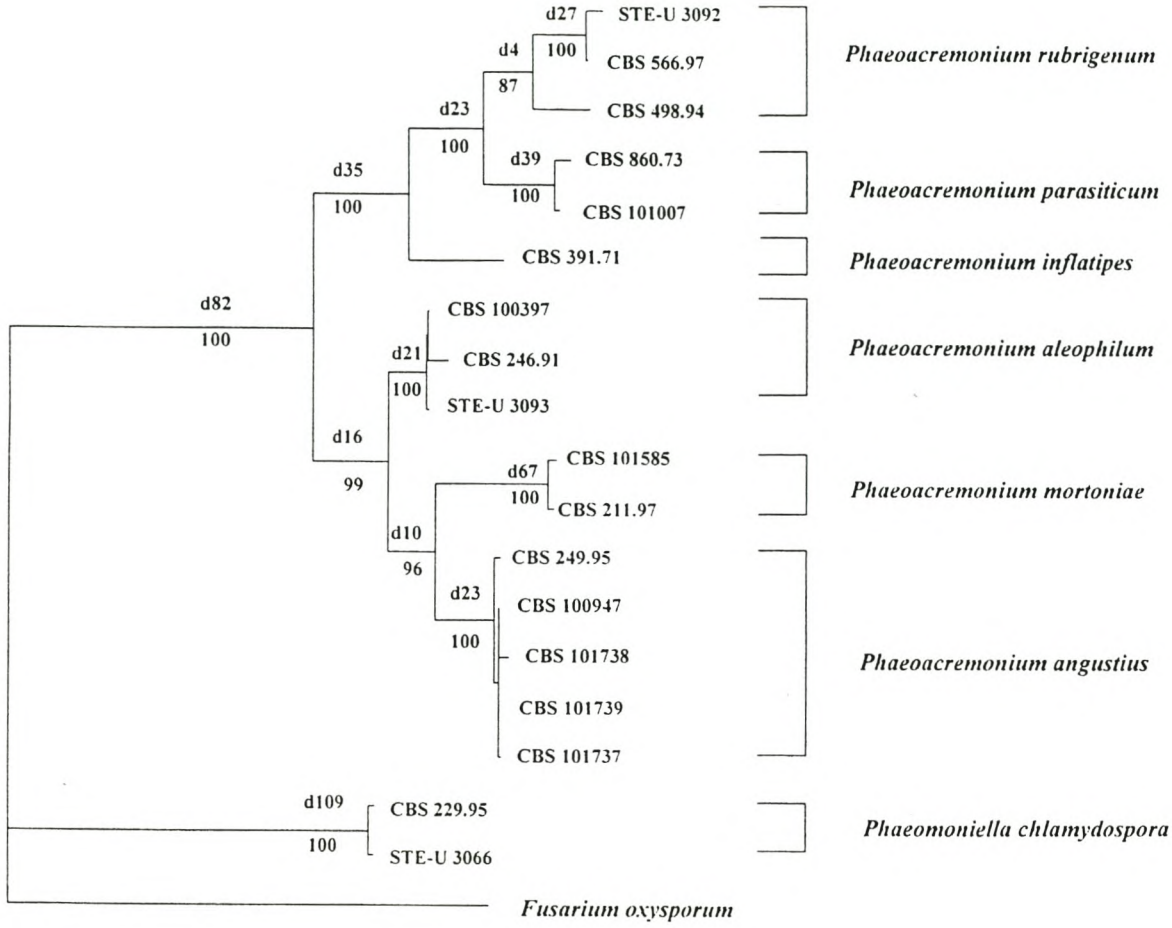
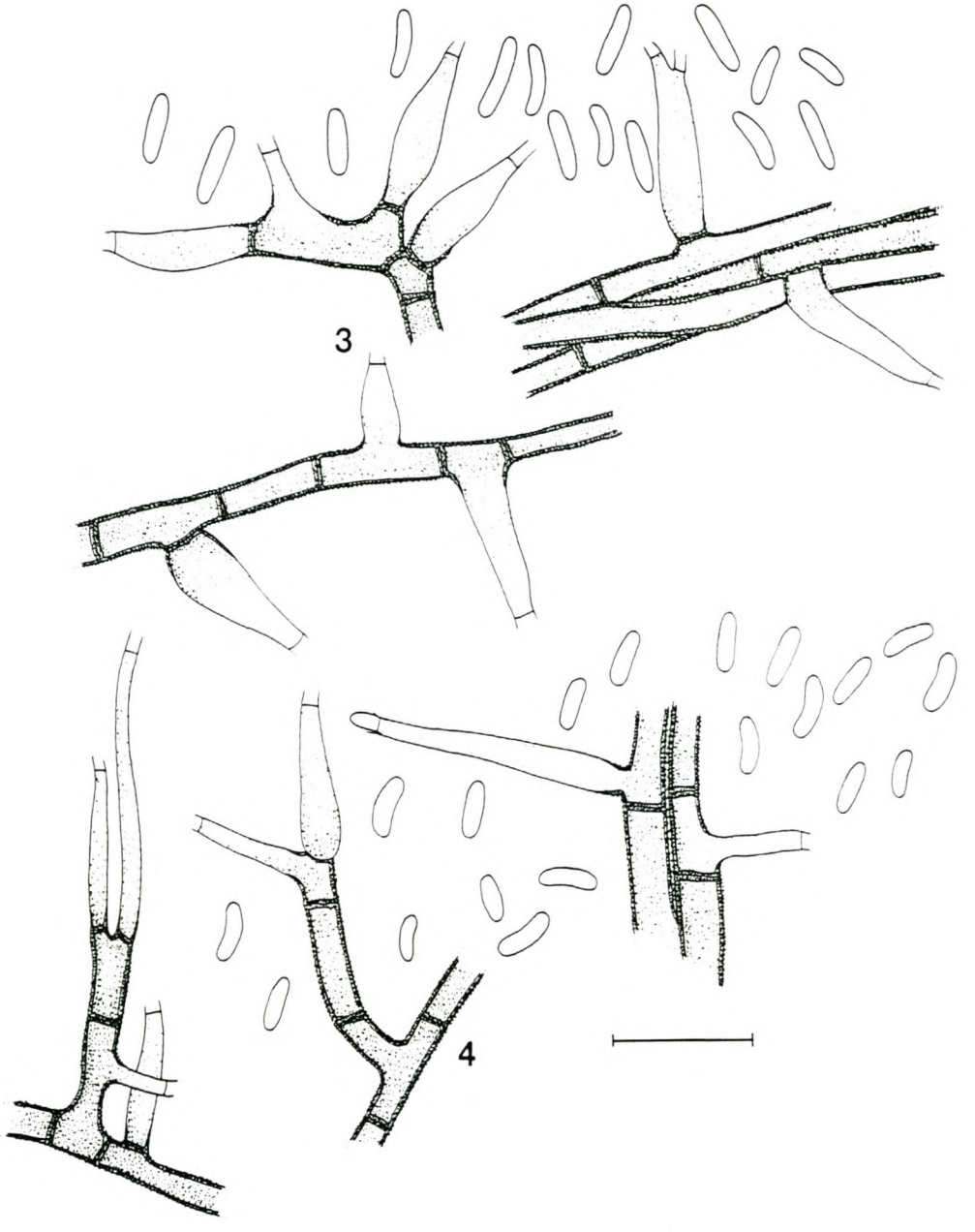


Fig. 2. One of three most parsimonious trees generated with PAUP* 4.0b2a from the combined data set of the aligned sequences of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions and the partial β-tubulin gene (tree length 1571, CI = 0.837, RI = 0.865, RC = 0.724, HI = 0.163). *Fusarium oxysporum* (AF132800 & U34424) was used as outgroup.



Figs. 3 & 4. Conidiophores and conidia of *Phaeoacremonium mortoniae* on MEA. Fig. 3. CBS 211.97 from *Fraxinus*. Fig. 4. CBS 101585 from *Vitis* (ex-type culture).

4. A PCR-BASED METHOD FOR THE DETECTION OF *PHAEOMONIELLA CHLAMYDOSPORA* IN GRAPEVINES

ABSTRACT

The oligonucleotide primers, PCL1 and PCL2, were synthesised for *Phaeomoniella chlamydospora* from the variable internal transcribed spacers ITS1 and ITS2 of the ribosomal DNA, respectively. Polymerase chain reaction (PCR amplification) with primers PCL1 and PCL2 produced a 325 bp fragment from isolates of *Pa. chlamydospora*. Amplification of this fragment was achieved from as little as 16 pg of fungal DNA. The specific primers amplified a 325 bp fragment from infected grapevine tissue. Fungal DNA from closely related genera, *Phaeoacremonium* and *Phialophora*, as well as several other fungi commonly occurring in grapevine stems, showed no amplification with the species-specific primers.

INTRODUCTION

Phaeomoniella chlamydospora is associated with grapevine diseases such as esca, apoplexy, black measles, slow die-back, and has recently been identified as the causal organism of Petri grapevine decline (Crous & Gams, 2000). Esca and related diseases have an extensive range of symptoms and have been described from most countries where grapevines are cultivated (Petri, 1912; Viala, 1926; Chiarappa, 1959; Dubos & Larignon, 1988; Ferreira *et al.*, 1994; Morton, 1995; Mugnai *et al.*, 1996; Scheck *et al.*, 1998; Mugnai *et al.*, 1999). In 1959 Chiarappa concluded that black measles of grapevines was in fact the same as apoplexy, and was caused by a *Cephalosporium* sp. which he consistently isolated from diseased vines. Several years later, Ajello *et al.* (1974) described a new species isolated from a subcutaneous phaeohyphomycotic infection of a human patient as *Phialophora parasitica*. In a subsequent study of this fungus, Hawksworth *et al.* (1976) reported that it had been associated with various woody hosts, but noted that the *Vitis* isolate originally collected by Chiarappa (1959) had some morphological differences. In South Africa, Ferreira *et al.* (1994) conducted pathogenicity tests with similar grapevine isolates, and demonstrated that this species (as *P. parasitica*) could cause wood discoloration, as well as extensive plugging of xylem tissue.

As more isolates of the *Phialophora parasitica* complex were obtained from diseased grapevines world-wide, it became obvious that these strains represented a group morphologically distinct from *Phialophora*. The fact that they had pigmented phialides with inconspicuous, non-flaring collarettes, resembling pigmented forms of *Acremonium*, resulted in the name *Phaeoacremonium* to be introduced to accommodate these fungi (Crous *et al.*, 1996). The separation of *Phaeoacremonium* from *Phialophora* was also supported by ITS sequence data published by Yan *et al.* (1995). In a further molecular circumscription of the genus, however, Dupont *et al.* (1998) presented ITS sequence data demonstrating that *Phaeoacremonium* was polyphialetic. *Pm. chlamydosporum* appeared to be more closely related to *Phialophora sensu stricto* (Herpotrichiellaceae), than other species of *Phaeoacremonium* (Magnaporthaceae). In subsequent research conducted in our laboratory, two respective data sets, namely of the ITS1, 5.8S and ITS2 region, as well as the beta-tubulin gene (M. Groenewald *et al.*, in prep.) supported the phylogenetic study of Dupont *et al.* (1998).

A re-examination of numerous *Pm. chlamydosporum* isolates from diverse geographical regions also showed several prominent morphological differences to be present between this species and others accommodated in *Phaeoacremonium sensu stricto*. Based on the molecular, morphological and pathological differences, a new genus, *Phaeomoniella* was therefore introduced, typified by *Pa. chlamydospora* (Crous & Gams, 2000).

Although grapevine decline has always been a problem in older vines, it has recently also become a problem in young vines (Morton, 1995; Scheck *et al.*, 1998; Ferreira *et al.*, 1998; Mugnai *et al.*, 1999). In isolations conducted from diseased young vines during the last two years in our laboratories, *Pa. chlamydospora* has regularly been isolated. Furthermore, during a survey of four grapevine nurseries, Ferreira (1998) found that the disease occurred in all the rootstocks, irrespective of the scion. In diseased vines, black vascular streaking was observed from the roots upwards, indicating possible infection through the roots, thus suggesting that *Pa. chlamydospora* could also be soil-borne. Furthermore, *Pa. chlamydospora* can also occur as a latent pathogen (or endophyte) in apparently healthy propagation material. This was first reported from Italy by Bertelli *et al.* (1998) and has

subsequently also been confirmed from apparently healthy South African nursery material (M. Groenewald, unpublished data).

Planting grapevine plants already infected with *Pa. chlamydospora* is fatal for the successful establishment of young vineyards. Petri grapevine decline can take years to develop, and therefore it is crucial to know that the propagation material received by farmers is healthy to begin with. To ensure that only healthy material is planted, nursery material should be screened for the fungus. The isolation of *Pa. chlamydospora* from symptomless grapevine wood is difficult and time-consuming. *Pa. chlamydospora* is a slow-growing fungus, and positive identification from infected material can take up to four weeks. Therefore, a rapid, sensitive method was necessary for the detection of the pathogen in nursery material, which could form the basis for the establishment of a nursery certification scheme for farmers. The aim of this study, therefore, was to develop species-specific primers for the detection of *Pa. chlamydospora* in symptomless grapevines. The ribosomal RNA genes occur in high numbers, consisting of variable and conserved areas, which can be amplified by universal primers (Bruns *et al.*, 1991). The rDNA also consists of multi-copy target sequences allowing greater sensitivity. This area has successfully been used for designing species-specific primers for many fungal taxa (Nazar *et al.*, 1991; Brown *et al.*, 1993; Screenivasaprasat *et al.*, 1996; Johanson *et al.*, 1998). In this study I describe the generation of species-specific primers for the detection of *Pa. chlamydospora* in grapevine tissue.

MATERIALS AND METHODS

Fungal isolates

Cultures of *Phaeoacremonium* spp. and *Pa. chlamydospora* were either obtained from symptomatic vines in South Africa or from the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands. Cultures or sequence data of related *Phialophora* spp. were respectively obtained from CBS or Genbank (Table 1). Several endophytic and plant pathogenic fungi isolated from grapevine wood were also used in this study to test the specificity of the species-specific primers.

Inoculation of grapevine plants with *Pa. chlamydospora*

Five tissue culture plants were inoculated by spraying a spore suspension of *Pa. chlamydospora* (1 ml, 1 x 10^6 spores/ml) onto the growth medium of each plant. An additional five plants were wounded by injecting a drop of spore suspension into their stems. Control plants were treated in a similar manner, using sterile water. Plants were maintained in a growth room at 22°C with a 24 h cool fluorescent white light photoperiod.

Extraction of DNA from fungal cultures and grapevine plants

Single conidial isolates were grown on malt extract agar (MEA; Biolab, Midrand, Johannesburg) plates and incubated at 25°C for 1-4 wks, depending on the growth rate of each isolate. DNA was extracted from growing cultures using the Promega Kit (Promega Corporation, Madison, Winconsin). The Nuclei lysis solution was substituted with SDS extraction buffer (20 % SDS, 2 M Tris-HCl (pH 8.0), 1 M NaCl, 0.5 M EDTA). Total DNA from inoculated grapevine plants was extracted using the modified CTAB (Hexadecyltrimethylammonium bromide) method of Doyle & Doyle (1987).

PCR amplification

The rDNA region of *Phaeoacremonium* and *Phaeomoniella* isolates (Table 1) was amplified with primers ITS1 and ITS4 (White *et al.*, 1990). The amplified region included the 5.8S ribosomal gene and the two internal transcribed spacers (ITS1 and ITS2), flanking the gene. PCR reactions (total volume of 25 µL) were performed using 1.5 units Biotaq (Bioline, London, UK), 1 mM deoxynucleoside triphosphates, 4 mM $MgCl_2$, 0.5 µM of each primer, and approximately 10 to 30 ng of fungal genomic DNA. Reactions were performed on a Perkin Elmer thermal cycler (Gene Amp PCR system 2400) programmed as follows: an initial denaturation for 4 min at 95°C, followed by 30 cycles of 1 min at 96°C, 30 s at 50°C and 1 min at 72°C.

DNA sequencing and data analysis

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, California). A Dye Terminator Cycle Sequencing Ready Reaction Kit containing AmpliTaq DNA Polymerase (Perkin Elmer) was used for the sequencing

reaction. rDNA was sequenced with the forward and reverse primers, ITS1 and ITS4, using the ABI Prism 377 DNA Sequencer (Perkin Elmer, Norwalk, Connecticut). A consensus sequence was created on Sequence Navigator for each isolate. The sequences of *Pa. chlamydospora*, *Phaeoacremonium* spp. (including all the type strains) and *Phialophora verrucosa* (sequences from Genbank), were aligned using the DNA and Protein Sequence Alignment (DAPSA) programme developed by Harley (1998). A phylogenetic analysis of the partial data set was performed using the PAUP* (Phylogenetic Analysis Using Parsimony) 4.0b1 programme (Swofford, 1998). Confidence intervals were determined using a 1000 bootstrap replication and *Amphisphaeria umbrinis* (AF009805) was used as outgroup.

Design of species-specific primers

After aligning the sequence data, the nucleotide sequences of the various species were compared. Regions in ITS1 and ITS2 where *Pa. chlamydospora* isolates varied from all the other species were identified. Two species-specific primers, corresponding to bases 160-180 of ITS1 and 465-485 of ITS2, of *Pa. chlamydospora* (CBS 229.95 / AF197973) were designed using 'Primer Designer Version 1.01'. These primers were synthesised by the DNA Synthesis Laboratory, Department of Biochemistry, UCT, South Africa, and designated as PCL1 and PCL2 (Fig. 1).

Testing of species-specific primers

The primers were tested for *Pa. chlamydospora* specificity with purified genomic DNA extracted from the isolates shown in Table 1. Other species of fungi, some related to *Pa. chlamydospora*, and some routinely isolated from grapevines were also tested. These included *Phialophora verrucosa*, *P. richardsiae*, *P. americana*, *Eutypa lata*, *Phomopsis* spp. (including *P. viticola*), *Cylindrocarpon destructans* and *Botryosphaeria* spp. (including *B. obtusa* and *B. dothidea*). Genomic DNA of tissue culture grapevine plants, inoculated with *Pa. chlamydospora* (wounded and unwounded), were also tested with primers PCL1 and PCL2. PCR amplification with primers PCL1 and PCL2 was performed on a Perkin Elmer thermal cycler (Gene Amp PCR System 2400), essentially as described earlier, but with different cycling conditions: 30 s at 96°C, followed by 30 cycles of 30 s at 96°C, 30 s at 68°C, 35 s at 75°C and a 5 min extension step at 75°C to complete the reaction. The PCR products

(25 µL) were separated on a 2% agarose gel containing ethidium bromide, and visualised using an UV transilluminator.

RESULTS AND DISCUSSION

Genomic DNA of *Pa. chlamydospora* was tested with PCL1 and PCL2. A fragment of 325 base pairs (bp) was visualised, cleaned and sequenced. Sequencing of this fragment confirmed the PCR product as the DNA area between primers PCL1 and PCL2 of *Pa. chlamydospora*. *Pa. chlamydospora* isolates from different geographical areas were also tested with primers PCL1 and PCL2. All isolates showed a fragment of 325 bp on a 2% agarose gel. Amplification was achieved from genomic DNA of *Pa. chlamydospora* as low as 16 pg.

The nucleotide sequence data of the *Phaeoacremonium* spp. were deposited in Genbank (Table 1). Although all isolates were tested with primers PCL1 and PCL2, a positive amplification was obtained with *Pa. chlamydospora* only. A phylogenetic tree was constructed for the five *Phaeoacremonium* spp., as well as *Pa. chlamydospora* and the closely related *Phialophora verrucosa* (Fig. 2). Based on this phylogeny, as well as the original report by Dupont *et al.* (1998) that *Pa. chlamydospora* appeared more closely related to *P. verrucosa* than to *Phaeoacremonium*, the primers were also tested against closely related *Phialophora* species (Yan *et al.*, 1995). *P. verrucosa*, *P. americana* and *P. richardsiae* showed no amplification with primers PCL1 and PCL2. Furthermore, the common fungal taxa normally isolated from grapevines (listed earlier) also failed to amplify with these primers. These results indicate that the primers PCL1 and PCL2 are specific to *Pa. chlamydospora*, as no amplification products were obtained from genomic DNA of any of the other taxa tested.

The grapevine plants inoculated with *Pa. chlamydospora* (both wounded and unwounded) were also tested with the primers PCL1 and PCL2. DNA was extracted from the asymptomatic grapevines 2 wks after inoculation. PCR banding patterns of inoculated vines corresponded to those of *Pa. chlamydospora* pure fungal genomic DNA (Fig. 3). No fragment was observed from the control grapevine DNA. A portion of the inoculated plant tissue, which was used for DNA isolation, was also plated onto MEA plates. *Pa. chlamydospora* was detected on these plates 2-3 wks

after inoculation. In contrast, using the specific primers, the presence of *Pa. chlamydospora* was detected within 24 hrs. In conclusion, this diagnostic PCR test allows rapid and accurate identification of *Pa. chlamydospora* in grapevines. Further testing with nursery material should now be conducted on a larger scale to further refine the process. The application of these primers for detection of *Pa. chlamydospora* in woody material should be evaluated to determine the role that these primers can play in future certification schemes for grapevine farmers in South Africa.

REFERENCES

Ajello, L., Georg, L.K., Steigbigel, R.T. & Wang, C.J.K. 1974. A case of Phaeohyphomycosis caused by a new species of *Phialophora*. *Mycologia* **66**, 490-498.

Bertelli, E., Mugnai, L. & Surico, G. 1998. Presence of *Phaeoacremonium chlamydosporum* in apparently healthy rooted grapevine cuttings. *Phytopathologia Mediterranea* **37**, 79-82.

Brown, A.E., Muthumeenakshi, S., Sreenivasaprasad, S., Mills, P.R. & Swinburne, T.R. 1993. A PCR primer-specific to *Cylindrocarpon heteronema* for detection of the pathogen in apple wood. *FEMS Microbiology Letters* **108**, 117-120.

Bruns, T.D., White, T.J. & Taylor, J.W. 1991. Fungal molecular systematics. *Annual Review of Ecology and Systematics* **22**, 525-564.

Chiarappa, L. 1959. Wood decay of the grapevine and its relationship with black measles disease. *Phytopathology* **49**, 510-519.

Crous, P.W. & Gams, W. 2000. *Phaeomoniella chlamydospora* gen. et comb. nov., the causal organism of Petri grapevine decline. *Phytopathologia Mediterranea* **39**, 112-118.

Doyle, J.J. & Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bulletin* **19**, 11-15.

Dubos, B. & Larignon, P. 1988. Esca and Black Measles. In *Compendium of grape diseases* (ed. R.C. Pearson & A.C. Goheen), pp. 34-35. APS Press: St. Paul, Minnesota, U.S.A.

Dupont, J., Laloui, W. & Roquebert, M.F. 1998. Partial ribosomal DNA sequences show an important divergence between *Phaeoacremonium* species isolated from *Vitis vinifera*. *Mycological Research* **102**, 631-637.

Ferreira, J.H.S., Van Wyk, P.S. & Venter, E. 1994. Slow dieback of grapevine: Association of *Phialophora parasitica* with slow dieback of grapevines. *South African Journal of Enology and Viticulture* **15**, 9-11.

Ferreira, J.H.S. 1998. *Phialophora* terugsterwing - 'n Algemene probleem by jong wingerde. *Wynboer* **3**, T6-T8.

Harley, E.H. 1998. Software Programme, DNA and Protein Sequence Alignment (DAPSA). Department of Chemical Pathology, University of Cape Town, Medical School, Observatory, South Africa.

Hawksworth, D.L., Gibson, A.S. & Gams, W. 1976. *Phialophora parasitica* associated with disease conditions in various trees. *Transactions of the Britsh Mycological Society* **66**, 427-431.

Johanson, A., Turner, H.C., McKay, G.J. & Brown, A.E. 1998. A PCR-based method to distinguish fungi of the rice sheath-blight complex, *Rhizoctonia solani*, *R. oryzae* and *R. oryzae-sativae*. *FEMS Microbiology Letters* **162**, 298-294.

Morton, L. 1995. Mystery diseases hit young vines. *Wines and Vines* **76**, 46-47.

Mugnai, L., Surico, G. & Esposito, A. 1996. Microflora associata al mal dell'esca della vite in Toscana (Fungal microflora associated with 'esca' grapevine disease in Tuscany). *Informatore Fitopatologico* **46**, 49-55.

Mugnai, L., Graniti, A. & Surico, G. 1999. Esca (Black measles) and brown wood-streaking: Two old and elusive diseases of grapevines. *Plant Disease* **83**, 404-418.

Nazar, R.N., Hu, X., Schmidt, J., Culham, D. & Robb, J. 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of verticillium wilt pathogens. *Physiological and Molecular Plant Pathology* **39**, 1-11.

Petri, L. 1912. Osservazioni sopra le alterazioni del legno della vite in seguito a ferite. *Le Stazioni Sperimentali Agrarie Italiane* **45**, 501-547.

Scheck, H.J., Vasquez, S.J. & Gubler, W.D. 1998. First report of three *Phaeoacremonium* spp. causing young grapevine decline in California. *Plant Disease* **82**, 590.

Screenivasaprasad, S., Sharada, K., Brown, A.E. & Mills, P.R. 1996. PCR-based detection of *Colletotrichum acutatum* on strawberry. *Plant Pathology* **45**, 650-655.

Swofford, D.L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods), 4.0b1. Computer programme. Sunderland, Massachusetts, U.S.A.

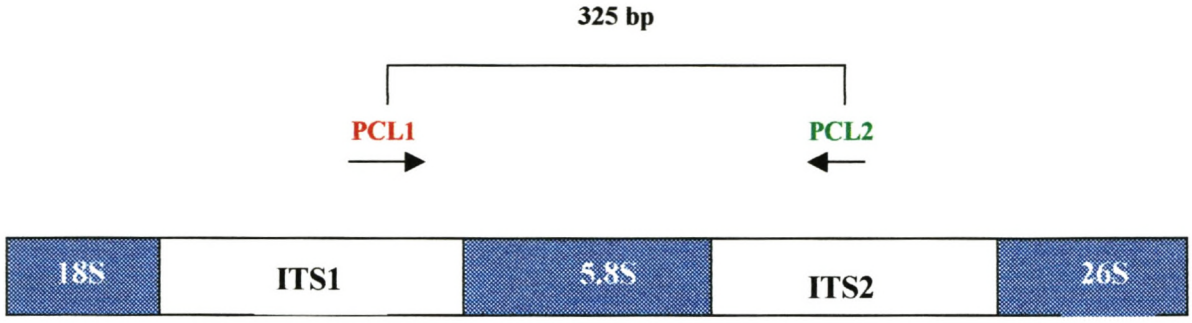
Viala, P. 1926. Recherches sur les maladies de la vigne: Esca. *Annales des Epiphyties* **12**, 1-108.

White, T.J., Burns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. In *PCR protocols. A guide to methods and application* (ed. M.A. Innis, D.H. Gelfand, J. Shinsky & T.J. White), pp. 315-322. Academic Press: San Diego, U.S.A.

Yan, Z.H., Rogers, S.O. & Wang, C.J.K. 1995. Assessment of *Phialophora* species based on ribosomal DNA internal transcribed spacers and morphology. *Mycologia* **87**, 72-83.

Table 1. Isolates of *Phaeoacremonium* spp. and *Phialophora verrucosa* compared with *Phaeomoniella chlamydospora* in the studies

Isolates	Genbank accession no.	Culture no.	Substrate
P. verrucosa	U31848	NIH 8701 / CBS 224.97	Human
	U31847	CDC-B 2152 / CBS 225.97	Human
	U31846	NYS 303 / CBS 226.97	Human
Pm. aleophilum	AF017651	CBS 246.91	*Vitis vinifera*, Yugoslavia
	AF197981	CBS 100397	*Vitis vinifera*, Italy
	AF197991	CBS 100399	*Vitis vinifera*, Italy
	AF197992	CBS 100400	*Vitis vinifera*, Italy
	AF197993	CBS 101358	*Actinidia chinensis*, Italy
	AF197982	CBS 100401	*Vitis vinifera*, Italy
	AF197994	CBS 100402	*Vits vinifera*, Italy
	AF197983	CBS 100548	*Olea europaea*, Italy
	AF197984	CBS 101568	*Vitis vinifera*, California
	AF197996	STE-U 3094 (MT78)	*Vitis vinifera*, South Africa
	AF197985	STE-U 3093 (MT79)	*Vitis vinifera*, South Africa
	AF197995	STE-U 3095 (MT80)	*Vitis vinifera*, South Africa
Pm. angustius	AF197974	CBS 249.95	*Vitis vinifera*, California
	AF197977	CBS 101739	*Vitis vinifera*, France
	AF197976	CBS 101737	*Vitis vinifera*, France
	AF197975	CBS 100947	*Olea europaea*, Italy
Pm. parasiticum	AF197980	CBS 101007	*Antinidia chinensis*, Italy
	U31842	CBS 860.73	Human
	U31842	HD 337 / CBS 513.82	Human
Pm. rubrigenum	AF197988	CBS 498.97	Human
	AF197978	STE-U 3092 (MT11)	*Vitis vinifera*, South Africa
	AF197979	CBS 566.97	Human
	AF197989	CBS 729.97	Human
Pm. inflatipes	AF197990	CBS 391.71	*Quercus virginiana*
	U31843	CBS 166.75	*Nectandra* sp.
Pa. chlamydospora	AF197973	CBS 229.95	*Vitis vinifera*
	AF197986	STE-U 3066	*Vitis vinifera*, South Africa
	AF197987	STE-U 3067	*Vitis vinifera*, South Africa
	AF017652	LCP 873550	*Vitis vinifera*



PCL1 **5'** TACATGTGACGTCTGAACGG **3'**
PCL2 **5' AGGACCACCTCAGTGTATGC 3'**

Fig. 1. Primers PCL1 and PCL2 designed for *Phaeomoniella chlamydospora.*

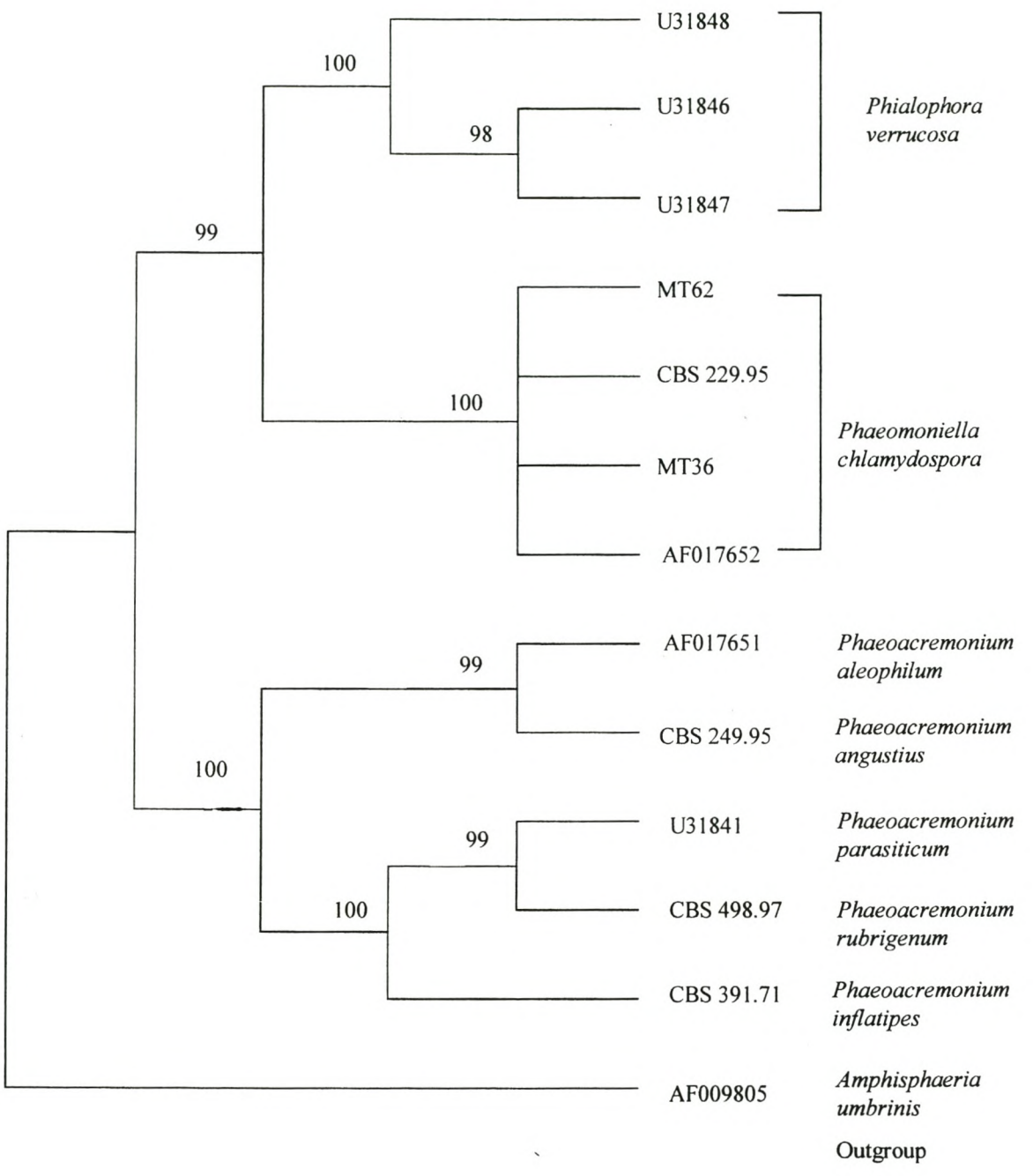


Fig. 2. One of four most parsimonious trees generated with PAUP* 4.0b1 from aligned sequences of the 5.8S gene and flanking ITS1 and ITS2 regions (tree length 712, CI=0.889, RI=0.929, RC=0.826, HI=0.11).

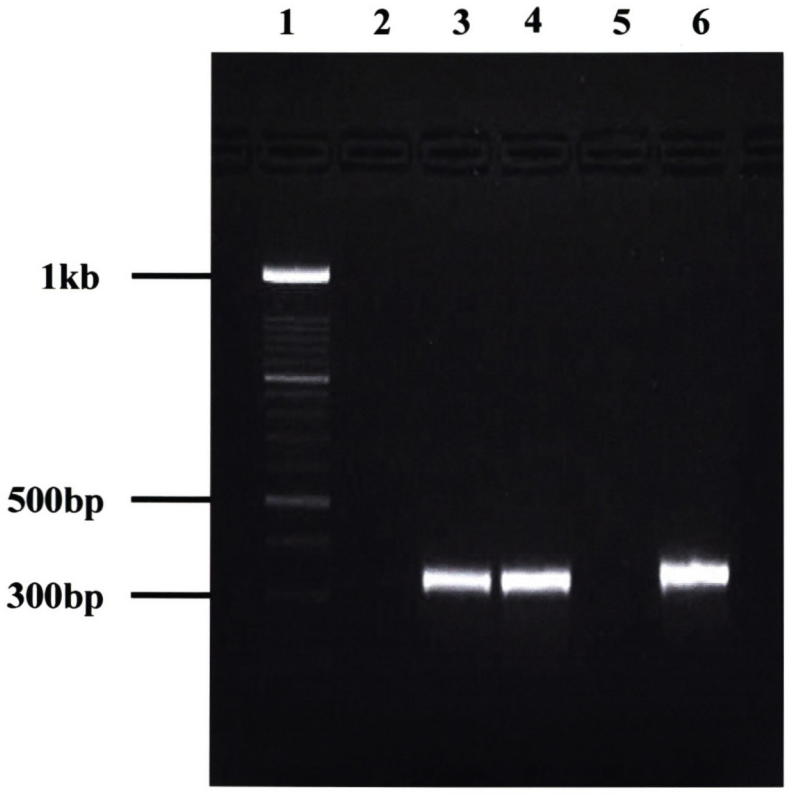


Fig. 3. Banding patterns of PCR amplification with PCL1 and PCL2. 1) 100 bp ladder, 2) negative control, 3) positive control, 4) genomic DNA of *Phaeomoniella chlamydospora*, 5) control plant DNA, 6) inoculated plant DNA.