CHARACTERISATION OF *CYLINDROCARPON* SPP. ASSOCIATED WITH BLACK FOOT DISEASE OF GRAPEVINE

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Dissertation presented for the degree of Doctor of Philosophy in Agriculture at the University of Stellenbosch

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December 2005

DECLARATION

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

Signature: Date:

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SUMMARY

During the past few years a drastic reduction has been noted in the survival rate of grafted grapevines in nurseries, as well as in young vineyards in the Western Cape Province of South Africa. Circumstantial evidence suggested that *Cylindrocarpon* spp., which cause black foot disease of grapevine, were associated with this decline. Black foot disease of grapevine is a relatively new, and as yet poorly known disease affecting vines in various countries where grapevines are cultivated. Primary aims of this research have been (1) to conduct nursery surveys in order to determine which fungi are involved in the decline phenomenon, with special reference to the involvement of *Cylindrocarpon* spp., (2) to identify and characterise the organisms believed to be the causal organisms of black foot disease, and (3) the development of control and/or management strategies to prevent or eradicate *Cylindrocarpon* infections.

Nursery grapevines were sampled at different stages from three commercial nurseries in the Wellington area of the Western Cape Province and were investigated during the 1999–2000 season by means of destructive sampling. The first samples were taken in September from callused cuttings prior to planting in nurseries. After planting, asymptomatic rooted cuttings were selected from nurseries after 3, 6 and 9 months. Isolation studies clearly demonstrated that different "*Cylindrocarpon* spp." infected cuttings from nursery soils. These species rarely occurred in rootstock propagation material prior to planting. At the time of planting, the susceptible basal ends (especially the pith area) of most of the nursery cuttings are partly or even fully exposed. Callus roots also break during the planting process, resulting in small wounds susceptible to infection by soilborne pathogens. The isolation studies revealed that the first infections occurred in the roots, followed by infections of the rootstocks. These infections increased progressively during the course of the growing season.

Substantial variation in cultural and morphological characters was observed among the *Cylindrocarpon* isolates obtained from the nursery survey, as well as from isolations that were made from diseased grapevines. Morphological and phylogenetic studies were conducted to identify these "*Cylindrocarpon* spp." and to establish their association with black foot disease. Sequences of the partial nuclear large subunit ribosomal DNA (LSU rDNA), internal transcribed

spacers 1 and 2 of the rDNA including the 5.8S rDNA gene (ITS), and partial β-tubulin gene introns and exons were used for phylogenetic inference. Phylogenetic analyses confirmed the diversity observed among the isolates and four *Cylindrocarpon*-like species were identified. One of these species was initially identified as *Cylindrocarpon destructans*. However, further research revealed *C. destructans* to represent a species complex. Grapevine isolates of "*C. destructans*" proved to be identical to the ex-type strain of *Cylindrocarpon liriodendri*, which also produced a teleomorph, *Neonectria liriodendri* in culture. A second species was newly described in this study as *Cylindrocarpon macrodidymum* (*Neonectria macrodidyma*). The two remaining *Cylindrocarpon*-like species were placed in a new genus, *Campylocarpon*. The two species were named *Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare*. Pathogenicity studies confirmed that all four species were able to reduce root and shoot mass significantly.

Knowledge obtained pertaining to the disease cycle of black foot disease suggest that suitable management strategies should focus on prevention of primary infection in nurseries. However, at present, no fungicides are registered for control of this disease in South African vineyards or nurseries. Thirteen fungicides were screened *in vitro* for mycelial inhibition of these pathogens. Prochloraz manganese chloride, benomyl, flusilazole and imazalil were the most effective fungicides tested, and were subsequently included in semi-commercial field trials. Basal ends of grafted cuttings were dipped (1 min) in various chemical and biological treatments prior to planting in open-rooted nurseries. Black foot pathogens were not isolated from grafted cuttings prior to planting in nurseries. Additional treatments involved soil amendments with *Trichoderma* formulations and hot water treatment (50°C for 30 min) of dormant nursery grapevines. Field trials were evaluated after a growing season of eight months. The incidence of black foot pathogens was not significantly and/or consistently reduced by the majority of chemical or biological treatments. However, these pathogens were not isolated from uprooted plants that were subjected to hot water treatment. It is therefore recommended that hot water treatment of dormant nursery plants be included in an integrated strategy for the proactive management of black foot disease in grapevine nurseries.

KARAKTERISERING VAN *CYLINDROCARPON* SPP. GEASSOSIEER MET SWARTVOETSIEKTE VAN WINGERD

OPSOMMING

Gedurende die afgelope paar jaar is ‘n drastiese afname waargeneem in die sukses van geënte wingerdplante in kwekerye, sowel as jong wingerde van die Wes-Kaap. Omstandigheidsgetuienis dui daarop dat *Cylindrocarpon* spp., wat die wingerdsiekte swartvoet veroorsaak, geassosieer word met hierdie agteruitgang. Swartvoet is ‘n relatiewe nuwe siekte waarvan daar baie min inligting bekend is, alhoewel dit voorkom in verskeie lande waar wingerd verbou word. Die primêre doel van navorsing was (1) om opnames in wingerdkwekerye uit voer om te bepaal watter swamme betrokke is by die verskynsel van agteruitgang, met spesiale verwysing na die betrokkenheid van *Cylindrocarpon* spp., (2) om die organismes te identifiseer en te karakteriseer wat daarvan verdink word dat hulle die siekte swartvoet veroorsaak, en (3) om beheer en/of bestuurspraktyke te ontwikkel om *Cylindrocarpon* infeksies te voorkom of uit te wis.

Kwekeryplantjies in drie kommersiële kwekerye in die Wellington omgewing van die Wes-Kaap is gedurende verskillende tye gedurende die groeiseisoen gemonitor. Die opnames het plaasgevind gedurende die 1999–2000 seisoen deur middel van destruktiewe monsterneming. Die eerste monsters is geneem in September nadat die stokkies geënt en gekallus is en voordat dit in die kwekery geplant is. Na plant is asimptomatiese, gewortelde plante vanuit die kwekerye na 3, 6 en 9 maande uitgehaal. Isolasiestudies dui duidelik daarop dat verskillende “*Cylindrocarpon* spp.” plante vanuit die kwekerygrond geïnfekteer het. Hierdie spesies het selde voorgekom in onderstok-voortplantingsmateriaal voor plant. Tydens plant is die vatbare basale gedeelte, veral die pit, van die meeste geënte stokkies gedeeltelik of selfs volledig blootgestel. Kalluswortels breek ook tydens plant wat wonde laat vir infeksie deur grondgedraagde siektes. Die isolasiestudies dui ook daarop dat die eerste infeksies in die wortels plaasgevind het, gevolg deur infeksies van die onderstokke. Hierdie infeksies het toenemend voorgekom gedurende die verloop van die groeiseisoen.

Substansiële variasie in kultuur- en morfologiese eienskappe is waargeneem in die *Cylindrocarpon* isolate wat tydens die kwekeryopnames versamel is, sowel as van isolasies wat gemaak is uit siek plante. Morfologiese en filogenetiese studies is uitgevoer om hierdie

"*Cylindrocarpon* spp." te identifiseer en hul betrokkenheid by die siekte swartvoet uit te klaar. Gedeeltelike DNS volgordes van die groot ribosomale subeenheid ("LSU rDNA"), interne getranskribeerde spasiëerderarea ("ITS1, "ITS2"), insluitend die 5.8S rRNS geen, en gedeeltelike β-tubilien geen introns and eksons is gebruik vir filogenetiese analise. Filogenetiese analises het die diversiteit wat waargeneem is tussen die verskillende isolate bevestig deurdat vier *Cylindrocarpon*-agtige spesies geïdentifiseer is. Een van hierdie spesies is aanvanklik geïdentifiseer as *Cylindrocarpon destructans*. Verdere navorsing het egter daarop gedui dat *C. destructans* 'n spesie-kompleks verteenwoordig. "*C. destructans*" afkomstig van wingerd blyk identies te wees aan die ex-tipe isolaat van *Cylindrocarpon liriodendri*, wat ook 'n teleomorf, *Neonectria liriodendri* in kultuur vorm. 'n Tweede spesie is nuut beskryf in hierdie studie as *Cylindrocarpon macrodidymum* (*Neonectria macrodidyma*). Die twee oorblywende *Cylindrocarpon*-agtige spesies is geplaas in 'n nuwe genus, *Campylocarpon*. Die twee spesies staan bekend as *Campylocarpon fasciculare* en *Campylocarpon pseudofasciculare*. Patogenisiteitstudies het bevestig dat al vier spesies die vermoë het om wortel- en lootmassa van wingerdplant drasties te verlaag.

Kennis wat opgedoen is rakende die lewensiklus van swartvoet dui daarop dat bestuurspraktyke daarop moet fokus om primêre infeksies in wingerdkwekerye te voorkom. Op die oomblik is daar egter geen fungisiedes geregistreer vir die beheer van die siekte in Suid-Afrikaanse wingerde of kwekerye nie. Dertien fungisiedes is *in vitro* geëvalueer om te bepaal of dit miseliumgroei van hierdie swamme kan inhibeer. Prochloraz mangaan chloried, benomyl, flusilasool en imazalil was die effektiefste fungisiedes wat ondersoek is, en is gevolglik ingesluit in semi-kommersiële veldproewe. Die basale gedeelte van geënte stokkies is gedoop (1 min) in verskeie chemies en biologiese behandelings voordat dit geplant is in die kwekerye. Patogene wat geassosieer word met swartvoet is nie vanuit geënte stokkies geïsoleer voordat dit in die kwekerye geplant is nie. Addisionele behandelings het bestaan uit grondtoevoegings met *Trichoderma* formulasies, sowel as warmwaterbehandeling (50°C vir 30 min) van dormante kwekeryplante. Die veldproewe is geëvalueer na 'n groeiseisoen van 8 maande. Die voorkoms van swartvoet patogene is nie betekenisvol/konstant verlaag deur die meeste chemies en biologiese behandelings nie. Hierdie patogene is egter nie vanuit plante geïsoleer wat na uithaal aan warmwaterbehandeling blootgestel is nie. Dit word dus aanbeveel dat warmwaterbehandeling van dormante kwekeryplante deel word van 'n geïntegreerde strategie vir die pro-aktiewe beheer van swartvoet in wingerdkwekerye.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to the following:

Prof. Pedro Crous (CBS), my promoter, and Dr. Paul Fourie (US), my co-promoter, for their guidance, advice, experience and knowledge which were essential in the successful completion of this research.

Dr. Ilze Trautmann (former manager of the Plant Disease Division, ARC Infruitec-Nietvoorbij) for her leadership, advice, and most of all encouragement.

Mr. Frikkie Calitz (ARC Infruitec-Nietvoorbij) for statistical analyses.

Zane Sedeman, Linda Nel, Carine Vermeulen and Julia Marais (ARC Infruitec-Nietvoorbij) for technical assistance.

Winetech, ARC and University of Stellenbosch for financial assistance.

The Table Grape Forum of the South African Society for Enology and Viticulture for sponsoring a visit to CBS.

Koos Malan, Petro Malan, Niel Malan, Kobus Smit, Jannie Bosman, Johan Wiese and Andrew Teubes for assistance with nursery procedures and execution of nursery surveys and control trials.

KWV Vititec, especially Dirk Visser, for assistance with hot water treatment, as well as Nico Spreeth for assistance with nursery procedures.

My parents for their support throughout my studies.

CONTENTS

1. A REVIEW OF BLACK FOOT DISEASE OF GRAPEVINE*

SUMMARY

Black foot disease of grapevine is a relatively new, and as yet poorly known disease affecting vines in various countries where grapevines are cultivated. The causal organisms, their distribution, associated symptoms, known epidemiology and possible management strategies are discussed. Specific attention is also given to the taxonomy of the fungi involved, and the detection methods being developed to facilitate rapid identification of these pathogens.

INTRODUCTION

Species of *Cylindrocarpon* Wollenw. are common soil inhabitants, occurring as saprobes or weak pathogens, often associated with roots of herbaceous and woody plants (Brayford, 1993). However, two species, *C. destructans* (Zinnsm.) Scholten and *C. obtusisporum* (Cooke & Harkn.) Wollenw., have been reported as the causal agents of black foot disease of grapevines (*Vitis* spp. L.). Scheck *et al.* (1998a) proposed that the common name Cylindrocarpon black foot disease be used with both species, as the disease symptoms were similar. The first record of *C. destructans* on grapevine was made in France in 1961 (Maluta and Larignon, 1991). Since then it has been isolated from diseased vines in Tasmania (Sweetingham, 1983), Sicily (Grasso, 1984), Portugal (Rego, 1994; Rego *et al*., 2000, 2001a) and Pennsylvania, U.S.A. (Gugino and Travis, 2003). *Cylindrocarpon obtusisporum* has been identified as the causal agent of this disease in Sicily (Grasso and Magnano di San Lio, 1975) and California, U.S.A. (Scheck *et al*., 1998a). Various unidentified species of *Cylindrocarpon* have also been isolated from young vines and from declining vines with basal rot or root necrosis in Australia (Edwards and Pascoe, 2004), Chile (Auger *et al*., 1999), Greece (Rumbos and Rumbou, 2001), Spain (Armengol *et al*., 2001) and South Africa (Fourie *et al*., 2000; Fourie and Halleen, 2001a). In a recent taxonomic study of the *Cylindrocarpon* spp. associated with black foot disease of grapevines, the primary causal organism was identified as *C. destructans*, while a second species was newly described as *C. macrodidymum* Schroers, Halleen & Crous (Halleen *et al*., 2004a; Chapter 3). Furthermore, two species were found to represent an undescribed genus that was *Cylindrocarpon*-like in

* Accepted for publication in Phytopathologia Mediterranea.

morphology, namely *Campylocarpon* Halleen, Schroers & Crous (*Campylocarpon fasciculare* Schroers, Halleen & Crous and *Campyl. pseudofasciculare* Halleen, Schroers & Crous). All four species have been implicated in this disease complex (Halleen *et al*., 2004a; Chapter 3).

SYMPTOMS

According to the literature regarding *Cylindrocarpon* spp. associated with grapevine diseases, two scenarios are evident. These scenarios might also be related to the initial source of infection and are therefore treated as 'nursery infections' and 'vineyard infections'.

Nursery infections. This scenario relates to nursery vines or younger vines shortly after transplantation where typical symptoms of vascular streaking are evident. Grasso and Magnano di San Lio (1975) described black foot symptoms from nursery plants with black discolouration of, and gum inclusions in, xylem vessels of affected rootstocks (225 Ruggeri). Scheck *et al*. (1998a) also described dark-brown to black streaking in the vascular tissue of young (2–5-year-old) grapevines investigated in California. Affected vines showed reduced vigour with small-sized trunks, shortened internodes, uneven wood maturity, sparse foliage, and small leaves with interveinal chlorosis and necrosis. Other symptoms included a reduction in root biomass and root hairs with sunken, necrotic root lesions. The pith of the affected vines was also compacted and discoloured (Scheck *et al*., 1998a). Whilst investigating rootstock nurseries in Portugal, Rego *et al*. (2000) also observed black discolouration and brown to dark streaks in wood, mainly at the base of the rootstock. Investigation of older vines (2–8-year-old) also revealed the presence of *C. destructans* in the basal end of the rootstocks (Rego *et al*., 2000).

Vineyard infections. This scenario relates to infections of 2–10-year-old grapevines. Sweetingham (1983) described the death of mature vines (5 years and older) caused by *C. destructans* in Tasmania. Disease symptoms were noticed early in the growing season as affected vines achieved poor new growth, failed to form shoots after winter dormancy, and died by mid-summer. Vines with reduced vegetative growth also died during the subsequent dormant winter period. A dark brown discolouration of the wood in the trunk at ground level was observed. This discolouration extended up to 15 cm above ground level, and throughout the below-ground portion of the trunk, and sometimes extended from the trunk into the larger roots for distances up to 10 cm. Sections through symptomatic tissue revealed that the

majority of the xylem vessels were plugged with thick-walled tyloses or brown gum, and functional phloem elements were plugged with gum. Further microscopic examination of infected tissue revealed the presence of fungal hyphae in the ray cells of the phloem and younger xylem. Hyphae were not visible in the xylem vessels and rarely in the functional phloem. The presence of hyphae in the ray cells declined towards the centre of the trunk in the discoloured tissue and they were not visible in tissue beyond the zone of discolouration or in tissue of healthy vines. Starch reserves are mainly stored in the ray cells, providing a readily metabolisable carbon source for *C. destructans*, which can produce extracellular amylases (Sweetingham, 1983). Larignon (1999) described black foot disease as a disease affecting mainly young vines between 2 and 8 years of age. Observations in California also support this, and according to Gubler *et al*. (2004) vines up to 10 years old might succumb to the disease. When young vines are infected, death occurs quickly, but as the vine ages, infection results in a more gradual decline and death might occur only after a year (Gubler *et al*., 2004). Larignon (1999) described symptoms similar to those reported by Sweetingam (1983) where diseased vines characteristically displayed abnormal, weak vegetation and in some cases did not sprout at all. Often shoots also dried and died during the summer. Furthermore, below-ground symptoms included abnormal root development characterised by shallow growth parallel to the soil surface. A second crown of roots may develop on an upper level of the rootstock to compensate for the loss of functional roots further below (Larignon, 1999; Fourie *et al*., 2000). Roots of the basal crown become necrotic. In some cases the rootstock diameter of older vines is thinner below the second tier (Fourie and Halleen, 2001b). Removal of rootstock bark reveals a brown to black zone beginning at the base of the rootstock extending up along the rootstock. A cross section through the affected area reveals internal necrosis which develops from the bark to the pith (Larignon, 1999; Fourie and Halleen, 2001a).

TAXONOMY AND PHYLOGENY

Teleomorphs with *Cylindrocarpon* anamorphs were traditionally classified in *Nectria* (Fr.) Fr., but are now considered to belong to *Neonectria* Wollenw. (Rossman *et al*., 1999; Mantiri *et al*., 2001, Brayford *et al*., 2004). Wollenweber based the generic name upon *Neon. ramulariae* Wollenw. (1916). The reintroduction of *Neonectria* resulted from the realisation that *Nectria* was too broadly defined and that its segregation into numerous teleomorphic genera could be corroborated by anamorphic, phylogenetic, and ecological character patterns

(Rehner and Samuels 1995; Rossman *et al*., 1999). Some pre-phylogenetic classification schemes had segregated the teleomorphs of *Cylindrocarpon* species into four infrageneric *Nectria* groups, based on perithecial wall anatomy and ascospore morphology; these groups were centred on "*Nectria*" *radicicola* Gerlach & L. Nilsson, "*Nectria*" *coccinea* (Pers. : Fr.) Fr., "*Nectria*" *mammoidea* W. Phillips. & Plowr., and "*Nectria*" *rugulosa* Pat. & Gaillard (Booth, 1959; Samuels and Brayford, 1990; Samuels and Brayford, 1994). Wollenweber (1917, 1928) created the sections *Chlamydospora* Wollenw. and *Ditissima* Wollenw. for species with and without chlamydospores, respectively. Booth (1966) schematically segregated *Cylindrocarpon* species into four groups based on the presence or absence of microconidia and chlamydospores. *Cylindrocarpon magnusianum* (Sacc.) Wollenw., which is the anamorph of the type species of *Neonectria, C. cylindroides* Wollenw., which is the type species of the genus *Cylindrocarpon*, *C. destructans*, which is the anamorph of *Neonectria radicicola*, and members of *Cylindrocarpon* species predominantly connected with teleomorphs of the "*Nectria*" *mammoidea* group were core members of the anamorphic groups delineated by Booth (1966). *Cylindrocarpon obtusisporum* was originally described from the U.S.A. (California) as occurring on *Acacia* sp., where it was observed to form macroconidia and chlamydospores (Booth, 1966). *Cylindrocarpon obtusisporum* strains identified by Booth (1966) originated from a broad range of host plants in Europe, New Zealand, North America, and, at least partly, formed microconidia. Currently, representatives of all "*Nectria*" groups with *Cylindrocarpon* anamorphs have been transferred into *Neonectria* (Rossman *et al*., 1999; Mantiri *et al*., 2001; Brayford *et al*., 2004). Mantiri *et al*. (2001) and Brayford *et al*. (2004) analysed mitochondrial small subunit (SSU) ribosomal DNA (rDNA) sequence data of some of the species and concluded that the *Neonectria*/*Cylindrocarpon* species grouped together by this reclassification were monophyletic. However, these authors also found that this overall *Neonectria*/*Cylindrocarpon* clade included distinct subclades corresponding to at least three of the four groups delineated by Booth (1966). Significant molecular variation among taxa with *Cylindrocarpon*-like anamorphs was found by Seifert *et al*. (2003) in a study on fungi causing root rot of ginseng (*Panax quinquefolius* L.) and other hosts. The dendrograms in this study, based on partial β-tubulin gene, and nuclear ribosomal internal transcribed spacer (ITS) region sequences, suggested that subclades including (i) *Neon. radicicola*, which consisted of numerous phylogenetically distinct units, (ii) *Neon. macroconidialis* (Samuels & Brayford) Seifert, and (iii) a subclade comprising two distinct isolates, one from *Vitis vinifera* in

Ontario, Canada and the other from *Picea* sp. in Quebec, Canada, were monophyletic. Other *Cylindrocarpon* species appeared to be excluded from this monophyletic group.

Significant variation in cultural and morphological characters was observed among *Cylindrocarpon* strains isolated from grapevines in nurseries and vineyards in South Africa, France, New Zealand, and Australia (Halleen *et al*., 2003; Halleen *et al*., 2004b; Halleen *et al*., unpublished). Halleen *et al*. (2004a; Chapter 3) used morphological characters and DNA sequences to characterise these taxa taxonomically and phylogenetically. Sequences were compared with those of members of the *Neon. radicicola* complex published by Seifert *et al.* (2003) and various other *Neonectria*/*Cylindrocarpon* species deposited at the CBS Fungal Biodiversity Centre (CBS, Utrecht, The Netherlands). Sequences of the partial nuclear large subunit ribosomal DNA (LSU rDNA), internal transcribed spacers 1 and 2 of the rDNA including the 5.8S rDNA gene (ITS), and partial β-tubulin gene introns and exons were used for phylogenetic inference. *Neonectria*/*Cylindrocarpon* species clustered in mainly three groups. One monophyletic group consisted of three subclades comprising (i) members of the *Neonectria radicicola*/*Cylindrocarpon destructans* complex, which contained strains isolated from grapevines in South Africa, New Zealand, and France; (ii) a *Neonectria*/*Cylindrocarpon* species isolated from grapevines in South Africa, Canada (Ontario), Australia (Tasmania), and New Zealand, described as *Cylindrocarpon macrodidymum*; and (iii) an assemblage of species closely related to strains identified as *Cylindrocarpon cylindroides*, the type species of *Cylindrocarpon*. This monophyletic group excluded two other groups, which comprised (i) members of the *Neonectria mammoidea* complex, with anamorphs characterised by curved macroconidia, violet or purple pigments in cultures of most of its members, and the lack of microconidia and chlamydospores; and (ii) two *Campylocarpon* species, *Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare*, isolated from grapevines in South Africa. The latter two clades formed a paraphyletic group in LSU rDNA analysis, but were supported as a monophyletic group in ITS and β-tubulin gene analysis. Analyses of Halleen *et al*. (2004a; Chapter 3) therefore excluded *Campylocarpon* and members of the *Neonectria mammoidea* group from *Neonectria*/*Cylindrocarpon*, contradicting the transfer of the mammoidea group to *Neonectria* by Brayford *et al*. (2004). *Campylocarpon* species, though similar in macroconidial morphology to members of the *Neonectria mammoidea* group, can be distinguished by the formation of typical brownish rather than violaceous cultures, as well as by production of brownish hyphae, often in strands, and in *Campyl. pseudofasciculare* by formation of chlamydospores (Halleen *et al*., 2004a; Chapter 3).

Strains of the *Neonectria radicicola*/*Cylindrocarpon destructans* complex isolated from grapevines matched those currently placed in *C. destructans* based on morphology and DNA sequences. However, as shown by previous phylogenetic studies (Seifert *et al*., 2003; Halleen *et al*., 2004a; Chapter 3), *C. destructans* represents a species complex. Furthermore, it appears that within this complex, different woody hosts have their own unique species, some of which are more host-specific than others. Although Halleen *et al*. (2004a; Chapter 3) referred to the primary causal organism of black foot rot of grapevines as "*C. destructans*", recent research has revealed that the grapevine pathogen is in fact *C. liriodendri* J.D. MacDon. & E.E. Butler (Halleen *et al*., 2006; Chapter 4).

A second species described from grapevines, *C. macrodidymum*, formed micro- and macroconidia, but rarely formed chlamydospores. Its predominantly 3-septate macroconidia were more or less straight, minutely widening towards the tip, and had an apical cell slightly bent to one side. Its teleomorph, *Neonectria macrodidyma*, was obtained in mating experiments, and was characterised by smooth to finely warted ascospores, smooth to finely warted perithecia, and moderately sized angular to subglobose cells in the outer region of the perithecial wall. *Campylocarpon* spp. were characterised by mostly 3–5-septate, curved macroconidia, and by the lack of microconidia (Halleen *et al*., 2004a; Chapter 3).

What happened to *C. obtusisporum*? The possibility that Grasso and Magnano di San Lio (1975) and Scheck *et al*. (1998a) misidentified *C. obtusisporum* and that it was in fact *C. macrodidymum* was raised by Halleen *et al*. (2004a; Chapter 3). Macroconidia of *C. macrodidymum* measure (26–)34–36–38(–45) × (4–)5.5–6–6.5(–8) µm (Halleen *et al*., 2004a; Chapter 3), whereas those of the type of *C. obtusisporum* measure 30–35 × 4–5 µm (Cooke, 1884). However, the shape of the macroconidia distinguishes *C. macrodidymum* from the type of *C. obtusisporum*, which Cooke (1884) described as having conidia with obtuse ends. Booth (1966) described macroconidia of similar shape in *C. obtusisporum*. According to Booth, however, 2–3-septate macroconidia of C. *obtusisporum* measure 34–50 × 6–7.5 µm. *C. obtusisporum* isolates obtained from California formed perithecia when cross-inoculated with *C. macrodidymum*, giving further evidence to support the misidentification theory (Halleen *et al*., unpublished data). This was also confirmed by sequence comparisons (Ulrike Damm, University of Stellenbosch, pers. comm).

MOLECULAR DETECTION

Hamelin *et al*. (1996) designed species-specific primers (Dest1 and Dest4) to detect *C. destructans* from conifer seedlings. Using these primers in direct PCR assays on DNA extracted from *C. destructans* cultures obtained from grapevines in Portugal, Nascimento *et al*. (2001) obtained a DNA fragment of 400 bp. However, Nascimento *et al*. (2001) were unable to distinguish between *C. destructans* and *C. obtusisporum* when using these primers, because an amplification of the same size was obtained for isolates of *C. obtusisporum*. Furthermore, these primers could also not detect *C. destructans* from artificially inoculated potted grapevines. The nested PCR assay developed by Hamelin *et al*. (1996) was therefore modified by Nascimento *et al*. (2001). The universal primer ITS4 and the fungus-specific primer ITS1F were used in a first-stage fungus-specific amplification, followed by a second-stage amplification with the primers Dest1 and Dest4 using the PCR product from stage one. This is a simple and reliable method for detection of *Cylindrocarpon* spp. directly from infected grapevines (Nascimento *et al*., 2001). Damm *et al*. (2005) developed a method for the extraction of fungal DNA from soil to study the epidemiology of grapevine trunk disease pathogens in South African grapevine nurseries and vineyards. The extracted DNA was tested for *Cylindrocarpon* spp. by using the primers Dest1 and Dest4. *Cylindrocarpon* spp. were detected in 66% of the samples investigated (Damm *et al*., 2005). Species-specific primers are currently being developed for detection of all the species involved in black foot disease in South Africa (Halleen *et al*., in prep.).

EPIDEMIOLOGY

Investigation of diseased vines in Tasmania showed that wood discolouration did not originate from the base of the trunk (Sweetingham, 1983). In fact, the discolouration and fungal hyphae first became evident in the buried portion of the trunk, 2-12 cm below ground surface (Sweetingham, 1983), suggesting that infection occurred at a later stage in the vineyard. Gubler *et al*. (2004) was also of the opinion that the presence of the pathogens in vineyards probably plays a larger role in disease development than infected nursery material.

Rego *et al*. (1998) speculated that rootstock nurseries might be the origin of these infections in Portugal, since severe outbreaks only occurred in vineyards where the rootstocks were sourced from the same region or even the same nursery. Surveys of rootstock nurseries

located in Ribatejo-Oeste and Beira Litoral confirmed that infected rootstocks were the most likely way in which the pathogens are disseminated, although the initial source of infection was still unknown (Rego *et al*., 2000). Investigation on the occurrence of decline pathogens in canes of rootstock mother vines in Portugal and South Africa revealed extremely low levels of *Cylindrocarpon* spp. (Rego *et al*., 2001b; Fourie and Halleen, 2002). A survey of 34 certified rootstock mother blocks in six production areas, where isolations were made from the basal and pruning wound ends of 2-year-old pruning stubs, again revealed the low incidence (av. 0.17%) of *Cylindrocarpon* spp. inside rootstock mother vines (Fourie and Halleen, 2004a). An investigation of fungi occurring in asymptomatic nursery vines supported these findings in that *Cylindrocarpon* spp. were hardly ever isolated from callused grafted cuttings prior to planting in nurseries (Halleen *et al*., 2003; Chapter 2). However, once planted in the nurseries, *Cylindrocarpon* spp. were isolated from the roots, rootstocks and graft unions. Infection of the roots occurred first, followed by infection of the rootstocks. At the time of planting, the basal ends (especially the pith area) of most of the cuttings are partly or even fully exposed for infection by soilborne pathogens. Callus roots often break during the planting process, resulting in small wounds susceptible to infection. The presence of *Cylindrocarpon* spp. in graft unions might be explained by the nursery practice where graft unions are covered with soil for a 5-week-period to prevent drying of the callus tissue (Halleen *et al*., 2003; Chapter 2). *Cylindrocarpon* spp. occurred in graft unions of 15% of nursery grapevines investigated by Aroca and Raposo (2005). This suggests that the recommendation of Stamp (2001), namely that the graft union should be fully healed when a vine is removed from the callusing chamber 2–4 weeks after grafting, is not always followed in practice.

The production of chlamydospores would also allow *Cylindrocarpon* spp. to survive for extended periods in soil (Booth, 1966; Halleen *et al*., 2004a; Chapter 3). However, very little information is currently available regarding the survival of these pathogens, and the role of chlamydospores during subsequent infections. In a related hypocrealean genus, *Cylindrocladium* Morgan, chlamydospores were shown to remain viable up to 15 years (Crous, 2002), which suggests that this could indeed be a very important aspect to consider in further epidemiological studies of *Cylindrocarpon*.

Rumbos and Rumbou (2001) argued that fungal infection alone could not be the sole reason of young grapevine decline in Greece, since the incidence of decline pathogens

(*Cylindrocarpon* spp., *Pa. chlamydospora* W. Gams, Crous, M.J. Wingf. & L. Mugnai, *Phaeoacremonium* spp. and *Botryosphaeria* spp.) were too low, and were present in too low a percentage of young vines. *Cylindrocarpon* spp. were isolated from only 1–4% of young vines. It was therefore speculated that abiotic factors such as lesions from improperly healed rootstock disbudding sites, and graft unions made in the nursery, as well as improper storage and transportation conditions of propagated material, could also play a role in enhancing grapevine decline (Rumbos and Rumbou, 2001).

PATHOGENESIS

As is the case with many other *Cylindrocarpon* species that cause disease on other crops, environmental factors and host stress may also play an important part in disease development (Brayford, 1993). Stress conditions that favour development of black foot disease include malnutrition, poor water drainage, soil compaction, heavy crop loads on young plants, planting of vines in poorly prepared soil and improper plant holes (Larignon, 1999; Fourie *et al*., 2000; Fourie and Halleen, 2001a; Halleen *et al*., 2004a; Chapter 3). Soil compaction and/or poor soil preparation will most likely contribute to poor root development (J-rooting and pothole effect) (Fourie *et al*., 2000; Halleen *et al*., 2004a). High temperatures during summer also play an important role in symptom expression. The deficient root system and altered vascular system of infected vines would not be able to supply enough water to compensate for the high transpiration rate during high temperatures (Larignon, 1999). *Cylindrocarpon* species are often part of disease complexes with other fungi or nematodes (Brayford, 1993). The example of apple replant disease is well-documented. In the case of declining vineyards, *Cylindrocarpon* spp. are often isolated together with other pathogens from the same diseased vines. These pathogens include *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp. (Petri disease pathogens), *Botryosphaeria* spp., *Phomopsis* spp., *Pythium* spp. and *Phytophthora* spp. (Fourie *et al*., 2000; Fourie and Halleen, 2001c; Oliveira *et al*., 2004; Edwards and Pascoe, 2004). Disease symptoms associated with these pathogens overlap in many respects, thereby making correct diagnosis based on visual symptoms nearly impossible.

Grasso and Magnano di San Lio (1975) induced black discolouration of wood in the basal area of rooted cuttings (225 Ruggeri) similar to the symptoms observed in diseased nursery vines 60 days after artificial inoculation with *C. obtusisporum*. Scheck *et al*. (1998a)

completed Koch's postulates by dipping the roots of cv. Carignane seedlings in a spore suspension of *C. obtusisporum*. Typical black foot symptoms appeared on 92% of the plants after 8 weeks. In the same experiment 67% of the plants developed symptoms after inoculation with *Phaeomoniella chlamydospora,* and 71% with *Phaeoacremonium inflatipes* W. Gams, Crous & M.J. Wingf. (recently re-identified as *Pm. aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai), demonstrating its virulence despite the fact that *Cylindrocarpon* spp. are generally recognised as relatively weak pathogens (Scheck *et al*., 1998b).

The first pathogenicity study with *C. radicicola* (= *C. destructans*) on grapevines was actually conducted on berries of grape variety Gordo Blanco when the fungus was consistently isolated from small, black necrotic spots on pedicels and blossom ends of Ohanez berries (Taylor, 1956). However, the inoculated fungus could invade green berries only when the skin was first ruptured and was therefore considered to be a secondary invader of already damaged tissue. Sweetingham (1983) failed to initiate infection of the basal trunk region and roots of potted 'Cabernet Sauvignon' vines when potting media were amended with *C. destructans*, despite the presence of *C. destructans* on the surface of below-ground parts. Mycelium plugs inserted into scalpel wounds in the vascular tissue of the buried portion of the trunk also resulted in no infection beyond the inoculation site. However, when 6-month-old own-rooted 'Cabernet Sauvignon' vines were inoculated with a spore suspension applied to the potting mixture directly adjacent to the trunk and the plants were then subjected to waterlogged treatments, symptoms appeared within 90 days. Leaves became chlorotic and some abscised, and vascular discolouration extending upward from the base of the cuttings was also observed in some plants. Rego *et al*. (2000) conducted pathogenicity studies with rooted cuttings of '99R' rootstock by dipping the roots in a conidial spore suspension of *C. destructans*. Typical black foot symptoms including root lesions, vascular discolouration and necrosis developed within two months. Similar results were obtained in studies conducted with rooted cuttings of cv. 'Seara Nova' (Oliveira *et al*., 1998) and cv. 'Periquita' (Rego *et al*., 2001a). However, in the latter study 13 *C. destructans* isolates, collected over a period of seven years, were used. Although all the isolates proved to be pathogenic, variation in virulence was observed and it was not correlated with the age of the cultures. All the isolates significantly reduced plant height and most significantly reduced the number of roots. In most cases the stunting could be explained by the shortened internodes, although it appeared as if the most virulent strains reduced the number of internodes. Auger *et al*. (1999) also observed dark streaking of vascular elements in roots of 'Flame Seedless' vines inoculated

with a *Cylindrocarpon* sp. Inoculation of 6-month-old potted grapevine rootstocks ('Ramsey') with *C. destructans*, *C. macrodidymum*, *Campyl. fasciculare* and *Campyl. pseudofasciculare* resulted in death, as well as reduced root and shoot mass of inoculated plants (Halleen *et al*., 2004a; Chapter 3).

DISEASE MANAGEMENT

Curative control. No fungicides are registered for the control of black foot disease in vineyards. Recommendations to farmers have thus far been based on the prevention and/or correction of predisposing stress factors.

Plant material. Plant material should be sourced from reputable nurseries that are subjected to standards as certified by the plant improvement associations in the different countries. Good quality planting material would ensure that nursery defects such as small and incomplete root systems, rootstock lesions, incomplete graft unions, etc., which are all detrimental to field performance, be limited (Stamp, 2001).

Very little information is currently available regarding rootstock susceptibility. Gubler *et al*. (2004) reported that the rootstocks *Vitis riparia* 'O39-16' and 'Freedom' appear to show some resistance towards *C. destructans*.

Soil preparation and vineyard activities. Soil compaction might be natural in some soils or may be the consequence of certain cultural practices. Compacted layers should be broken up during the soil preparation stages for new establishments to make the subsoil accessible to roots (Larignon, 1999). Plant holes should be deep and big enough to facilitate proper root development (Fourie *et al*., 2000). Excessive movement of farm vehicles result in soil compaction, especially when the soil is wet or poorly drained, and this should therefore be avoided (Larignon, 1999; Halleen *et al*., 2004a; Chapter 3). New vineyards should not be established on heavy, poorly drained soils (Larignon, 1999; Gubler *et al*., 2004). Drainage in heavy soils can be improved by planting on berms and moving drip irrigation emitters away from the vine (Gubler *et al*., 2004). Waterlogged situations can also be the consequence of drip irrigation systems where the drippers are positioned in such a way that the trunk is maintained in a waterlogged environment for most of the year, especially in excessive irrigation regimes (Sweetingham, 1983). Planting of certified vines according to best practice

procedures and thereafter carefully managed in such a way that roots can develop properly to such an extent that they can carry a decent crop, should go a long way towards ensuring successful establishment of a new vineyard.

Soil health is another important aspect to take into consideration. Preliminary results regarding the suppression of *C. destructans* by means of composted soil amendments have recently been published. Several microorganisms isolated from the compost have demonstrated antagonism towards *C. destructans* (Gugino and Travis, 2003).

Fluctuations in soil organic matter may result in changes to the populations of bacteria and actinomycetes able to produce anti fungal antibiotics (Whitelaw-Weckert, 2004). Whitelaw-Weckert (2004) investigated the effect of mulch and organic matter from herbicide treated weeds on the populations of vineyard soil bacteria and actinomycetes and their effect on *C. destructans*. *In vitro* evaluations revealed that 70% of the bacteria and actinomycetes from a herbicide inter-row treatment inhibited *C. destructans*. Populations of these microorganisms were also seven times higher in soil from this treatment compared to the herbicide under-vine only and no herbicide treatments.

Nursery practices. As mentioned previously, research has shown that black foot disease fungi infect grapevine cuttings when planted in infested nursery soils (Halleen *et al*., 2003; Chapter 2). Control methods should therefore focus on preventing or eradicating infection in the basal ends of these cuttings. *In vitro* studies conducted in South Africa revealed that benomyl, flusilazole and prochloraz manganese chloride were the most effective fungicides (Halleen *et al*., 2005; Chapter 5). Nursery trials were conducted to evaluate the effectiveness of various physical, chemical and biological treatments aimed at protecting the basal ends of rootstocks against infection. After callusing, the basal ends of grafted cuttings were dipped in various treatments prior to planting. Additional treatments involved soil amendments with *Trichoderma* formulations and hot water treatment (50°C for 30 min) of dormant nursery grapevines. Nursery plants were uprooted after eight months (Halleen *et al*., 2004c; Chapter 5). The incidence of black foot disease pathogens in the basal ends was not significantly and/or consistently reduced by the majority of chemical and biological treatments investigated. However, no black foot disease fungi were isolated from the plants that were subjected to hot water treatment (Halleen *et al*., 2005; Chapter 5). Halleen *et al*. (2005; Chapter 5) therefore recommended that hot water treatment of dormant nursery

grapevines be included in an integrated strategy for the proactive management of black foot disease in grapevine nurseries. Previously this treatment was also recommended for the eradication of several pests and diseases from dormant propagation material and/or nursery grapevines, including *Meloidogyne javanica* (Treub) Chitwood (Barbercheck, 1986), *Phytophthora cinnamomi* Rands (Von Broembsen and Marais, 1978), phytoplasmas (Caudwell *et al*., 1997), and the causal organism of Pierce's disease (Goheen *et al*., 1973). It was also found to be effective in reducing crown gall (Ophel *et al*., 1990), as well as *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. that cause Petri disease of grapevines (Fourie and Halleen, 2004b).

The following fungicides inhibited mycelial growth of *C. destructans in vitro*: prochloraz, benomyl, cyprodinil + fludioxonil and carbendazim + flusilazole, whilst tebuconazole and difenoconazole were less effective (Rego *et al*., 2005). Cyprodinil + fludioxonil, azoxystrobin, tryfloxistrobin and tolyfluanide effectively reduced spore germination. *In vivo* studies on potted grapevines proved that benomyl, tebuconazole, carbendazim + flusilazole and cyprodinil + fludioxonil significantly improved plant growth and decreased disease incidence compared with non-treated vines (Rego *et al*., 2005).

In South Africa, the same soil in grapevine nurseries has been used for decades. Standard nursery practice of a two-year rotation system, whereby cuttings are planted every second year, alternated with a cover crop, might have led to a build-up of soilborne pathogens such as species of *Cylindrocarpon* (Halleen *et al*., 2003; Chapter 2). In earlier studies these species appeared insignificant (Marais 1979, 1980). The duration of this rotation period and the type of cover crop should therefore be investigated to establish its effect on pathogen populations.

Biological control. Gubler *et al*. (2004) reported that the mychorrizal fungus *Glomus intraradices* Schenck & Smith provided excellent control against black foot disease if applied to grapevines in advance of inoculation with *Cylindrocarpon* spp.

The growth stimulating attributes of *Trichoderma* Pers. treatments (dips, soil amendments and drenches with *Trichoderma* products containing propagules of selected strains of *Trichoderma harzianum* Rifai, Agrimm Technologies Ltd., Christchurch, New Zealand), and the effect thereof on the occurrence of decline pathogens including

Cylindrocarpon spp. were investigated in South African nurseries (Fourie *et al*., 2001). The treatments consisted of rootstock drenches with Trichoflow-T™ before and directly after grafting, planting of grafted vines in planting furrows pre-inoculated with Trichopel™, and monthly root drenches with Trichogrow™. The treatments reduced the incidence of *Cylindrocarpon* spp. in nursery grapevines and significantly improved root development, which would undoubtedly make plants more tolerant when subjected to stress (Fourie *et al*., 2001).

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2. FUNGI ASSOCIATED WITH HEALTHY GRAPEVINE CUTTINGS IN NURSERIES, WITH SPECIAL REFERENCE TO PATHOGENS INVOLVED IN THE DECLINE OF YOUNG VINES*

ABSTRACT

Little information is presently available on the disease aetiology and epidemiology of the fungi involved in the decline of young vines. To address this question, four rootstock-scion combinations, originating from three commercial nurseries in the Wellington area of the Western Cape Province of South Africa were investigated during the 1999–2000 season. The first isolations were made in September from callused cuttings prior to planting in ~~the~~ nurseries. After planting, asymptomatic rooted cuttings were selected from nurseries after 3, 6 and 9 months, respectively. Isolations were made from the roots, rootstock, grafting union and scion. Isolations from callused cuttings prior to planting clearly demonstrated that primary pathogens associated with Petri disease, such as *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. were already present in the apparently healthy rootstock propagation material as endophytes. However, *Cylindrocarpon* spp., which cause black foot disease, rarely occurred in propagation material at this time. Species of this genus were isolated at higher percentages later during the season. Less than 1% of the plants were infected with *Cylindrocarpon* spp. before planting in the nursery (October), whereas 50% or more of the plants were infected at the end of the season (June). These findings suggest that the low percentage survival of vine plants observed in recent years is partly due to infected propagation material, and to new infections being established in nurseries.

INTRODUCTION

Over the last few years, a drastic reduction has been noted in the survival rate of vine cuttings due to a decline disease present in nurseries, as well as in young vineyards, in the Western Cape Province of South Africa (Ferreira, 1998). The low average take percentages (40–60%) of young vines can be attributed to several factors, including fungal, bacterial and viral diseases, insect and nematode pests, abiotic factors, as well as nutritional deficiencies and toxicities (Ferreira, 1999). Petri disease, caused by *Phaeomoniella* (Pa) *chlamydospora*

* Published in Australasian Plant Pathology 32: 47-52 (2003).

(W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams (= *Phaeoacremonium chlamydosporum* W. Gams, Crous, M.J. Wingf. & L. Mugnai), as well as several species of *Phaeoacremonium* (Pm), has been implicated as a major contributor to the decline of young vines in South Africa (Ferreira *et al*., 1994; Ferreira, 1998; Fourie *et al*., 2000*a*, 2000*b*; Fourie and Halleen, 2001; Groenewald *et al*., 2001). Other than *Pa. chlamydospora*, several species of *Phaeoacremonium*, including *Pm. aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai and *Pm. rubrigenum* W. Gams, Crous & M.J. Wingf., have been isolated from diseased vines in South Africa (Groenewald *et al*., 2001). The situation may be even more complex than presently accepted, however, as several additional species such as *Pm. inflatipes* W. Gams, Crous & M.J. Wingf. (Mugnai *et al*., 1999), *Pm. angustius* W. Gams, Crous & M.J. Wingf. (Chicau *et al*., 2000), *Pm. parasiticum* W. Gams, Crous & M.J. Wingf. (Gatica *et al*., 2001) and *Pm. mortoniae* Crous & W. Gams (Groenewald *et al*., 2001) have also been associated with Petri disease in other parts of the world.

Furthermore, *Cylindrocarpon* spp., which cause black foot disease of grapevine (Maluta and Larignon, 1991), have also been found to be associated with the decline of young vines in South Africa (Fourie *et al*., 2000*b*; Fourie and Halleen, 2001). Species of *Cylindrocarpon* are common soil inhabitants, occurring as saprobes or weak pathogens, often associated with roots of herbaceous and woody plants (Brayford, 1993). Two species, *C. destructans* (Zinnsm.) Scholten and *C. obtusisporum* (Cooke & Harkn.) Wollenw., have been reported as the causal agents of black foot disease of grapevines. The first record of *C. destructans* on grapevines was made in France in 1961 (Maluta and Larignon, 1991). Since then it has been isolated from diseased vines in Tasmania (Sweetingham, 1983), Italy (Grasso, 1984) and Portugal (Rego *et al*., 2000). In Sicily (Grasso and Magnano di San Lio, 1975) and California (Scheck *et al*., 1998), the causal agent of this disease was again identified as *C. obtusisporum*.

Little information is presently available on the aetiology of the decline of young vines, as well as the epidemiology of the various plant pathogens involved. Furthermore, other fungi, or combinations thereof, could presumably also play a role in this disease complex. The present study was therefore undertaken to identify the fungi already established in apparently healthy mother vine grapevine material prior to them being propagated for planting in nurseries. A further aim was to re-examine the fungi occurring as endophytes or latent

pathogens in apparently healthy plants, but only after they had been cultivated in commercial nurseries prior to being sold to farmers.

METHODS

Plant samples. Four rootstock-scion combinations, originating from three commercial nurseries in the Wellington area of the Western Cape Province were investigated during the 1999–2000 season. The combinations were Richter 99/Pinotage, 101-14 Mgt./Pinotage, Ramsey/Sultana and 143 B Mgt./Sultana. Rootstock and scion propagation material are propagated in various mother blocks as specified by the Vine Improvement Association of South Africa (VIA, P.O. Box 166, Paarl 7622, South Africa) and then bought by the individual nurseries for grafting. For the material studied here, grafting occurred in each of the three nurseries during June 1999, after which the callused material was planted during October according to standard nursery practices (Van der Westhuizen, 1981).

The first isolations were made in September from callused cuttings prior to planting in the nurseries. Apparently healthy, rooted cuttings were subsequently selected from the nurseries after 3, 6 and 9 months. All the cuttings were visually healthy according to the Plant Improvement Act (Act 53 of 1976) standards as specified by the Vine Improvement Association of South Africa. At each of the four sampling dates, 10 plants per combination were collected randomly from each nursery and immediately taken to the laboratory for surface sterilization (30 s in 70% ethanol, 5 min in 0.35% sodium hypochlorite and 30 s in 70% ethanol) before isolations were made. Vines were split lengthwise to reveal the xylem and pith regions. Isolations were made from the roots, rootstock (within 5 cm of the basal end), grafting union and scion (2 cm above the graft union). Twelve pieces of tissue (approximately 0.5 x 2 mm in size) were removed from each of the four isolation zones and placed in Petri dishes containing 2% potato-dextrose agar (PDA, Biolab, Midrand, Johannesburg) amended with chloramphenicol (250 mg/l) to reduce bacterial growth. Dishes were incubated in an incubation growth room at approximately 25°C. Fungal growth from plated tissue pieces was monitored daily, identified, or hyphal-tipped and transferred to PDA slants for later identification. The presence of bacteria and yeasts was also recorded.

Data analysis. The relative importance values (RI) of species isolated were computed (Ludwig and Reynolds, 1988). After standardisation of the RI values within each sample by

assigning the most frequent species the value of 100%, the other RI values were computed as percentages of it. For ordination analysis, a simple correspondence analysis was performed using the data referring only to those fungi with a standardised RI value of at least 1%. The data were pooled by site and time of isolation, as it was felt this would be the best way of characterising the occurrence of *Cylindrocarpon*, *Phaeoacremonium* and *Phaeomoniella* in the course of the growth of the plants. Simple correspondence analysis was performed on the reduced matrix of the raw data with the package XLSTAT ver. 4.3 (Kovach Computing Services, Anglesey, Wales, UK).

RESULTS

The fungi most frequently isolated from grapevine cuttings in the three nurseries over the four isolation dates are listed in descending order according to their relative importance values in Table 1. Results of the correspondence analysis showed that the first two axes accounted for 48% and 21% of the variance in the data, which indicated a good fit of the ordination to the data set (not shown). The ordination grouped the fungi into four main clusters, associated with the sampling times at which they were most abundant. One cluster contained *Pestalotiopsis*, *Epicoccum*, *Alternaria*, *Cladosporium* and the yeasts, which were most abundant in isolations prior to planting, but were isolated in only low numbers at later samplings. *Ulocladium*, *Aspergillus*, *Cytosphaera* and *Fusarium* formed a cluster of taxa that were most frequently isolated at 3 months. *Trichoderma* was intermediate between these two clusters, being isolated at high frequencies prior to planting and at 3 months, but at much lower numbers at 6 and 9 months. *Cylindrocarpon*, *Phoma*, *Phomopsis viticola* (Sacc.) Sacc., *Phialophora*, *Tetracoccosporium* and *Rhizoctonia solani* Kühn formed a cluster of fungi that were more frequently isolated at 6 and 9 months than at the earlier two samplings. The fourth cluster contained *Phaeomoniella*, *Phaeoacremonium*, *Acremonium*, *Clanostachys*, *Paecilomyces* and *Botryosphaeria*, which were isolated at similar frequencies at all sampling times. The correspondence analysis also suggested that apart from nursery J prior to planting, which was characterised by relatively high numbers of *Trichoderma* and low numbers of *Cladosporium* isolates, the spectrum of fungi isolated was similar at all three nurseries at each sampling time.

Cylindrocarpon was by far the most frequently isolated taxon (RI = 47.5%). Other known grapevine pathogens included *Phaeoacremonium* spp. (RI = 9.8%), *Pa.*

chlamydospora (RI = 5.7%), *Botryosphaeria* spp. (RI = 5.4%), *Rhizoctonia solani* (RI = 5.4%) and *Phomopsis viticola* (RI = 2.1%). Although species of *Fusarium* were also isolated in relatively high numbers (Table 1), a previous study conducted on South African grapevines by Marais (1979) suggested that they were of less importance in this disease complex, and hence they were excluded from further consideration. The frequencies at which the three selected taxa, *Cylindrocarpon*, *Phaeoacremonium* and *Phaeomoniella* were isolated were generally very similar for all three nurseries and four isolation dates (Table 1). *Cylindrocarpon* spp. were isolated only twice from callused cuttings prior to planting in the nurseries, but were more frequently isolated from rooted grapevine cuttings 3, 6 and 9 months after planting in the nurseries. Species of *Phaeoacremonium* and *Phaeomoniella*, however, were isolated both from callused cuttings prior to planting, as well as from rooted cuttings 3, 6 and 9 months after planting (Table 1). *Cylindrocarpon* spp. were isolated from more than 50% of all plants at the final sampling date. Further analysis to characterise the occurrence of the three selected taxa, *Cylindrocarpon*, *Phaeoacremonium* and *Phaeomoniella* in the nursery plants over time, is presented in Tables 2. *Cylindrocarpon* spp. were mostly isolated from the roots, followed by isolations from the rootstocks (Table 2). Although they rarely occurred in the graft unions and scions, they were more frequently isolated from the roots and rootstocks as the season progressed. On the other hand, *Pa. chlamydospora* was most frequently isolated from the rootstocks and graft unions, followed by isolations from the scions (Table 2), and rarely occurred in the roots. The isolations from the roots were made later during the season, after six and nine months. The frequency of *Pa. chlamydospora* isolations did not fluctuate much during the growing season. *Phaeoacremonium* spp. were most frequently isolated from the graft unions followed by isolations from the rootstocks (Table 2), and rarely occurred in the scions and roots. The frequency of *Phaeoacremonium* spp. also did not fluctuate much during the growing season.

DISCUSSION

The only previous comparable study in South African grapevine nurseries was conducted by Marais (1980), who chiefly isolated from soil and roots of dead, dying or stunted vines. Marais (1980) found that species of *Pythium* and *Phytophthora* were the most frequently isolated pathogens, with *Phytophthora cinnamomi* Rands being the most virulent

root rot pathogen. No mention was made of *Cylindrocarpon* spp. and species of *Fusarium* also appeared not to play any major role in nursery disease. An earlier study reported, however, that low numbers of *Cylindrocarpon* spp. were isolated from roots and rhizospheres of grapevines showing decline symptoms in several commercial vineyards (Marais, 1979).

Of the dominant fungal genera isolated in the present study, *Phaeomoniella chlamydospora* and species of *Cylindrocarpon* and *Phaeoacremonium* have in recent years been positively linked to the decline of young vines (Scheck *et al*., 1998; Fourie *et al*., 2000*b*; Rego *et al*., 2000). The present study demonstrates clearly that species of *Cylindrocarpon*, which cause black foot disease, rarely occurred in propagation material being sold to commercial nurseries. Species of this genus were isolated at higher percentages later during the season. Less than 1% of the plants were infected with *Cylindrocarpon* spp. prior to planting in nurseries (October), whereas 50% or more of the plants were infected in the nurseries by the end of the season (June). Infection of the roots occurred first, followed by infection of the basal end of the rootstock in some plants. At the time of planting, the basal ends (especially the pith area) of most of the cuttings are partly or even fully exposed for infection by soilborne pathogens, since callus tissue does not normally cover the entire area. The young callus roots often break during the planting process, resulting in small wounds susceptible to infection by these pathogens. The occurrence of *Cylindrocarpon* spp. in the graft union might be explained by the nursery practice of covering graft unions with soil for a period of approximately five weeks to prevent drying of the callus tissue.

In contrast to these data for the species associated with black foot disease, primary pathogens associated with Petri disease such as *Pa. chlamydospora* and *Phaeoacremonium* spp. were already present in the healthy rootstock propagation material as endophytes. It was also recently shown that these pathogens could infect plants via pruning wounds on the mother vines (Larignon and Dubos, 2000). Plants were infected, therefore, even before the cuttings were sold to nurseries for grafting. *Pa. chlamydospora* and *Phaeoacremonium* spp. were isolated most frequently from the rootstock and graft union. The high frequency of these pathogens occurring in the graft unions might be explained by the availability of sufficient weakened plant tissue as a result of the grafting process, or aerial contamination during the grafting process. These fungi rarely occurred in the roots, which suggests they are not primary soilborne pathogens. Bertelli *et al*. (1998) hypothesised about the soilborne nature of *Pa. chlamydospora*, since the fungus produces chlamydospores in artificial media

(Crous *et al*. 1996). Petri disease is commonly attributed to *Pa. chlamydospora* and several species of *Phaeoacremonium* (Mugnai *et al*., 1999). In South Africa, however, Groenewald *et al.* (2001) found *Pm. aleophilum* to be the most frequently isolated *Phaeoacremonium* species from vines. Several other as yet unidentified species of *Phaeoacremonium* have also subsequently been isolated from local vines (Mostert and Crous, unpublished data), and their potential role in this disease remains to be further elucidated.

Several species of *Botryosphaeria* were isolated in the present study. *Botryosphaeria obtusa* (Schwein.) Shoemaker, *B. ribis* Grossenb. & Duggar and *B. dothidea* (Moug.: Fr.) Ces. & De Not. are known to occur in grapevines in South Africa (Crous *et al*., 2000). *Botryosphaeria obtusa* is the causal agent of black dead arm disease in France (Larignon and Dubos, 2001), and has also been linked to various other symptoms including black to brown streaks, brown-red wood, altered pith, brown necrosis, white decay, brown-red margin of decayed wood and even healthy wood (Mugnai *et al*., 1999). It has also been associated with vine decline and dieback symptoms in Australian vineyards (Castillo-Pando *et al*., 2001). Isolates from the *B. ribis/B. dothidea* species complex are associated with Macrophoma rot, occurring on blighted stems, and have been reported to cause stem blighting and stem cankers on numerous hosts (Milholland, 1994). A recent molecular study (Zhou *et al*., 2001) has, however, shown that these taxa must be seen as two separate species. *Botryosphaeria dothidea* is also associated with black dead arm in France (Larignon and Dubos, 2001) as well as excoriose in Portugal (Phillips, 1998, 2000). The precise roles of the different species found in grapevines are not yet clearly understood, but it is possible that they are primary pathogens of stressed wood in some cases and secondary invaders of wood infected by other fungi in other cases (Pascoe, 1998).

Rhizoctonia solani is a soilborne pathogen with a wide host and distribution range (Carling and Summer, 1992) that was also encountered during the course of this study. Its relative importance to grapevine cultivation in South Africa presently remains unclear, although Marais (1979) reported it to be pathogenic to local vines.

In summary, our results suggest that the low percentage survival of vine plants observed in recent years might be due to infected propagation material, as well as to new infections established in nurseries. Further studies should thus investigate various culture and molecular techniques to screen plants (grafted nursery plants as well as mother vines used as

propagation material) that are visually disease free, thereby confirming their status as healthy plants. In South Africa the same nursery soil has been used for decades. Standard nursery practice of a two-year rotation system, whereby cuttings are planted every second year, alternated with a cover crop, might have led to a build-up of soilborne pathogens such as species of *Cylindrocarpon* that appeared insignificant in earlier studies (Marais, 1979, 1980). The duration of this rotation period should, therefore, be investigated to establish the effect on pathogen populations. A method to protect the basal end of the cuttings at the time of planting, such as dipping the basal end in a suitable fungicide or biological control agent, should also be investigated.

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Table 1. Fungi most frequently isolated from grapevine cuttings[a]

Taxon	L0	V0	J0	L3	V3	J3	L6	V6	J6	L9	V9	J9
Cylindrocarpon spp.	0	2	0	73	86	62	177	117	164	201	163	243
Fusarium spp.	15	2	6	121	100	133	59	19	98	68	27	81
Trichoderma sp.	77	34	119	69	48	171	12	2	15	3	5	19
Acremonium spp.	28	21	7	51	62	42	34	46	57	28	44	62
Phoma spp.	9	3	5	23	48	32	68	93	30	52	43	45
Alternaria spp.	152	72	10	34	9	14	7	19	5	8	19	12
Phialophora spp.	7	0	8	17	9	12	40	15	61	75	11	103
Aspergillus spp.	0	0	0	27	93	79	25	33	15	26	10	17
Clonostachys spp.	20	20	14	40	20	29	28	13	0	44	17	39
Phaeoacremonium spp.	15	22	20	11	18	15	18	52	20	18	34	24
Phaeomoniella chlamydospora	8	27	1	6	3	5	37	12	8	35	10	2
Cladosporium sp.	80	51	5	4	1	1	2	0	0	3	1	1
Botryosphaeria spp.	23	2	23	1	0	8	20	6	3	24	23	13
Pestalotiopsis sp.	56	7	11	50	2	8	8	0	1	1	1	0
Rhizoctonia solani	0	0	0	15	8	13	41	25	10	18	11	4
Yeast	38	29	16	11	0	15	2	1	7	6	3	16
Ulocladium sp.	4	0	0	28	26	10	0	0	0	2	0	0
Cytosphaera sp.	0	0	0	12	20	9	0	2	12	1	8	5
Tetracoccosporium sp.	0	0	0	0	1	0	14	9	18	5	3	9
Phomopsis viticola	0	5	2	3	8	1	1	23	0	3	8	3
Paecilomyces sp.	7	1	0	15	3	2	2	2	5	4	1	10
Epicoccum sp.	24	4	4	5	1	10	0	0	0	0	0	0

[a] Raw frequencies are given for three nurseries and four isolation dates. The figures are the total number of isolates from a given nursery and isolation date, e.g. L0, V0 and J0 indicate isolates from callused cuttings prior to planting in nurseries L, V and J respectively; L3, V3 and J3 indicate isolates from rooted cuttings three months after planting in nurseries L, V and J respectively. Only those fungi with a relative importance value of more than 1% have been considered.

Table 2. Isolations of *Cylindrocarpon* spp., *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. from different portions of the roots and stems of vine cuttings sampled prior to planting in the nurseries (time = 0) and 3, 6 and 9 months after planting in the nurseries

	Time (months)				
Isolation zone	**0**	**3**	**6**	**9**	**Sum of fungal species isolated**
***Cylindrocarpon* spp.**					
Scion	0	0	0	2	2
Graft	0	0	11	4	15
Rootstock	2	2	41	80	125
Roots	0	219	406	521	1146
Sum of *Cylindrocarpon* spp. isolated	2	221	458	607	1288
Phaeomoniella chlamydospora					
Scion	2	1	19	7	29
Graft	13	4	29	12	58
Rootstock	21	9	8	25	63
Roots	0	0	1	3	4
Sum of *Phaeomoniella chlamydospora* isolated	36	14	57	47	154
***Phaeoacremonium* spp.**					
Scion	3	4	2	10	19
Graft	14	16	61	51	142
Rootstock	40	22	22	14	98
Roots	0	2	5	1	8
Sum of *Phaeoacremonium* spp. isolated	57	44	90	76	267

3. NOVEL SPECIES OF *CYLINDROCARPON* (*NEONECTRIA*) AND *CAMPYLOCARPON* GEN. NOV. ASSOCIATED WITH BLACK FOOT DISEASE OF GRAPEVINES (*VITIS* SPP.)*

ABSTRACT

Four *Cylindrocarpon* or *Cylindrocarpon*-like taxa isolated from asymptomatic or diseased *Vitis vinifera* plants in nurseries and vineyards of South Africa, New Zealand, Australia, and France were morphologically and phylogenetically compared with other *Neonectria*/*Cylindrocarpon* taxa. Sequences of the partial nuclear large subunit ribosomal DNA (LSU rDNA), internal transcribed spacers 1 and 2 of the rDNA including the 5.8S rDNA gene (ITS), and partial β-tubulin gene introns and exons were used for phylogenetic inference. *Neonectria*/*Cylindrocarpon* species clustered in mainly three groups. One monophyletic group consisted of three subclades comprising (i) members of the *Neonectria radicicola*/*Cylindrocarpon destructans* complex, which contained strains isolated from grapevines in South Africa, New Zealand, and France; (ii) a *Neonectria*/*Cylindrocarpon* species isolated from grapevines in South Africa, Canada (Ontario), Australia (Tasmania), and New Zealand, described here as *Cylindrocarpon macrodidymum*; and (iii) an assemblage of species closely related to strains identified as *Cylindrocarpon cylindroides*, the type species of *Cylindrocarpon*. This monophyletic group excluded two other groups, which comprised (i) members of the *Neonectria mammoidea* complex, with anamorphs characterised by curved macroconidia, violet or purple pigments in cultures of most of its members, and lack of microconidia and chlamydospores; and (ii) two undescribed *Cylindrocarpon*-like species, both from grapevines in South Africa. The latter two clades formed a paraphyletic group in LSU rDNA analysis but were supported as a monophyletic group in ITS and β-tubulin gene analysis. Strains of the *Neonectria radicicola*/*Cylindrocarpon destructans* complex isolated from grapevines matched *C. destructans* in morphology and DNA sequences. *Cylindrocarpon macrodidymum* formed micro- and macroconidia, but rarely formed chlamydospores. Its mostly 3-septate macroconidia were more or less straight, minutely widening towards the tip, and had an apical cell slightly bent to one side. Its teleomorph, *Neonectria macrodidyma*, was obtained in mating experiments, and was characterised by smooth to finely warted ascospores, smooth to finely warted perithecia, and moderately sized

* Published in Studies in Mycology 50: 431-455 (2004).

angular to subglobose cells in the outer region of the perithecial wall. The other two undescribed *Cylindrocarpon*-like species mentioned above were characterised by mostly 3–5-septate, curved macroconidia, and by the lack of microconidia. Both species differed from members of the *Neonectria mammoidea* group by brownish colonies and by brownish hyphal strands formed in the aerial mycelium. For these species a new genus, *Campylocarpon* gen. nov., is proposed. It comprises the new species *Campylocarpon fasciculare* and *Campylocarpon pseudofasciculare*, respectively. Inoculation of 6-month-old potted grapevine rootstocks (cv. Ramsey) with selected isolates of *Cylindrocarpon destructans*, *Neonectria macrodidyma*, *Campylocarpon fasciculare*, and *Campylocarpon pseudofasciculare* resulted in a reduced root and shoot mass of inoculated plants and appearance of symptoms typical of black foot disease.

Taxonomic novelties: *Neonectria macrodidyma* Halleen, Schroers & Crous sp. nov. (anamorph *Cylindrocarpon macrodidymum* Schroers, Halleen & Crous sp. nov.), *Campylocarpon* Halleen, Schroers & Crous gen. nov., *Campylocarpon fasciculare* Schroers, Halleen & Crous sp. nov., *Campylocarpon pseudofasciculare* Halleen, Schroers & Crous sp. nov.

INTRODUCTION

Species of *Cylindrocarpon* Wollenw. are common and may be isolated as soil inhabitants, saprobes on dead plant material, root colonizers or pathogens, or weak pathogens of various herbaceous and woody plants (Brayford, 1993). *Cylindrocarpon destructans* (Zinnsm.) Scholten [anamorph of *Neonectria radicicola* (Gerlach & L. Nilsson) Mantiri & Samuels] and *C. obtusisporum* (Cooke & Harkn.) Wollenw. have frequently been described as the agents of root rots of various hosts (Booth, 1966; Seifert *et al*., 2003b), and a black foot disease of grapevines (*Vitis vinifera* L.). The first record of *C. destructans* on grapevines was made in France in 1961 (Maluta and Larignon, 1991). Since then this species has been isolated from diseased vines in Tasmania (Sweetingham, 1983), Sicily (Grasso, 1984), and Portugal (Rego *et al*., 2000, 2001). *Cylindrocarpon obtusisporum* has been identified as the causal agent of this disease in Sicily (Grasso and Magnano di San Lio, 1975) and California (Scheck *et al*., 1998a). Various species of *Cylindrocarpon*, preliminarily identified as "*Cylindrocarpon* sp.", have also been isolated from young vines and from vines with basal rot or root necrosis in Spain (Armengol *et al*., 2001) as well as from diseased grapevines in South

Africa (Fourie *et al*., 2000; Fourie and Halleen, 2001). The black foot disease of grapevines was described by Sweetingham (1983), Larignon (1999), and Fourie and Halleen (2001) as mainly affecting young vines between two and eight years of age. The symptoms described were weak or absent vegetation, drying and dying of shoots during summer, abnormal development of roots with growth parallel to the soil surface, necrotic root crowns, development of secondary root crowns, brown to black wood of rootstocks, and internal necrosis extending from the bark to the pith in diseased parts of the plants. Additional symptoms, described by Grasso and Magnano di San Lio (1975) and Scheck *et al*. (1998a) include black discoloration of the wood, gum inclusions in xylem vessels and black streaks in the vascular tissue.

Teleomorphs with *Cylindrocarpon* anamorphs were traditionally classified in *Nectria* (Fr.) Fr., but are now considered to belong to *Neonectria* Wollenw. (Rossman *et al*., 1999; Mantiri *et al*., 2001; Brayford *et al*., 2004). Wollenweber based this name on *Neon. ramulariae* Wollenw. (1916). The reintroduction of *Neonectria* resulted from the realization that *Nectria* was too broadly defined and that its segregation into numerous teleomorphic genera could be corroborated by anamorphic, phylogenetic, and ecological character patterns (Rehner and Samuels, 1995; Rossman *et al*., 1999). Some pre-phylogenetic classification schemes had segregated the teleomorphs of *Cylindrocarpon* species into four infrageneric *Nectria* groups based on perithecial wall anatomy and ascospore morphology; these groups were centred on "*Nectria*" *radicicola* Gerlach and L. Nilsson, "*Nectria*" *coccinea* (Pers. : Fr.) Fr., "*Nectria*" *mammoidea* Phill. & Plowr., and "*Nectria*" *rugulosa* Pat. & Gaillard (Booth, 1959; Samuels and Brayford, 1990; Samuels and Brayford, 1994). Wollenweber (1917, 1928) created the sections *Chlamydospora* Wollenw. and *Ditissima* Wollenw. for species with and without chlamydospores, respectively. Booth (1966) schematically segregated *Cylindrocarpon* species into four groups based on the presence or absence of microconidia and chlamydospores. *Cylindrocarpon magnusianum* (Sacc.) Wollenw., which is the anamorph of the type species of *Neonectria*, *C. cylindroides* Wollenw., which is the type species of the genus *Cylindrocarpon*, *C. destructans*, which is the anamorph of *Neonectria radicicola*, and members of *Cylindrocarpon* species predominantly connected with teleomorphs of the "*Nectria*" *mammoidea* group were core members of the anamorphic groups delineated by Booth (1966). *Cylindrocarpon obtusisporum* was originally described from the U.S.A. (California) as occurring on *Acacia* sp., where it was observed to form macroconidia and chlamydospores (Booth, 1966). *Cylindrocarpon obtusisporum* strains

identified by Booth (1966) originated from a broad range of host plants in Europe, New Zealand, and North America, and, at least partly, formed microconidia.

Currently, representatives of all "*Nectria*" groups with *Cylindrocarpon* anamorphs have been transferred into *Neonectria* (Rossman *et al*., 1999; Mantiri *et al*., 2001; Brayford *et al*., 2004). Mantiri *et al*. (2001) and Brayford *et al*. (2004) analysed mitochondrial small subunit (SSU) ribosomal DNA (rDNA) sequence data of some of the species and concluded that the *Neonectria*/*Cylindrocarpon* species grouped together by this reclassification were monophyletic. However, these authors also found that this overall *Neonectria*/*Cylindrocarpon* clade included distinct subclades corresponding to at least three of the four groups delineated by Booth (1966). Significant molecular variation among taxa with *Cylindrocarpon*-like anamorphs was found by Seifert *et al*. (2003b) in a study on fungi causing root rot of ginseng (*Panax quinquefolius* L.) and other hosts. The dendrograms in this study, based on partial β-tubulin gene, and nuclear ribosomal internal transcribed spacer (ITS) region sequences, suggested that subclades including (i) *Neon*. *radicicola*, which consisted of numerous phylogenetically distinct units, (ii) *Neon*. *macroconidialis* (Samuels & Brayford) Seifert, and (iii) a subclade comprising two distinct isolates, one from *Vitis vinifera* in Ontario, Canada and the other from *Picea* sp. in Quebec, Canada, were monophyletic. Other *Cylindrocarpon* species appeared to be excluded from this monophyletic group.

A great variation in cultural and morphological characters was recently observed among *Cylindrocarpon* strains isolated from grapevines in nurseries and vineyards in South Africa (Halleen *et al*., 2003; Fourie and Halleen, 2004), France (Larignon, 1999), New Zealand, and Australia (Halleen, unpublished data). Some of the isolates could not be identified to species level because of unusual character combinations and poorly sporulating cultures. Combining cultures of some of the strains yielded a teleomorph that also could not be identified in literature, although it was most similar to teleomorphs in the *Neon*. *radicicola* group (Samuels and Brayford, 1990). In the present study, morphological characters and DNA sequences were used to characterise these *Cylindrocarpon*-like taxa from diseased and asymptomatic grapevines taxonomically and phylogenetically. Sequences of these taxa were compared with those of members of the *Neon*. *radicicola* complex published by Seifert *et al.* (2003) and various other *Neonectria*/*Cylindrocarpon* species deposited at the CBS Fungal Biodiversity Centre (CBS, Utrecht, The Netherlands). Some of these strains had earlier been deposited, studied, or identified by H.W. Wollenweber, C. Booth, W. Gams, & G.J. Samuels.

MATERIALS AND METHODS

Fungal cultures. Strains were isolated from both diseased and healthy looking, asymptomatic grapevines (Fig. 1) in nurseries and vineyards in South Africa, Australia, New Zealand, and France. They are listed in Tables 1, 2. Symptoms included various forms of decline as well as typical black foot symptoms (Table 1). The strains are stored at CBS and at the Department of Plant Pathology, University of Stellenbosch, South Africa (collection designation STE-U). Additional species of *Neonectria*/*Cylindrocarpon* were obtained from CBS (Table 2; Anonymous, 2001).

DNA isolation, sequencing and phylogenetic analyses. Mycelium was grown in tubes with 2 ml of complete medium (Raper and Raper, 1972) and DNA was extracted using the FastDNA® Kit (Bio 101, Carlsbad, CA, U.S.A.). PCR for the partial β-tubulin gene introns and exons was performed as described by Schroers *et al*. (2004). ITS and partial large subunit (LSU) rDNA was amplified using the primer pair V9G/LR5 (de Hoog and Gerrits van den Ende, 1998; Vilgalys and Hester, 1990). The same PCR system was used as for the partial β-tubulin gene and the following PCR program: an initial denaturation step at 94°C for 2 min, 35 cycles of 94°C for 35 s, 55°C for 50 s, 72°C for 2 min, and a final extension at 72°C for 6 min. The vials of 50 µl contained 1 µl genomic DNA extract, 25 pmol of each of the primers, 200 µmol of each of the dNTPs (Amersham Biosciences Europe GmbH, Freiburg, Germany), 1 U of Taq polymerase (Super Taq, HT Biotechnology Ltd, Cambridge, UK), and 1× standard PCR buffer supplied together with the Taq polymerase.

PCR fragments were purified using GFX^{TM} purification kit (Amersham Pharmacia Biotech Inc., Roosendaal, The Netherlands). Sequencing was done on an ABI Prism 3700 instrument (Applied Biosystems, Foster City CA, U.S.A.) with a BigDye terminator cycle sequencing kit (Applied Biosystems) or DYEnamicET dye terminator (Amersham Biosciences) following the conditions recommended by the vendors. PCR products were sequenced using the primers ITS1 and ITS4 (White *et al*., 1990) for the ITS; LR5, NL1, and NL4 (O'Donnell, 1993) for the LSU rDNA; and T1 and T2 (O'Donnell and Cigelnik, 1997) for the partial region of the β-tubulin gene.

Strains of which sequences were newly generated and their host and origin are listed in Tables 1, 2. Newly generated sequences have been deposited in GenBank (Tables 1, 2). The following taxa and published (i) ITS, (ii) LSU, and (iii) β-tubulin sequences, indicated by GenBank accession numbers, were included in the analyses: (i) *C. cylindroides*: CR6 (AY295301) (Seifert *et al*., 2003b), *C. destructans*: UAMH 4907 (University of Alberta Microfungus Collection, Edmonton, AB, Canada) (AF172261) (Iwen *et al*., unpublished), *Cylindrocarpon* sp.: 94-1356 (AY295304), 94-1685 (= CCFC 226730 [Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada, Ottawa, ON, Canada]) (AY295334), CD1401 (AY295335) (Seifert *et al*., 2003b), *Nectria cinnabarina* (Tode : Fr.) Fr.: CBS 279.48 (AF163025) (Lee *et al*., 2000), *Neon. coprosmae* (Dingley) Seifert: G.J.S. 85-182 (AF220971), CTR 73-152 (AF220970) (Schoch *et al*., 2000), G.J.S. 85-39 (AY295326) (Seifert *et al*., 2003b), *Neon. macroconidialis*: G.J.S. 83-162 (AY295327) (Seifert *et al.,* 2003b), *Neon. radicicola*: IMI 061536 (CABI Biosciences, Egham, U.K.) (AJ007354), IMI 375717 (AJ007355), IMI 375719 (AJ007356), IMI 376403 (AJ007351), IMI 376404 (AJ007357), IMI 376408 (AJ007352), IMI 376409 (AJ007353) (Langrell, unpublished), AR 2553 (AF220968), CTR 71-322 (AF220969) (Schoch *et al*., 2000), CCFC 139398 (AY295330), CCFC 144524 (AY295332), CCFC 150670 (AY295319), CD1557 (AY295329), CD1666 (AY295331), CD999 (AY295312), CR20 (AY295317), CY9801 (AY295310), IMI 3133237 (AY295333), JAT1378 (AY295328), NSAC-SH-1 (AY295311), NSAC-SH-2 (AY295313), NSAC-SH-2.5 (AY295314) (Seifert *et al*., 2003b); (ii) *Albonectria albosuccinea* (Pat.) Rossman & Samuels: NRRL 20459 (National Center for Agricultural Utilization Research, U.S. Dept. of Agriculture, Peoria, IL, USA) (U34554) (O'Donnell and Cigelnik, 1997), *Albonectria rigidiuscula* (Berk. & Broome) Rossman & Samuels: NRRL 13412 (U88104) (O'Donnell, 1993), *Calonectria morganii* Crous *et al*.: ATCC 11614 (American Type Culture Collection, Bethesda, MD, U.S.A.) (U17409) (Rehner and Samuels, 1995), *Cosmospora episphaeria* (Tode : Fr.) Rossman & Samuels: NRRL 20687 (U88100) (O'Donnell, 1993), *Cosmospora vilior* (Starbäck) Rossman & Samuels: ATCC 16217 (U57348) (Glenn and Bacon, unpubl.), *C. cylindroides*: CCFC 226722 (AY283551) (Seifert *et al*., 2003b), *Cylindrocladium floridanum* Sobers & C.P. Seymour: ATCC 22677 (U17408) (Rehner and Samuels, 1995), *Fusarium culmorum* (W.G. Smith) Sacc.: NRRL 25475 (AF006322) (O'Donnell *et al*., 1998), *Fusarium fujikuroi* Nirenberg: NRRL 13566 (U34528) (O'Donnell and Cigelnik, 1997), *Fusarium oxysporum* Schlecht. : Fr.: NRRL 26409 (AF060383) (Cigelnik, unpubl.), *Fusarium solani* (Mart.) Sacc.: NRRL 22292 (L36629) (O'Donnell and Gray, 1995), *Fusarium verticilloides* (Sacc.) Nirenberg: NRRL 22172

(U34526), (O'Donnell and Cigelnik, 1997), *Haematonectria haematococca* (Berk. & Broome) Samuels & Nirenberg: NRRL 22141 (L36623) (O'Donnell and Gray, 1995), *Leuconectria clusiae* (Samuels & Rogerson) Rossman, Samuels & Lowen: AR2706 (U17412), (Rehner and Samuels, 1995), *Nectria cinnabarina*: G.J.S. 89-107 (U00748) (Rehner and Samuels, 1994), *Nectria pseudotrichia* Berk. & M.A. Curtis: AR1755 (U17410) (Rehner and Samuels, 1995), "*Nectria*" *mariannaeae* Samuels and Seifert: CCFC 226709 (AY283553) (Seifert *et al*., 2003a), "*Nectria*" *ventricosa* Appel & Wollenw.: NRRL 20846 (L36613) (O'Donnell, 1993), *Neocosmospora endophytica* Polishook *et al*.: AR2674 (U17411) (Rehner and Samuels, 1995), *Neon. coccinea*: NRRL 20485 (U88124) (O'Donnell, 1993), *Neon. galligena* (Bres.) Rossman & Samuels: NRRL 20487 (U88126) (O'Donnell, 1993), *Neon. radicicola*: AR 2553 (U17415) (Schoch *et al*., 2000), CCFC 226721 (AY283552), (Seifert *et al*., 2003b), *Verticillium dahliae* Kleb.: ATCC 16535 (U17425) (Rehner and Samuels, 1995), *Viridispora diparietispora* (J.H. Miller *et al*.) Samuels & Rossman: ATCC 13214 (U17413) (Rehner and Samuels, 1995); (iii) *C. cylindroides*: CR21 (AY297211), CR6 (AY297172) (Seifert *et al*., 2003b), *Cylindrocarpon* sp.: 94-1356 (AY297175), 94-2057 (= CCFC 226735) (AY297176) (Seifert *et al*., 2003b), *Fusarium* sp.: NRRL 22900 (U34422) (O'Donnell and Cigelnik, 1997), *Neon. coprosmae*: G.J.S. 85-39 (AY297192) (Seifert *et al*., 2003b), *Neon. galligena*: KAS1224 (AY297216) (Seifert *et al*., 2003b), *Neon. macroconidialis*: G.J.S. 83-162 (AY297193) (Seifert *et al*., 2003b), *Neon. radicicola*: 20M15 (AY297215), 94-1628 (AY297185), CCFC 139398 (AY297196), CCFC 144524 (AY297198), CD1557 (AY297195), CD1561 (AY297179), CD1596 (AY297217), CD1598 (AY297202), CD1636 (AY297205), CD1666 (AY297197), CD842 (AY297214), CD999 (AY297184), CR20 (AY297187), CR26 (AY297188), CR29 (AY297212), CR36 (AY297213), IFO 31882 (NITE Biological Resources Centre, Chiba, Japan) (AY297191), IMI 3133237 (AY297194), NSAC-SH-1 (AY297181), NSAC-SH-2 (AY297182) (Seifert *et al*., 2003b).

The sequences were aligned automatically using the software ClustalX 1.81 (Jeannmougin *et al*., 1998). Alignments were adjusted manually using the software Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit) (TreeBASE S1207, M2082–6). Sequences of strains from grapevines were compared with those of hypocrealean taxa characterised by multiseptate macroconidia typical of the genera *Fusarium* Link and *Cylindrocarpon*. A few other hypocrealean taxa such as "*Nectria*" *mariannaeae* Samuels & Seifert, *Leuconectria clusiae* (Samuels & Rogerson) Rossman, Samuels & Lowen, *Calonectria* De Not., and

Nectria sensu stricto were also included. In phylogenetic trees, downloaded sequences are indicated by their GenBank accession numbers; newly generated sequences are indicated by CBS strain numbers. Five datasets were created and analysed separately (Figs 2–6). Phylogenetic relationships were estimated from the aligned sequences by the maximum parsimony criterion as implemented in PAUP 4.0b10 (Swofford, 2002). Heuristic searches were performed using parsimony-informative, unordered, and equally weighted characters. Gaps were treated as missing characters. A maximum of 1000 trees was allowed. Branch robustness in the analyses was tested by 1000 heuristic search replications, each on bootstrapped data sets. Simple sequence addition was used.

Morphological examination. Strains were grown in darkness or under continuous near-UV light (400–315 nm) (Sylvania Blacklight-Blue, Osram Nederland B.V., Alphen aan den Rijn, The Netherlands) at 20°C. Media used were synthetic nutrient-poor agar (SNA) with and without the addition of a 1 × 3 cm piece of filter-paper to the colony surface (Nirenberg, 1976), potato-dextrose agar (Difco PDA, Becton Dickinson, Sparks, MD, U.S.A.), oatmeal agar (OA, Gams *et al*., 1998), and a diluted V8-juice agar ($V8_{50}$, as described in Gams *et al*., 1998, diluted 1:1 with water agar), and malt extract agar (MEA) (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) using 9 cm diam Petri dishes. Growth rates and colony diameters of cultures incubated in darkness were measured on SNA and PDA. Characters such as size and shape of conidia, phialides, and chlamydospores were measured from strains grown on SNA, OA, PDA, or $V8_{50}$ after 14–21 days. For measurements, water was used as mounting medium in microscopic slides. Images were taken from slides mounted in water or lactic acid. Measurements in the description are given as described by Schroers (2001). Macroscopic characters of colonies were described after 14 days; colour names are from Kornerup and Wanscher (1978). Cardinal temperatures for growth were assessed on PDA incubated for 7 days in the dark at 4, 10, 15, 20, 25, 30, and 35°C. Mating experiments were performed on carnation leaf agar (CLA) at 23°C using the methods and conditions outlined by Schoch *et al*. (1999). Three replicates were done for each cross. Petri dishes were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI, U.S.A.) and observed at weekly intervals for a total period of 60 days. Two strains were considered sexually compatible if they produced perithecia with viable, exuding masses of ascospores within this time.

Pathogenicity. A pathogenicity study was conducted with 6-month-old potted grapevine rootstocks (cv. Ramsey) in a glasshouse. Due to the lack of "disease-free" nursery plants, tissue culture plants were prepared by the Breeding and Evaluation Division, ARC Infruitec-Nietvoorbij (The Fruit, Vine and Wine Institute of the Agricultural Research Council, Stellenbosch, South Africa). Four isolates, CBS 112597 (F.H. c33), CBS 112604 (F.H. c10), CBS 112614 (F.H. c79) and CBS 112679 (F.H. c108), which represented the four *Cylindrocarpon*-like species from grapevines delineated in phylogenetic analyses, were used. The isolates were selected based on the results of preliminary pathogenicity screenings (results not shown). The plants, 20 per isolate, were inoculated by dipping the roots (Scheck *et al*., 1998b) in a 1×10^6 conidial suspension for 30 min. Control plants were dipped in sterile water. Inoculated plants were planted individually in pots containing sterilised potting mixture and placed in a glasshouse at 24°C in a completely randomised design. Evaluation was conducted after 4.5 months by counting dead plants, as well as by making isolations from the stem bases of the remaining plants to confirm the continuing presence of the inoculated fungus. The experiment was repeated once.

The data was subjected to analysis of variance using SAS version 8.2 (SAS, 1999). Levene's test for homogeneity of variance was performed to test if the experimental variability in observations was of comparable magnitude. A Shapiro-Wilk test was performed to test for non-normality (Shapiro and Wilk, 1965). The Student's t-Least Significant Difference was calculated at the 5% confidence level to compare treatment means. Levene's test for homogeneity of variance ($P > 0.05$) indicated that the experimental variability in observations was of comparable magnitude, and hence a combined analysis could be conducted. There was no significant ($P > 0.05$) evidence for any experiment × isolate interactions, and therefore the means of the main effects were studied.

RESULTS

Phylogenetic analyses. Heuristic parsimony analyses of aligned partial LSU sequences of representatives of selected hypocrealean taxa and *Verticillium dahliae* (*Phyllachorales*), which was used as outgroup (**dataset 1**; 529 bp alignment) yielded four equally most parsimonious trees, of which one is shown in Fig. 2. The four trees, based on 98 parsimony-informative characters (PIC), were 371 steps long and had a consistency index (CI) of 0.380 and a retention index (RI) of 0.749. The trees differed by minor changes mainly

within the clade that included *Haematonectria*, *Neocosmospora*, *Cosmospora*, and "*Nectria*" *ventricosa*. Taxa with *Cylindrocarpon*-like anamorphs were found in three distinct clades. The largest of these clades consisted of a group of species centred around *Cylindrocarpon cylindroides*, including strains originally studied by Wollenweber (Anonymous, 2001), such as CBS 151.29 (*C. magnusianum*), CBS 183.36 (*C. obtusisporum*), CBS 226.31 [*C. willkommii* (Lindau) Wollenw.], CBS 232.31 [*C. heteronema* (Berk. & Broome) Wollenw.], CBS 237.29 [*C. candidum* (Link : Fr.) Wollenw.], and CBS 242.29 [*C. album* (Sacc.) Wollenw.]. Also included were CBS 217.67, which is the ex-type strain of *C. faginatum* C. Booth, as well as additional strains identified as *Neon. coccinea* (NRRL 20485, as U88124), "*Nectria*" *fuckeliana* C. Booth (CBS 112466), *Neon. galligena* (NRRL 20487, as U88126), and *C. cylindroides* (CBS 189.61; CCFC 226722, as AY283551). Additional fungi associated with the same clade were *Neon. macrodidyma*, a species newly recognised in this paper, and members of the *Neonectria radicicola*/*Cylindrocarpon destructans* complex including the ex-type strain of *Neon. radicicola*, CBS 264.65 (Tables 1, 2). Neither the overall *Neonectria*/*Cylindrocarpon* clade nor any of its three subclades, however, received support in bootstrap analyses. *Neonectria*/*Cylindrocarpon* formed the sister group to several other, phenotypically dissimilar genera of the *Nectriaceae* (*Hypocreales*) such as *Nectria* (anamorph: *Tubercularia* Tode), *Calonectria* (anamorph: *Cylindrocladium* Morgan), "*Nectria*" (anamorph: *Mariannaea* Arnaud ex Samson), and *Leuconectria* Rossmann, Samuels & Lowen, and was also the sister group of a large clade comprising taxa with *Fusarium*-like anamorphs. This last clade included *Albonectria* Rossman & Samuels, *Gibberella* Sacc., *Neocosmospora* E.F. Sm., *Haematonectria* Samuels & Nirenberg, and *Cosmospora* Rabenh. *Neonectria*/*Cylindrocarpon* appeared to be unrelated to two other clades with *Cylindrocarpon*-like anamorphs that were found near the base of the tree. One of these two clades comprised taxa of the "*Neon.*" *mammoidea* group (Samuels *et al*., 1990, as *Nectria discophora* group; Rossman *et al*., 1999; Brayford and Samuels, 1993; Samuels and Brayford, 1993; Brayford *et al*., 2004), while the other clade contained the genus *Campylocarpon*, which is newly described in this paper.

In ITS analysis, it was found that the lengths of the included sequences differed by up to 80 bp, which necessitated the introduction of multiple gaps in the alignment. Three regions with these multiple gaps, alignment positions 85–110, 399–419, and 439–461, were excluded from the analyses of **dataset 2**, which included representatives of the three major clades with *Cylindrocarpon*-like fungi analysed in Fig. 2, as well as *Nectria cinnabarina* and *Fusarium*

solani, of which the latter was used as outgroup. Dataset 2 formed a 588 bp alignment. Heuristic parsimony analyses of aligned included regions yielded 10 equally most parsimonious trees, of which one is shown in Fig. 3, based on 143 PIC 479 steps in length with a CI of 0.547 and a RI of 0.838. The 10 trees differed by minor changes within the *Neon*. *radicicola* complex and the clade referred to as "*Cylindrocarpon cylindroides* and other species". The analyses supported the existence of three clades containing taxa with *Cylindrocarpon*-like anamorphs.

The monophyly of species of the "*Neon*." *mammoidea* group was moderately supported (bootstrap value = 78%). The monophyly of *Neonectria*/*Cylindrocarpon* was strongly supported with a bootstrap value of 96%, as was that of *Campylocarpon* with 100% bootstrap support. *Neonectria*/*Cylindrocarpon* appeared excluded from the *Campylocarpon* and the "*Neon*." *mammoidea* group but the phylogeny of these three groups remained unresolved. Relatedness of the "*Neon*." *mammoidea* group to *Campylocarpon* was strongly supported in bootstrap analyses (bootstrap value = 96%), a result contradicting that obtained in LSU analysis. Monophyly of members of the *Neon*. *radicicola* complex, including *Neon*. *macrodidyma*, was relatively strongly supported (bootstrap value = 87%). Inclusion of *Neon*. *macrodidyma* within this clade was mainly due to the sequence of strain IMI 376409 (AJ007353) (S. Langrell, unpublished, referred to as *Neon*. *radicicola*), which contained apomorphic characters typical of the *Neon*. *radicicola* as well as other apomorphies seen in *Neon*. *macrodidyma*. Analyses done without this sequence placed *Neon*. *macrodidyma* as a sister to the *Neon*. *radicicola* complex (not shown). A long branch of 16 steps (Fig. 3) indicated that both *Neon*. *macrodidyma* and IMI 376409 differed from members of the *Neon*. *radicicola* complex. A subclade referred to as "*Cylindrocarpon cylindroides* and other species" contained strains identified as *Cylindrocarpon cylindroides*, *C*. *magnusianum*, *C*. *obtusisporum*, *C. faginatum*, *C. album*, *C. heteronema*, and *Cylindrocarpon* sp.; its monophyly was moderately supported (bootstrap value = 70%).

Heuristic parsimony analyses of aligned ITS sequences of **dataset 3** (533 bp alignment), which was restricted to members of the *Neon*. *radicicola* complex and closely related species as well as *Nectria cinnabarina* and *Fusarium solani*, of which *Fusarium solani* was used as outgroup, yielded 14 equally most-parsimonious trees, of which one is shown in Fig. 4, based on 75 PIC 174 steps in length with a CI of 0.626 and a RI of 0.925. The trees differed by minor changes within the *Neon*. *radicicola* complex and the clade referred to as

"*Cylindrocarpon cylindroides* and other species". All trees placed *Neon*. *macrodidyma* as a sister taxon to members of the *Neon*. *radicicola* complex. Relatedness of *Neon*. *macrodidyma* and members of the *Neon*. *radicicola* complex was moderately supported in bootstrap analyses (bootstrap value = 78%). Both these clades together formed the sister group to a clade including representatives of *C*. *magnusianum*, *C*. *cylindroides*, *C*. *album*, *C*. *faginatum*, *C*. *heteronema*, and *C*. *obtusisporum*. Monophyly of members of the latter group was weakly supported (bootstrap value = 70%). Monophyly of the conjunction of this clade, *Neon*. *macrodidyma*, and the *Neon*. *radicicola* complex was strongly supported (bootstrap value = 99%). In these analyses, however, *Neon*. *macroconidialis* (G.J.S. 83-162, AY295327) and the above-mentioned unusual *Neon*. *radicicola* isolate IMI 376409 (AJ007353) were excluded. Inclusion of these two taxa resulted in an inconclusive analysis yielding more than 1000 equally parsimonious trees, in which a clade containing *Neon*. *macrodidyma* and IMI 376409 was placed on a long branch of 18 steps among members of the *Neon*. *radicicola* complex, similar to the arrangement shown in Fig. 3. Strain DAOM 144524 (Seifert *et al*., 2003b), isolated from grapevine in Ontario, Canada, had ITS sequences identical to those of *Neon*. *macrodidyma* strains originating from South African, Australian, and New Zealand grapevines. All *Neon*. *macrodidyma* strains had identical ITS sequences. The species clade for *Neon*. *macrodidyma* was strongly supported (bootstrap value = 100%). Members of *Neon*. *radicicola* formed a weakly supported monophyletic clade (bootstrap value = 66%) that comprised relatively variable sequences. Forty percent of the PIC encountered in the dataset applied only to this clade. Analysis of the variation resulted in several, moderately to strongly supported subclades (see also Seifert *et al*., 2003b). One of the moderately supported subclades (bootstrap value = 74%) contained strains from grapevines in South Africa, France, and New Zealand. However, this clade also contained a strain isolated from *Cyclamen* in the Netherlands (CBS 301.93). The variation within this clade did not appear to correlate with geographical patterns or host diversity.

Heuristic parsimony analyses of 500–550 bp long sequences of the β-tubulin gene, flanked by primers T1 and T2 (**dataset 4**), resulted in a 572 bp alignment and yielded one most parsimonious tree (Fig. 5) based on 167 PIC 404 steps in length with a CI of 0.681 and a RI of 0.918. Taxa analysed in this dataset include those isolated from vines as well as additional *Cylindrocarpon*-like strains. A *Fusarium* sp. isolate was used as outgroup. Members of the "*Neon*." *mammoidea* group were excluded from the analyses. *Neonectria*/*Cylindrocarpon*, represented by members of the *Neon*. *radicicola* complex, *Neon*.

macrodidyma, and "*Nectria*" *fuckeliana*, fell into one strongly supported monophyletic clade (bootstrap value = 96%).

Unlike what was seen in LSU and ITS analyses, members of the *Neon. radicicola* complex formed a strongly supported monophyletic group (bootstrap value = 100%). Strains of *C. destructans* from grapevines in South Africa, France, and New Zealand, as well as CBS 301.93 from *Cyclamen* in the Netherlands, had identical partial β-tubulin sequences. Among the South African strains of *C. macrodidymum* that originated from seven different locations, five different scions, and six different rootstocks (Table 1), four variable sites were encountered in the partial β-tubulin gene. The variation within these strains did not appear to correlate with geographical patterns or host diversity.

A major, strongly supported clade (bootstrap value = 95%) comprised representatives of the "*Neon*." *mammoidea* group and *Campylocarpon*. This clade was only remotely related to *Neonectria*/*Cylindrocarpon*. Within this clade the "*Neon*." *mammoidea* group and *Campylocarpon* were accommodated in different subclades, with the former obtaining 84% and the latter 100% bootstrap support. Within the three representatives of the "*Neon*." *mammoidea* complex, considerable variation was encountered, consisting of numerous substitutions and indels. The isolates of *Campyl. fasciculare* had identical partial β-tubulin sequences. Between the two strains of *Campyl. pseudofasciculare* 1 indel and 9 substitutions were observed.

Dataset 5 comprised β-tubulin gene sequences of grapevine isolates as well as of selected *Cylindrocarpon*-like isolates published by Seifert *et al.* (2003b) that were not included in dataset 4. Heuristic parsimony analyses of this dataset was based on *ca*. 350–380 bp long sequences (386 bp alignment), flanked by primers T10 (O'Donnell and Cigelnik, 1997) and T2. They yielded 360 equally most parsimonious trees, of which one is shown in Fig. 6, based on 118 PIC 286 steps in length with a CI of 0.640 and a RI of 0.942. The trees differed by minor changes mainly within the *Neon. radicicola* complex. The highly supported monophyletic *Neonectria*/*Cylindrocarpon* clade (bootstrap value = 100%) contained three well supported subclades. One, supported by a 94% bootstrap value, contained *Neon. galligena* and *C. cylindroides* (note that various species shown to be related to *C. cylindroides* in ITS analysis were not included in β-tubulin analysis). A second clade consisted of *Neon. macrodidyma* (bootstrap value = 100%), and the third contained the *Neon.*

radicicola complex (bootstrap value = 99%). Single sequences of *Neon. macroconidialis* and "*Nectria*" *fuckeliana* included in the analysis also belonged to *Neonectria*/*Cylindrocarpon*, but within this group their phylogenetic positions remained unresolved. In contrast to the ITS analysis (Fig. 3), the present analysis only weakly supported close relatedness of *Neon. macroconidialis* to the *Neon. radicicola* complex.

Morphology. Isolates from grapevines in South Africa, France, and New Zealand identified as *C. destructans* showed micro- and macromorphological characters that were consistent overall. Conidiophores on SNA were mostly free-standing, unbranched or sparsely branched, and several times septate, as well as sporodochial, relatively short, and irregularly branched. Phialides borne on unbranched conidiophores had well-developed collarettes. Other characters included aseptate microconidia; predominantly 3-septate, mostly straight, sometimes slightly curved, apically rounded macroconidia; and chlamydospores abundantly formed on SNA within 14 days. Following Booth (1966) and Samuels and Brayford (1990), these strains were judged to be morphologically indistinguishable from *Neon. radicicola*/*C. destructans*.

Neonectria macrodidyma/*C. macrodidymum* is characterised by details of the shape of its macroconidia, the apex or apical cells, which are typically slightly bent to one side. Similar apical cells have also been described for *C. didymum* (Hartig) Wollenw., which differs from *Neon. macrodidyma* by forming smaller, 0–2-septate conidia (Domsch *et al*., 1980). Macroconidia of *C. destructans* and *C. obtusisporum* typically have an obtuse apex (Samuels and Brayford, 1990; Booth, 1966). *Neonectria macrodidyma* forms free-standing conidiophores that are mostly unbranched and several times septate, as well as sporodochial conidiophores that are relatively short and irregularly branched. Macroconidia were produced by both kinds of conidiophores. *Neon. macrodidyma* typically formed brown colonies on PDA and OA. In contrast to what is observed in some *Cylindrocarpon* species, however, it frequently shows a yellow pigmentation at the margin of PDA and OA colonies and below the filter paper of SNA colonies. The presence of micro- and macroconidia and the rare formation of chlamydospores would tend to place *Neon. macrodidyma* in groups 1 or 3 in Booth (1966). In *Neon. macrodidyma*, teleomorphic characters are reminiscent of those seen in the *Neon. radicicola* complex (Samuels and Brayford, 1990). As with members of that complex, this species has perithecial walls that are at least slightly verruculose, and also has a principal perithecial wall region composed of angular to globose cells. *Neonectria*

macrodidyma differs from *Neon. radicicola* in narrower perithecial walls [mostly less than 30 µm in *Neon. macrodidyma*, (20–)35–50(–60) µm in *Neon. radicicola* (Samuels and Brayford, 1990)], smaller cells in the outer region of the perithecial wall [mostly less than 20 × 15 µm diam in *Neon. macrodidyma*, (10–)20–50(–60) µm diam in *Neon. radicicola* (Samuels and Brayford, 1990)], and in longer ascospores [mostly longer than 14 µm in *Neon. macrodidyma*, mostly shorter than 13 µm in *Neon. radicicola* (Samuels and Brayford, 1990)]. Perithecia of *Neon*. *macrodidyma* are solitary or formed in loose aggregates, as in the *Neon*. *radicicola* complex, whereas *Neon*. *galligena* and *Neon*. *coccinea* typically form crowded perithecia in large numbers on a well developed stroma.

Campylocarpon fasciculare and *Campyl. pseudofasciculare*, both isolated from grapevines in South Africa, form only multi-septate macroconidia and are therefore reminiscent of members of Booth's group 2 (1966), which accommodates most anamorphs of the "*Neon*." *mammoidea* group (Brayford *et al*., 2004). In *Campyl. pseudofasciculare*, aerial chlamydospores have been observed; any formation of such structures is atypical for Booth's group 2. *Campylocarpon fasciculare* is particularly distinguished by fascicles of aggregated conidiophores formed on brownish strands of aerial hyphae and on unusually broad hyphae near the agar surface. A similar morphology has been described for the anamorph of *Neon*. *phaeodisca* (Rossman) Samuels & Brayford (Samuels and Brayford, 1993), which Brayford *et al*. (2004) placed among other members of the "*Neon*." *mammoidea* species group. The two species differ in their colony pigment, which is violaceous-brown or pale violaceous in *C*. *phaeodiscum* (Samuels and Brayford, 1993) but rather dark brown in *Campyl. fasciculare*, and in the length ranges of their macroconidia [62.5–91 µm in *C*. *phaeodiscum* (Samuels and Brayford, 1993) but typically less than 60 µm long in *Campyl. fasciculare*]. Brownish strands of aerial hyphae have also been observed in *Campyl. pseudofasciculare*. In this species, however, only solitary, mostly branched conidiophores have been seen.

TAXONOMY

Neonectria macrodidyma Halleen, Schroers & Crous, **sp. nov.** MycoBank MB500113. Figs 7a–l, 8.

Anamorph: *Cylindrocarpon macrodidymum* Schroers, Halleen & Crous, sp. nov.

Etymology: *Makrós* (Greek), large, referring to the macroconidia, which resemble those of *Cylindrocarpon didymum* but are significantly longer.

Neonectriae radicicolae similis sed ascosporis levibus vel verruculosis, et peritheciis levibus vel verruculosis distincta.

Perithecia formed heterothallically *in vitro*, disposed solitarily or in groups, developing directly on the agar surface or on sterile pieces of carnation leaf, ovoid to obpyriform, dark red, becoming purple red in 3% KOH (positive colour reaction), smooth to finely warted, 200–250 µm diam, up to 270–300 µm high when rehydrated; without recognizable stroma; perithecial wall consisting of two poorly distinguishable regions; outer region 20–30 µm thick, composed of 1–3 layers of angular to subglobose cells, 9–20 × 7–15 µm (n = 15); cell walls up to 1 µm thick; inner region around 5 µm thick, composed of cells that are flat in transverse optical section and angular to oval in subsurface optical face view; walls in the outer and inner region sometimes locally thinning to form pseudopores in conjunction with matching structures in adjacent cells; perithecial warts consisting of globose to subglobose cells, 7–16.5 × 4.5–13.5 µm (n = 20), that have walls up to 2.5 µm thick. *Asci* clavate to narrowly clavate, *ca.* 65 × 10 µm, 8-spored; apex rounded, with a minutely visible ring. *Ascospores* divided into two cells of equal size, ellipsoidal to oblong ellipsoidal, somewhat tapering towards the ends, smooth to finely warted, (12–)14–15–16(–18) × (3.5–)4(–4.5) µm (n = 37).

Cylindrocarpon macrodidymum Schroers, Halleen & Crous, **sp. nov.** MycoBank MB500114. Fig. 9a–y.

Anamorphe a *Cylindrocarpo didymo* conidiis plerumque 3-septatis distincta, a *C. destructante* chlamydosporis paucissimis differt. Microconidia 0–1-septata, ellipsoidea vel ovoidea, plus minusve recta, hilo plus minusve laterali, (5.5–)8–9.5–10.5(–12.5) × (3.5–)4–4–4.5(–4.5) µm; macroconidia copiosiora, 1–3(–4)-septata, plus minusve recta, cylindrica vel sursum paulo expansa, cellula apicali modice unilateraliter curvata et exigue rostrata; macroconidia 3-septata (26–)34–36–38(–45) × (4–)5.5–6–6.5(–8) µm.

Conidiophores simple or complex, sporodochial. Simple conidiophores arising laterally or terminally from the aerial mycelium or erect, arising from the agar surface, solitary to loosely aggregated, unbranched or sparsely branched, 1–4-septate, rarely consisting only of the phialide, 55–120 µm long; phialides monophialidic, cylindrical, slightly tapering toward the base, 16.5–30 µm long, 2.5–3.5 µm wide at base, 2–2.5 µm near aperture (n = number of measurements = 28). Complex, sporodochial conidiophores aggregated in pionnote sporodochia, repeatedly, irregularly branched; phialides cylindrical but slightly tapering towards the tip or narrowly flask-shaped, mostly with widest point near the middle, (15.5–)17.5–20–21(–28.5) µm long, (2.5–)3(–3.5) µm wide at the base, (2.5–)3.5(–4) µm at the widest point, and (1.5–)2(–2.5) µm near the aperture (n = 58). *Micro-* and *macroconidia* present on both types of conidiophores. *Macroconidia* predominating, formed by both types of conidiophores, predominantly 1–3(–4)-septate, straight or sometimes slightly curved, cylindrical or typically minutely widening towards the tip, therefore appearing somewhat clavate, particularly when still attached to the phialide, with apex or apical cell typically slightly bent to one side and minutely beaked, mostly with a visible, slightly laterally displaced hilum; 1-, 2-, and 3-septate macroconidia of similar size range; 1-septate macroconidia 24–32 × 5–7 µm (n = 10); 3-septate macroconidia (26–)34–36–38(–45) × (4–)5.5–6–6.5(–8) µm (n = 116). *Microconidia* sparsely produced on SNA, moderately common on OA, 0–1-septate, ellipsoidal to ovoidal, more or less straight, with a minutely or clearly laterally displaced hilum; aseptate microconidia (5.5–)8–9.5–10.5(–12.5) × (3.5–)4(–4.5) µm (n = 43). *Conidia* formed in heads on simple conidiophores or as white (OA) or unpigmented (SNA) masses on simple as well as complex conidiophores. *Chlamydospores* sometimes occurring, mostly in short, intercalary chains, 7–12.5 × 6–10 µm (n = 25).

Holotypes: For the anamorph: dried SNA colony of CBS 112615 (herb. CBS 6588); for the teleomorph: dried CLA colony with perithecia formed by crossing CBS 112594 and CBS 112603 (herb. CBS. 6589).

Cardinal temperatures for growth: Minimum temperature not determined, < 4°C; optimum temperature 20–25°C, at which PDA colonies reach 30–45 mm diam after 7 days in the dark; maximum temperature ≤ 30°C. *Colonies* at 20°C reaching 10–20 mm diam on SNA and 10–17 mm diam on PDA after 4 days, 30–45 mm diam on SNA and 25–35 mm diam on PDA after 7 days. *Aerial mycelium* on SNA only developed near or on the filter paper, highly diffuse, consisting of single hyphae, overall white, or, if accumulating on the filter paper

yellowish; on OA and PDA abundantly formed, covering the whole colony or sectors thereof, resulting in a felty appearance; on OA white to yellowish, on PDA predominately yellowish, strongly vacuolated. *Colony reverse* on SNA not pigmented; on OA mustard-brown to cinnamon-brown (5E6–6E6) or Pompaeian to honey-yellow (5C6–5D6), sometimes with a pale yellow to mustard-yellow (3A5–3B6) margin; on PDA in central parts of the colony burnt umber (6E7–6F7), towards the margin raw Sienna (6D7), reddish golden (6C7), or brownish yellow (5C7), typically with a pale yellow to amber-yellow (3A5–4B6) margin; pigmentation similar on PDA incubated in darkness or under continuous near UV-light.

Strains studied: CBS 112615 (STE-U 3976, F.H. c98), CBS 112593 (STE-U 3990, F.H. c107), CBS 112603 (STE-U 4007, F.H. c8), CBS 112609 (STE-U 3969, F.H. a), CBS 112608 (STE-U 3987, F.H. c62), CBS 112594 (STE-U 3991, F.H. c111), CBS 112598 (STE-U 3997, F.H. c115), CBS 112605 (STE-U 3984, F.H. c106) (Table 1).

Fertile matings: Perithecia observed mostly within 3 or 4 weeks in crossings of strains CBS 112603 × CBS 112615, CBS 112603 × CBS 112609, CBS 112603 × CBS 112605, CBS 112603 × CBS 112598, CBS 112601 × CBS 112605, CBS 112594 × CBS 112601, CBS 112594 × CBS 112603.

Distribution: Australia, Canada, New Zealand, South Africa.

Habitat: Roots and rootstocks of grapevines, causing black foot disease.

Phylogenetic affinity: *Nectriaceae, Hypocreales*.

Campylocarpon Halleen, Schroers & Crous, **gen. nov.** MycoBank MB500115.

Etymology: *Kampylos* (Greek), referring to the curved macroconidia, the second part matching *Cylindrocarpon*.

Cylindrocarpo simile, microconidiis carens, chlamydosporis raris vel absentibus, macroconidiis vulgo curvatis. Conidiophora e latere hypharum aeriarum singularium vel fasciculatarum vel ex hyphis in superficie substrati crescentium oriunda, singula vel laxe vel arcte aggregata, capitula conidiorum mucida pionnoti similia formantia. Cellulae basilares

stipitis conidiophori ad 16 µm latae, nonnullas phialides vel penicillum irregularem ramorum brevium portantes; rami ultimi 1 vel complures phialides portantes. Macroconidia sicut in *Cylindrocarpo*, sed typice curvata, ad 6-septata; cellula apicalis obtusa, basilaris obtusa vel hilo inconspicuo praedita.

Type: *Campylocarpon fasciculare* Schroers, Halleen & Crous, sp. nov.

Similar to *Cylindrocarpon*. *Microconidia* absent, *chlamydospores* rare or absent, macroconidia mostly curved. *Conidiophores* arising laterally from single or fasciculate aerial hyphae or from creeping substrate hyphae, singly or in loose or dense aggregates; conidial heads forming pionnotes-like aggregates. *Conidiophore* stipe base to 16 µm wide, bearing several phialides or a penicillus of irregular branches; terminal branches bearing 1 or several phialides. *Macroconidia* as in *Cylindrocarpon*, but typically curved, up to 6-septate; apical cell obtuse, basal cell obtuse or with inconspicuous hilum.

Phylogenetic affinity: Apparently a sister taxon of the "*Neonectria*" *mammoidea* group (*Nectriaceae, Hypocreales*).

Campylocarpon fasciculare Schroers, Halleen & Crous, **sp. nov.** MycoBank MB500116. Fig. 10a–o.
Teleomorph: Not known.

Etymology: *Fasciculus* (Latin), a little bundle, referring to the compact, densely branched conidiophores that arise from short supporting cells.

Cylindrocarpo phaeodisco simile sed conidiis paulo minoribus et coloniis brunneis, pigmento purpureo carentibus distinctum. Conidiophora compacta, ex una vel compluribus cellulis stipitariis et semel vel compluries irregulariter ramoso penicillo composita, ex hyphis aeriis saepe brunneis, fasciculatis vel ex hyphis in substrato repentibus ad 16 µm latis oriunda. Microconidia nulla, macroconidia cylindrica, recta vel modice curvata, (1–)3–4(–5)-septata, utrinque angustata et obtusa, hilo vix visibili; 3-septata (29–)38–41.5–44.5(–53) × (5.5–)6.5–7.5–8(–9) µm, 4-septata (39–)47–49–51.5(–58) × (6.5–)7.5–8–8.5(–9). Chlamydosporae absentes.

Conidiophores initially simple, consisting of single phialides, or phialides in whorls of up to three members, that are formed on short supporting cells situated laterally on hyphae of the aerial mycelium; later arranged in dense fascicles, 40–60 µm high and 80–140 µm diam, arising laterally from unpigmented hyphae of the aerial mycelium, unpigmented hyphae growing near the agar surface, or brownish pigmented hyphal strands of the aerial mycelium. Hyphal cells supporting the conidiophore fascicles 15–40 × 6–16 µm (n = 12); fascicles 1–3 times branched, with basal cells 8–35 × 5.5–16 µm and metulae 7.5–13.5 × 3.5–5.5 µm (n = 10). Phialides narrowly flask-shaped, mostly with widest point near the middle, (13–)16–18–19(–29) µm long, (2–)2.5–3(–3.5) µm wide at the base, (2–)3.5–4(–4.5) µm at the widest point, and 2(–2.5) µm near the aperture (n = 52). *Macroconidia* mostly 3–4-septate, also 1-, 2-, and 5-septate, cylindrical, slightly to moderately curved, with minutely tapering, obtuse ends, sometimes somewhat more strongly tapering at the base; base with or without an obscure hilum; when 1-septate 24–32 × 5–7 µm (n = 5), when 2-septate (28.5–)35–38–43.5(–47) × (6–)6.5–7–7.5(–9) µm (n = 34), when 3-septate (29–)38–41.5–44.5(–53) × (5.5–) 6.5–7.5–8(–9) µm (n = 126), when 4-septate (39–)47–49–51.5(–58) × (6.5–)7.5–8–8.5(–9) µm (n = 33), and when 5-septate 44.5–54 × 7.5–9 µm (n = 5). *Microconidia* not observed. *Conidial masses* off-white, hemispherical, with shape arising from formation on fascicles borne on hyphal strands; or alternatively pionnote-like, covering the agar surface. *Chlamydospores* not observed.

Holotype: Dried MEA colony of CBS 112613 (herb. CBS 6590).

Cardinal temperatures for growth: Minimum temperature 10°C; optimum temperature 30°C, at which PDA colonies reach *ca.* 30–45 mm diam after 7 days in the dark; maximum temperature not determined, ≥ 35°C. *Colonies* reaching 10–18 mm diam on SNA and 8–16 mm diam on PDA after 4 days , 20–30 mm diam on SNA and PDA after 7 days. *Aerial mycelium* on SNA essentially absent; on OA and PDA abundantly formed, covering the whole of colony or sectors thereof, white, thick, cottony to felty, intermingled with or giving rise to erect, white or brown hyphal strands up to 8 mm long and 20–70 µm thick; these strands sometimes partly covered by off-white slime. Aerial mycelium on $V8_{50}$ sparsely formed or absent; surface on $V8_{50}$ smooth to waxy or covered by slimy domes up to 300 µm in diam consisting of conidial masses. *Colony reverse* on SNA not pigmented; on OA and PDA

chocolate-brown (6F4) to dark brown (7F4–7F8) or, in some sectors, camel (6D4); pigmentation similar on PDA incubated in darkness or under continuous near-UV light.

Strains studied: CBS 113560 (STE-U 3972, F.H. c120), CBS 113559 (STE-U 4006, F.H. c119), CBS 112614 (STE-U 3973, F.H. c79), CBS 112613 (STE-U 3970, F.H. c76), CBS 112612 (STE-U 3966, F.H. c134), CBS 112611 (STE-U 3965, F.H. c147), CBS 112600 (STE-U 3981, F.H. c132) (Table 1).

Distribution: South Africa.

Habitat: Roots, rootstock and trunk of grapevines, causing black foot disease.

Campylocarpon pseudofasciculare Halleen, Schroers & Crous, **sp. nov.** MycoBank MB500117. Fig. 11a–m.
Teleomorph: Not known.

Etymology: *Pseudo* (Greek), false, referring to its similarities to *Campyl. fasciculare*.

Cylindrocarpo fasciculari simile sed conidiophoris singulis, simplicibus vel irregulariter ramosis, e latere hypharum aeriarum singularium vel fasciculatarum saepe brunnearum oriundis distinctum. Macroconidia cylindrica, plus minusve curvata, plerumque magis curvata in parte distali, utrinque exigue angustata, obtusa, hilo vix visibili; (2–)3–5(–6)-septata, 3-septata (29–)37.5–44–48.5(–68.5) × (6–)6.5–7–7.5(–9.5) µm; 4-septata (40.5–)46.5–51–53.5(–62) × (6.5–)7–8–8.5(–9.5); 5-septata (36.5–)51–55–59(–68) × (6.5–)7.5–8–8.9(–10) µm. Chlamydosporae rarae, plerumque 3–5 aggregatae, intercalares vel in ramis brevibus lateralibus, rotundatae vel modice angulares, (6–)8–9.5–11(–13) × (4.5–)7–7.5–8.5(–11) µm.

Conidiophores rarely simple, consisting of single phialides, or phialides in whorls of up to 3 members, that are formed on short supporting cells or on more highly branched structures that develop laterally on hyphae or hyphal strands. Most basal cells of conidiophores 12–19 × 5–7 µm; metulae 10–17 × 3–5.5 µm (n = 10). Phialides narrowly flask-shaped, mostly with widest point near the middle, (14–)16.5–19–20.5(–24.5) µm long, (2–)3(–3.5) µm wide at the

base, (3.5–)4(–5) µm at the widest point, and (1.5–)2(–2.5) µm near the aperture (n = 18). *Macroconidia* cylindrical, slightly to moderately curved; typically somewhat more curved at the tip than at the base; with minutely tapering, obtuse ends, somewhat more strongly tapering at the base than at the apex, and with or without an obscure hilum; 3–5-septate, also 2- and 6-septate; when 2-septate 24–36 × 6–7 µm (n = 2), when 3-septate (29–)37.5–44–48.5(–68.5) × (6–)6.5–7–7.5(–9.5) µm (n = 59), when 4-septate (40.5–)46.5–51–53.5(–62) × (6.5–)7–8–8.5(–9.5) µm (n = 29), when 5-septate (36.5–)51–55–59(–68) × (6.5–)7.5–8–8.9(–10) µm (n = 119), and when 6-septate 59.8–61.45 × 8.5–9 µm (n = 2). Chlamydospores sparse, typically in clusters of 3–5, intercalary or borne on short side-branches, round to somewhat angular, (6–)8–9.5–11(–13) × (4.5–)7–7.5–8.5(–11) µm.

Holotype: Dried PDA colony of CBS 112679 (herb. CBS 6591).

Cardinal temperatures for growth: Minimum temperature 10°C; optimum temperature 30°C, at which PDA colonies reach 45 mm diam after 7 days in the dark; maximum temperature not determined, ≥ 35°C. *Colonies* reaching 10–15 mm diam on SNA and around 10 mm diam on PDA after 4 days, 25 mm diam on SNA and PDA after 7 days. *Aerial mycelium* on SNA essentially absent; on $V8_{50}$ sparsely formed or absent; on OA and PDA abundant, covering the whole or sectors of the colony, white to off-white or slightly brownish, thickly cottony to felty, intermingled with or giving rise to erect white or brown hyphal strands up to 10 mm long and 10–80 µm thick. *Colony reverse* on SNA not pigmented; on OA and PDA chocolate-brown (6F4) to dark brown (7F4–7F8); pigmentation similar on PDA whether incubated in darkness or continuous near-UV light.

Isolates studied: CBS 112592 (STE-U 3988, F.H. c89), CBS 112679 (STE-U 5472, F.H. c108) (Table 1).

Distribution: South Africa.

Habitat: Roots of asymptomatic nursery grapevines.

Pathogenicity. None of the control plants died, while 35%, 27.5%, 22.5% and 17.5% of the plants inoculated with CBS 112604 (F.H. c10) (*Neon. macrodidyma*), CBS 112597

(F.H. c33) (*Cy. destructans*), CBS 112614 (F.H. c79) (*Ca. fasciculare*) and CBS 112679 (F.H. c108) (*Ca. pseudofasciculare*) died, respectively. The corresponding isolates were re-isolated from 81–100% of the remaining plants. No *Cylindrocarpon* or *Campylocarpon* species were isolated from any of the control plants. Inoculations resulted in a dramatic reduction of root ($P < 0.0001$) and shoot mass ($P = 0.0087$) of the potted grapevines (ANOVA table not shown). Compared to the uninoculated control plants, CBS 112597, CBS 112604, CBS 112614 and CBS 112679 reduced root mass by 57.5%, 48%, 42.1%, and 40.7%, respectively. Statistically, however, there was no difference between the isolates. Compared to the uninoculated control plants, CBS 112597, CBS 112614, CBS 112679 and CBS 112604, reduced shoot mass by 39.4%, 27.5%, 22.5%, and 17.8%, respectively.

DISCUSSION

Our phylogenetic analyses suggest that the circumscription of *Cylindrocarpon* must be restricted to anamorphs within the *Neon. radicicola* complex, the anamorph of *Neon. macrodidyma*, and an assemblage of species including *C. cylindroides* (the type species of *Cylindrocarpon*), *C. magnusianum*, *C. obtusisporum*, *C. willkommii*, *C. heteronema*, *C. candidum*, *C. album*, and *C. faginatum*, as well as the anamorphs of *Neon. coccinea*, "*Nectria*" *fuckeliana*, *Neon. galligena*, and *Neon. macroconidialis*, plus some additional species not included in these analyses. The connection of *Neonectria* as the holomorphic genus corresponding to species related to *Cylindrocarpon sensu stricto* is based on the work of Wollenweber (1928), who identified *C. magnusianum* as the anamorph of *Neon. ramulariae*, the type species of *Neonectria*. Among the species of *Neonectria*/*Cylindrocarpon*, "*Nectria*" *fuckeliana* and *Neon. macroconidialis* deviated most strongly in sequence from the other included species.

The analyses exclude *Campylocarpon* species and members of the former "*Nectria*" *mammoidea* group (Booth, 1959; Samuels and Brayford, 1993) from *Neonectria/Cylindrocarpon*, contradicting the recent transfer of the latter group to *Neonectria* (Brayford *et al*., 2004). Because these species are phylogenetically not closely related to *Neonectria*/*Cylindrocarpon*, the generic designation of the "*Neon*." *mammoidea* group has been rendered in quotation marks throughout this paper.

The perithecial wall of species within the *Neon. radicicola* complex, as well as that of *Neon. macrodidyma*, consists mainly of angular to subglobose cells (Samuels and Brayford, 1990; this study). This anatomy differs from that of the perithecial wall of teleomorphs within the "*Neon.*" *mammoidea* group; walls of this group are characterised by a region of elongate cells perpendicularly oriented to the surface of the perithecial wall (Samuels and Brayford, 1993; Brayford *et al*., 2004). However, a similar perithecial wall region has also been described for *Neon. ramulariae* (Rossman *et al*., 1999) and "*Nectria*" *fuckeliana* (Brayford *et al*., 2004). As the former species is, as mentioned previously, the type species of *Neonectria*, this deprives this character of significance at the generic level.

Species here accepted for *Neonectria*/*Cylindrocarpon* represent Booth's groups 1, 3, and 4 (Booth, 1966). They feature cylindrical to slightly curved, multi-septate macroconidia but only some of them form microconidia or chlamydospores. Microconidia and chlamydospores therefore appear not to be important in the distinction of *Neonectria*/*Cylindrocarpon* from related taxa. The excluded taxa consist of some members of Booth's group 4 such as *C. olidum* (Wollenw.) Wollenw., which clusters among members of the "*Neon.*" *mammoidea* group (Fig. 3), most taxa of Booth's group 2, which includes anamorphs of species of the "*Neon.*" *mammoidea* group, and the two *Campylocarpon* species. These species are characterised by typically curved, rarely straight macroconidia, and they lack microconidia. Although chlamydospores have not been described in Booth's group 2 (Booth, 1966; Brayford *et al*., 2004), they are known to be formed in one species of *Campylocarpon* and in *C. olidum* (Booth, 1966). Monophyly of *Campylocarpon* and the "*Neon.*" *mammoidea* group is supported in ITS and partial β-tubulin analysis. However, there is considerable genetic variation within the "*Neon.*" *mammoidea* group, giving rise to long terminal and subterminal branches for species such as *C. olidum* var. *crassum* (CBS 216.67), "*Neon.*" *trachosa* (CBS 112467), "*Neon.*" *lucida* (CBS 112456), and *C. ianthothele* var. *majus* (CBS 328.81). *Campylocarpon* species, though similar in macroconidial morphology to members of the "*Neon.*" *mammoidea* complex, can be distinguished by the formation of typically brownish rather than violaceous cultures, as well as by production of brownish hyphae, often in strands, and, in *Campyl. pseudofasciculare*, by formation of chlamydospores.

To address their strong molecular as well as morphological differences from anamorphs classified in *Cylindrocarpon*, a new genus, *Campylocarpon*, is proposed for *Campyl. fasciculare* and *Campyl. pseudofasciculare*. The fasciculate conidiophore aggregates

formed by *Campyl. fasciculare* have also been described for *C. phaeodiscum* Samuels & Brayford (Samuels and Brayford, 1993), the anamorph of "*Neon.*" *phaeodisca*, but not for any other *Cylindrocarpon* species. These two species differ in their colony pigment and in the length of their macroconidia. *Cylindrocarpon phaeodiscum* is phylogenetically closely related to "*Neon.*" *discophora*, "*Neon.*" *lucida*, and other species of the "*Neon.*" *mammoidea* group (Brayford *et al*., 2004), and therefore could also be related to *Campylocarpon*, as can be inferred from ITS and β-tubulin analyses. It is possible that *Campylocarpon* and members of the "*Neon.*" *mammoidea* group should be considered congeneric. The conidiophores of *Campyl. fasciculare* are similar to those of *C. phaeodiscum,* and *Campylocarpon* macroconidia resemble those formed by anamorphs of the "*Neon.*" *mammoidea* group (Brayford *et al*., 2004). In addition no microconidia are formed in either of these groups. *Campylocarpon fasciculare* and *Campyl. pseudofasciculare* are similar in forming conidiophores from sometimes brownish mycelial strands, as well as curved, mostly 3–5-septate macroconidia, and brownish pigmented cultures. Because they are phylogenetically closely related (Figs 2, 3, 5, 6), we initially expected also to find conidiophore fascicles in *Campyl. pseudofasciculare*, but instead, only separate, mostly branched conidiophores (Fig. 11b–d) were observed. *Campylocarpon pseudofasciculare*, however, sporulated poorly in cultures, and the absence of fascicles might have been an artifact of suboptimal culture conditions.

Ostensible *C. destructans* isolates from diseased grapevines on different continents were found to contain an additional, readily phylogenetically distinguished member of the *Neon. radicicola* complex *sensu* Seifert *et al*. (2003b). No morphological character was found to segregate these grapevine isolates from *C. destructans* as described by Samuels and Brayford (1990). This cryptic taxon is commonly associated with the typical black foot symptoms as described by Larignon (1999), and particularly affects young grapevines and frequently causes decline during the first year after planting. While this species seems to be particularly aggressive to grapevines and has mainly been isolated from that source, it is interesting that one strain originated from a *Cyclamen* sp. in the Netherlands.

Neonectria macrodidyma, though forming conidiophores like those of *C. destructans*, forms relatively fewer chlamydospores. Also, *Neon. macrodidyma* macroconidia have a slightly bent apical cell, while the corresponding cells in *C. destructans* are rather broadly rounded (Samuels and Brayford, 1990). Slightly bent apical cells have also been described

particularly for *C. didymum* (Domsch *et al*., 1980), but this species has 0–2-septate conidia that are smaller than those of *Neon. macrodidyma*. Similar conidia have been illustrated for a *C. obtusisporum* isolate from a *Tilia* branch (Wollenweber 1916, no. 465). Macroconidia of this isolate, however, were 20–40 × 3.5–5.75 µm, while those of *Neon. macrodidyma* appeared wider, measuring (26–)34–36–38(–45) × (4–)5.5–6–6.5(–8) µm. The shape of the macroconidia also distinguishes *Neon. macrodidyma* from the type of *C. obtusisporum*, macroconidia which were described by Cooke (1884) as obtuse at both ends and measuring 30–35 × 4–5 µm. Booth (1966) described macroconidia of similar shape in *C. obtusisporum*. According to Booth, however, 2–3-septate macroconidia of C. *obtusisporum* measure 34–50 × 6–7.5 µm. *Cylindrocarpon obtusisporum* has been identified as the cause of black foot disease on grapevines (Grasso and Magnano di San Lio, 1975; Scheck *et al*., 1998a) and on some other hosts (Booth, 1966). It is not known if these *C. obtusisporum* were correctly identified or if they were actually *Neon. macrodidyma* isolates.

Isolates of *Neon. macrodidyma* were derived from asymptomatic grapevines, and from vines displaying a range of decline symptoms, including typical black foot symptoms. The fact that many of these isolates were derived from nursery plants may be of great concern to the South African grapevine propagation industry, since a previous study (Halleen *et al*., 2003) has already shown that these infections occur in nurseries. Isolates of *Campylocarpon* were mostly derived from asymptomatic nursery plants, although they were also obtained from some plants with decline and black foot symptoms. A significant conclusion of our investigations is that black foot symptoms are caused by a cryptic species in the *C. destructans* complex, as well as *Neon. macrodidyma*, and possibly some *Campylocarpon* strains. This information might be useful in the improvement of control methods, since the fungi involved may differ in their susceptibility to selective fungicides as well as in epidemiology. Studies are currently underway to investigate fungicide responses among these isolates. The present study also impacts on the development of molecular detection strategies, since most of the primers currently used would not detect the two species of *Campylocarpon*.

Most of the isolates from grapevines included in this study were derived from diseased plants brought in by individual farmers or consultants, and therefore we make no claim to have adequately documented the geographic distribution of these taxa. However, it was noted that the South African strains of the *C. destructans* genotype that were aggressively pathogenic to grapevines originated from relatively warm and dry viticultural areas, which

normally have relatively dry soils. This pattern of occurrence might be explained by the use in such areas of a drip-irrigation practice whereby the roots and the belowground region of the stem are kept moist for long periods, creating conditions favourable for disease development.

Most South African nurseries are confined within a few limited areas where conditions are suitable, and the same soils have been used for many years. These "old" soils are hardly ever replaced by fresh soil, and fumigation practices are not a viable option. Standard nursery practice includes a 2-year rotation system, whereby cuttings are planted every second year and alternated with a cover crop. This might also favour the build-up of soilborne pathogens including the pathogens studied here. Future research needs to be focused on the distribution of these taxa in local nurseries, with an eye to establishing the extent to which cultivation practices such as rotation systems and cover crops, play a role in disease prevalence. The susceptibility of different grapevine rootstocks towards the four taxa also needs to be investigated.

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Fig. 1a–i. Symptoms associated with black foot disease. a. Eight-year-old grapevines showing severe decline symptoms including absence of budding, abnormal, weak vegetation, and summer wilting. b. Cross-section through infected rootstock revealing necrosis extending from the bark to the pith. c. Part of trunk with bark removed, showing a brown, subcortical zone beginning at the base of the rootstock running up along the trunk in severely affected vines. d. Cross section of an infected root. e. Dark vascular streaking seen in longitudinal section of trunk. f. Soil compaction and poor water drainage resulting from excessive movement of farm vehicles. g. Poor root development (J-rooting) resulting from soil compaction and poor soil preparation. h. Poor root development (pothole effect) resulting from soil compaction and poor soil preparation. i. Second layer of roots, growing parallel to the soil surface, formed by the plant in order to compensate for the loss of functional roots further below. Rootstocks are also thinner below the second root layer.

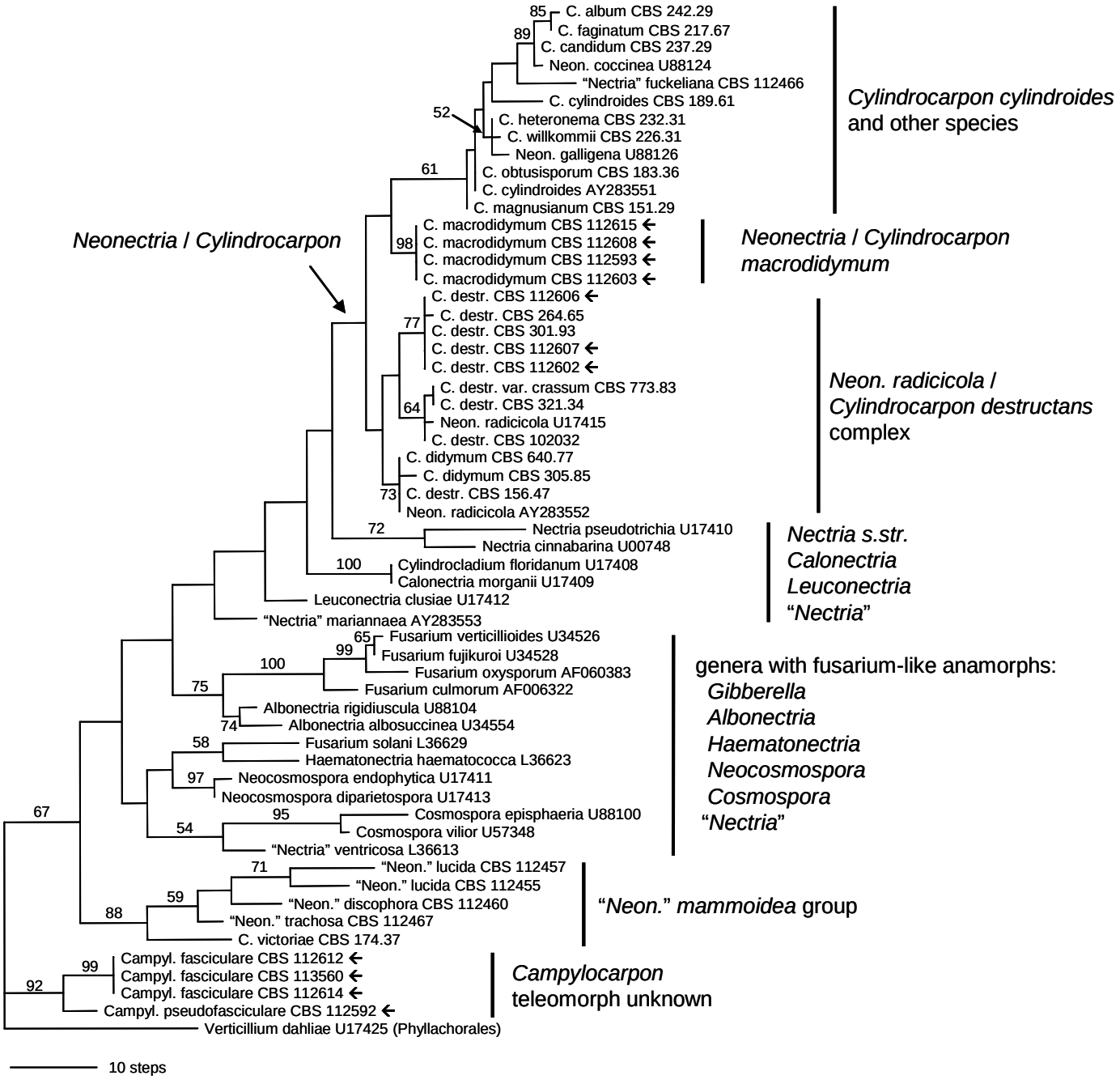

Fig. 2. One of four equally parsimonious phylograms (dataset 1) inferred from partial LSU rDNA sequences. Bootstrap values are indicated near nodes. Strains isolated from grapevines are marked by arrows.

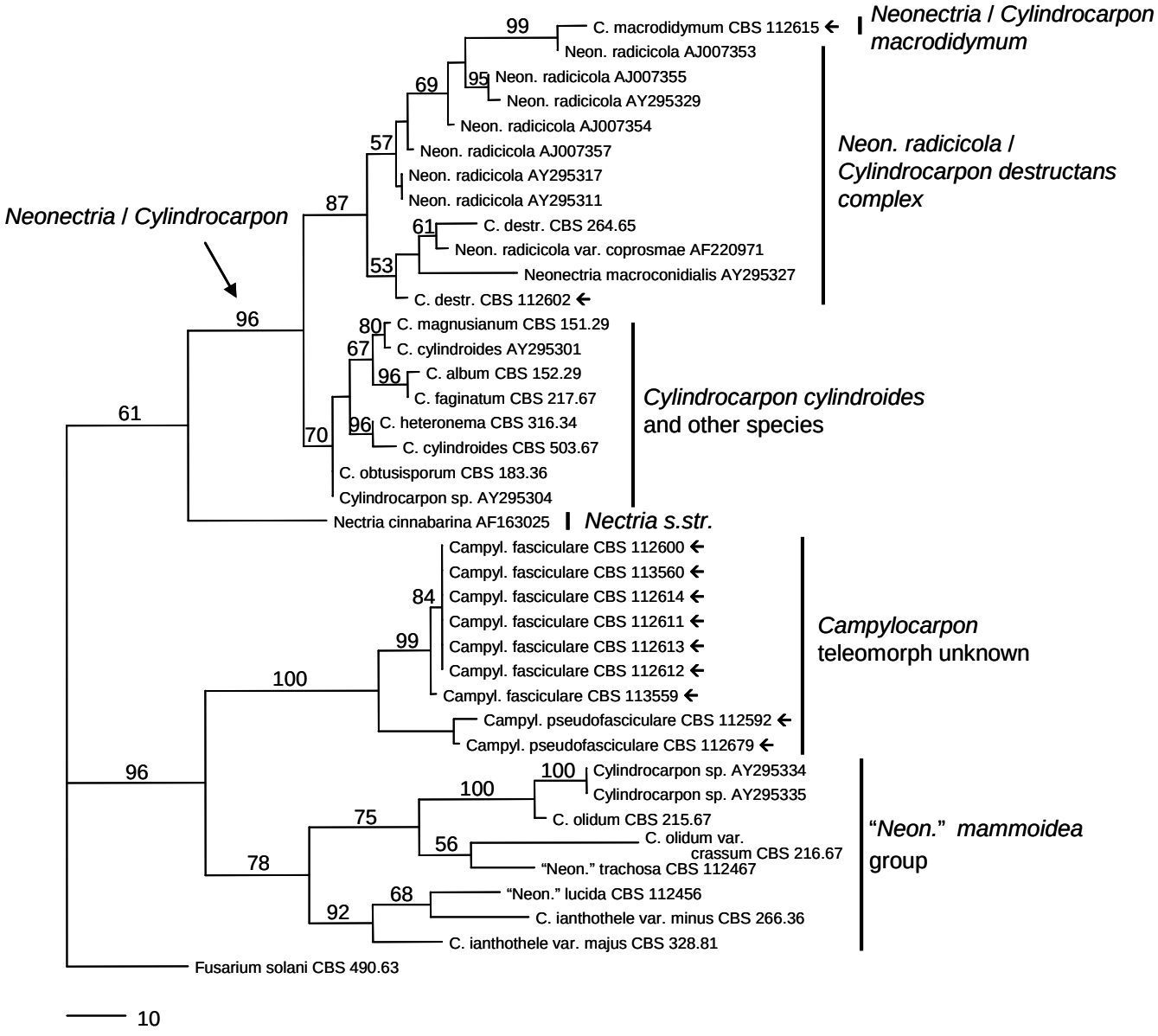

Fig. 3. One of 10 equally parsimonious phylograms (dataset 2) inferred from sequences of the internal transcribed spacer region 1, 5.8S rDNA and internal transcribed spacer region 2. Bootstrap intervals are indicated above nodes. Strains isolated from grapevines are marked by arrows.

Fig. 4. One of 14 equally parsimonious phylograms of a wider range of *Cylindrocarpon* isolates (dataset 3) inferred from sequences of the internal transcribed spacer region 1, 5.8S rDNA and internal transcribed spacer region 2. Bootstrap values are indicated above nodes. Strains isolated from grapevines are marked by arrows.

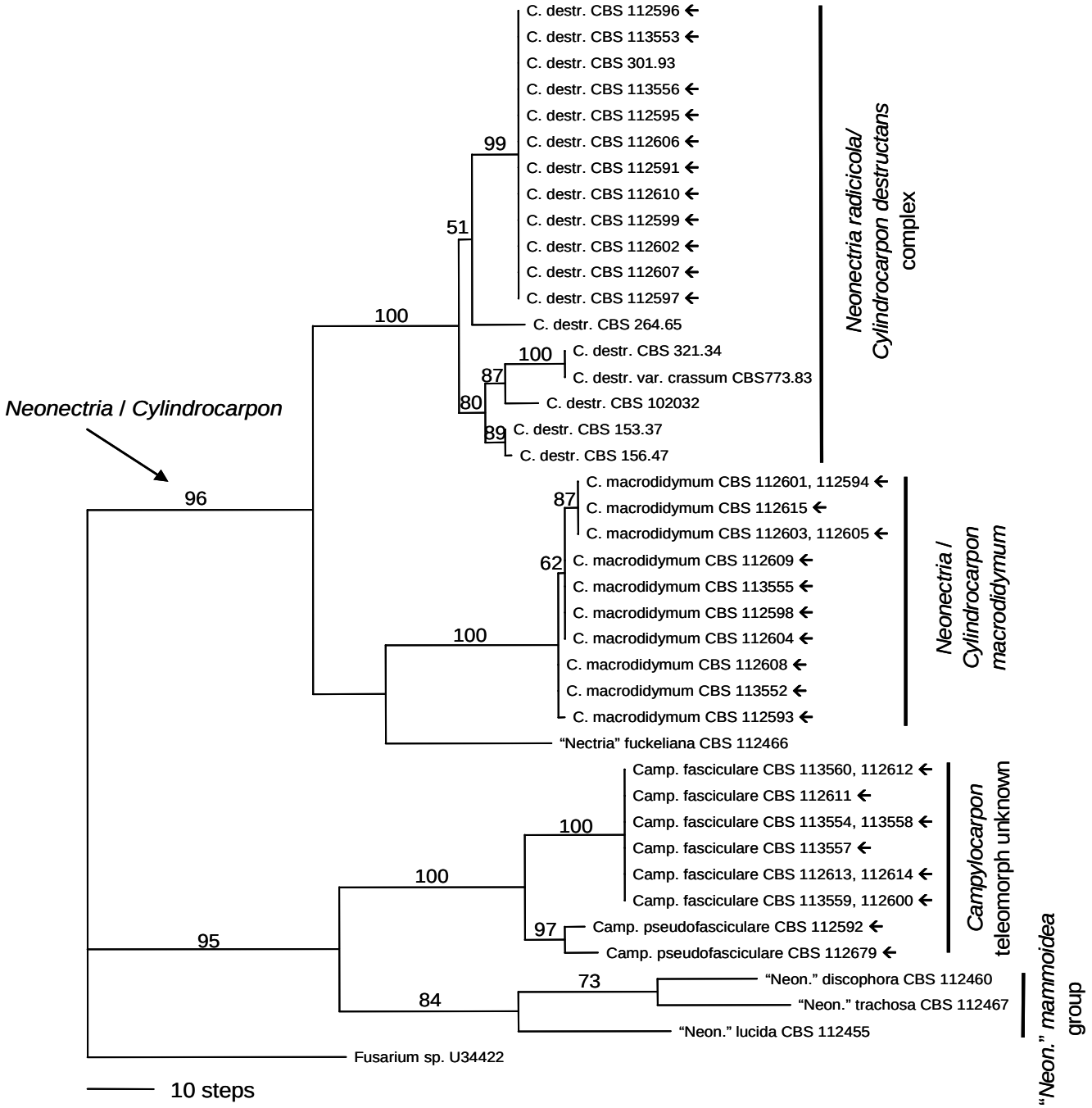

Fig. 5. Single most parsimonious phylogram for *Cylindrocarpon* and *Campylocarpon* isolates (dataset 4) inferred from sequences of a 572 bp alignment of the β-tubulin gene. Bootstrap values are indicated above the nodes. Strains isolated from grapevines are marked by arrows.

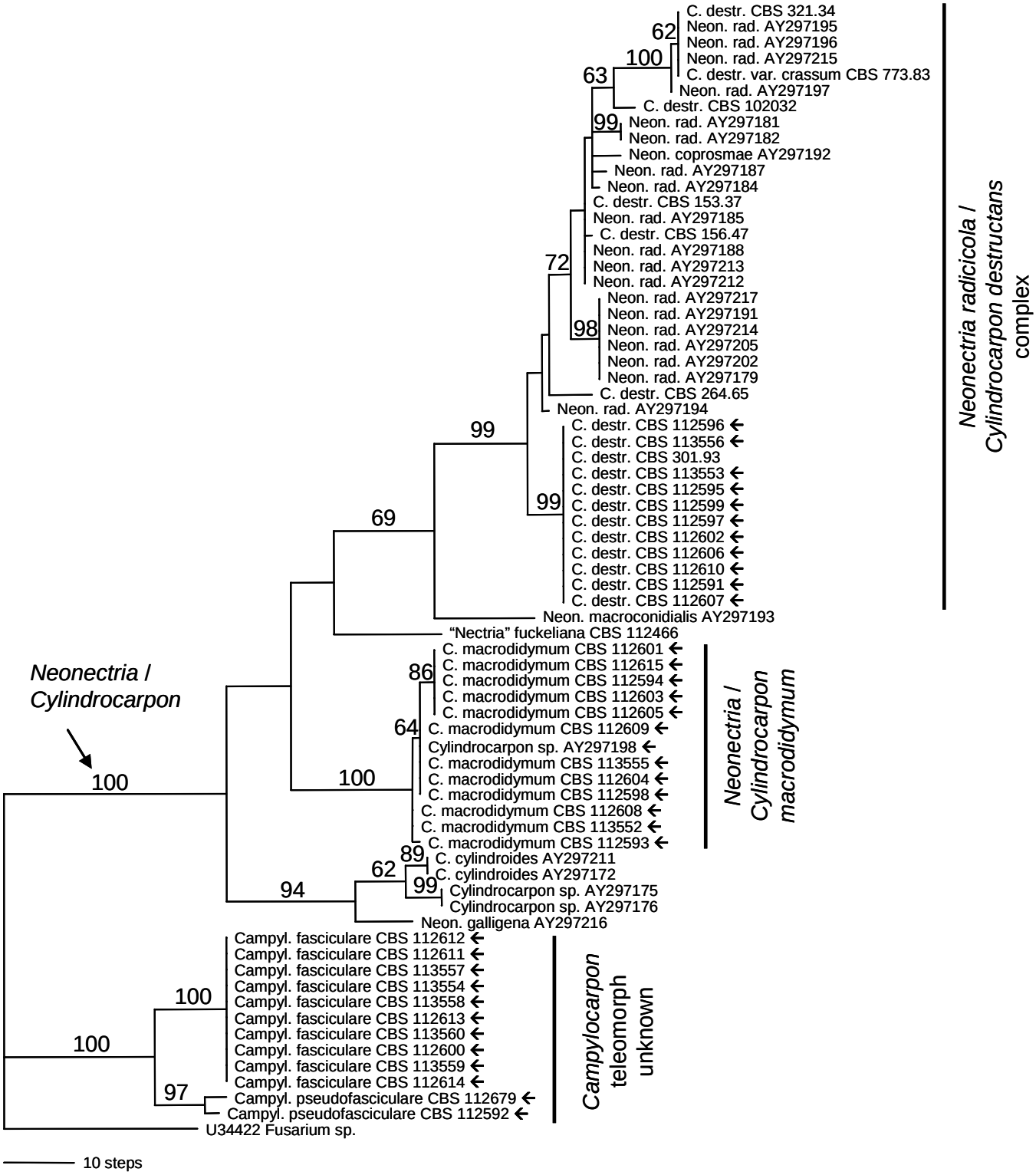

Fig. 6. One of 360 equally parsimonious phylograms for mainly grapevines isolates of *Cylindrocarpon* and *Campylocarpon* isolates (dataset 5) inferred from sequences of a 386 bp alignment of the β-tubulin gene. Bootstrap values are indicated above the nodes. Strains isolated from grapevines are marked by arrows.

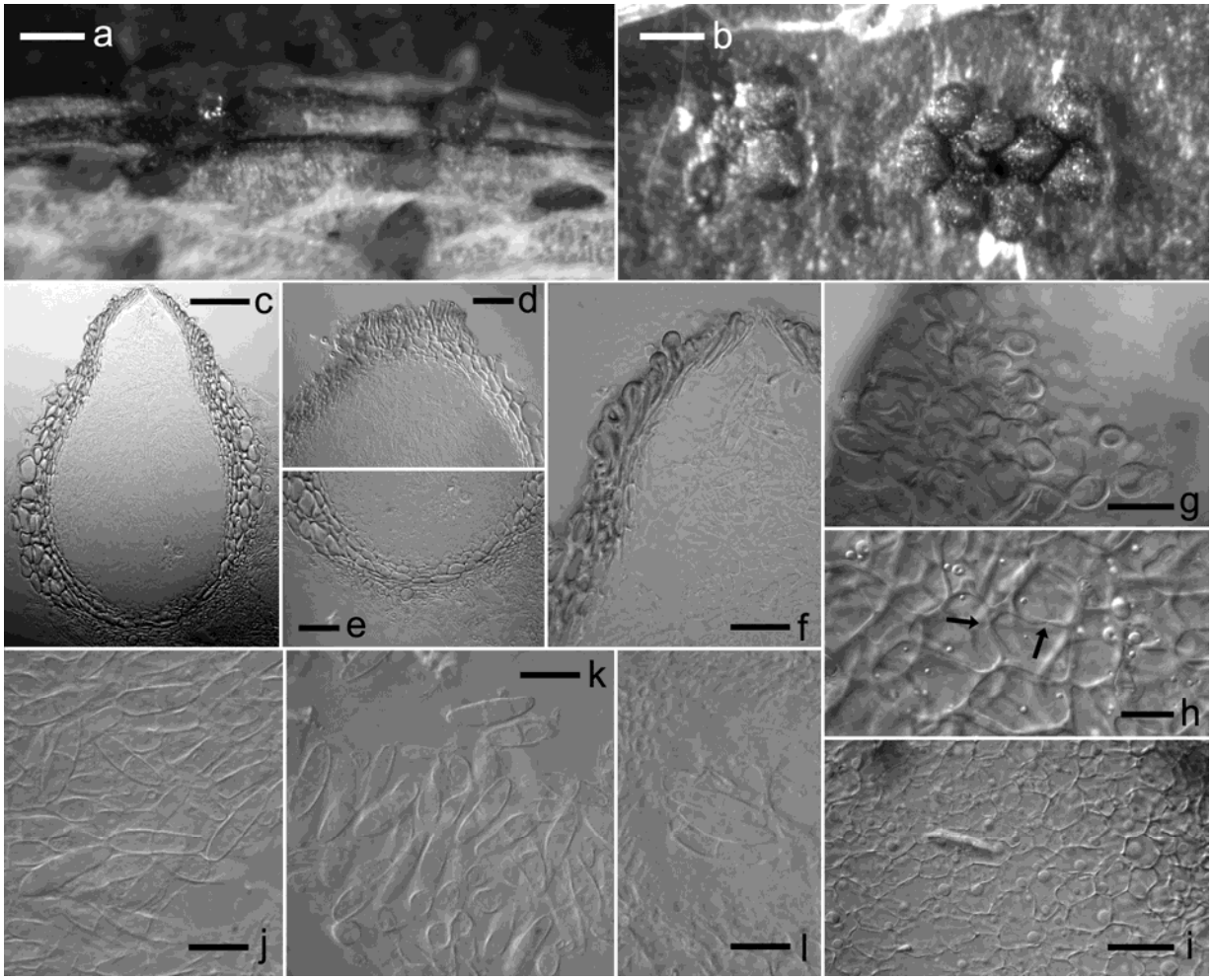

Fig. 7a–l. *Neonectria macrodidyma*/*Cylindrocarpon macrodidymum*. a, b. Solitary to aggregated development of perithecia. c–f. Longitudinal sections of perithecia showing details of wall, apex and base. g–i. Surface (g) and subsurface (h) optical face view of outer perithecial wall region; subsurface optical face view of inner perithecial wall region (i); main perithecial wall region consisting of cells with walls showing "pseudopores" (arrows in h). j–l. Ascospores. All from a crossing of CBS 112594 with CBS 112603 on CLA. Scale bars: a, b = 200 µm; c = 50 µm; d, e = 30 µm; f, g, i = 20 µm, h, j–l = 10 µm.

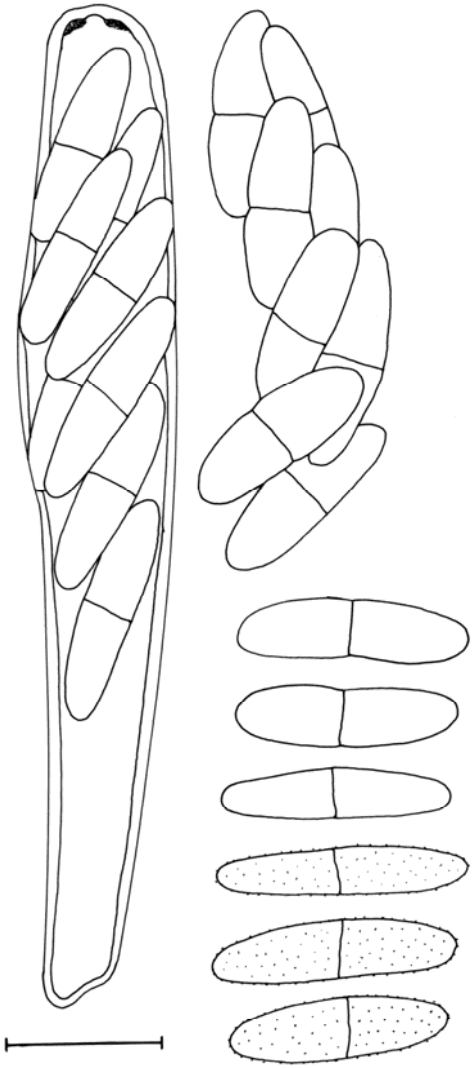

Fig. 8. Ascus and ascospores of *Neonectria macrodidyma*/*Cylindrocarpon macrodidymum*. All from a crossing of CBS 112594 with CBS 112603 on CLA. Scale bar = 10 µm.

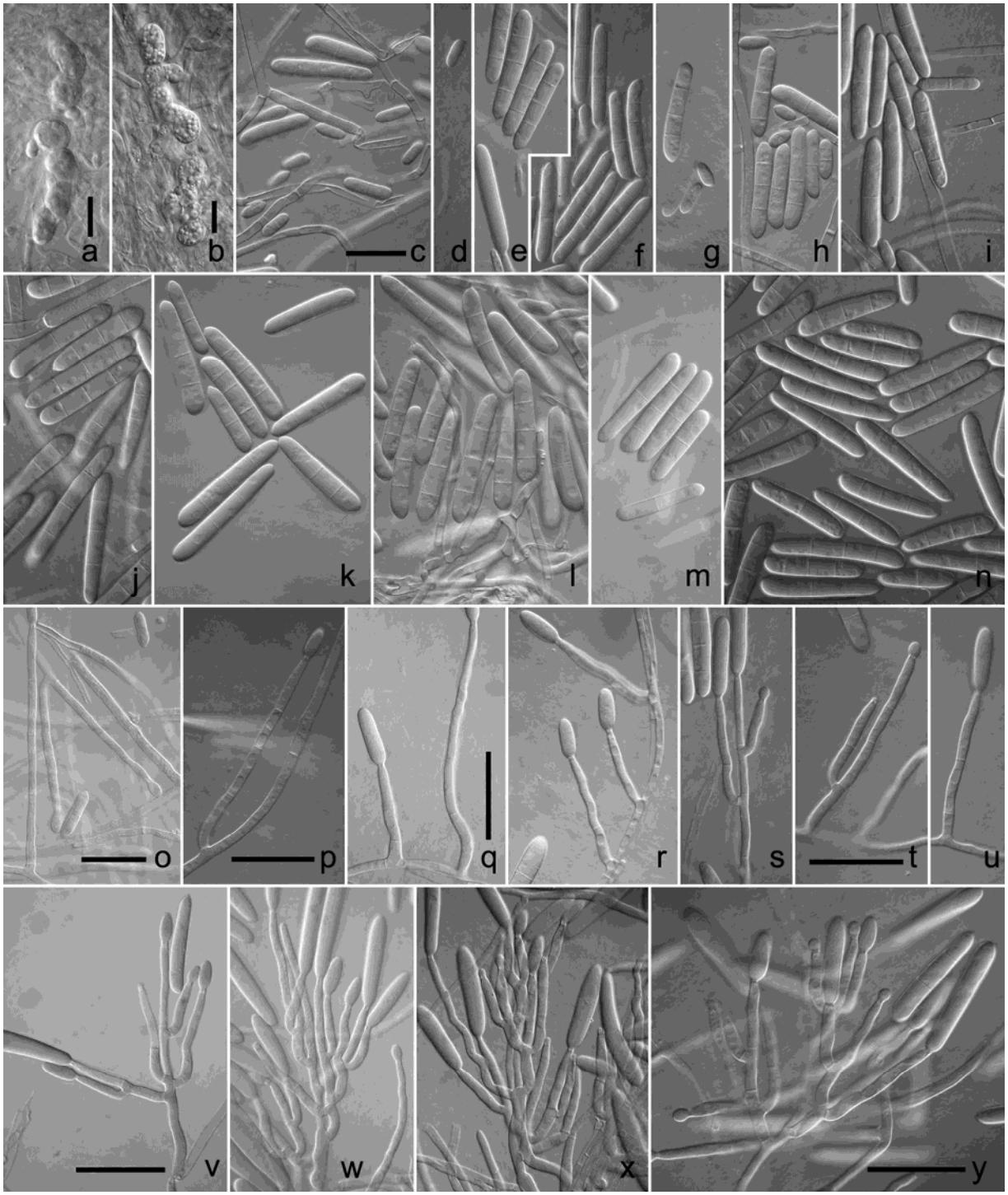

Fig. 9a–y. *Neonectria macrodidyma*/*Cylindrocarpon macrodidymum*. a, b. Chlamydospores. c–i. Micro- and macroconidia formed on SNA. j–n. Macroconidia formed on sporodochial conidiophores on OA. o–u. Simple, unbranched or sparsely branched, septate conidiophores on SNA. v–y. Complex, sporodochial conidiophores. a, b from CBS 112609, c–y from CBS 112615; a–i, o–y from 10-d-old SNA culture; j–n all 10-d-old OA culture. Scale bars: a, b = 10 µm; c–n = 20 µm, o–y = 30 µm.

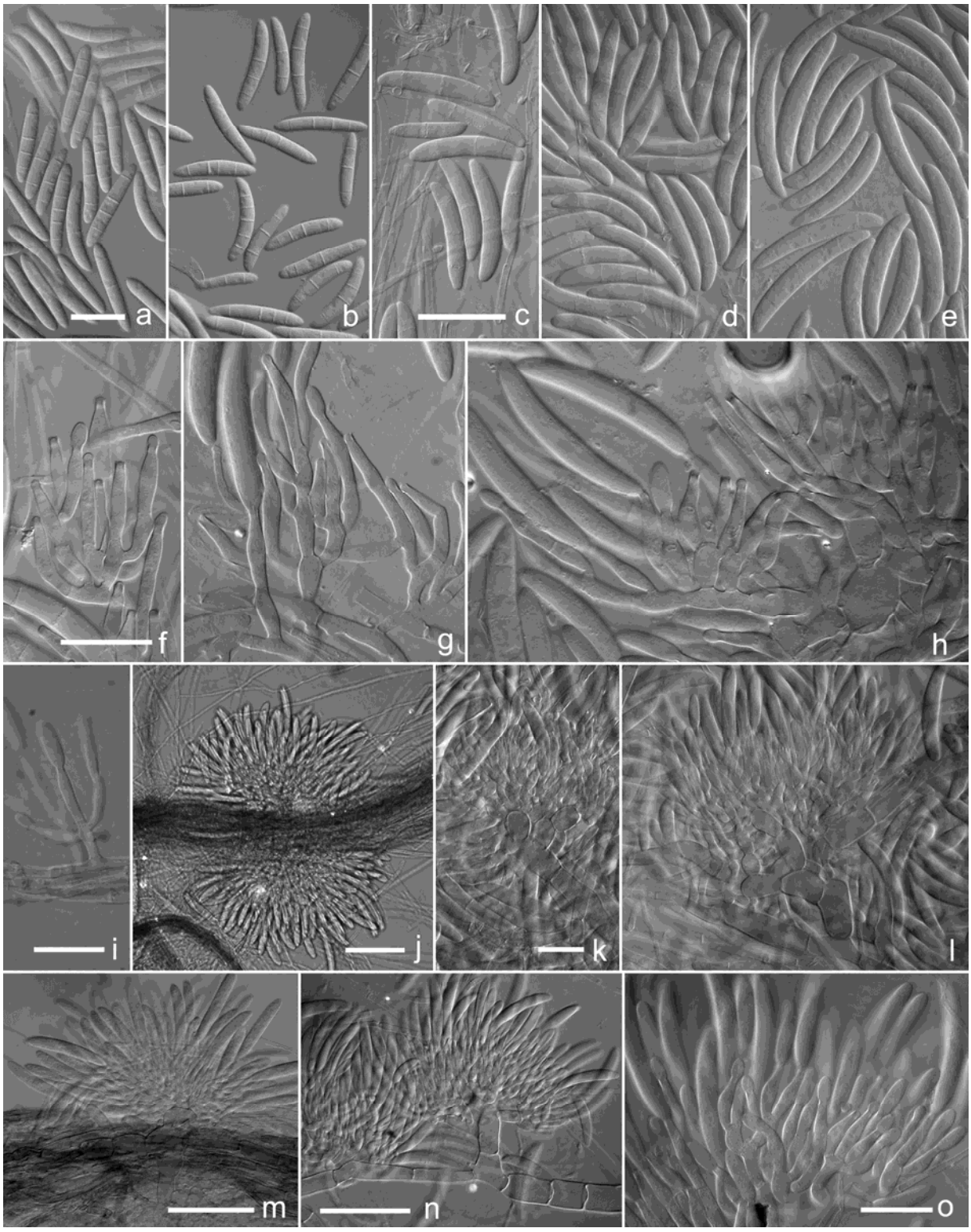

Fig. 10a–o. *Campylocarpon fasciculare*. a–e. Macroconidia. f–h. Branched conidiophores. i. Branched conidiophore arising from apical part of hyphal strand. j–n. Fascicles of branched conidiophores arising from brownish hyphal strands (j, m) or from the agar surface (k, l, n). o. Whorls of phialides of a fascicle of conidiophores. All from CBS 112613; a–o from 21-d-old $V8_{50}$ cultures. Scale bars: a–e = 30 µm, f–i, k, l, o = 20 µm, j, m, n = 50 µm.

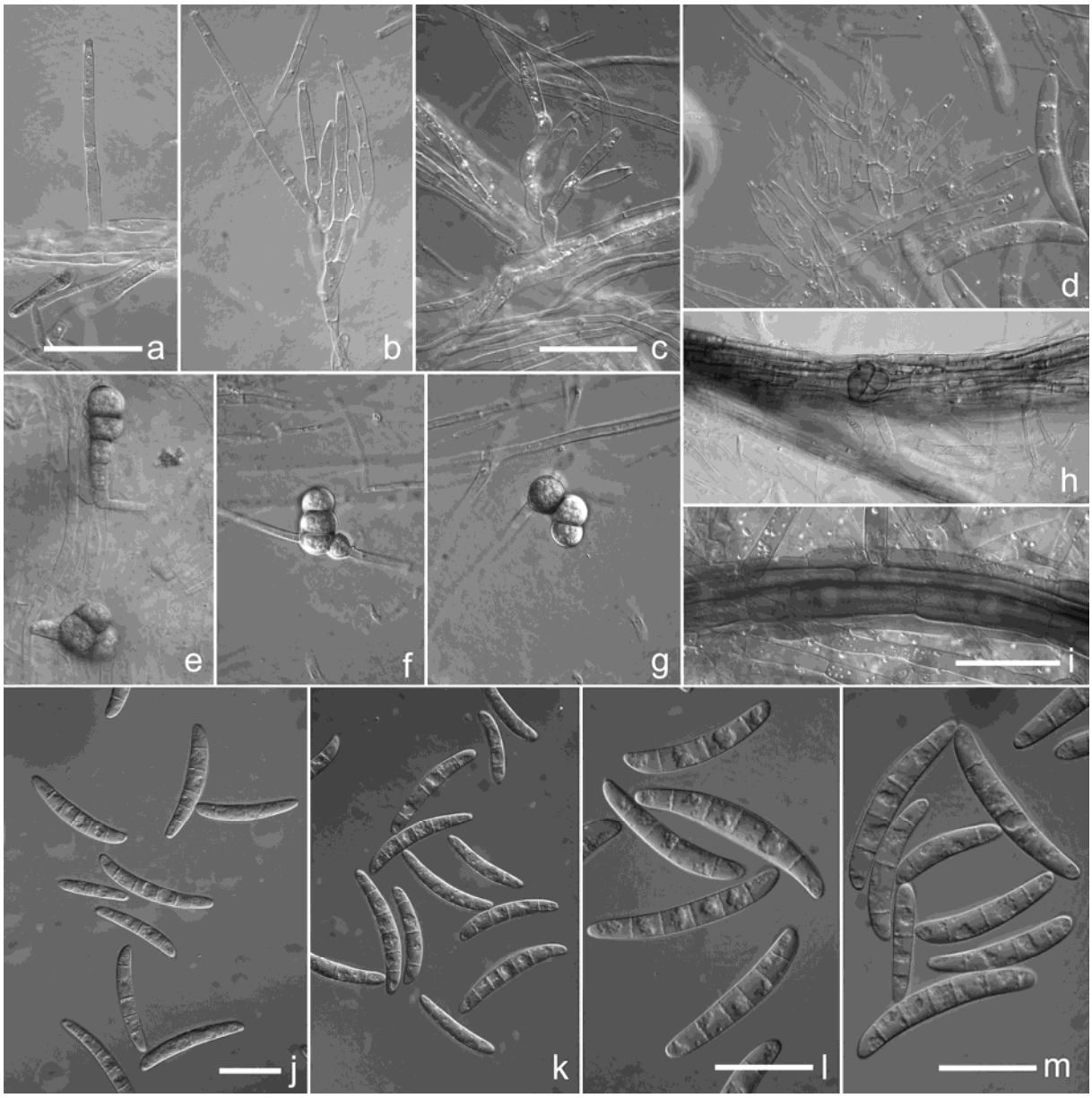

Fig. 11a–m. *Campylocarpon pseudofasciculare*. a. Simple, unbranched, septate conidiophore. b–d. Branched conidiophores. e–g. Chlamydospores. h–i. Brownish hyphal strand formed in the aerial mycelium. j–m. Macroconidia. All from CBS 112679; a–d, h–m from 21-d-old PDA culture; e from 21-d-old OA culture; f, g from 21-d-old SNA culture. Scale bars: a–h, j–m = 30 µm, i = 20 µm.

Table 1. *Cylindrocarpon* strains isolated from grapevines (*Vitis* spp.).

Taxon	CBS no [a]	Primary isolation no. [b]	STE-U no. [c]	Isolation date	Plant zone	Scion/rootstock	Origin [d]	Symptoms on host	GenBank accession no.		
									ITS1, 5.8S, ITS2 rDNA	LSU rDNA	β-tubulin
C. destructans	112606	C20	3985	9/12/1999	Roots	Semillon/unknown	South Africa, Bonnievale	Young vines die	AY677268	AY677314	AY677246
	112607	C81	3986	28/2/2000	Basal end of trunk	Merlot/101-14 Mgt	South Africa, Robertson	Black foot	AY677269	AY677316	AY677241
	112595	C17	3993	26/11/1999	Trunk	Cinsaut/101-14 Mgt	South Africa, Worcester	3-yr-old vines die	AY677263	–	AY677247
	112596	C14	3994	16/11/1999	Roots	Cabernet Sauvignon/Richter 99	South Africa, De Wet	Young vines die	AY677264	–	AY677239
	112597	C33	3995	16/11/1999	Trunk	Cabernet Sauvignon/Richter 110	South Africa, Tulbagh	Decline. Poor root development	AY677265	–	AY677240
	112602	C25	3998	26/11/1999	Roots	Chenin blanc/Richter 99	South Africa, Worcester	Old vines die	AY677267	AY677315	AY677242
	112599	F2	3672	1995	Trunk	Ugni blanc/ungrafted	France, Balan, Bordeaux	Black foot	AY677266	–	AY677243
	112591	F3	3673	1995	Trunk	Ugni blanc/ungrafted	France, Ile de Re, Bordeaux	Black foot	AY677262	–	AY677245
	112610	F4	3674	1995	Trunk	Ugni blanc/ungrafted	France, Fougerat, Bordeaux	Black foot	AY677270	–	AY677244
	113553	NZ C 59	5714	10/2/2003	Basal end of trunk	Pinot noir/101-14 Mgt	New Zealand, Canterbury	Blackening areas in wood and roots	–	–	AY677250
	113556	NZ C 65	5717	12/3/2003	Basal end of trunk	Unknown	New Zealand, Marlborough	Decline	–	–	AY677248
Neon. macrodidyma	112604	C10	4004	2/12/1999	Roots	Cabernet Sauvignon/101-14 Mgt	South Africa, Paarl	Decline	AY677286	–	AY677227
	112615	C98	3976	2/3/2000	Roots	Sultana/143-B Mgt	South Africa, Wellington (J)	Asymptomatic nursery plant	AY677290	AY677322	AY677233
	112601	C82	3983	24/2/2000	Roots	Pinotage/US 8-7	South Africa, Tulbagh	Black foot	AY677284	–	AY677229
	112605	C106	3984	2/3/2000	Rootstock (within 5 cm of the basal end)	Sultana/143-B Mgt	South Africa, Wellington (J)	Asymptomatic nursery plant	AY677287	–	AY677230
	112608	C62	3987	27/1/2000	Roots	Chardonnay/101-14 Mgt	South Africa, Citrusdal	Black foot	AY677288	AY677325	AY677235
	112593	C107	3990	7/3/2000	Roots	Pinotage/101-14 Mgt	South Africa, Wellington (V)	Asymptomatic nursery plant			

Table 1 (continue). *Cylindrocarpon* strains isolated from grapevines (*Vitis* spp.).

Neon. macrodi-dyma	112594	C111	3991	2/3/2000	Roots	Pinotage/Richter 99	South Africa, Wellington (J)	Asymptomatic nursery plant	AY677282	–	AY677231
	112598	C115	3997	14/3/2000	Roots	Sultana/Ramsey	South Africa, Wellington (L)	Asymptomatic nursery plant	AY677283	–	AY677228
	112603	C8	4007	20/12/1999	Trunk	Sauvignon blanc/Richter 110	South Africa, Darling	Decline of young vines	AY677285	AY677323	AY677232
	112609	A	3969	1979	Trunk	Cabernet Sauvignon/ungrafted	Australia, Bream Creek, Tasmania	Dark brown discoloration in trunk	AY677289	–	AY677226
	113552	NZ C 41	5713	31/1/2003	Rootstock	Unknown	New Zealand, Gisborne	Declining of nursery plants (dead rootstocks)	–	–	AY677237
	113555	NZ C 60	5715	10/2/2003	Basal end of trunk	Pinot noir/101-14 Mgt	New Zealand, Canterbury	Blackening areas in wood and roots	–	–	AY677234
Campyl. fasciculare	113554	C171 (3O/C20)	5719	24/6/2003	Rootstock (within 5 cm of the basal end	Cabernet Sauvignon/ 101-14 Mgt	South Africa, Wellington (V)	Vascular streaking from base of rootstock	–	–	AY677223
	113558	C172 (310)	5720	23/6/2003	Rootstock (within 5 cm of the basal end	Cabernet Sauvignon/ 101-14 Mgt	South Africa, Wellington (V)	Vascular streaking from base of rootstock	–	–	AY677224
	113557	C173 (3I4)	5721	6/6/2003	Rootstock (within 5 cm of the basal end	Cabernet Sauvignon/ 101-14 Mgt	South Africa, Wellington (V)	Vascular streaking from base of rootstock	–	–	AY677222
	112611	C147	3965	8/3/2000	Rootstock (within 5 cm of the basal end)	Pinotage/Richter 99	South Africa, Wellington (V)	Asymptomatic nursery plant	AY677299	–	AY677225
	112612	C134	3966	14/3/2000	Roots	Sultana/Ramsey	South Africa, Wellington (L)	Asymptomatic nursery plant	AY677300	AY677307	AY677216
	112613	C76	3970	1/2/2000	Trunk	Cabernet Sauvignon/Richter 99	South Africa, Riebeeck Kasteel	Young vines die	AY677301	–	AY677221
	113560	C120	3972	2/3/2000	Roots	Pinotage/Richter 99	South Africa, Wellington (J)	Asymptomatic nursery plant	AY677304	AY677309	AY677217
	112614	C79	3973	17/2/2000	Trunk	Pinotage/Richter 99	South Africa, Stellenbosch	Black foot	AY677302	AY677308	AY677220
	112600	C132	3981	1/3/2000	Roots	Pinotage/101-14 Mgt	South Africa, Wellington (J)	Asymptomatic nursery plant	AY677298	–	AY677219
	113559	C119	4006	9/3/2000	Roots	Sultana/ 143-B Mgt	South Africa, Wellington (V)	Asymptomatic nursery plant	AY677303	–	AY677218

Table 1 (continue). *Cylindrocarpon* strains isolated from grapevines (*Vitis* spp.).

Campyl. pseudofas-ciculare	112679	C108	5472	3/3/2000	Roots	Sultana/Ramsey	South Africa, Wellington (J)	Asymptomatic nursery plant	AY677306	–	AY677214
	112592	C89	3988	2/3/2000	Roots	Pinotage/Richter 99	South Africa, Wellington (J)	Asymptomatic nursery plant	AY677305	AY677310	AY677215

[a] CBS strain numbers, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

[b] Strains F2, F3, and F4 collected by Philippe Larignon (INRA, France).

Strain A collected by Mark Sweetingham (Australia).

Strains NZ C 41, 59, 60, 64, 65, and 72 collected by Rod Bonfiglioli (New Zealand).

All other strains collected by Francois Halleen (F.H.).

[c] STE-U strain numbers, University of Stellenbosch, South Africa.

[d] (J), (L) and (V) = three grapevine nurseries, Western Cape Province, South Africa.

Table 2. Other taxa with *Cylindrocarpon*-like anamorphs, of which sequences were newly generated.

Taxon	Strain no. [a]	Location/host, substrate	GenBank accession no.		
			ITS1, 5.8S, ITS2 rDNA	LSU rDNA	β-tubulin
"*Neonectria*" *trachosa* Samuels & Brayford	CBS 112467 (= G.J.S. 92-45)	Scotland/conifer bark	AY677297	AY677337	AY677258
C. album (teleomorph: "*Nectria*" *punicea* (Schmidt : Fr.) Fr.	CBS 242.29	Germany/*Rhamnus* sp.	–	AY677326	–
	CBS 152.29	Germany/*Solanum tuberosum* tuber	AY677260	–	–
C. candidum	CBS 237.29	Norway/*Ulmus* sp.	–	AY677327	–
C. cylindroides (teleomorph: *Neon. neomacrospora* (C. Booth & Samuels) Mantiri & Samuels	CBS 189.61	France/*Abies alba*	–	AY677331	–
	CBS 503.67	Norway/*Abies alba*	AY677261	–	–
C. faginatum (teleomorph: "*Nectria*" *coccinea* var. *faginata* Lohman *et al.*)	CBS 217.67	Canada/*Cryptococcus fagi* nymph on *Fagus grandifolia*	AY677277	AY677328	–
C. heteronema (teleomorph: *Neonectria galligena*	CBS 316.34	Canada/*Betula lutea*	AY677278	–	–
	CBS 232.31	Germany/*Fraxinus excelsior*	–	AY677329	–
C. willkommii	CBS 226.31	Germany/*Fagus sylvatica*	–	AY677330	–
"*Nectria*" *fuckeliana* C. Booth	CBS 112466 (= G.J.S. 90-31)	Switzerland/*Picea* sp.	–	AY677334	AY677238
"*Neonectria*" *discophora* (Mont.) Mantiri & Samuels (anamorph: *C. ianthothele* var. *majus* Wollenw.)	CBS 328.81	Switzerland/unknown	AY677279	–	–
	CBS 112460 (= G.J.S. 85-45)	New Zealand/root of indet. Tree	–	AY677338	AY677257
"*Neonectria*" *lucida* (Höhnel) Samuels & Brayford (anamorph: *Cylindrocarpon lucidum* Booth)	CBS 112455 (C.T.R. 72-71)	Venezuela/bark	–	AY677336	AY677259
	CBS 112457 (= G.J.S. 85-27)	New Zealand/bark	–	AY677335	–
	CBS 112456 (= G.J.S. 96-35)	U.S.A., Puerto Rico/bark of recently dead *Cecropia* sp.	AY677296	–	–
C. ianthothele var. *minus* Reinking	CBS 266.36	Germany/unknown	AY677280	–	–
C. victoriae (Henn. ex Wollenw.) Wollenw.	CBS 174.37	Germany/unknown	–	AY677339	–

Table 2 (continue). Other taxa with *Cylindrocarpon*-like anamorphs, of which sequences were newly generated.

C. destructans	CBS 264.65	Sweden/*Cyclamen persicum*	AY677273	AY677318	AY677256
	CBS 321.34	Tunisia/*Loroglossum hircinum*	AY677275	AY677313	AY677253
	CBS 102032	Venezuela/bark		AY677311	AY677255
	CBS 156.47	Belgium/*Azalea indica*	AY677272	AY677320	AY677252
	CBS 153.37	France/dune sand	AY677271		AY677251
	CBS 301.93	Netherlands/*Cyclamen* sp.	AY677274	AY677317	AY677249
C. destructans var. *crassum* (Wollenw.) C. Booth	CBS 773.83	Netherlands/water, in aquarium with *Anodonta*	AY677276	AY677312	AY677254
C. didymum	CBS 640.77	France/*Abies alba*	–	AY677319	–
	CBS 305.85	Netherlands/*Lilium* sp.	–	AY677321	–
C. obtusisporum (teleomorph: *Neonectria tawa* Dingley)	CBS 183.36	Germany/*Solanum tuberosum*	AY677292	AY677332	–
C. magnusianum (teleomorph: *Neonectria ramulariae* Wollenw.	CBS 151.29	UK England/*Malus sylvestris*	AY677291	AY677333	–
C. olidum var. *crassum* Gerlach	CBS 216.67	Germany/*Zygocactus* sp.	AY677294	–	–
C. olidum var. *olidum* (Wollenw.) Wollenw.	CBS 215.67	Germany/Rotting rhizome of *Asparagus officinalis*	AY677293	–	–

[a] CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; G.J.S.: G.J. Samuels; C.T.R.: C.T. Rogerson.

4. *NEONECTRIA LIRIODENDRI* SP. NOV., THE MAIN CAUSAL AGENT OF BLACK FOOT DISEASE OF GRAPEVINES

ABSTRACT

Black foot disease of grapevines is a serious disease of this crop in most areas where grapevines are grown. Recent investigations have identified two species of *Cylindrocarpon*, as well as two species of *Campylocarpon* to be associated with the disease. One of these taxa, namely *C. destructans*, has been shown to be a species complex. The aim of the present study was thus to elucidate the taxonomic status of the *C. destructans* isolates associated with black foot disease of grapevines. Isolates were thus studied morphologically, mated in all combinations *in vitro* to try and induce the teleomorph, and also subjected to DNA analysis of their ITS and partial β-tubulin genes. Data from this study revealed grapevine isolates to be morphologically and phylogenetically identical to the ex-type strain of *C. liriodendri*, which also produced a teleomorph, *Neonectria liriodendri* sp. nov. in culture. *Cylindrocarpon liriodendri* is distinct from *Neonectria radicicola*, which again appears to be distinct from its purported anamorph, *C. destructans*, which encompasses several phylogenetic distinct species awaiting further study.

Taxonomic novelty: *Neonectria liriodendri* Halleen, Rego & Crous sp. nov.

INTRODUCTION

In recent years, three species of *Cylindrocarpon* Wollenw. have been associated with black foot disease of grapevines (*Vitis* spp. L.). *Cylindrocarpon destructans* (Zinnsm.) Scholten [anamorph of *Neonectria radicicola* (Gerlach & L. Nilsson) Mantiri & Samuels] was first recorded on grapevine in France in 1961 (Maluta and Larignon, 1991). Since then it has been isolated from diseased grapevines in Tasmania (Sweetingham, 1983), Sicily (Grasso, 1984), Portugal (Rego, 1994; Rego *et al*., 2000, 2001), Pennsylvania, U.S.A. (Gugino and Travis, 2003), New Zealand and South Africa (Halleen *et al*., 2004). *Cylindrocarpon obtusisporum* (Cooke & Harkn.) has been identified as the causal agent of this disease in Sicily (Grasso and Magnano di San Lio, 1975) and California (Scheck *et al*., 1998). Various unidentified species of *Cylindrocarpon* have also been isolated from young grapevines and

from declining grapevines with basal rot or root necrosis in Australia (Edwards and Pascoe, 2004), Chile (Auger *et al*., 1999), Greece (Rumbos and Rumbou, 2001), Spain (Armengol *et al*., 2001) and South Africa (Fourie *et al*., 2000, Fourie and Halleen, 2001). In a recent taxonomic study, a third species, newly described as *C. macrodidymum* Schroers, Halleen & Crous (anamorph of *Neonectria macrodidyma* Halleen, Schroers & Crous) was associated with the disease. These isolates were obtained from grapevines in South Africa, Tasmania, New Zealand and Canada (Halleen *et al*., 2004). *Campylocarpon* Halleen, Schroers & Crous, a newly described genus that is *Cylindrocarpon*-like in morphology, has also been associated with the disease in South Africa (*Campylocarpon fasciculare* Schroers, Halleen & Crous and *Campyl. pseudofasciculare* Halleen, Schroers & Crous) (Halleen *et al*., 2004).

Booth (1966) segregated *Cylindrocarpon* species into four groups based on the presence or absence of microconidia and chlamydospores. *Cylindrocarpon magnusianum* (Sacc.) Wollenw., which is the anamorph of the type species of *Neonectria* Wollenw., *C. cylindroides* Wollenw., which is the type species of the genus *Cylindrocarpon*, *C. destructans*, which is the purported anamorph of *Neon. radicicola*, and members of *Cylindrocarpon* species predominantly connected with teleomorphs of the *Nectria mammoidea* W. Phillips & Plowr. group were core members of the anamorphic groups delineated by Booth (1966). Rossman *et al*. (1999), Mantiri *et al*. (2001) and Brayford *et al*. (2004) recently transferred representatives of all "*Nectria*" groups with *Cylindrocarpon* anamorphs into *Neonectria*. Mantiri *et al*. (2001) and Brayford *et al*. (2004) analysed mitochondrial small subunit (SSU) ribosomal DNA (rDNA) sequence data of some of the species and concluded that the *Neonectria*/*Cylindrocarpon* species grouped together by this reclassification were monophyletic. However, these authors also found that this overall *Neonectria*/*Cylindrocarpon* clade included distinct subclades corresponding to at least three of the four groups delineated by Booth (1966). Significant molecular variation among taxa with *Cylindrocarpon*-like anamorphs was found by Seifert *et al*. (2003) in a study on fungi causing root rot of ginseng (*Panax quinquefolius*) and other hosts. The dendrograms in this study, based on partial β-tubulin gene, and nuclear ribosomal internal transcribed spacer (ITS) region sequences, suggested that subclades including (i) *Neon. radicicola*, (ii) *Neon. macroconidialis* (Samuels & Brayford) Seifert, and (iii) a subclade comprising two distinct isolates, one from *Vitis vinifera* in Ontario, Canada and the other from *Picea* sp. in Quebec, Canada, were monophyletic. Other *Cylindrocarpon* species appeared to be excluded from

this monophyletic group. Halleen *et al*. (2004) found that *Neonectria*/*Cylindrocarpon* species clustered in mainly three groups. One monophyletic group consisted of three subclades comprising (i) members of the *Neon. radicicola*/*C. destructans* complex; (ii) *C. macrodidymum*; and (iii) an assemblage of species closely related to strains identified as *C. cylindroides*, the type species of *Cylindrocarpon*. This monophyletic group excluded two other groups, which comprised (i) members of the *Neon. mammoidea* complex; and (ii) the two *Campylocarpon* species. The latter two clades formed a paraphyletic group in LSU rDNA analysis, but were supported as a monophyletic group in ITS and β-tubulin gene analysis. Analyses of Halleen *et al*. (2004) therefore excluded *Campylocarpon* and members of the *Neon. mammoidea* group from *Neonectria*/*Cylindrocarpon*, contradicting the transfer of the *Neon. mammoidea* group to *Neonectria* by Brayford *et al*. (2004).

Strains of the *Neon. radicicola*/*C. destructans* complex isolated from grapevines matched those currently placed in *C. destructans* based on morphology and DNA sequences (Halleen *et al*., 2004). However, as shown by previous phylogenetic studies (Seifert *et al*., 2003), *C. destructans* represents a species complex. Seifert *et al*. (2003) observed a high amount of sequence divergence among this group and suggested the existence of several phylogenetic species in this complex. Although Halleen *et al*. (2004) referred to the primary causal organism of black foot disease of grapevine as *C. destructans*, the ex-type strain of *N. radicicola* CBS 264.65 did not form part of the clade comprising of grapevine isolates, except in the LSU rDNA analyses. The aim of the present study, therefore, was to determine the correct identity of the "*C. destructans*" species occurring on grapevines, and establish if it had a teleomorph. To do this grapevine isolates from several countries were subjected to DNA analysis of their ITS and β-tubulin genes, and subjected to *in vitro* mating studies.

MATERIALS AND METHODS

Isolates. *Cylindrocarpon destructans* isolates, previously isolated from diseased grapevines in Portugal (Rego, 1994; Rego *et al*., 2001), France, South Africa and New Zealand (Halleen *et al*., 2004), were obtained from the collection of the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands (CBS) (Table 1). Disease symptoms associated with these isolates include various forms of decline as well as typical black foot symptoms.

DNA phylogeny. Mycelium was grown in tubes with 2 ml of complete medium (Raper and Raper, 1972) and DNA was extracted using the FastDNA® Kit (Bio 101, Carlsbad, CA, U.S.A.). PCR amplification and sequencing of the partial β-tubulin gene introns and exons, as well as ITS rDNA, was performed as described by Halleen *et al*. (2004). Newly generated sequences have been deposited in GenBank (Table 1).

Sequences were manually aligned using Sequence Alignment Editor v. 2.0a11 (Rambaut, 2002). Additional sequences were obtained from GenBank and added to the alignment. In phylogenetic trees, downloaded sequences are indicated by their GenBank accession numbers; newly generated sequences are indicated by CBS strain numbers. Two datasets were created and analysed separately using PAUP 4.0b10 (Swofford, 2002) as described by Halleen *et al*. (2004), but implementing a 100 random taxon addition.

Morphology. Strains were grown in darkness or under continuous near-ultraviolet (nuv) light (400–315 nm) (Sylvania Blacklight-Blue, Osram Nederland B.V., Alphen aan den Rijn, The Netherlands) at 20°C. Media used were synthetic nutrient-poor agar (SNA) with and without the addition of a 1 × 3 cm piece of filter-paper to the colony surface, potato-dextrose agar (Difco PDA, Becton Dickinson, Sparks, MD, U.S.A.), oatmeal agar (OA, Gams *et al*., 1998), and carnation leaf agar (CLA, Gams *et al*., 1998), and malt extract agar (MEA) (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) using 9 cm diam Petri dishes. Growth rates and colony diameters of cultures incubated in darkness were measured on PDA. Characters such as size and shape of conidia, phialides, and chlamydospores were determined from strains grown on SNA, PDA, or CLA after 14–21 days. Structures were mounted in lactic acid, and 30 measurements at × 1000 magnification were made of each structure. The 95% confidence levels were determined, and the extremes of spore measurements given in parentheses. Images were taken from slides mounted in lactic acid. Macroscopic characters of colonies were described after 14 days; colour names are from Rayner (1970). Cardinal temperatures for growth were assessed on PDA incubated for 7 days in the dark at 4, 10, 15, 20, 25, 30 and 35°C. Mating experiments were performed on minimal salt medium at 25°C, using autoclaved birch toothpicks as explained by Guerber and Correll (2001). Three replicates were done for each cross. Petri dishes were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI, USA), incubated in a single layer under a mixture of cool-white fluorescent and nuv light, and observed at weekly intervals for a total period of 8 weeks. Two

strains were considered sexually compatible if they produced perithecia with viable, exuding masses of ascospores within this time.

RESULTS

DNA phylogeny. For ITS and β-tubulin approximately 520 and 600 bases were determined, respectively, for the isolates listed in Table 1. The manually adjusted ITS alignment contained 59 taxa (including the outgroup) and 472 characters including alignment gaps. Two gaps of more than 10 characters each (caused by the outgroup sequence) were each coded as a single character (TreeBASE accession SN2458). Of the 472 characters, 47 were parsimony-informative, 60 were variable and parsimony-uninformative, and 365 were constant. Parsimony analysis of the ITS data yielded 61 most parsimonious trees (TL = 224 steps; CI = 0.795; RI = 0.924; RC = 0.734), one of which is shown in Fig. 1. The topology of the trees generated with neighbour-joining analysis using the uncorrected "p", Jukes-Cantor and HKY85 substitution models were identical to each other and were also similar to the trees obtained using parsimony (data not shown). In the tree (Fig. 1), isolates of *C. liriodendri* grouped with a bootstrap support value of 85% and a clade (99% bootstrap support) of two isolates of *Neon. radicicola* forms the closest sister clade (75% bootstrap support). Isolates of "*C. destructans*" form two poorly supported clades (57 and 54%, respectively) separated by *Neon. coprosmae*. The final clade is in a basal position and contains isolates of *N. galligena* (94% bootstrap support) with *N. ramulariae* as closest sister (82% bootstrap support). The manually adjusted β-tubulin alignment contained 55 taxa (including the outgroup) and 327 characters including alignment gaps. Of the 327 characters, 33 were parsimony-informative, 39 were variable and parsimony-uninformative, and 255 were constant. Parsimony analysis of the β-tubulin data yielded 180 most parsimonious trees (TL = 103 steps; CI = 0.864; RI = 0.959; RC = 0.829), one of which is shown in Fig. 2. The topology of the trees generated with neighbour-joining analysis using the uncorrected "p", Jukes-Cantor and HKY85 substitution models and the trees obtained using parsimony only differed in the order of the isolates in the "*C. destructans*" clade (data not shown). As with the ITS tree, isolates of *C. liriodendri* form a well-defined clade (bootstrap support value of 93%), but the two isolates of *Neon. radicicola* group together in a poorly supported clade (56% bootstrap support). Isolates in the "*C. destructans*" complex clade (75% bootstrap support) are mainly in basal positions

with a small number of defined clades with high bootstap support values. *Neonectria coprosmae* is also included in this clade.

Taxonomy

Neonectria liriodendri Halleen, Rego & Crous, **sp. nov.** Fig 3A–V.

Anamorph: ***Cylindrocarpon liriodendri*** J.D. MacDon. & E.E. Butler, Plant Disease 65: 156. 1981.

Neonectriae radicicolae similis sed ascosporis levibus vel verruculosis, et peritheciis levibus vel verruculosis distincta. *Ascosporae* (7–)9–11(–14) × (2.5–)3–3.5(–4) µm.

Perithecia formed heterothallically *in vitro*, disposed solitarily or in groups of up to six, developing directly on the agar surface or on sterile pieces of beach wood or pine needles, ovoid to obpyriform, with a flattened apex, up to 70 µm wide, orange to red, becoming purple-red in 3% KOH (positive colour reaction), smooth to warted, up to 300 µm diam and high; with minute stroma of dark red pseudoparenchymatal cells; perithecial wall consisting of two regions; outer region 15–30 µm thick, composed of 1–3 layers of angular to subglobose cells, 10–25 × 8–17 µm; cell walls up to 1 µm thick; inner region 10–15 µm thick, composed of cells that are flat in transverse optical section and angular to oval in subsurface optical face view, 7–15 × 3–5 µm; perithecial warts consisting of globose to subglobose cells, 15–30 × 15–20 µm in surface view. *Asci* narrowly clavate to cylindrical, 45–60 × 5–6 µm, 8-spored; apex subtruncate, with a minutely visible ring. *Ascospores* medianly 1-septate, ellipsoidal to oblong ellipsoidal, somewhat tapering towards both ends, smooth to finely warted, (7–)9–11(–14) × (2.5–)3–3.5(–4) µm.

Conidiophores simple or complex, sporodochial. Simple conidiophores arising laterally or terminally from the aerial mycelium or erect, arising from the agar surface, solitary to loosely aggregated, unbranched or sparsely branched, 1–6-septate, rarely consisting only of the phialide, 40–160 µm long; phialides monophialidic, cylindrical, 20–40 × 3–4 µm, 2–2.5 µm near aperture. Sporodochial conidiophores aggregated in pionnote sporodochia, irregularly branched; phialides cylindrical, mostly widest near the base, 15–30 × 2.5–3.5 µm, 2–2.5 µm wide near the aperture. *Micro-* and *macroconidia* present on both types of conidiophores.

Macroconidia predominating, formed by both types of conidiophores, predominantly (1–)3-septate, straight or sometimes slightly curved, cylindrical, mostly with a visible, basal or slightly laterally displaced hilum; 3-septate macroconidia, (24–)35–40(–55) × (4.5–)5.5–6(–6.5) µm (n = 116). *Microconidia* sparsely produced on all media, 0–1-septate, ellipsoidal to subcylindrical to ovoid, more or less straight, with a minutely or clearly lateral hilum; aseptate subcylindrical to ellipsoidal microconidia, 5–15 × 2.5–4 µm; aseptate ovoid microconidia, 3–5 × 3–4 µm, formed predominently on dense, penicillately branched conidiophores on CLA and twigs, and then also without subcylindrical to ellipsoidal microconidia, which again occur on other media as a mixture with ovoid microconidia. *Conidia* formed in heads on simple conidiophores, as hyaline masses on simple as well as complex conidiophores. *Chlamydospores* common, mostly in short, intercalary chains, 10–20 × 10–17 µm.

Specimens examined: Heterothallic mating between two opposite mating types. **France**, *Vitis vinifera*, P. Larignon, CBS 112591 × South Africa, F. Halleen, *Vitis vinifera*, CBS 112596, **holotype** specimen herb. CBS 17776; **Portugal**, *Vitis vinifera*, C. Rego, CBS 117526 × **Portugal**, C. Rego, *Vitis vinifera*, CBS 117527, herb. CBS 17781; **Portugal**, *Vitis vinifera*, C. Rego, CBS 117527 × California, **U.S.A.**, *Liriodendron tulipifera*, CBS 110.81, herb. CBS 17780; **Portugal**, *Vitis vinifera*, C. Rego, CBS 117527 × **South Africa**, F. Halleen, *Vitis vinifera*, CBS 112596, herb. CBS 17779; **Portugal**, *Vitis vinifera*, C. Rego, CBS 117527 × **South Africa**, F. Halleen, *Vitis vinifera*, CBS 112602, herb. CBS 17778; **Portugal**, *Vitis vinifera*, C. Rego, CBS 117527 × **France**, P. Larignon, *Vitis vinifera*, CBS 112610, herb. CBS 17777.

Mating studies: Perithecia observed within 3–4 weeks in crossings of strains: CBS 117526 × CBS 117527; CBS 117527 × CBS 110.81 (ex-type of *C. liriodendri*); CBS 117527 × CBS 112602; CBS 117527 × CBS 112610; CBS 117527 × CBS 112596; CBS 112591 × CBS 112596.

Cultural characteristics: Colonies on PDA (surface and reverse) cinnamon to sepia, with sparse aerial mycelium. On OA dark brick to fawn (surface and reverse). Minimum temperature for growth < 4°C; optimum temperature 20–25°C, at which PDA colonies reach 30–42 mm diam after 7 days in the dark; maximum temperature between 30–35°C.

Host range and distribution: *Vitis vinifera* (France, Portugal, New Zealand, South Africa), The Netherlands (*Cyclamen* sp.), *Liriodendron tulipifera* (U.S.A., California).

Habitat: Roots and rootstocks of grapevines, causing black foot disease. The ex-type culture was described from *Liriodendron tulipifera* in California, while another was associated with bulb rot of a *Cyclamen* sp. in the Netherlands, so it is possible that this pathogen has a much wider host range than that reported here.

Phylogenetic affinity: *Nectriaceae, Hypocreales*.

DISCUSSION

Species of *Cylindrocarpon* Wollenw. are commonly isolated from soil and generally regarded to be saprobes or weak pathogens of a wide range of herbaceous and woody plants (Brayford, 1993). Since *C. destructans* was first reported from grapevine in France in 1961, it has been recognised as a pathogen of this host from numerous countries where vines are cultivated (Sweetingham, 1983; Grasso, 1984; Rego, 1994; Fourie *et al*., 2000; Gugino and Travis, 2003). In the recent taxonomic study revising the *Cylindrocarpon* spp. associated with black foot disease of grapevines, the primary causal organism was identified as "*C. destructans*", while a second species was newly described as *C. macrodidymum* (Halleen *et al*., 2004). Furthermore, two new species were also found to represent an undescribed genus of fungi that was *Cylindrocarpon*-like in morphology, namely *Campylocarpon*. Although all four species have been implicated in this disease complex, "*C. destructans*" proved to be the most commonly isolated *Cylindrocarpon* species isolated from diseased vines, and might therefore be considered as the most important pathogen of this complex (Halleen *et al*., 2004).

Several pathogenicity studies have previously been conducted with isolates from the "*C. destructans*" clade. Oliveira *et al*. (1998) inoculated rooted cuttings of the grapevine cultivar Seara Nova by dipping the roots in a spore suspension of "*C. destructans*" (Cy1 = CBS 117640). Typical black foot disease symptoms were observed within 60 days. Similar results were obtained when rooted cuttings of 99Richter rootstock were inoculated with 12

"*C. destructans*" isolates, two of which were Cy 68 (CBS 117526) and Cy 76 (CBS 117527). Inoculation significantly reduced plant height and the number of roots, whilst isolate Cy 68 was considered to be one of the most virulent isolates evaluated (Rego *et al*., 2001). Inoculation of 6-month-old potted grapevine rootstocks (cv. Ramsey) with isolate CBS 112597 resulted in death of 27.5% of the plants 60 days after inoculation, whilst the remaining plants suffered a dramatic reduction in root and shoot mass (Halleen *et al*., 2004).

Cylindrocarpon liriodendri was first associated with root rot of tulip poplar (*Liriodendron tulipifera*) in California. Affected plants appeared severely stunted and the root systems were covered with black, dry, scabby lesions that completely girdled or rotted off distal portions of some roots (MacDonald and Butler, 1981). Although reported to lack microconidia (MacDonald and Butler, 1981), and thus accepted to not be part of the *C. destructans*-complex, we were surprised to find that based on DNA sequence of the ITS and β-tubulin gene regions, the ex-type strain (CBS 110.81) was identical to other isolates from grapevines formerly identified as "*C. destructans*", and that it also had the ability to form microconidia in culture. If the "*C. destructans*" species occurring on grapevines was in fact *C. liriodendri*, this raised the question as to the identity of *Neon. radicicola* and its purported anamorph, *C. destructans*. *Neonectria radicicola* was originally described from rotting bulbs of *Cyclamen persicum* collected in Sweden, of which an ex-type culture was available for study (CBS 264.65) (Gerlach and Nilsson, 1963). The anamorph linked to this species, however, is *C. destructans*, which Booth (1966) based on a North American neotype from Kentucky, collected on *Panax ginseng* (CUP 11985), for which there is no culture available. In a recent study, however, Seifert *et al*. (2003) showed that there was more than one species on *Panax*, and that one of these would probably represent *C. destructans*. If one accepts that one of the *Panax* morphotypes is *C. destructans*, the issue is further complicated by our finding that *Neon. radicicola* is not its teleomorph (Figs. 1–2). Notwithstanding this fact, however, *C. liriodendri* is a name available for the grapevine pathogen, which clusters in its own well supported clade, for which the name *Neon. liriodendri* is introduced to accommodate its teleomorph.

To fully resolve the taxonomic status of the species present in the *C. destructans* species complex, however, detailed mating studies with all clades in this complex, and

additional sequence data of other loci need to be generated. This work is currently in progress, and will be reported on in future studies.

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Table 1. *Cylindrocarpon* and *Neonectria* isolates included in this study.

Species	Accession number[1]	Host	Country	Collector	GenBank numbers[3] (ITS, TUB)
N. liriodendri / *C. liriodendri*	CBS 110.81[2]; IMI 303645	*Liriodendron tulipifera*	U.S.A.	—	DQ178163, DQ178170
	CBS 112591; CPC 3673	*Vitis vinifera*	France	P. Larignon	AY677262, AY677245
	CBS 112596; CPC 3994	*Vitis vinifera*	South Africa	F. Halleen	AY677264, AY677239
	CBS 112602; CPC 3998	*Vitis vinifera*	South Africa	F. Halleen	AY677267, AY677242
	CBS 112610; CPC 3674	*Vitis vinifera*	France	P. Larignon	AY677270, AY677244
	CBS 117526; Cy 68	*Vitis vinifera*	Portugal	C. Rego	DQ178164, DQ178171
	CBS 117527; Cy 76	*Vitis vinifera*	Portugal	C. Rego	DQ178165, DQ178172
	CBS 117640; IMI 357400; Cy 1	*Vitis vinifera*	Portugal	C. Rego	DQ178166, DQ178173
N. galligena / *C. heteronema*	CBS 117751; KIS 10463	*Malus* sp.	Slovenia	H.J. Schroers & R. Mavec	DQ178167, —
	CBS 117752; KIS 10462	*Malus* sp.	Slovenia	H.J. Schroers & R. Mavec	DQ178168, —
	CPC 12078	*Malus* sp.	The Netherlands	P.W. Crous	DQ178169, —

[1] CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS; IMI: International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.; KIS:

[2] Ex-type cultures.

[3] ITS: internal transcribed spacer region, TUB: partial beta-tubulin gene.

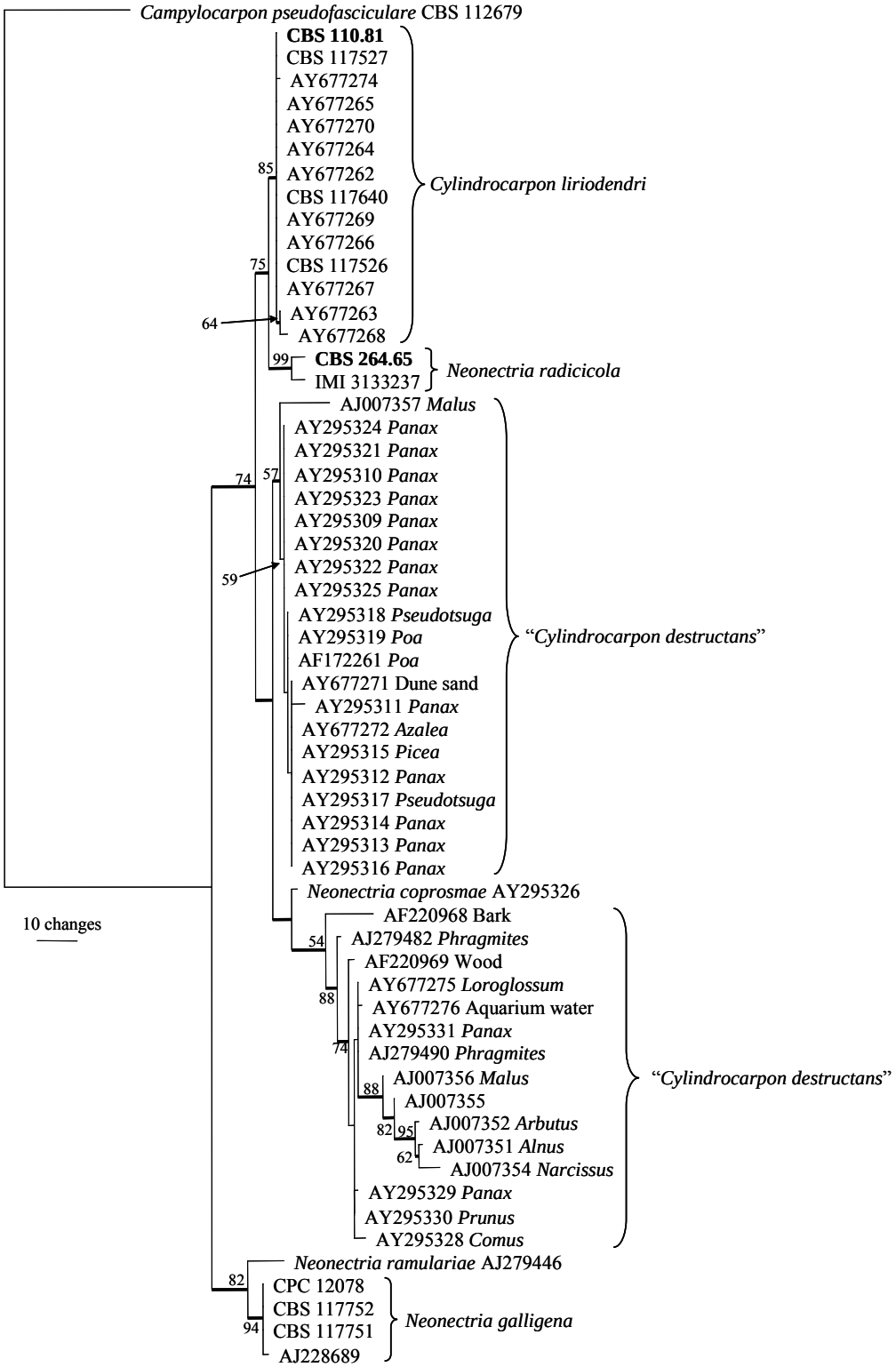

Fig. 1. One of 61 most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment. The scale bar shows 10 changes and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and type strains are shown in bold print. The host genus or source is indicated next to the GenBank accession numbers for the taxa in the "*C. destructans*" complex. The tree was rooted to *Campylocarpon pseudofasciculare* AY677306.

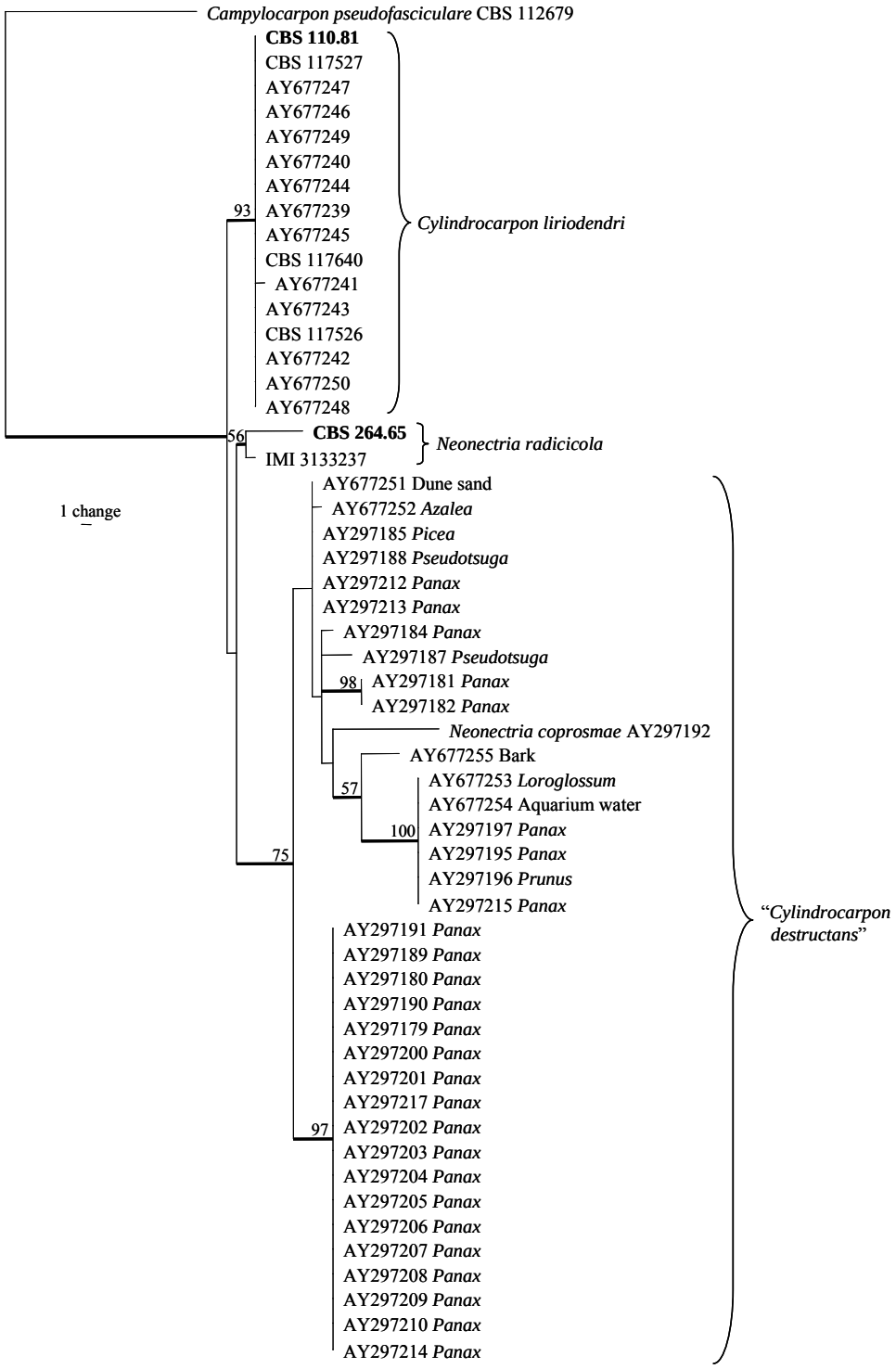

Fig. 2. One of 180 most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the β-tubulin sequence alignment. The scale bar shows a single change and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and type strains are shown in bold print. The host genus or source is indicated next to the GenBank accession numbers for the taxa in the "*C. destructans*" complex. The tree was rooted to *Campylocarpon pseudofasciculare* AY677214.

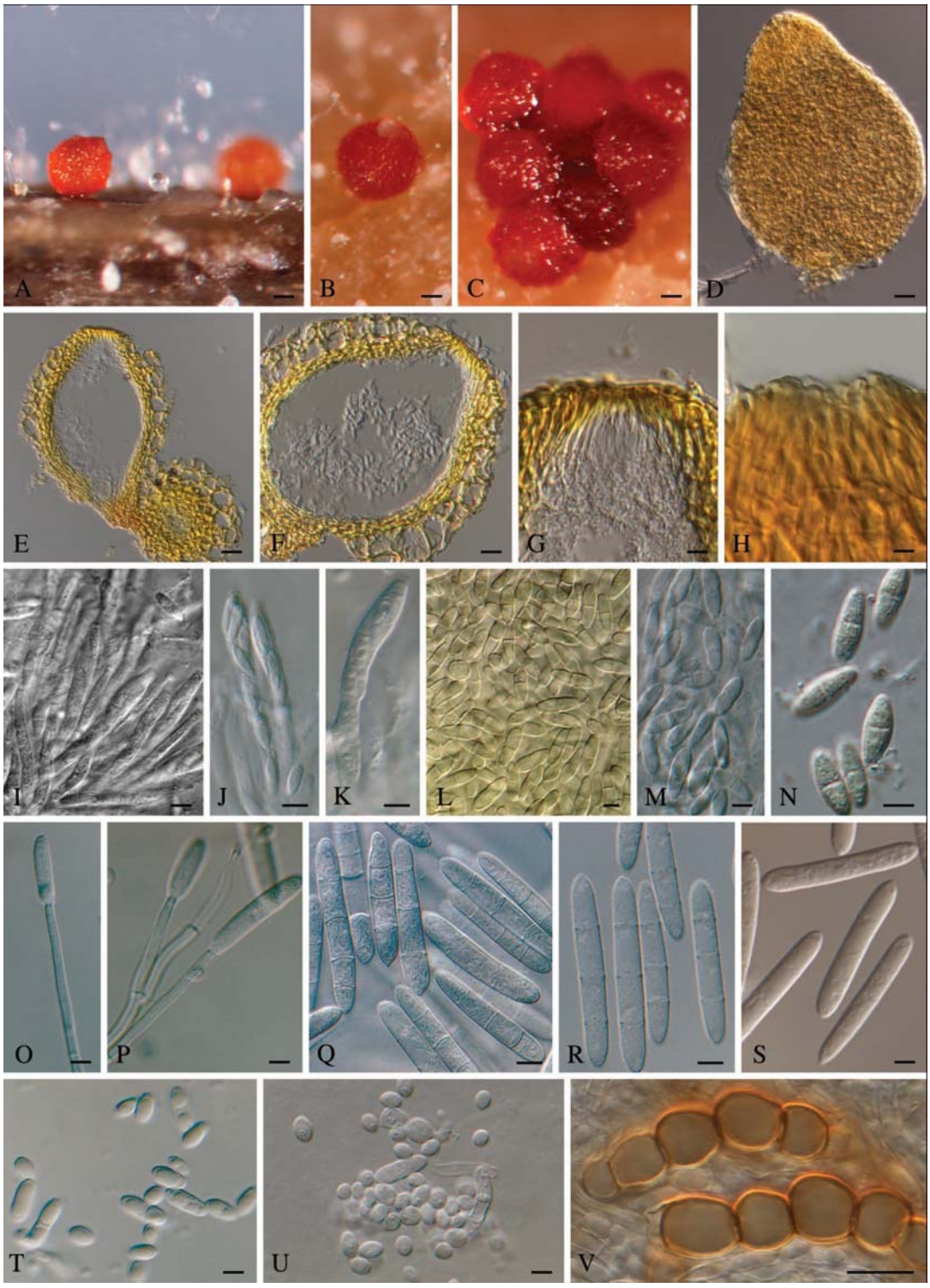

Fig. 3. *Neonectria liriodendri* and its *Cylindrocarpon liriodendri* anamorph. A–C. Perithecia on beach toothpicks. D. Perithecium mounted in lactic acid. E–H. Sections though perithecia, showing wall anatomy and ostiolar area. I–K. Asci. L–N. Ascospores. O–P. Conidiophores. Q–S. Macroconidia. T–U. Subcylindrical, ellipsoid and ovoid microconidia. V. Chlamydospores in chains. Scare bars: A = 90 µm, B–C = 70 µm, D = 30 µm, E = 80 µm, F = 26 µm, G–H = 20 µm, I–K = 5 µm, L–N = 3 µm, O–S = 5 µm, T–U = 4 µm, V = 15 µm.

5. CONTROL OF BLACK FOOT DISEASE IN GRAPEVINE NURSERIES

ABSTRACT

Black foot disease of grapevines is a decline and dieback disease caused by a soilborne pathogen complex including *Cylindrocarpon liriodendri*, *C. macrodidimum*, *Campylocarpon fasciculare* and *Campyl. pseudofasciculare*. These pathogens cause primary infections of roots and basal ends of grafted cuttings in nursery soils. The aim of the present study was to evaluate chemical, biological and physical strategies to prevent or eradicate these infections. Thirteen fungicides were screened *in vitro* for mycelial inhibition of these pathogens. Prochloraz manganese chloride, benomyl, flusilazole and imazalil were the most effective fungicides tested, and were subsequently included in semi-commercial field trials. Basal ends of grafted cuttings were dipped (1 min) in various chemical and biological treatments prior to planting in open-rooted nurseries. Black foot pathogens were not isolated from grafted cuttings prior to planting in the nurseries. Additional treatments involved soil amendments with *Trichoderma* formulations and hot water treatment of dormant nursery grapevines. Field trials were evaluated after a growing season of eight months. None of the treatments had a negative effect on root or shoot mass, although some treatments caused a reduction in the certifiable plant yield compared with that of the untreated control. Isolations from uprooted plants revealed low levels of black foot pathogens in the roots of untreated control plants, and significantly higher levels in basal ends of rootstocks. The incidence of black foot pathogens, as well as that of Petri disease pathogens (*Phaeomoniella chlamydospora*, *Phaeoacremonium* spp.) was not significantly and/or consistently reduced by the majority of chemical or biological treatments. However, these pathogens were not isolated from uprooted plants that were subjected to hot water treatment. It is therefore recommended that hot water treatment of dormant nursery plants be included in an integrated strategy for the proactive management of these diseases in grapevine nurseries.

INTRODUCTION

Black foot disease, caused by species of *Cylindrocarpon* and *Campylocarpon*, is a relatively newly described disease affecting mainly young grapevines (Grasso and Di San

Lio, 1975; Maluta and Larignon, 1991; Scheck *et al*., 1998a, 1998b; Larignon, 1999; Halleen *et al*., 2004; Chapter 3). Two species, *Cylindrocarpon obtusisporum* (Cooke & Harkn.) Wollenw. and *C. destructans* (Zins.) Scholten have previously been associated with this disease. *Cylindrocarpon obtusisporum* caused a decline of rooted grapevine cuttings in a Sicilian nursery (Grasso and Di San Lio, 1975). The decline was characterised by stunting, black discolouration of the wood and gum inclusions of xylem vessels. This species has also been associated with black foot disease in Californian vineyards (Scheck *et al*., 1998b). In France, the disease is caused by a different species, *C. destructans* (Maluta and Larignon, 1991) and losses of 50% or more have been recorded in young 2–8-year-old vineyards (Larignon, 1999). A third species, *C. macrodidymum* Schroers, Halleen & Crous, as well as two newly described *Campylocarpon* species, *Campyl. fasciculare* Schroers, Halleen & Crous and *Campyl. pseudofasciculare* Halleen, Schroers & Crous, have recently been associated with the disease (Halleen *et al*., 2004; Chapter 3). Although "*Cylindrocarpon destructans*" appears to be the dominant species associated with black foot disease (Halleen *et al*., 2004; Chapter 3), recent research has revealed that this taxon is actually a species complex (Samuels and Brayford, 1990; Seifert *et al*., 2003), and that the dominant grapevine pathogen is *C. liriodendri* J.D. MacDon. & E.E. Butler (Halleen *et al*., 2006; Chapter 4), and not *C. destructans* as reported previously (Halleen *et al*., 2004; Chapter 3).

In spring, affected plants are characterised by the absence of budding, or delayed budding with abnormal, weak vegetation that often wilts during early summer. Roots of infected plants become necrotic brown to black. To compensate for the loss in root mass, a second crown of horizontally growing roots is sometimes formed close to the soil surface (Larignon, 1999; Fourie *et al*., 2000). In older vines, the rootstock diameter below this second crown might also be thinner (Fourie and Halleen, 2001a). When bark of severely affected vines is removed, a brown necrotic lesion originating at the base of the rootstock can be seen. A cross section through this necrosis reveals the development of this necrosis from the bark to the pith (Larignon, 1999; Fourie and Halleen, 2001a). Sweetingham (1983) also described the discolouration of the phloem from which *C. destructans* was isolated. Furthermore, fungal hyphae were visible in the ray cells of the phloem and younger xylem of the discoloured tissue. Functional phloem was plugged with gum and xylem vessels were plugged with thick-walled tyloses or gum.

Isolation studies conducted in South African grapevine nurseries clearly demonstrated that black foot pathogens infected grafted grapevines from soils, once planted in open-rooted nurseries (Halleen *et al*., 2003; Chapter 2). Black foot pathogens rarely occurred in rootstock propagation material prior to planting (Fourie and Halleen, 2002; Halleen *et al*., 2003; Chapter 2). At the time of planting, the susceptible basal ends (especially the pith area) of most of the nursery cuttings are partly or even fully exposed. Callus roots also break during the planting process, resulting in small wounds susceptible to infection by soilborne pathogens (Halleen *et al*., 2003; Chapter 2).

Environmental factors and host stress play an important part in the development of black foot disease. Stress conditions that favour development of the disease include malnutrition, poor water drainage, soil compaction, heavy crop loads on young plants, planting of vines in poorly prepared soil and improper plant holes (Larignon, 1999; Fourie *et al*., 2000; Fourie and Halleen, 2001b; Halleen *et al*., 2004; Chapter 3). Soil compaction and/or poor soil preparation would also contribute to poor root development (J-rooting and pothole effect) (Fourie *et al*., 2000; Halleen *et al*., 2004; Chapter 3). High temperatures during summer also play an important role in symptom expression, since the compromised root and vascular system of diseased plants would not be able to supply enough water to compensate for the high transpiration rate during high temperatures (Larignon, 1999). *Cylindrocarpon* species are often part of disease complexes with other fungi or nematodes (Brayford, 1993). In the case of declining grapevines, black foot pathogens are often isolated in association with other pathogens. These pathogens include *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams, *Phaeoacremonium* (Petri disease pathogens), *Botryosphaeria*, *Phomopsis*, *Pythium* and *Phytophthora* spp. (Fourie *et al*., 2000; Fourie and Halleen, 2001c; Edwards and Pascoe, 2004; Oliveira *et al*., 2004).

Presently, no measures are known for control of black foot disease in South African vineyards or nurseries (Nel *et al*., 2003). Disease management in South African vineyards mainly involves the prevention and/or correction of predisposing stress situations (Fourie and Halleen, 2001a). However, knowledge obtained pertaining to the disease cycle of black foot disease suggests that suitable management strategies should focus on prevention of primary infection in nurseries. Rego *et al*. (2005) conducted *in vitro* fungicide screenings and found that prochloraz, benomyl, cyprodinil + fludioxonil and carbendazim + flusilazole inhibited mycelial growth of *C. liriodendri* (as *C. destructans*), whilst tebuconazole and difenoconazole

were less effective. Cyprodinil + fludioxonil, azoxystrobin, tryfloxistrobin and tolyfluanide effectively reduced spore germination (Rego *et al*., 2005). Results from subsequent *in vivo* studies conducted on potted grapevines in Portugal proved that benomyl, tebuconazole, carbendazim + flusilazole and cyprodinil + fludioxonil significantly improved plant growth and decreased disease incidence (Rego *et al*., 2005). Biological control measures have also been tested in glasshouse trials with potted grapevines, and the mychorrizal fungus *Glomus intraradices* Schenck & Smith provided excellent control against black foot disease when applied preventatively (Gubler *et al*., 2004). However, none of these treatments have been tested under field conditions. In a semi-commercial nursery trial, Fourie *et al.* (2001) demonstrated the growth stimulating attributes of *Trichoderma* Pers. treatments (dips, soil amendments and drenches with *Trichoderma* products containing propagules of selected strains of *Trichoderma harzianum* Rifai, Agrimm Technologies Ltd., Christchurch, New Zealand), as well as the effect thereof on natural infection by decline pathogens such as *Cylindrocarpon* spp. Low levels of *Cylindrocarpon* spp. were recorded, but the *Trichoderma* treatments notably reduced their incidence in roots of nursery grapevines. It furthermore significantly improved root development, which would undoubtedly make plants more tolerant to black foot disease when subjected to stress (Fourie *et al*., 2001). In a study aimed at the proactive management of Petri disease in grapevine nurseries, Fourie and Halleen (2004) found that hot water treatment (30 min at 50°C) of rootstock cuttings prior to grafting or dormant nursery plants after uprooting proved to be the most effective means of reducing infection levels of Petri disease pathogens in rootstocks of nursery plants. The latter treatment appeared to eradicate black foot pathogens from rootstocks, but infection levels were unfortunately too low for this finding to be conclusive (Fourie and Halleen, unpublished results).

In the present study, fungicides were evaluated for their ability to inhibit black foot pathogens *in vitro*. Semi-commercial nursery trials were subsequently conducted to evaluate the effectiveness of selected fungicides, *Trichoderma* treatments and hot water treatment in preventing or eradicating natural infection of the roots and basal ends of nursery grapevines. Means to prolong residual activity of fungicides were also investigated. As naturally infected material was used in the field trials, the effect of these treatments on black foot pathogens as well as Petri disease pathogens was recorded.

MATERIALS AND METHODS

In vitro evaluation of fungicides

Isolates. *Cylindrocarpon liriodendri* (CBS 112607, CBS 112597, CBS 112606), *C. macrodidymum* (CBS 112615, CBS 112603, CBS 112594), *Campylocarpon fasciculare* (CBS 112611, CBS 112612, CBS 112613) and *Campyl. pseudofasciculare* (CBS 112592, CBS 112679) isolates used in this study were previously isolated from asymptomatic grapevine nursery plants, as well as from diseased grapevines (Halleen *et al*., 2004). During this study, isolates were maintained on 2% potato-dextrose agar (PDA, Biolab, Midrand, Johannesburg) slants at 4°C and transferred onto PDA plates when needed. Petri dishes were subsequently incubated at room temperature for 1 week, at which time there was sufficient growth to transfer to fungicide-amended media.

Fungicides. Thirteen fungicides (Table 1), representing 10 different chemical classes, were screened for *in vitro* mycelial inhibition of *Cylindrocarpon destructans*, *C. macrodidymum*, *Campylocarpon fasciculare* and *Campyl. pseudofasciculare*. The fungicides were: thiram (Thiram 750WP, Dow Agrosciences, Silverton, South Africa); benomyl (Benomyl 500WP, Dow Agrosciences); flusilazole (Olymp 100EW, Du Pont de Nemours International Societe Anonyme, Halfway House, South Africa); mancozeb (Penncozeb 750WG, Du Pont); kresoxim-methyl (Stroby 500WG, BASF, Halfway House, South Africa); spiroxamine (Prosper 500EC, Bayer, Isando, South Africa); pyrimethanil (Scala 400SC, Bayer); propineb (technical grade, 83.1% a.i., Bayer); prochloraz manganese chloride (Octave 500WP, Bayer); procymidone (Sumisclex 250SC, Philagro, Menlo Park, South Africa); captab (Kaptan 500WP, Applied Chemical Products, Witfield, South Africa); hydroxyquinoline sulphate (Chinosol 67% a.i., Algro-Chem, Rynfield, South Africa) and imazalil (Magnate 800EC, Makhteshim, Brackenfell, South Africa). The fungicides were suspended in sterile distilled water and added to molten PDA at approximately 50°C in amounts to achieve final concentrations of 0, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 µg/ml. Mycelial plugs (4.5 mm in diameter), obtained from the margins of actively growing cultures, were transferred to fungicide amended plates. Three mycelial plugs (three different isolates) were placed at an equal distance from each other on each plate. There were three replicates of each fungicide concentration, and the experiment was repeated once. Concentrations of 50 and 100 µg/ml were added for less effective fungicides when the experiment was repeated. The dishes

were incubated for 6 days at 23°C in the dark, whereafter the diameter of each colony was measured twice perpendicularly.

Statistical analyses. The experimental design was completely randomised with an 4×13×10 factorial and three random replications. The factors were 4 species, 13 fungicides and 10 concentrations. The percentage inhibition for each isolate at each concentration was calculated as a percentage of the control treatment (unamended PDA). The percentage inhibition data of both experiments were pooled and linear regressions were fitted over the natural log concentrations for each isolate and fungicide separately. For more accurate estimations of the EC_{50} values (concentration at which 50% of the mycelial growth was inhibited), a linear regression equation [% Inhibition = a + b x Ln(Concentration), where a = intercept and b = slope] was fitted on two points in the log regression that included the 50% inhibition value. EC_{50} values were calculated as follows: $EC_{50} = \exp(b/(50-a))$. The EC_{50} values were subjected to analysis of variance and the Student's t-LSD (Least Significant Difference) was calculated at a 5% significance level to compare means (SAS, 1990). The Shapiro-Wilk test was performed to test normality on residuals (Shapiro and Wilk, 1965). Isolates with outliers were discarded until the residuals were normally distributed.

Field trials

Field trials were conducted at two nurseries in Wellington during the 2002–2003 and 2003–2004 seasons. In total, 4825 Cabernet Sauvignon (clone CS163B) / 101-14 Mgt (clone AA26A) cuttings were hand grafted (September 2002 and 2003). All grafting took place at the same nursery according to standard nursery practices (Le Roux, 1988) as conducted by this specific nursery. None of the plants received any treatment at this stage. Graftlings were placed in callusing boxes (300/box) filled with moist sawdust and allowed to callus according to the standard method for cold callusing (Le Roux, 1988) used by this specific nursery.

Treatments. Graftlings were removed from callusing boxes on 10 October 2002 and 21 October 2003. Basal ends (bottom 5–10 cm) of the rootstocks were dipped for 1 min in various treatments (300 grafted cuttings/treatment). The treatments were: benomyl (1 g/l); flusilazole (0.5 ml/l); prochloraz manganese chloride (1 g/l); imazalil (1.32 ml/l); Trichoflow-T™ (selected strains of *Trichoderma harzianum*, Agrimm Technologies, NZ; 2 g/l); carnauba wax (liquid wax derived from palm trees that is used in the citrus industry as a fruit wax and as carrier for fungicides like imazalil; Novon Crop Protection, Nelspruit, South Africa;

formulation strength); prochloraz manganese chloride (3 g/l) + carnauba wax (1 L); benomyl (3 g/l) + carnauba wax (1 l); flusilazole (1.5 ml/l) + carnauba wax (1 L); Nu-Film 17 (an organic compound derived from Pinolene, a natural pine resin, used as a sticker, spreader and extender; Miller Chemical South Africa; 0.25 ml/l); benomyl (3 g/l) + Nu-Film 17 (0.25 ml/l); flusilazole (1.5 ml/l) + Nu-Film 17 (0.25 ml/l); and prochloraz manganese chloride (3 g/l) + Nu-Film 17 (0.25 ml/l). Control plants were dipped in water. Treated graftlings were allowed to air dry on a dripping rack. Graftlings from the various treatments were divided into two groups and taken to two separate nursery fields. Graftlings from the various treatments were further divided into three groups of 50 each. The trial layout was a randomised block design with two sites, three blocks and 16 treatments. Graftlings were planted at 5 cm spacing within rows, and 60 cm between rows. Normal nursery practices (irrigation, nutrition, cultivation practices and disease and pest management) were followed for the duration of each growing season.

Additional treatments involved soil amendments with *Trichoderma* formulations and hot water treatment of dormant nursery grapevines. For the soil amendment treatment Trichopel-R™ (selected strains of *T. harzianum*, Agrimm Technologies, NZ) were added to the planting furrows at 20 g/m. After planting, the root zones were drenched with Trichogrow™ (selected strains of *T. harzianum*, Agrimm Technologies, NZ) at monthly intervals (1.5 kg/ha), with a total of six applications. Dormant vines were subjected to hot water treatment (50°C for 30 min followed by 30 min in cold water) after uprooting. These plants did not receive any treatment before planting.

Ratings. Isolations were also made from grafted cuttings prior to planting to determine whether rootstocks were infected with *Cylindrocarpon*, *Campylocarpon* or any other pathogen. After callusing, prior to planting, 25 graftlings were taken to the laboratory where isolations were made from the rootstock (within 1–3 cm of the basal end). The basal end section was removed and surface sterilised (30 s in 70% ethanol, 5 min in 0.35% sodium hypochlorite and 30 s in 70% ethanol) before isolations were made. These sections were split lengthwise to reveal the xylem and pith regions. Four pieces of tissue (approximately 0.5 x 1 mm in size) were removed and placed in Petri dishes containing PDA amended with chloramphenicol (250 mg/l) to reduce bacterial growth. Dishes were incubated in an incubation growth room at ±25°C for 4 weeks. Fungal growth from plated tissue pieces was

monitored daily, identified, or hyphal-tipped and transferred to PDA slants for later identification.

Growth of nursery plants was monitored throughout the season. The nursery plants were uprooted after 7 months, classed by the respective nurseries (according to SA Plant Improvement Regulations), and the certifiable plant yield determined (number of Class 1 certified vines as a percentage of grafted cuttings that were planted). Twenty-five vines per replicate were randomly selected, total root and shoot mass of each determined, and used for fungal isolation as described above. The latter was done to determine the level of natural infection by *Cylindrocarpon*, *Campylocarpon* and other grapevine pathogens following planting in two field nurseries. The incidence of *Trichoderma* spp. was also recorded to provide an indication of the extent of colonisation following treatment with the *Trichoderma* formulations. Isolations were made from the rootstock (within 1–3 cm of the basal end) as well as from the roots (within 1 cm from the basal end of the rootstock).

Statistical analyses. Experimental design was a randomised block design with 17 treatments, three repetitions per treatment, randomised in two field nurseries, each with three blocks. The blocks and nurseries were combined as six blocks and the repeated measurements over seasons as a subplot factor. The incidence of fungi present in each rootstock and root was determined as a percentage of the four isolated segments colonised. Data were subjected to analysis of variance using SAS version 8.1 (SAS, 1990). Student's t-Least Significant Difference was calculated at the 5% confidence level to compare treatment means.

RESULTS

In vitro evaluation of fungicides

According to the analysis of variance of EC_{50} values, significant fungicide × species interaction ($P < 0.0001$) was observed (ANOVA not shown). Mean EC_{50} values for reduction in mycelial growth of *C. destructans*, *C. macrodidymum*, *Campyl. fasciculare* and *Campyl. pseudofasciculare* are given in Table 1. Prochloraz manganese chloride was consistently the fungicide that most effectively reduced mycelial growth with lowest EC_{50} values for all four species (0.01–0.29 µg/ml). Benomyl was the only other fungicide that reduced mycelial growth of all four species (0.8–1.1 µg/ml). Flusilazole and imazalil were effective in

reducing mycelial growth of the *Cylindrocarpon* spp. (0.2–0.8 µg/ml for flusilazole and 0.6–1.5 µg/ml for imazalil), but not the *Campylocarpon* spp. (12.2–12.6 µg/ml for flusilazole and 61.2–100 µg/ml for imazalil). All the other fungicides were markedly less effective in inhibiting mycelial growth with EC_{50} values ranging from 19.8 to >100 µg/ml. Based on these results, prochloraz manganese chloride, benomyl, flusilazole and imazalil were selected to be included in nursery trials for further evaluation.

Field trials

Certifiable plant yield. Analysis of variance of certifiable plant yield percentages indicated that no significant interaction was observed between season and treatment (P = 0.0710; ANOVA not shown). Certifiable plant yields were significantly (P < 0.0001) higher during the 2002–2003 season (78.4%) compared with the 2003–2004 season (71.3%). Significant differences were furthermore observed between means for treatments (P < 0.0001). The prochloraz manganese chloride / Nu-Film 17 (73.3%), flusilazole / Nu-Film 17 (70.4%), carnauba wax (68.0%), flusilazole / carnauba wax (68.0%) and prochloraz manganese chloride (66.7%) treatments caused reductions of 7.6% to 15.9% in certifiable plant yield compared with the water treated control plants (79.3%). None of the other treatments (73.5% to 84.0%) differed significantly from the water treated control plants (results not shown).

Root and shoot mass. Analyses of variance for root and shoot mass data indicated significant differences between seasons (P = 0.0331 and P < 0.0001, respectively; ANOVA not shown), which might be attributed to climatic differences between seasons and/or differences in nursery practices. However, no significant treatment × season interaction was observed for root or shoot mass (P = 0.8071 and P = 0.8577, respectively). None of the treatments yielded plants with roots or shoots lower in mass than the water treated controls. However, the carnauba wax treated plants had significantly heavier roots (mean of 35.1 g) than the water control (29.2 g), whereas the prochloraz manganese chloride (26.5 g), benomyl / carnauba wax (26.6 g), carnauba wax (28.3 g) and flusilazole / Nu-Film 17 (28.4 g) treated plants had significantly heavier shoots than the water treated control (22.8 g; results not shown). The increase in root and shoot mass might be attributed to the low certifiable plant yields of respective treatments, which results in reduced competition between plants. However, the common nursery practice where shoots are regularly topped should also be considered in the interpretation of the data.

Incidence of black foot pathogens. Significant treatment × season interaction ($P < 0.0001$; Table 2) occurred for the mean incidence of *Cylindrocarpon* and *Campylocarpon* spp. in rootstocks, and datasets for the two seasons could therefore not be combined. No black foot pathogens were isolated from any of the control plants prior to planting in the nurseries (Table 4), but infection levels in the water treated control plants were significantly higher at the end of each growing season, and significantly higher during the 2002–2003 season (45.3%) compared with the 2003–2004 season (16.8%). Imazalil (31.3%), benomyl / Nu-Film 17 (29.3%), benomyl / carnauba wax (15.3%) and hot water treatment (0.0%) were the only treatments that differed significantly from the water treated control (45.3%) in the 2002–2003 season, whilst only the benomyl / Nu-Film 17 (4.6%) and hot water treatment (0.0%) harboured significantly lower levels of black foot pathogens compared with the control (16.8%) in the 2003–2004 season.

In roots, no significant interaction was observed between season and treatment ($P = 0.3725$; Table 3) and the datasets of the two seasons were combined. Significant differences ($P < 0.0001$; Table 3) were observed between seasons and contrary to what was observed in rootstocks, significantly lower incidences were observed in the 2002–2003 season (2.8%) compared with the 2003–2004 season (5.7%). Significant differences ($P = 0.0067$) were also observed between the treatments. Isolations that were made from uprooted grapevines at the end of the growing season revealed very low levels (mean incidence of 4.1%) of black foot pathogens in the water treated control plants (Table 4). Significantly more black foot pathogens were isolated from roots of flusilazole treated plants (8.3%) compared with the control plants. No black foot pathogens were isolated from roots of plants that were subjected to hot water treatment, while none of the other treatments differed significantly from the control plants.

Incidence of Petri disease pathogens. Significant treatment × season interaction ($P < 0.0001$; Table 2) was observed for the incidence of *Pa. chlamydospora* in rootstocks. *Pa. chlamydospora* was not isolated from any of the control plants prior to planting (Table 5). Higher levels were, however, recorded from the water treated control plants at the end of the growing season, with significantly more in the 2003–2004 season (21.7%) than the 2002–2003 season (5.3%). All the treatments (mean incidence of 0.0–10.7%), except Trichoflow-T™ (18.0%) and flusilazole (15.3%), reduced infection during the 2003–2004 season

compared with the water treated control. None of the treatments differed from the water treated control during the 2002–2003 season. It should, however, be noted that *Pa. chlamydospora* was not isolated from hot water treated plants in any of the seasons. In roots, significant treatment × season interaction was also observed (P = 0.0355; Table 3). *Pa. chlamydospora* levels in roots of control plants were approximately 7-fold less than those observed for rootstocks (Table 5). None of the treatments differed significantly from the water treated control during the 2002–2003 season. However, all the treatments, except carnauba wax (2.7%), flusilazole / carnauba wax (2.0%) and Nu-Film 17 (2.0%) reduced infection during the 2003–2004 season compared with the water treated control (3.5%; Table 5). As in rootstocks, *Pa. chlamydospora* was not isolated from roots of hot water treated plants (Table 5).

For *Phaeoacremonium* spp. isolated from rootstocks, treatment × season interaction was not significant (P = 0.1586; Table 2). Contrary to what was observed for *Pa. chlamydospora*, significantly (P = 0.0239) less *Phaeoacremonium* spp. were isolated from rootstocks during the 2003–2004 season (mean incidence of 3.2%) compared with 4.8% during the 2002–2003 season. Significant differences (P < 0.0014) were also observed between the treatments. No *Phaeoacremonium* spp. were isolated from any of the control plants prior to planting in the nurseries, whilst these species were isolated from the basal ends of 6% of the water treated control plants after uprooting (Table 5). None of the other treatments, except for the hot water treatment (0.3%) differed significantly from the water treated control. In roots, significant treatment × season interaction was observed (P = 0.0050; Table 3). *Phaeoacremonium* spp. were not isolated from the roots of plants that were subjected to hot water treatment, which was the only treatment that reduced the incidence of *Phaeoacremonium* spp. compared with the control plants during both seasons (6.0 and 5.3%, respectively; Table 5). The flusilazole / carnauba wax treatment (18%) caused an increase in *Phaeoacremonium* incidence during the 2002–2003 season, whereas it effected a reduction during 2003–2004 (2.7%; Table 5).

Incidence of *Trichoderma* spp. No interaction was observed between season and treatment (P = 0.1098; Table 2). Significantly higher *Trichoderma* incidences (P = 0.0022) were observed during the 2002–2003 season (mean of 1%) compared with the 2003–2004 season (0.3%). Significant differences (P = 0.0052) were also observed between the

treatments. *Trichoderma* spp. were not isolated from any of the control plants prior to planting in the nurseries (Table 6). Isolations from uprooted grapevines revealed very low levels (0.3%) of *Trichoderma* spp. in the basal ends of water treated control plants. Only the Trichoflow-T^{TM} and prochloraz manganese chloride / carnauba wax treatments differed from the water treated control plants (2.3% and 1.8%, respectively). In roots, significant treatment × season interaction occurred ($P = 0.0208$; Table 3). *Trichoderma* spp. were not isolated from any roots in 2002–2003, while flusilazole / Nu-Film 17 treatment (3.2%) was the only treatment that differed from the water treated control (0%) during the 2003–2004 season (Table 6). *Trichoderma* spp. were not isolated from roots of plants subjected to *Trichoderma* treatments during both seasons.

DISCUSSION

Results from the *in vitro* studies indicated that prochloraz manganese chloride, and benomyl were the most effective in inhibiting mycelial growth of the *Cylindrocarpon* and *Campylocarpon* spp. tested, while flusilazole and imazalil were highly effective against the *Cylindrocarpon* spp. only. This is the first report of *in vitro* fungicide sensitivity of *C. macrodidymum*, *Campyl. fasciculare* and *Campyl. pseudofasciculare*, while the findings support earlier findings for *C. liriodendri* / *C. destructans* by Rego *et al.* (2005). Benomyl is registered for the control of grapevine powdery mildew (*Erysiphe necator* Schwein.) and Botrytis rot (*Botrytis cinerea* Pers.) and flusilazole for the control of grapevine powdery mildew (Nel *et al.*, 2003). Prochloraz manganese chloride is registered in South Africa for use on apricots, peaches, plums, roses, mushrooms and potatoes (Nel *et al*., 2003). Imazalil is registered for the control of post harvest decay on citrus and cucurbits. On citrus, it is generally applied in wax or in water before waxing of fruit as a spray-on or brush-on application (Nel *et al*., 2003). The latter two compounds are, however, not registered for use on grapevines. Previous studies have shown that prochloraz manganese chloride, benomyl and flusilazole also effectively inhibited mycelial growth of *Pa. chlamydospora* (Groenewald *et al*., 2000; Jaspers, 2001), whereas prochloraz manganese chloride and benomyl also inhibited spore germination (Jaspers, 2001). *Pa. chlamydospora*, the causal organism of Petri disease, and black foot disease pathogens are regularly co-isolated from diseased grapevines (Fourie and Halleen, 2001b) and a fungicide that control both these pathogens could be of great benefit to the grapevine nursery industry, especially in nurseries where primary infection should be prevented.

The fact that black foot pathogens infect grapevine cuttings in nursery soils has been confirmed in this study, and clearly underlines the importance of suitable control measures to prevent or eradicate these infections. However, inconsistent results were obtained with the various chemical and biological treatments tested regarding prevention of infection by black foot and Petri disease pathogens. Generally low infection levels in the roots and low infection levels in rootstocks by these pathogens during the 2003–2004 and 2002–2003 seasons, respectively, might have contributed to the inconsistent results. The benomyl / Nu-Film 17 and benomyl / carnauba wax treatments can nonetheless be identified for further investigation, since it effected marginal to significant reductions in the incidences of these pathogens in rootstocks and/or roots. Reasons for the generally poor performance of fungicides are not clear, although several factors might have contributed. Firstly, the poor performance could be due to the inability of the fungicides to penetrate the xylem tissue. Additionally, breakdown of fungicide residue on the rootstock surface would have resulted in a limited period of protection only. Prochloraz manganese chloride and imazalil are active against various seed- and soilborne pathogens (Kuck *et al*., 1995). Although registered in various countries for use as soil drenches and seed treatments, they nonetheless proved ineffective in this study. Disappointingly, the addition of Nu-film 17 and carnauba wax to improve adhesion and prolong efficacy of the fungicide treatments proved largely ineffective. The addition of these products to benomyl did, however, lead to a marked improvement of the fungicide's efficacy.

An interesting observation emerged during this study regarding the incidences of *Pa. chlamydospora* in rootstocks: *Pa. chlamydospora* is reported to occur in low levels in rootstock cuttings harvested from infected mother plants (Zanzotto *et al*., 2001: Fourie and Halleen, 2002, 2004; Edwards and Pascoe, 2004; Whiteman *et al*., 2004; Retief *et al*., 2005), but was not isolated from the control plants prior to planting in the nurseries. This might be attributed to low levels of infection that might not have been detected by the isolation technique used in this study, or to the small sample size used for these isolations. Significantly higher levels were, however, isolated at the end of each growing season, but were found to differ substantially in plants grown at different nursery fields during the 2003/2004 season (30% and 13.3%; results not shown). Rootstock and scion cuttings were obtained from the same source and evidence of differences in cultivation practices and environmental conditions between sites could not be found. Therefore, these differences in

Phaeomoniella incidence can only be attributed to infections from nursery soils. A possible soilborne phase for this pathogen has been postulated by other researchers (Crous *et al*., 1996; Adalat *et al*., 2000; Whiteman *et al*., 2004; Damm and Fourie, 2005; Gaforio *et al*., 2005; Retief *et al.*, 2005) and should be investigated further.

The *Trichoderma* treatments proved inefficient or at most inconsistent, as they prevented infection of *Phaeomoniella* in one season only (Trichopel/Trichogrow treatment in 2003/2004). This might be attributed to insufficient systemic colonisation of the basal ends of rootstocks as was shown by the low re-isolation percentages observed for *Trichoderma* spp. in plants treated with this biological control agent. In this regard the Trichoflow treatment prior to planting proved to be more effective than the Trichopel-R™ / Trichogrow™ treatment, although *Trichoderma* was isolated from a mere 2.3% of rootstocks. It is therefore recommended that the duration of this treatment (1 min dip) be re-evaluated in an attempt to improve colonisation and prolonged protection. In previous studies these treatments reduced the incidence of *Cylindrocarpon* spp. in nursery grapevines and significantly improved root development (Fourie *et al*., 2001). Although it was not observed in this study, this growth stimulating effect would undoubtedly make plants more tolerant when subjected to stress, and due to its environmentally safe nature would be of great importance to the nursery industry.

The reduction in black foot and Petri disease pathogens caused by the hot water treatment clearly demonstrated the potential of this control measure to eradicate pathogen infections from dormant nursery vines. Previously this treatment was also recommended for the eradication of *Phytophthora cinnamomi* (Von Broembsen and Marais, 1978), *Pa. chlamydospora* (Crous *et al*., 2001; Fourie and Halleen, 2004) and *Meloidogyne javanica* (Barbercheck, 1986) from dormant nursery grapevines. It is therefore recommended that hot water treatment of dormant nursery grapevines be included in an integrated strategy for the proactive management of diseases and pests in grapevine nurseries.

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Table 1. EC_{50} values for inhibiting mycelial growth of *Cylindrocarpon* and *Campylocarpon* spp. by fungicides representing different chemical classes

	EC_{50} values (µg/ml ai)[a]				
Fungicide	***C. liriodendri***	***C. macrodidymum***	***Campyl. fasciculare***	***Campyl. pseudofasciculare***	**LSD** (*P*=0.05)
Benomyl	0.89 [b]	1.06 [a]	0.80 [b]	1.11 [a]	0.09
Captab	87.25 [a]	43.27 [b]	81.60 [a]	75.49 [a]	17.70
Flusilazole	0.80 [b]	0.21 [b]	12.63 [a]	12.15 [a]	3.42
Hydroxyquinoline sulphate	>100	>100	>100	>100	Not Determined
Imazalil	1.53 [c]	0.62 [c]	100.00 [a]	61.23 [b]	4.04
Kresoxim-methyl	88.89 [a]	100.00 [a]	100.00 [a]	100.00 [a]	18.78
Mancozeb	62.87 [b]	29.33 [c]	51.33 [b]	91.76 [a]	15.66
Prochloraz manganese chloride	0.04 [c]	0.01 [d]	0.26 [b]	0.29 [a]	0.02
Procymidone	100.00 [a]	88.89 [a]	71.43 [a]	100.00 [a]	32.95
Propineb	71.30 [a]	19.77 [c]	47.30 [b]	72.51 [a]	5.75
Pyrimethanil	100.00 [a]	98.40 [a]	100.00 [a]	100.00 [a]	1.60
Spiroxamine	64.77 [c]	100.00 [a]	100.00 [a]	78.29 [b]	8.04
Thiram	58.01 [b]	24.84 [c]	78.64 [a]	54.17 [b]	13.51

[a] Values in the same row followed by the same letter do not differ statistically ($P = 0.05$).

Table 2. Analysis of variance for effects of block, nursery, season and treatment on the incidence (percentage) of selected taxa isolated[x] from the basal ends of nursery grapevines treated[y] with various chemical and biological agents prior to planting in two field nurseries in Wellington (2002-2003 and 2003-2004)

	***Cylindrocarpon* spp. and *Campylocarpon* spp.**			***Phaeomoniella chlamydospora***			***Phaeoacremonium* spp.**			***Trichoderma* spp.**		
Source of variation	**Df**	**MS**	**SL (=*P*)**	**Df**	**MS**	**SL (=*P*)**	**Df**	**MS**	**SL (=*P*)**	**Df**	**MS**	**SL (=*P*)**
Nursery	1	1064.34	0.0487	1	37.40	0.2458	1	7.08	0.5737	1	0.50	0.5151
Block (Nursery)	4	135.60	0.2716	4	20.26	0.5956	4	18.92	0.5325	4	0.98	0.8550
Treatment	16	1462.47	<0.0001	16	155.00	<0.0001	16	66.93	0.0014	16	5.25	0.0052
Block (Trt x Nursery)	80	64.04	0.9838	80	31.13	0.3760	80	24.13	0.4778	80	2.17	0.9158
Season	1	36412.52	<0.0001	1	375.64	0.0005	1	126.17	0.0239	1	29.55	0.0022
Treatment x Season	16	535.91	<0.0001	16	128.58	<0.0001	16	33.53	0.1586	16	4.51	0.1098
Error	84	103.23		84	29.04		84	23.84		84	2.95	
Corrected total	202			202			202			202		

[x] Percentage incidence was determined after uprooting in May-June 2003 and 2004.

[y] Graftlings were treated on 10 October 2002 and 21 October 2003 by dipping the basal ends of the graftlings in various treatments for 1 minute.

Table 3. Analysis of variance for effects of block, nursery, season and treatment on the incidence (percentage) of selected taxa isolated[x] from roots of nursery grapevines treated[y] with various chemical and biological agents prior to planting in two field nurseries in Wellington (2002-2003 and 2003-2004)

	***Cylindrocarpon* spp. and *Campylocarpon* spp.**			***Phaeomoniella chlamydospora***			***Phaeoacremonium* spp.**			***Trichoderma* spp.**		
Source of variation	**Df**	**MS**	**SL (=*P*)**	**Df**	**MS**	**SL (=*P*)**	**Df**	**MS**	**SL (=*P*)**	**Df**	**MS**	**SL (=*P*)**
Nursery	1	6.97	0.2059	1	2.27	0.6286	1	109.98	0.0343	1	0.35	0.5709
Block (Nursery)	4	3.06	0.9590	4	8.28	0.0045	4	11.03	0.7167	4	0.91	0.3815
Treatment	15	31.81	0.0067	15	4.58	0.0309	15	77.85	0.0001	15	1.51	0.0678
Block (Trt x Nursery)	75	13.24	0.9525	75	2.35	0.2541	75	21.51	0.4547	75	0.88	0.4445
Season	1	425.26	<0.0001	1	35.91	<0.0001	1	335.11	0.0001	1	3.03	0.0637
Treatment x Season	15	21.33	0.3725	15	3.84	0.0355	15	51.81	0.0050	15	1.68	0.0288
Error	79	19.44		79	2.02		79	20.96		79	0.86	
Corrected total	190			190			190			190		

[x] Percentage incidence was determined after uprooting in May-June 2003 and 2004.

[y] Graftlings were treated on 10 October 2002 and 21 October 2003 by dipping the basal ends of the graftlings in various treatments for 1 minute.

Table 4. Mean incidences of pathogens associated with black foot disease isolated from grapevine nursery plants that were treated with various chemical and biological control agents prior to planting, as well as hot water treatment of plants after uprooting

Treatment[x]	Rootstock[y]		Roots[y]
	2002-2003	2003-2004	Combined
Benomyl	40.7 bcde	12.0 gh	5.2 b
Benomyl /Carnauba wax	15.3 gh	6.9 ghi	4.6 b
Benomyl/Nu-Film 17	29.3 ef	4.6 hi	4.2 b
Carnauba wax	38.0 cde	11.4 ghi	4.0 b
Control	45.3 abc	16.8 g	4.1 b
Control (isolations before planting)	0.0 i	0.0 i	-
Flusilazole	54.0 a	15.2 gh	8.3 a
Flusilazole/Nu-Film 17	48.7 abc	10.4 ghi	4.7 b
Flusilazole/Carnauba wax	41.3 bcd	8.7 ghi	3.3 b
Hot Water Treatment	0.0 i	0.0 i	0.0 c
Imazalil	31.3 de	16.7 g	5.3 ab
Nu-Film 17	54.7 a	15.7 gh	3.1 b
Prochloraz manganese chloride	53.3 a	12.2 gh	4.3 b
Prochloraz manganese chloride/Carnauba wax	45.0 abc	10.0 ghi	4.7 b
Prochloraz manganese chloride/Nu-Film 17	49.3 abc	11.3 ghi	4.0 b
Trichoflow	40.7 bcde	10.7 ghi	3.7 b
Trichopel/Trichogrow	50.0 ab	18.1 fg	4.2 b
LSD (P = 0.05)	11.67		2.97

[x] Graftlings were treated on 10 October 2002 and 21 October 2003 by dipping the basal ends of the graftlings in various treatments for 1 minute.

[y] Percentage incidence was determined by means of isolations after uprooting in May-June 2003 and 2004. Values within the respective columns followed by the same letter do not differ significantly (P = 0.05).

Table 5. Mean incidences of Petri disease pathogens isolated from grapevine nursery plants that were treated with various chemical and biological control agents prior to planting, as well as hot water treatment of plants after uprooting

	***Pa. chlamydospora*[y]**				***Phaeoacremonium* spp.[y]**		
	Rootstock		**Roots**		**Rootstock**	**Roots**	
Treatment[x]	**2002-2003**	**2003-2004**	**2002-2003**	**2003-2004**	**Combined**	**2002-2003**	**2003-2004**
Benomyl	4.0 efgh	5.3 defgh	0.0 d	1.5 bcd	3.3 cde	2.7 efgh	3.3 efgh
Benomyl /Carnauba wax	10.7 cd	2.0 fgh	1.3 bcd	0.0 d	2.5 de	6.7 bcdef	6.6 bcdef
Benomyl/Nu-Film 17	10.0 cde	4.3 efgh	0.0 d	0.0 d	2.0 de	6.7 bcdef	1.3 gh
Carnauba wax	2.0 fgh	10.7 cd	0.0 d	2.7 ab	2.7 cde	10.7 b	6.8 bcdef
Control	5.3 defgh	21.7 a	0.7 cd	3.5 a	6.0 abcd	6.0 bcdefg	5.3 cdefg
Control (isolations before planting)	0.0 h	0.0 h	.	.	0.0 e	.	.
Flusilazole	6.0 defgh	15.3 abc	0.0 d	1.3 bcd	5.3 abcd	7.3 bcde	6.0 bcdefg
Flusilazole/Carnauba wax	5.3 defgh	4.0 efgh	0.0 d	2.0 abc	5.3 abcd	18.0 a	2.7 efgh
Flusilazole /Nu-Film 17	9.3 cde	6.4 defg	0.0 d	0.0 d	4.7 abcd	6.0 bcdefg	3.2 efgh
Hot Water Treatment	0.0 h	0.0 h	0.0 d	0.0 d	0.3 e	0.0 h	0.0 h
Imazalil	6.7 defg	2.0 fgh	0.0 d	0.0 d	4.0 bcde	9.3 bc	2.7 efgh
Nu-Film 17	2.1 fgh	10.2 cde	0.7 cd	2.0 abc	4.6 abcd	6.1 bcdefg	3.3 efgh
Prochloraz manganese chloride (PMC)	0.7 gh	7.1 def	0.0 d	0.7 cd	8.1 a	5.3 cdefg	3.5 defgh
PMC / Carnauba wax	4.7 defgh	5.3 defgh	0.0 d	0.0 d	7.7 ab	6.9 bcdef	8.7 bcd
PMC / Nu-Film 17	5.3 defgh	6.7 defg	0.0 d	1.3 bcd	6.7 abc	4.7 cdefgh	5.3 cdefg
Trichoflow	6.7 defg	18.0 ab	0.0 d	1.3 bcd	2.3 de	6.0 bcdefg	1.3 gh
Trichopel/Trichogrow	4.0 efgh	7.5 def	0.0 d	0.0 d	2.3 de	2.0 fgh	2.0 fgh
LSD (P = 0.05)	6.19		1.63		4.00	5.26	

[x] Graftlings were treated on 10 October 2002 and 21 October 2003 by dipping the basal ends of the graftlings in various treatments for 1 minute.

[y] Percentage incidence was determined by means of isolations after uprooting in May-June 2003 and 2004. Values within the respective columns followed by the same letter do not differ significantly (P = 0.05).

Table 6. Mean incidence of *Trichoderma* spp. isolated from grapevine nursery plants that were treated with various chemical and biological control agents prior to planting, as well as hot water treatment of plants after uprooting.

	Rootstock[y]	Roots[y]	
Treatments[x]	**Combined**	**2002–2003**	**2003–2004**
Benomyl	1.0 [bc]	0.0 [b]	0.0 [b]
Benomyl /Carnauba wax	0.0 [c]	0.0 [b]	0.0 [b]
Benomyl/Nu-Film 17	0.3 [c]	0.0 [b]	0.7 [b]
Carnauba wax	1.0 [bc]	0.0 [b]	0.7 [b]
Control	0.3 [c]	0.0 [b]	0.0 [b]
Control (isolations before planting)	0.0 [c]	.	.
Flusilazole	1.0 [bc]	0.0 [b]	0.0 [b]
Flusilazole/Carnauba wax	0.7 [bc]	0.0 [b]	0.0 [b]
Flusilazole /Nu-Film 17	0.7 [bc]	0.0 [b]	3.2 [a]
Hot Water Treatment	0.0 [c]	0.0 [b]	0.0 [b]
Imazalil	0.0 [c]	0.0 [b]	0.0 [b]
Nu-Film 17	0.0 [c]	0.0 [b]	0.0 [b]
Prochloraz manganese chloride (PMC)	0.3 [c]	0.0 [b]	0.0 [b]
PMC / Carnauba wax	1.8 [ab]	0.0 [b]	0.0 [b]
PMC / Nu-Film 17	1.0 [bc]	0.0 [b]	0.0 [b]
Trichoflow	2.3 [a]	0.0 [b]	0.0 [b]
Trichopel/Trichogrow	0.7 [bc]	0.0 [b]	0.0 [b]
LSD (P = 0.05)	1.20	1.06	

[x] Graftlings were treated on 10 October 2002 and 21 October 2003 by dipping the basal ends of the graftlings in various treatments for 1 minute.

[y] Percentage incidence was determined by means of isolations after uprooting in May-June 2003 and 2004. Values within the respective columns followed by the same letter do not differ significantly (P = 0.05).