THE CHARACTERIZATION AND CONTROL OF PHOMOPSIS CANE AND LEAF SPOT ON VINE

LIZEL MOSTERT

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Supervisor: Prof. P.W. Crous

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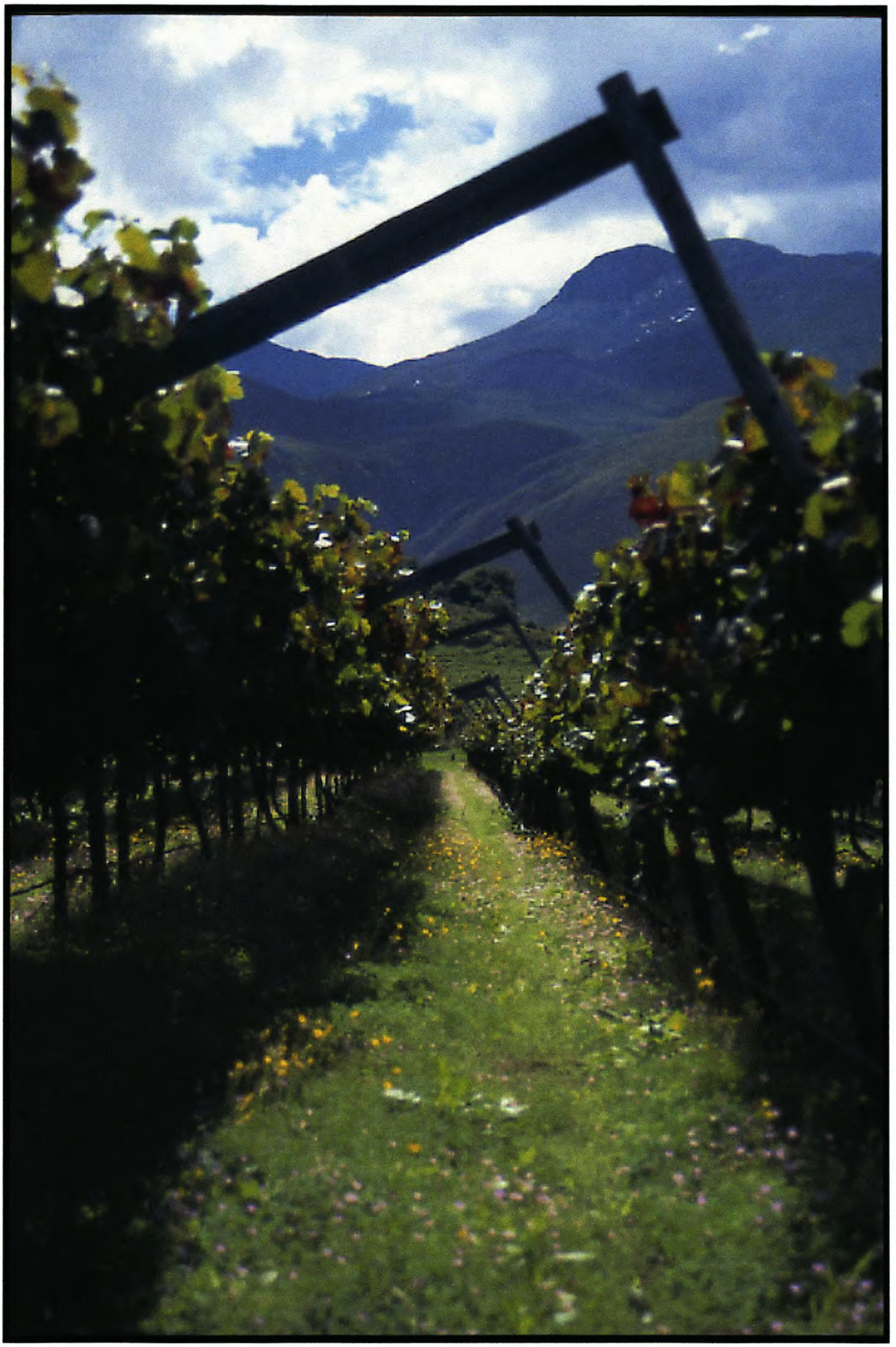

DECLARATION

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

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SUMMARY

Phomopsis cane and leaf spot disease of grapevine is an economically important disease in many of the vine-growing areas of the world. Four different *Phomopsis* spp. have previously been associated with this disease. The present study investigates the taxonomic significance of the different taxa found on grapevines in South Africa, as well as the endophytic growth and fungicide sensitivity of *Phomopsis viticola* isolates. The thesis is compiled of several different parts, which deal with specific, but related topics, and hence some duplication has been unavoidable.

Understanding the epidemiology of a disease is important for the correct timing of disease control. To investigate the endophytic growth of *P. viticola*, asymptomatic shoots were collected at eight different growth stages. Nodes, internodes, leaf petioles, leaves, tendrils and bunch peduncles were investigated. Two *Phomopsis* spp., taxon 1 and 2 were identified in this study. The *Phomopsis viticola*-complex had a relative importance of 9% and accounted for 3% of the isolations. *P. viticola* (taxon 2) is mainly isolated from the nodes and internodes. Inoculations of healthy, young vine tissue confirmed taxon 2 to be a virulent pathogen, suggesting that it is a latent pathogen rather than an endophyte. In contrast, taxon 1 appeared to be a true endophyte, and did not seem to be an important pathogen on vines.

The true identity of the causal organism of Phomopsis cane and leaf spot disease was investigated by collecting samples from 58 different vineyards in the grapevine growing areas of the Western Cape. *P. viticola* occurred in grapevine material collected from Lutzville to Swellendam, but was not found in the Oudtshoorn and Orange River grapevine areas. *Diaporthe perjuncta* (taxon 1), *P. viticola* (taxon 2), taxon 3 and a *Phomopsis* species commonly associated with shoot blight of peaches in the U.S.A., *P. amygdali*, were identified among the South African grapevine isolates. Examination of the Australian culture designated as taxon 4 found it to be a species of *Libertella*, thus excluding it from the *P. viticola*-complex. An Italian isolate was found to represent a species of *Phomopsis* not previously known from grapevines, and this was subsequently described as taxon 5. Species delimitation was based on morphological and cultural characteristics, stem inoculations and the formation of the teleomorph *in vitro*. The

identity of each morphological taxon was confirmed by means of phylogenetic analyses of the nuclear ribosomal DNA internal transcribed spacers (ITS1 and ITS2) and the 5' end partial sequence of the mitochondrial small subunit (mtSSU). *P. amygdali*, associated with peach shoot blight in the U.S.A., was isolated once only and appeared to be of lesser importance in this disease complex. Furthermore, taxa 1 (*Diaporthe perjuncta*) and 3 were also rarely encountered and proved to be non-pathogenic, indicating their non-functional role in Phomopsis cane and leaf spot disease. Taxon 2 (*Phomopsis viticola*) was common and widely distributed in diseased vineyards. This taxon was associated with the typical disease symptoms and proved to be pathogenic. Morphologically taxon 2 corresponded best with *P. viticola*, which was also neotypified in this study. Taxon 2 was mostly isolated from buds and nodes, indicating that these are important sites in which the fungus survives during winter. Molecular data indicated that taxon 3 and *P. amygdali* were not host specific to grapevine.

The currently used foliar fungicides were compared to the new strobilurin fungicides. The effects of nine fungicides (azoxystrobin, flusilazole, folpet, fosetyl-Al+mancozeb, kresoxim-methyl, mancozeb, penconazole, spiroxamine and trifloxystrobin) were tested *in vitro* on inhibition of mycelial growth. The following EC_{50} (µg/ml) values were obtained: azoxystrobin (0.350), flusilazole (0.007), folpet (4.489), fosetyl-Al+mancozeb (3.925), kresoxim-methyl (1.665), mancozeb (2.891), penconazole (0.023), spiroxamine (0.321) and trifloxystrobin (0.051). Additionally, azoxystrobin, folpet, kresoxim-methyl, mancozeb, propineb and trifloxystrobin were tested for their ability to inhibit spore germination *in vitro*. The subsequent EC_{50} (µg/ml) values were obtained: azoxystrobin 0.123), folpet (0.510), kresoxim-methyl (0.0037), mancozeb (0.250), propineb (0.156) and trifloxystrobin (0.003). The results reported in part 4 showed that the strobilurin fungicides inhibited the mycelial growth and spore germination of *P. viticola*. However, further trials need to be conducted to verify these findings under field conditions. In the present study taxa 1, 3 and *P. amygdali* were infrequently isolated, suggesting that they played a less prominent role in the *P. viticola*-complex.

OPSOMMING

Streepvleksiekte van wingerd is 'n ekonomies belangrike siekte wat in die meeste wingerdproduserende gebiede van die wêreld voorkom. Vier *Phomopsis* spesies is in die verlede met dié siekte geassosieer. Hierdie studie ondersoek die taksonomiese belangrikheid van die verskillende taksa wat op wingerd in Suid Afrika gevind word, asook die endofietiese groei en fungisiedsensitiwiteit van die *Phomopsis viticola* isolate. Hierdie tesis bestaan uit verskeie dele met spesifieke, maar verwante onderwerpe wat tot onafwendbare duplisering lei.

Dit is belangrik om die epidemiologie van 'n siekte te verstaan sodat korrekte en tydsberekende siektebeheer toegepas kan word. Die endofietiese groei van *P. viticola* is ondersoek deur simptoomlose lote by agt verskillende groei stadiums te versamel. Nodusse, internodusse, blaarstele, blare, rankies en trosstele is ondersoek. Twee *Phomopsis* spp., takson 1 en 2 is geïdentifiseer. Die *Phomopsis viticola*-kompleks het 3% van die isolasies uitgemaak en 'n relatiewe belangrikheid van 9% getoon. *P. viticola* (takson 2) is meestal uit die nodus en internodus geïsoleer. Inokulasies van gesonde, jong wingerdweefsel het bevestig dat takson 2 'n virulente patogeen is en dat die takson eerder 'n latente patogeen as 'n endofiet is. In teenstelling hiermee is takson 1 'n ware endofiet en 'n onbelangrike patogeen op wingerd.

Die ware identiteit van die veroorsakende organisme van streepvlek is ondersoek deur plantmateriaal vanaf 58 verskillende wingerde in die wingerproduserende gebiede van die Wes-Kaap te versamel. *P. viticola* is in wingerdmateriaal vanaf Lutzville tot Swellendam aangetref, maar nie in die Oudtshoorn en Oranjerivier wingerd produserende gebiede nie. *Diaporthe perjuncta* (takson 1), *P. viticola* (takson 2), takson 3 en *P. amygdali* is in die Suid Afrikaanse wingerdisolate geïdentifiseer. *P. amygdali* word met lootverskroeiing van perske bome in die V.S.A. geassosieer. Die Australiese isolaat wat benoem is as takson 4, is met die huidige ondersoek gevind om 'n spesie van *Libertella* te wees. Takson 4 is daarvolgens uit die *P. viticola*-kompleks gelaat. 'n Italiaanse isolaat het 'n nuwe spesie van *Phomopsis* op wingerd verteenwoordig en is vervolgens as takson 5 beskryf. Spesie-onderskeiding is op morfologiese en kulturele eienskappe, staminokulasies en die vorming van die teleomorf *in vitro* gebaseer. Die identiteit van

elke morfologiese takson is met behulp van filogenetiese analises van die nukleêre ribosomale DNS intern transkriberende spasieerders (ITS1 en ITS2) en die 5' punt gedeeltelike nukleotied volgorde van die mitochondriale klein subeenheid (mtSSU) bevestig. *P. amygdali* is slegs een keer geïsoleer en blyk van minder belang in die siektekompleks te wees. Takson 1 (*Diaporthe perjuncta*) en takson 3 het ook min voorgekom en is nie-patogenies, wat hul nie-funksionele rol in streepvleksiekte aandui. Takson 2 (*P. viticola*) is algemeen geïsoleer en kom wyd verspreid voor. Hierdie takson is geassosieer met die tipiese siektesimptome en is ook patogenies. Morfologies stem takson 2 met *P. viticola* ooreen en is ook geneotipifiseer in hierdie studie. Takson 2 is meestal vanaf die ogies en nodusse geïsoleer, wat daarop dui dat hierdie belangrike setels is waar die swam tydens die winter oorleef. Die molekulêre data toon aan dat takson 3 en *P. amygdali* nie gasheerspesifiek tot wingerd is nie.

Die swamdoders wat tans teen streepvlek gebruik word, is met die nuwe strobilurin swamdoders vergelyk. Die effek van nege swamdoders (azoksistrobin, flusilasool, folpet, fosetyl-Al + mancozeb, kresoxim-metiel, mankozeb, penconasool, spiroksamien en trifloksistrobin) is *in vitro* op die inhibisie van miseliumgroei getoets. Die volgende EK_{50}-waardes (g/ml) is verkry: azoxystrobin (0.350), flusilasool (0.007), folpet (4.489), fosetiel-Al + mankozeb (3.925), kresoxim-metiel (1.665), mankozeb (2.891), penkonasool (0.023), spiroksamien (0.321) en trifloxystrobin (0.051). Azoxystrobin, folpet, kresoxim-metiel, mankozeb, propineb en trifloksistrobin is ook *in vitro* getoets vir hul inhibisie op spoorontkieming. Die volgende EK_{50}-waardes is verkry: azoxystrobin (0.123), folpet (0.510), kresoxim-metiel (0.0037), mankozeb (0.250), propineb (0.156) en trifloxystrobin (0.003). Die resultate vervat in deel 4 toon dat die strobilurin swamdoders die miseliumgroei en spoorontkieming van *P. viticola* inhibeer. Toetsing in die veld word egter benodig om die effektiwiteit van die middels te bevestig. In hierdie studie is taksa 1, 3 en *P. amygdali* selde geïsoleer, wat aangedui het dat hierdie taksa 'n minder belangrike rol in die *P. viticola*-kompleks speel.

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CONTENTS

1. PHOMOPSIS CANE AND LEAF SPOT DISEASE OF GRAPEVINES: AN OVERVIEW

ABSTRACT

Phomopsis cane and leaf spot is an important disease of grapevines. Knowledge about the causal organism of a disease is important to understand the disease epidemiology and to improve disease control. The present review attempts, therefore, to summarize the taxonomic history, morphology, symptoms and epidemiology of *Phomopis viticola* and the four taxa identified in this species complex. The history and role of the teleomorph is also noted. The disease is placed in perspective in the South African context by discussing practical aspects such as cultivar resistance and disease control with cultural and chemical means.

INTRODUCTION

Phomopsis cane and leaf spot disease of *Vitis vinifera* L., caused by the fungus *Phomopsis viticola* (Sacc.) Sacc., can lead to significant yield losses. Serious losses and outbreaks have been reported in several countries, and in some instances losses of up to 50% have been reported (Pine, 1958; Berrysmith, 1962; Pscheidt & Pearson, 1989a). However, the actual economic loss from *P. viticola* is minor in most years (Chairman *et al.*, 1982). The disease occurs sporadically and does not spread rapidly, causing a slow die-back of vines (Emmet *et al.*, 1992). Losses can occur from shoots breaking off near the base where the lesions have developed, stunting of grapevinecs, loss of growth vigour, reduced bunch set and Phomopsis rot of fruit (Punithalingam, 1979; Chairman *et al.*, 1982; Nicholas *et al.*, 1994; Pearson & Goheen, 1994).

Phomopsis cane and leaf spot disease is widely distributed and found in most countries where grapevines are grown (Punithalingam, 1979). In South Africa, this disease was first noticed in 1935 and subsequently reported on by Du Plessis (1938). Although this disease occurs sporadically, serious disease incidences have been recorded (Synnott, 1958), specifically in the Helderberg, Firgrove, Somerset West, Rawsonville and Slanghoek production regions (Marais, 1981). The disease appears to be more

common in cooler regions, spreading during wet weather in spring (Nicholas *et al.*, 1994), and is sometimes restricted to coastal regions (Shea, 1961).

HISTORY AND TAXONOMY

Various names have been used for Phomopsis cane and leaf spot. In Europe this disease is sometimes referred to as 'excoriose' (Punithalingam, 1979). Reddick (1909) considered the names 'side arm' and 'black knot', but decided that 'necrosis of grapevine' described the symptoms best. Farmers referred to *Phomopsis* as 'dead arm', and this common name was subsequently used by Shear (1911). Various plant pathologists have since referred to the disease as 'dead arm' (Du Plessis, 1938; Shea, 1961; Berrysmith, 1962; Chamberlain *et al.*, 1963; Punithalingam, 1979). Locally, farmers still refer to this disease as 'dead arm'. This is largely based on the fact that *P. viticola* has been positively isolated from typical slow die-back symptoms of affected vines. Two fungi, *P. viticola* and *Eutypa lata* (Pers.: Fr.) Tul. & C. Tul. have been isolated alone or in combination from 'dead arm' symptoms (Dye & Carter, 1976). However, *E. lata* proved to be the most frequently isolated fungus from vines with typical dying arm symptoms. Moller and Kasimatis (1981) conducted Koch's postulates with mycelial cultures of *E. lata* and *P. viticola* and determined that only *E. lata* was capable of inducing the pruning wound cankers and chlorotic, stunted spring foliage that was associated with 'dead arm' disease. This canker and shoot die-back phase of what was known as 'dead arm' is presently called Eutypa die-back (Pearson *et al.*, 1994). *Phomopsis viticola* was seen as the causal organism of excoriose in Europe (Pearson *et al.*, 1994). However, *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & De Not. (anamorph *Fusicoccum aesculi* Corda) has also been associated with excoriose symptoms (Phillips, 1998).

The first description of *P. viticola* (as *Phoma viticola*) was by Saccardo in 1880 from grapevine canes collected in France (Saccardo, 1880). In 1885 Cooke described the same fungus from Britain as *Phoma viniferae* Cooke (Cooke, 1885). Other taxa such as *Phoma cordifolia* Brun. Champ. Saint. (Grove, 1917) and *Phoma desciscens* Oudem. (Petrak, 1916) from grapevine also corresponded with the description of *P. viticola*.

Although differences between *Phoma* Sacc. (Pleosporaceae) and *Phomopsis* (Sacc.) Bubák (Valsaceae) have not been clear, these genera could be differentiated based on several characters. The most prominent of these are differences in the conidiogenous cells and conidium types and sizes. *Phoma* has got very short, often non-recognisable conidiogenous cells, whereas *Phomopsis* has larger condiogenous cells, often occurring on conidiophores. *Phomopsis* species mostly have larger conidia and also have alpha, beta and gamma conidia, which are absent in species of *Phoma* (Grove, 1917). These differences were often overlooked in the past, and old type specimens therefore need to be re-examined to determine what genus they represent.

In a later treatment of *Phoma viticola* by Saccardo (1915), he listed an American specimen collected by H.D. House, and proposed a new combination in *Phomopsis*. Various other synonyms were subsequently named, namely *Phomopsis ampelopsidis* Petrak (Petrak, 1916), *Phomopsis viticola* Grove (Grove, 1917) and *Phomopsis viticola* Sacc. var. a*mpelopsidis* Grove (Grove, 1919). *P. viticola* was again correctly published as *Phomopsis viticola* (Sacc.) Sacc. (Saccardo, 1931).

Reddick (1909) collected grapevine canes with a fungus similar to *P. viticola*, which he described as *Fusicoccum viticolum* Reddick. Goidànich re-examined the morphological and cultural characteristics of the pathogen, and established a new combination in *Phomopsis* as *P. viticola* (Reddick) Goid. (Pine, 1958). However, the culture lodged by Goidànich proved to be *Coniella granati* (Sacc.) Petrak & Syd. (Merrin *et al.*, 1995). This was also confirmed by my own examination of this culture (CBS 252.38).

There is currently no type material of *Phomopsis viticola* (NY, BPI, IMI, K, PAD, B and CUP) and a new specimen needs to be chosen as neotype. Subsequent studies have shown that the concept of *P. viticola* is heterogeneous. Four taxa have thus far been distinguished in the *P. viticola* complex (Merrin *et al.*, 1995). Merrin *et al.* (1995) were of the opinion that taxon 1 most closely resembled *Phomopsis viticola* based on alpha spore morphology. They also stated that taxon 2 probably resembled *Fusicoccum viticolum*, and suggested that it could be a variety of *P. viticola*, or a distinct species of

Phomopsis (Merrin *et al.*, 1995). However, taxon 2 has been associated with the typical disease symptoms further complicating the nomenclature of *P. viticola*.

In general the teleomorph has only been reported for 18 –20 % of *Phomopsis* species (Uecker, 1988). The *Diaporthe* sp. associated with Phomopsis cane and leaf spot on grapevine has not been found in South Africa. The first *Diaporthe* species described from grapevine was *D. viticola* Nitschke, reported from Germany and Maine by Nitschke in 1870 (Nitschke, 1870), and *D. silvestris* Sacc. & Berl. in Italy (Saccardo & Berlese, 1885). Although teleomorphs of *Phomopsis* reside in the genus *Diaporthe* (Wehmeyer, 1933), was *Cryptosporella viticola* Shear described as the teleomorph of *Fusicoccum viticolum*, which is regarded to be a synonym of *P. viticola* (Shear, 1911). It is, however, doubtful whether this was a *Diaporthe* species, since the ascospores were continuous and aseptate. In addition, Shear (1911) found a *Diaporthe* species that was associated with *Phomopsis* pycnidia on dead grapevines, but its ascospores did not produce *F. viticolum* in culture. This could therefore be a different *Diaporthe* species. Recently, the teleomorph of the Australian *Phomopsis* taxon 1 was collected in Australia and attributed to *Diaporthe viticola* (Scheper *et al.*, 1995). This identification was confirmed by the International Mycological Institute in the UK (Scheper *et al.*, 2000). A *Diaporthe* sp. was subsequently also found on vines for taxon 1 in Portugal (Phillips, 1999), and attributed to *Diaporthe perjuncta*.

MORPHOLOGY

Phomopsis viticola produces black pycnidia ranging from 200 to 500 µm diam. The pycnidia, which occur primarily on stems, are initially immersed, later breaking through the epidermis. They are usually solitary, depressed-subglobose, uniloculate, sometimes having a convulated hymenium layer. The pycnidial wall is stromatic, composed of several layers of pseudoparenchymatic cells, the innermost layer is thin-walled and hyaline, the outermost layer thick-walled and heavily pigmented. An ostiole opens at the apex of a short neck. The opening of the pycnidium is usually round and smooth but may be irregular and sometimes ragged (Punithalingam, 1979; Pearson *et al.*, 1994). Wechtl

(1990) refers to the fruiting bodies of *Phomopsis* as 'stromatic pycnidia' since in the genus *Phomopsis* the stroma and pycnidia cannot be easily separated in general.

The conidiophores of the genus *Phomopsis* are branched, frequently multi-septate, occasionally short, filiform, hyaline, formed from the inner cells of the locular walls (Sutton, 1980). In the early descriptions of *P. viticola* conidiophores were referred to as sporophores and measurements given as 10-12 x 2.5 µm (Grove, 1917) and 15 x 2 µm (Uecker, 1988). Conidiophore structure was, however, not included in a synoptic key of *Phomopsis* species (Wechtl, 1990). The IMI description of *P. viticola* (Punithalingam, 1979), and the recent delimitation of the different taxa found in the *P. viticola* complex (Merrin *et al.*, 1995) also omitted conidiophore structure.

The conidiogenous cells of *P. viticola* are hyaline, simple, cylindrical, determinate phialidic, arising from the innermost layer of cells lining the pycnidial cavity (Punithalingam, 1979). Two types of conidiogenous cells are found; the one bearing the alpha conidia which has a pointed apex, 12-20 x 2 µm, and shorter conidiogenous cells bearing the beta conidia, 5-8 x 1.5 µm (Pearson *et al.*, 1994). In the generic description of the genus *Phomopsis*, the conidiogenous cell openings are described as apical on long or short lateral and main branches of the conidiophores, having collarettes and minute periclinal thickenings (Sutton, 1980).

Phomopsis viticola basically forms two types of conidia, namely alpha and beta conidia. In some cases intermediate or gamma conidia are also formed. The alpha conidia are hyaline, unicellular, ellipsoidal, truncate or rounded at the base, narrowed and pointed at the apex, sometimes slightly constricted in the mid region and normally have a large guttule at each end (6 -)7-9(-10) x 2-4 µm. The beta conidia are hyaline, filiform, curved or hamate 18-30 x 5-1 µm. The intermediate or gamma conidia are hyaline, ellipsoidal, narrowed at the apex, guttulate 12-17 x 1.5-2 µm. The conidia are exuded through the ostiole, either in long, curled, yellow to cream-coloured cirrhi, or in a gelatinous mass (Punithalingam, 1979; Pearson *et al.*, 1994). The formation of alpha and beta conidia in the same conidioma is commonly found in *Phomopsis* (Uecker, 1988), and both conidial types can occur in pycnidia of *P. viticola* (Pine, 1958).

The alpha conidia are fertile and can cause infection, while the beta conidia are sterile. Beta conidia do not germinate under ordinary cultural conditions in which the alpha conidia germinate (Shear, 1911). It has, however, been observed that the beta conidia form distinctive protrusions in spore dilutions on agar plates, although no cross walls or branching were noted (Pine, 1958). Saccardo (1884) described the beta conidia as spermatia that could play a role in the formation of the *Diaporthe* teleomorph. The function of the beta conidia has, however, not yet been clarified (Pearson *et al.*, 1994).

Studies have been conducted to understand the factors influencing the production of beta conidia. Generally less than 25 percent of conidia counted were found to be beta conidia when produced on sterile grapevine canes (Pine, 1957a). It was further reported that the number of beta conidia increased with an increase of dextrose (Nitimargi, 1935; Pine, 1957a). Other factors that have influenced the production of beta conidia include nitrogen levels, carbon dioxide, temperatures above 30°C, and the presence of *Bacillus subtilis* (Punithalingam, 1979).

In recent studies done on the conidium morphology of the *P. viticola* complex, two main groups were observed, namely the smaller, biguttulate alpha conidia of taxon 1 (3.8-)4.8-7.2(-9.0) x (1.1-)1.4-2.2(-2.9) µm, and the larger, multi-guttulate alpha conidia of taxon 2 (7.0-)8.0-11.8(-14.0) x (1.6-)2.0-3.2(-3.9) µm (Merrin *et al.*, 1995). It is important that cultures of different isolates are of the same age when studying alpha conidial features, because shape and guttulation change with age (Wechtl, 1990). The presence or absence of beta conidia was found to be typical of the different taxa associated with the *P. viticola* complex (Merrin *et al.*, 1995). Beta conidia varied unpredictably for isolates of taxa 1 and 2, while isolates of taxon 3 regularly produced beta conidia, and taxon 4 produced beta conidia only (Merrin *et al.*, 1995).

In culture mycelium of *P. viticola* is hyaline, septate, branched and forms a dense mat, often growing in concentric rings under alternating light-dark regimes. Portions of the mat or the margins of concentric rings turn black as the culture ages. The mats are often sectored in white and black. Pycnidia form irregularly in the dark parts of the mycelial mat, either singly of in clumps (Pearson *et al.*, 1994). Colonies on oat-meal agar have wool-like tufts, initially white, later becoming greyish white to yellow-white,

with numerous pycnidia distributed on the surface of agar amongst the mycelium. The reverse of the colony is colourless to pale greyish sepia, with dark brown to black spots (Punithalingam, 1979).

It is important to use a standard medium for cultural studies, and to keep the concentration of carbon constant, since noticeable changes in the cultural characteristics were found with an increase of carbon (Pine, 1957a). The colour, texture, mean growth after six d and the optimum temperatures for mycelium growth (on potato dextrose agar amended with lactic acid) were distinct for the four *P. viticola* taxa isolated from vines in Australia (Merrin *et al.*, 1995). Mycelial growth can therefore aid in the identification of groups within the *P. viticola* complex.

SYMPTOMOLOGY

Phomopsis viticola can produce disease symptoms on leaves, shoots, leaf petioles, rachides, pedicels and fruit of grapevines. The first symptoms of the disease are small, dark brown to black leaf spots (rarely more than 1 mm diam.) surrounded by 2-3 mm of light yellow halos (Fig. 1) (Chairman *et al.*, 1982; Nicholas *et al.*, 1994; Swart & De Kock, 1994). These spots first appear three to four wk following rain after bud break (Chairman *et al.*, 1982). The spots can coalesce to form large, brown areas. The necrotic spots may drop out of the leaf, causing a "shot hole" appearance (Pearson *et al.*, 1994). If there are many spots, leaves distort and whole leaf sections may be killed off. Severely infected basal leaves may become distorted and usually never develop to full size. Leaves on badly infected stems turn yellow and fall off prematurely (Chairman *et al.*, 1982; Nicholas *et al.*, 1994). Later in the season these leaves are hidden by the development of normal foliage (Chairman *et al.*, 1982). According to Du Plessis (1938), it appears that the leaf curl and malformation are not a result of direct infection, but rather of infections that took place from the affected internodes of the shoot from which these leaves arose.

The first signs of shoot infections are small, black lesions (Fig. 2), similar to those on the leaves. Symptoms are usually confined to the first four internodes of shoots (Fig. 3). The lesions expand and elongate into thin black lens-shaped cracks, 5-6 mm wide

(Nicholas *et al.*, 1994; Swart *et al.*, 1994). Where there are numerous spots, they coalesce and may ultimately give a scabby appearance to parts of the shoots (Chairman *et al.*, 1982). These fissures crack and scar as the canes swell and harden (Fig. 4) (Nicholas *et al.*, 1994). Cracks in the epidermis and cortex of the shoots tend to heal during the growing season and become rough as the tissues mature (Fig. 5) (Pearson *et al.*, 1994). Primary lesions on young shoots are superficial but coalescent ones are deep seated and often become perennial (Punithalingam, 1979). By midseason, symptoms on the shoots become less visible because of the vine growth and leaf cover (Du Plessis, 1938), and environmental conditions unfavourable for further development of the disease (Marais, 1981). Symptoms have also been found on internodes at intervals along the shoots, due to successive rain-induced infection periods later in the season (Marais, 1981; Pearson *et al.*, 1994).

Heavily infected shoots can be dwarfed or retarded and some may die (Chairman *et al.*, 1982). Eventually the whole arm dies off and even the whole vine, if proper remedial measures are not taken (Berrysmith, 1962). This rarely happens in one season since disease development on the shoots is fairly slow (Du Plessis, 1938). Other losses include the progressive desiccation of stocks and shoots, stunting of leaf bearing branches, flower abortion, drying of buds with successive reduced bud burst, death of spurs and scarcity of berries (Punithalingam, 1979; Marais, 1981; Merrin *et al.*, 1995). Scheper (1995) found that *P. viticola* was able to reduce bud burst up to 20%, and delay bud burst by an additional 20%.

In winter, the diseased canes are bleached white in areas between the black lesions (Fig. 6) (Chairman *et al.*, 1982; Nicholas *et al.*, 1994). Tissue around the original lesions and at the nodes become whitish with black speckling. The black specks are the pycnidia which develop during the dormant season, breaking through the surface as minute, black, pimple-like pustules (Chairman *et al.*, 1982; Pearson *et al.*, 1994). The pycnidia may be numerous and able to lift the epidermis and admit air under it, which gives the surface a white to silvery sheen (Pearson *et al.*, 1994). These pycnidia are the source of overwintering spores for the following season's inoculum (Chamberlain *et al.*, 1963).

During spring, spots similar to those on the shoots and leaves also appear on the bunch rachides and pedicels (Fig. 7). Occasionally the bunch rachides are so badly infected that bunches whither (Chairman *et al.*, 1982). These lesions become inactive in summer, but early fall rains (just before harvest) combined with cool weather may reactivate the pathogen and cause berry infections, resulting in berry and bunch rot (Chairman *et al.*, 1982). Infected fruit may abscise from the pedicel to leave a dry stem scar (Pearson *et al.*, 1994). When the grape cluster was considered, rachis infections were the most important, because they could cause the entire cluster or portions to fall from the vine before harvest (Pscheidt & Pearson, 1989b). Fruit symptoms have not been extensive, with isolated bunches affected on a vine (Chairman *et al.*, 1982). The infected berries develop light-brown spots that quickly enlarge, becoming dark brown with black pycnidia breaking through the skin, exuding yellow spore masses. Consequently the fungus causes the berry to shrivel and become mummified (Berrysmith, 1962; Chairman *et al.*, 1982; Pearson *et al.*, 1994). Although not common, berry infections have resulted in serious yield losses as observed in New Zealand and India (Lal & Arya, 1982; Nicholas *et al.*, 1994). Rotting of berries has been noted as rare in Australia (Nicholas *et al.*, 1994), and has also not been observed in South Africa due to the comparatively dry conditions prevailing during the grape ripening period (Du Plessis, 1938).

Differences in symptom appearance were associated with the taxa identified within the *P. viticola*-complex (Merrin *et al.*, 1995). Isolates of taxon 1 were associated with flattish, dark lesions with a black border, while isolates of taxon 2 were associated with heavy and obvious scars on woody tissue (Merrin *et al.*, 1995). Taxon 1 did not form symptoms on young growth (Merrin *et al.*, 1995). Latent infections of taxon 1 only became visible in the late winter as pycnidia on bleached canes (Whisson *et al.*, 1998). In subsequent studies it appeared that taxon 1 did not cause obvious damage to leaves and canes, but was commonly isolated from buds that did not burst (Brant *et al.*, 1999). This indicated that taxon 1 was probably involved in bud loss. Also bleaching symptoms were found to not be reliable to identify taxon 1 (Brant *et al.*, 1999).

Symptoms of *P. viticola* can also be confused with that of other grapevine diseases. Black spot or anthracnose caused by *Elsinoë ampelina* (de Bary) Shear forms stem lesions that are more circular and have rough, raised and callused edges (Nicholas *et*

al., 1994). *Botryosphaeria dothidea* causes berry rot that has been difficult to distinguish from the berry rot caused by *P. viticola* (Nicholas *et al.*, 1994). Symptoms of *B. dothidea* can be confused with *P. viticola*, since both cause similar symptoms associated with excoriose (Phillips, 1998). *Botryosphaeria stevensii* Shoem. is the causal organism of black dead arm (Pearson *et al.*, 1994). *B. stevensii* and *P. viticola* have been isolated in equal proportions from lesions on trunks and stubs (Chamberlain *et al.*, 1963). However, only *P. viticola* was isolated from green, growing tissue and no *B. stevensii* (Chamberlain *et al.*, 1963), indicating the different epidemiology of these two diseases. Black dead arm can also be distinguished from *Phomopsis* by the black streaks found in the xylem (Pearson *et al.*, 1994).

Phomopis viticola has also been associated with bacterial blight symptoms caused by *Xylophilus ampelinus*. Scanning electron microscopy revealed a close association between these two pathogens in plant tissues (Serfontein *et al.*, 1996).

PATHOGENICITY

The four taxa identified in the *P. viticola* complex differed in their ability to cause disease (Merrin *et al.*, 1995). Taxa 1 and 3 were not associated with symptoms on young growth while taxon 2 caused dark brown lesions on young shoots and petioles (Merrin *et al.*, 1995). The host response was also tested *in vitro* on detached leaf disks. Taxa 1, 3 and 4 caused approximately 90-95% less necrosis of leaf tissue 6 wk after inoculation than isolates of taxon 2 (Merrin *et al.*, 1995). These findings suggest that in general isolates of taxon 2 were more virulent than taxa 1, 3 and 4.

Phomopsis viticola has been found to be pathogenic on other hosts apart from *Vitis vinifera* (Lal *et al.*, 1982). The ability of *P. viticola* to infect other hosts suggests that inoculum could also be derived from alternate hosts growing nearby. Cross inoculations done with isolates of *P. viticola* obtained from diseased grape berries in India showed that the following were susceptible: apple, pear, peach, guava, mango, banana, orange, Indian gooseberry, papaya, plum, stone fruit, litchi, lemon, tomato, cape-gooseberry and water chestnut (Lal *et al.*, 1982). *Phomopsis longicolla* Hobbs isolated from weeds found in soybean fields, were also pathogenic to soybean (Roy *et al.*, 1997).

The ability of *Phomopsis* species to infect more that one host was also confirmed by phylogenetic analysis of nuclear ribosomal DNA internal transcribed spacer sequences (ITS1 and ITS2) of *Phomopsis* spp. isolated from diverse plant hosts and geographic origins (Rehner & Uecker, 1994). Furthermore, a wide host range has serious implications to the species concept of *Phomopsis*, since most species have been described as new based on their different hosts (Rehner *et al.*, 1994).

EPIDEMIOLOGY

Phomopsis viticola overwinters as mycelium and pycnidia within the woody parts on infected canes, spurs and pruned shoots (Nicholas *et al.*, 1994; Pearson *et al.*, 1994). It has also been reported to occur as mycelium in dormant buds (Pearson *et al.*, 1994). Most spores that cause infection come from the resting bodies on canes that were infected during the previous season (Nicholas *et al.*, 1994). Cool weather and rainfall are required for spore release and infection (Punithalingam, 1979). In spring, mature pycnidia erupt through the epidermis of canes and leaf petioles, through cracks in the bark on older diseased tissue and other dead, diseased parts hanging in the vine (Pearson *et al.*, 1994). During spring rains, spores exude from the pycnidia and are washed or splashed onto the young shoots (Nicholas *et al.*, 1994; Pearson *et al.*, 1994). At least ten hours of rain are required for spores to be produced (Nicholas *et al.*, 1994). Infection occurs when free moisture remains on the unprotected green tissue for eight to ten hours (Chairman *et al.*, 1982; Nicholas *et al.*, 1994). However, free water is not essential for conidial germination (Punithalingam, 1979). Berry infection is favoured by long (20-30 hour) wet periods at flowering (Nicholas *et al.*, 1994). Alpha conidia can germinate in a temperature range of 1-37°C. At the optimal temperature of 23°C, infection takes place within a few hours (one to three hours) in free water or 98% to 99% relative humidity (Marais, 1981; Pearson *et al.*, 1994).

Fruit infection might also occur early in the growing season, during bloom, and reside in the fruit as latent infections to cause symptoms later in the season when the fruit matures (Pscheidt *et al.*, 1989b). Most fruit infections appear to be associated with the lenticels as well as lesions on the rachis or pedicel tissue (Pearson *et al.*, 1994). Recent

research results indicated that berry and rachides tissues were increasingly susceptible to infection from pre-bloom through to veraison. This was in contrast to previous studies which indicated that berry infection occurred primarily during bloom and shortly after bloom, with decreasing levels of infection as fruit matured (Erincik *et al.*, 1999).

Phomopsis viticola was considered to be a wound parasite, entering through leaf stomata, pruning wounds and lesions on canes (Shea, 1961). However, it can also penetrate uninjured young shoots directly (Punithalingam, 1979). In recent studies conducted by Whisson *et al.* (1998), they observed papillae produced by the plant in response to conidia, which had penetrated the cuticle directly.

Symptoms usually appear 21 to 30 d after infection (Nicholas *et al.*, 1994; Pearson *et al.*, 1994). The fungus does not appear to be active during the warm summer months, but can become reactivated during cool, wet weather, and may remain active throughout the growing season in cooler climates (Pearson *et al.*, 1994). The optimum temperature for mycelial growth is 23°C (Punithalingam, 1979). During autumn and winter growth of *Phomopsis* resumes, the canes discolour and pycnidia develop (Nicholas *et al.*, 1994). The optimum temperature for pycnidium production is 20°C (Punithalingam, 1979), which correlates well with the time of season when pycnidia start to form.

With varying climatic conditions disease severity varies from year to year (Chairman *et al.*, 1982). Prolonged periods of rain and cold weather during early spring favour the development of disease epidemics (Pearson *et al.*, 1994). The spread of the fungus within a vineyard takes place slowly from a localised source of inoculum (Nicholas *et al.*, 1994; Pearson *et al.*, 1994). Mycelium and conidia can be carried over to the other vines through the splashing of spores, nematodes and insect larvae or pruning scissors (Punithalingam, 1979). Long distance spread may occur through transport of infected or contaminated propagation materials such as bud wood, cane cuttings, nursery stock (Pearson *et al.*, 1994) and possibly also through the wind dispersal of ascospores (Scheper *et al.*, 1995). Seedlings derived from diseased berries died, indicating that *P. viticola* taxa 1 and 2 can be transmitted in seed (Scheper *et al.*, 1996b).

ENDOPHYTIC GROWTH

Generally endophytes can be referred to as organisms that exist within plants (Clay, 1991). Petrini (1991) defines endophytes as: "all the organisms inhabiting plant organs that at some time in their life, can colonise internal plant tissues without causing apparent harm to their host". To identify endophytic fungi, healthy surface sterilised plant material are plated on to nutrient agar and the outgrowth of fungi observed (Carroll & Carroll, 1978). The fungi isolated with surface sterilised plant material are completely different from that obtained by methods sampling the microflora of plant surfaces (Carroll, 1990).

The genus *Phomopsis* has been frequently isolated as an endophyte from various plant hosts (Fisher & Anson, 1986; Carroll, 1988; Petrini *et al.*, 1992; Sinclair & Cerkauskas, 1996). *Phomopsis* species have been isolated as the most common endophyte from *Ulex europaeus* (Fisher & Petrini, 1987) and *Quercus ilex* L (Fisher *et al.*, 1994). Species of *Phomopsis* were also found to be one of the dominant fungal taxa in coniferous hosts (Carroll *et al.*, 1978).

Endophytes hold various advantages for plants. The endophyte-host relationship can be mutualistic (Carroll, 1988), where the host gains increased survival, growth and reproduction, and endophytes gain nutrition and a habitat (Clay, 1991). Endophytes have been found to enhance the plant growth (Leuchtmann & Clay, 1988) and increase the rate of grass seed germination (Clay, 1987). Some endophytes produce toxic metabolites which increase resistance to insect pests (Prestidge & Gallagher, 1988; Clark *et al.*, 1989; Clay, 1992; Findlay *et al.*, 1995). Endophytes could be used as mycoherbicides (Sieber *et al.*, 1990) in providing protection against pathogenic fungi (Matta, 1971; Wilson, 1993; Carroll, 1994). An example of herbivore resistance has been found with *Phomopsis oblonga* (Desm.) Traverso that disrupts beetle breeding and leads to a decline in beetle populations (Webber & Gibbs, 1984). However, not all *Phomopsis* spp. have shown promising advantages. In an analysis for the production of antibiotics from endophytic fungi, a *Phomopsis* sp. isolated from *Vaccinium myrtillus* L. did not show any antibiotic activity (Fisher *et al.*, 1984).

Pathogens that are living asymptomatically inside plant material have been classically described as latent (Stone, 1987). However, a fungus can be described as an

endophyte and a pathogen depending on the phase of its life cycle (Wilson, 1995). Wilson (1995) expands this interpretation to the infection strategy of fungi: 'endophyte and pathogen do not oppose each other but describe different infection strategies'. The differences between endophytes and pathogens have been researched on gene level and it has been found that the genetic difference between an endophyte and a pathogen can be ascribed to a mutation of a single genetic locus (Freeman & Rodriguez, 1993).

Various species of *Phomopsis* have been associated with latent infections in a variety of plants (Sinclair *et al.*, 1996). Latent infections of *P. viticola* have been identified by applying the paraquat technique on symptomless clusters collected from naturally infected vines (Pscheidt *et al.*, 1989b). According to Sieber *et al.* (1990) latency can last several d to many years depending on the fungus's virulence, host, climate and ecological conditions. These latent pathogens can, however, also be seen as endophytes in view of the definition that include asymptomatic growth and a pathogenic stage.

GENETIC VARIATION

The role of the teleomorph in the epidemiology of the disease is uncertain (Pearson *et al.*, 1994; Scheper *et al.*, 1995). Shear (1911) did not regard the ascogenous phase of the fungus as an important factor in the distribution of the disease. Scheper *et al.* (1997) stated that the long perithecial necks of *P. viticola* taxon 1 indicated that ascospores could be wind dispersed. This would then imply that these spores could be involved in long distance transmission of the disease (Scheper *et al.*, 1997). The slimy nature of the ascopores does not, however, make this a likely possibility (A.J.L Phillips, pers. comm.).

Sexual reproduction is an important mechanism for producing new genetic variants that could be more resistant to the more commonly used fungicides (Scheper *et al.*, 1997). It is therefore important to establish the extent of the occurrence of the teleomorph for the different taxa in the *P. viticola* complex.

The genetics and population dynamics of different *P. viticola* isolates have been researched through the use of phenotypic and molecular markers, mating types, vegetative compatibility groups, restriction fragment length polymorphism (RFLP)

markers and random amplified polymorphic DNA (RAPD) markers (Scheper *et al.*, 1996a; Scheper *et al.*, 1997). Different strains of *P. viticola* occurring on a single cane and high levels of variation found within a small geographic area indicated that the sexual stage of *P. viticola* may play an important role in the disease epidemiology (Scheper *et al.*, 1997). However, taxon 1 occurred predominantly in the coastal regions in Australia and taxon 2 occurred mostly inland, indicating the distinct genetically uniform geographical groupings of these two taxa (Merrin *et al.*, 1995).

HOST RESISTANCE

Phomopsis viticola has been isolated from *Vitis vinifera* L. (*Parthenocissus quinquefolia* = *Ampelopsis quinquefolia* = *Vitis hederacea*) and other *Vitis* spp. (Punithalingam, 1979). The species *Vitis vinifera* includes table grapes as well as wine grapes. Cultivars vary widely in their resistance towards *P. viticola*.

Cultivars highly susceptible to *Phomopsis* include: Kandahar, Olivette blance, Olivette noir, Rish Baba, Flame Tokay (Pearson *et al.*, 1994), Riesling, Sultana, Barbarossa, (Du Plessis, 1938), Cabernet Sauvignon, Chenin blanc, Sémillon, Riesling, Raisin blanc and Sultana (Marais, 1981). Also Grenache, Palomino, Muscadelle, Sultana (Nicholas *et al.*, 1994) Baroque, Gros Vert, Alphonse Lavallée, Cardinal and Chasselas (Doazan, 1974).

The following cultivars were found to be moderately susceptible: Carbernet, Barlinka, Hanepoort, Gros Colman, Black Prince, Ferdinand de Lesseps (Du Plessis, 1938) and Colombar, Merlot and Muscat de Alexandrie (Marais, 1981). The following table grape cultivars were also moderately susceptible, Waltham Cross, Purple Cornichon, Muscat Hamburg, Ohanez and Cardinal (Nicholas *et al.*, 1994).

Cultivars regarded as resistant include: Cabernet franc, Carignan, Grenache and Ugni blanc (Marais, 1981), Pinot meunier, Mourvèdre, Riesling, Cinsaut, Cabernet franc and Ugni blanc (Doazan, 1974). Field trials have not indicated complete resistant cultivars, but in France the variety Pinot Meunier has been classified as practically resistant to *P. viticola* (Punithalingam, 1979).

Isolates of taxon 1 have occurred predominantly on Chardonnay and Riesling (Merrin *et al.*, 1995). Further studies found fruiting bodies of *Diaporthe viticola* (taxon 1) also on Chardonnay, Riesling, Shiraz, Cabernet, Pinot noir (Scheper *et al.*, 1997). Taxon 2 occurred, however, on a wide range of cultivars.

CONTROL

Phomopsis cane and leaf spot can be effectively controlled through the combined use of cultural and chemical methods. Disease control is dependent on disease pressure that varies annually depending on the existing pathogen inoculum in the vineyard and weather conditions.

Cultural practices

Control measures should start at nurseries where selection and propagation of clean nursery stock must be combined with fungicide applications to prevent primary infection (Willison *et al.*, 1965). To avoid introducing *P. viticola* into the vineyard, pathogen-free propagation materials (cuttings, buds, rootings and grafted or budded nursery material) must be used for planting or replanting (Emmet *et al.*, 1992; Nicholas *et al.*, 1994; Pearson *et al.*, 1994).

Once the disease has appeared, diseased and dead wood should be removed during pruning (Willison *et al.*, 1965; Emmet *et al.*, 1992; Pearson *et al.*, 1994). Spur and cane lesions provide most of the inoculum for new infections and need to be reduced (Chairman *et al.*, 1982). The debris after pruning should be destroyed by shredding it, ploughing it into the soil or burning it (Nicholas *et al.*, 1994; Pearson *et al.*, 1994). For maximum control of badly infected vines, removal and renewal of these at ground level have been recommended (Punithalingam, 1979). Also, damaged canes and spurs should not be used to develop the framework of vines in the following season (Emmet *et al.*, 1992; Nicholas *et al.*, 1994).

Planting resistant cultivars is the best preventative measure. However, few grape cultivars are resistant to *Phomopsis* cane and leaf spot. Cultivar susceptibility also seems to vary with locality (Pearson *et al.*, 1994).

The difficulty in monitoring infection and growth of the pathogen in green tissue has led to the development of molecular detection techniques. PCR based identification can now be made of *P. viticola* taxon 1 and 2 with specific molecular markers allowing the identification of *P. viticola* directly from diseased grapevine tissue (Whisson *et al.*, 1998). This technology can also be applied to establish whether nursery stock material are clean from *P. viticola*.

Chemical control

Properly timed applications in the form of sprays, dips or soak treatments form an important part in the control of *Phomopsis* cane and leaf spot. Fungicides are grouped as eradicant fungicides (applied during dormancy), and protective fungicides (applied on the new plant growth) early in the growing season.

Fungicides that have given good control include: propineb, benomyl, Bordeaux, captafol, captab, chinosol, chloramine, dinoseb, dichlofluanid, dichlozoline, dinitrocarbolineum, dinosol, nabam, dithianon, foet, folpet, folpet+captafol, mancozeb, methylthiophanate, metiram, phenylmercuric chloride, perocin, sodium arsenite, thiram and triarimol (Punithalingam, 1979). Azoxystrobin, kresoxim-methyl and trifloxistrobyn represent new fungicide chemistry derived from naturally occurring strobilurins. In South Africa the following fungicides are registered against *Phomopsis*: copper oxychloride, copper oxychloride/sulphur, copper sulphate/lime, folpet, fosetyl-Al+mancozeb, mancozeb, propineb and sulphur (Nel *et al.*, 1999). Folpet, fosetyl-Al+mancozeb and mancozeb have been applied the most (Swart *et al.*, 1994).

Biological control has been investigated but further research is, however, required. *In vitro* studies showed that mycelial growth of *P. viticola* was inihibited by heat-killed spores of *Trichoderma viride* Pers.: Fr. (Ale-Agha *et al.*, 1974).

Phomopsis can be controlled on propagation material with fungicides. The current practice in Australia of preparing grapevines for propagation purposes involves soaking the cuttings in 8-hydroxyquinoline sulphate. The use of a systemic fungicide such as benomyl or protectant fungicides such as fluazinam and mancozeb for dipping

planting materials, appears also to be an efficient and safe method to reduce the spread of the pathogen (Castillo-Pando *et al.*, 1997).

In vineyards with a history of *Phomopsis*, control during dormancy can be implemented to reduce inoculum carry over and prevent infections of new shoots (Chairman *et al.*, 1982). Eradicant chemical sprays may be applied during late dormancy (two to three wk before bud swell) to kill the pycnidia and conidia on the surface of vine parts. Such fungicides include sodium arsenite and dinoseb (Chairman *et al.*, 1982). Results obtained during in situ studies indicated that benomyl, fluazinam, mancozeb and 8-hydroxyquinoline sulphate could be used for control during dormancy (Castillo-Pando *et al.*, 1997). Dormant applications of mancozeb and fluazinam significantly lowered the carry over of disease into the next season, and thereby reduced the disease pressure on spray programmes applied in spring (Castillo-Pando *et al.*, 1997). However, sodium arsenite and dinoseb require safety measures and can be toxic to vines if used with improper timing (Chairman *et al.*, 1982). Combining dormant and early growth fungicide applications has given good disease control (Pine, 1957b; Marais, 1981).

Phomopsis is mostly controlled by the application of protectant fungicides during the early shoot growth stages. It is important to spray at the correct time during the season to protect the new growth (Pine, 1957b). Different programs have been recommended for foliar sprays. Two applications of a protective chemical when shoots are 1-3 cm and 6-12 cm long are generally recommended (Matthee *et al.*, 1971; Pearson *et al.*, 1994). However, control can start as early as 25% bud-break (Swart *et al.*, 1994). A set application every 2 wk commencing at bud-burst has also provided satisfactory protection (Emmet *et al.*, 1992). When disease conditions favour the disease, up to five sprays might be needed of which the first two prove to be the most important. Various field studies have been conducted to compare the different foliar fungicides' efficacy (Pine, 1957b; Matthee *et al.*, 1971; Cucuzza & Sall, 1982; Gadoury *et al.*, 1994; Leavitt & Martin-Duvall, 1997b).

An integrated approach to control vineyard diseases is necessary to minimise the use of fungicides, which can be hazardous to the environment when applied extensively. Several fungicides are registered against Phomopsis cane and leaf spot disease, as well as

other diseases, or have shown promise as general fungicides during field trials (Emmet *et al.*, 1992; Swart *et al.*, 1994; Leavitt *et al.*, 1997a). By applying one fungicide for different grapevine diseases, farmers would be able to reduce the costs of disease control.

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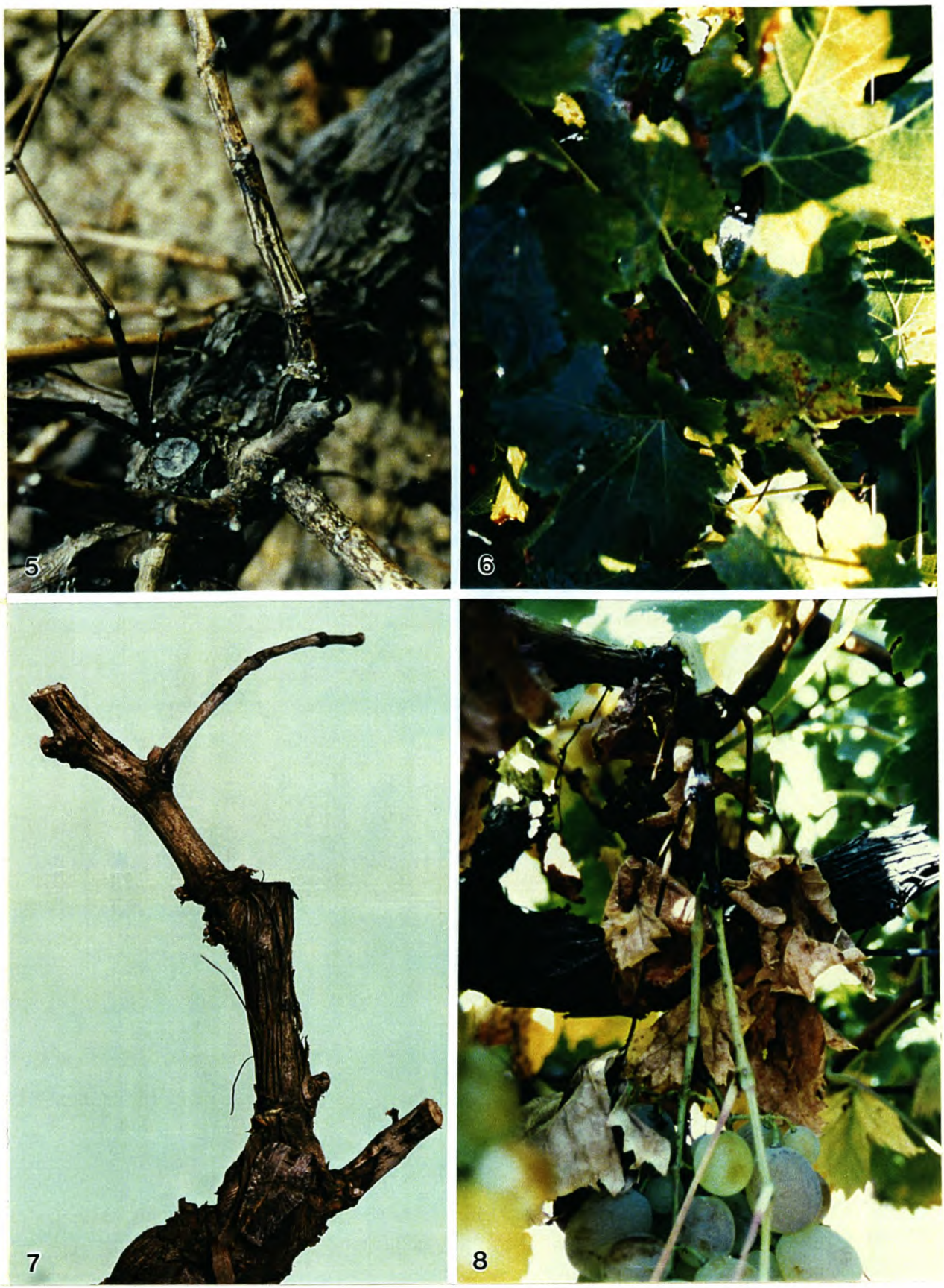
Figs 1-4. Leaf and shoot symptoms of Phomopsis cane and leaf spot disease. **Fig. 1.** Brown, irregular leaf spot symptoms. **Fig. 2.** Black stem lesions on green shoots. **Fig. 3.** Stem lesions developing from a node infection site. **Fig. 4.** Distinct lens-shaped, black lesions on infected canes.



Figs 5-8. Symptoms on older shoots and lesions on bunch peduncle. **Fig. 5.** Dormant vine canes covered with lesions that cracked open. **Fig. 6.** Black lesions occurring on younger shoot growth later in the season. **Fig. 7.** Infected canes bleached white during winter. **Fig. 8.** Black lesions on bunch peduncle and laterals.

2. ENDOPHYTIC FUNGI ASSOCIATED WITH SHOOTS AND LEAVES OF *VITIS VINIFERA*, WITH SPECIFIC REFERENCE TO THE *PHOMOPSIS VITICOLA* COMPLEX

ABSTRACT

The aims of this study were to investigate whether *Phomopsis viticola* grew endophytically in various vine tissues, to monitor its distribution over the growing season and to determine which endophytic fungi were associated with shoots and leaves of grapevines. Asymptomatic shoots were collected at eight different growth stages. Nodes, internodes, leaf petioles, leaves, tendrils and bunch rachides were surface sterilised, cut into smaller pieces and plated out on potato dextrose agar. After the fungal endophytes were identified, the relative importance (RI) values were calculated and used to perform a correspondence analysis. Of the 46 different fungal taxa found, 20 were present at relative importance values of more than 1%. An analysis of the relative importance of the different species showed that the most frequently isolated fungi were members of the *Alternaria alternata* complex (40%), and *Sphaeropsis* sp. (27%). The *P. viticola* complex had a relative importance of 9% and accounted for 3% of the isolations. Two different *Phomopsis* spp. were isolated, with 94% of the isolates representing *P. viticola* taxon 2, and the rest taxon 1. *P. viticola* was mainly isolated from the nodes and internodes, the plant parts in which the fungus usually causes disease symptoms. Isolations made from diseased vine material during 1997 and 1998 revealed taxon 2 to be dominant in vineyards in the Western Cape Province. Inoculations into healthy, young vine shoots also showed taxon 2 to be a pathogen, further suggesting that it is probably a latent pathogen rather than an endophyte. In contrast, taxon 1 appeared to be a true endophyte, and does not seem to be an important pathogen on grapevines.

INTRODUCTION

Phomopsis viticola (Sacc.) Sacc. is a well-known pathogen of *Vitis vinifera* L. (Reddick, 1909; Pearson & Goheen, 1994), causing an economically important disease, Phomopsis

cane and leaf spot, in many of the vine-growing areas of the world. Disease symptoms can be seen on the leaves as dark brown spots with yellow halos, followed by black lens-shaped lesions on the lower nodes of the green shoots during spring (Chairman *et al.*, 1982). Yield loss can occur due to stunting of vines, loss of vigor, reduced bunch set (Pscheidt & Pearson, 1989) and in severe cases, death and breaking off of shoots (Berrysmith, 1962). *P. viticola* overwinters as mycelium within the woody parts on infected canes, spurs, pruned shoots and dormant buds (Nicholas *et al.*, 1994; Pearson *et al.*, 1994). Inoculum can be carried over to the next season through mycelium or pycnidia in the cortex of diseased, one-year-old vine canes (Pearson *et al.*, 1994). In these cases the symptoms of the previous season's disease, whitening of the spurs or the presence of pycnidial fruiting bodies, would be visible indicators of inoculum that could cause disease under prolonged periods of rain in spring. *P. viticola*, however, has been isolated from healthy grape leaves after intensive surface sterilisation, suggesting that it is an endophyte of *V. vinifera* (Cardinali *et al.*, 1994). Presently no information is available pertaining to its endophytic distribution within vines, and whether it can be a source of inoculum in healthy looking vines. Futhermore, in Australia up to four different *Phomopsis* taxa have been associated with Phomopsis cane and leaf spot disease (Merrin *et al.*, 1995), some which could possibly be endophytes.

Several well-known pathogens have frequently been reported from endophyte studies (Sieber *et al.*, 1990; Johnson *et al.*, 1992; Smith *et al.*, 1996). These observations can be understood in the light of Petrini's (1991) and Wilson's (1995) definition that a fungus can be endophytic as well as pathogenic, depending on the phase of its life cycle.

The aims of this study were, therefore, to determine which *Phomopsis* taxa occurred endophytically in apparently healthy South African grapevines, to determine their distribution within the tissue, and to establish potential sources of inoculum for new vine growth.

MATERIALS AND METHODS

Endophyte study

Plant samples

Ten plants without any Phomopsis cane and leaf spot symptoms were selected for this study in a vineyard at Nietvoorbij, Stellenbosch, planted with the cultivar Riesling. The disease was present within the vineyard, however, and was noted on specific vines in the spring of 1997-1999. For the duration of this study penconazole (Topaz EW, Novartis) was applied for the control of powdery mildew according to the recommendation of the manufacturer. Canes were sampled at eight different stages of shoot development coded according to the Eichhorn-Lorenz system (Pearson & Goheen, 1994): winter dormancy – stage 01 (08.09.97), bud burst – stage 05 (18.09.97), five to six leaves unfolded – stage 12 (29.09.97), beginning of flowering - stage 19 (29.10.97), berries pea-sized – stage 31 (25.11.97), beginning of berry ripening – stage 35 (19.01.98), berries ripe for harvest – stage 38 (11.02.98) and after harvest – stage 41 (26.02.98). The vineyard was also monitored for Phomopsis cane and leaf spot symptoms from August 1997 to February 1998.

The following plant tissues were investigated (Fig. 1): nodes (N), internodes (I), leaf petiole (Lp), leaf (L), tendril (T) and bunch peduncle (Bp). Berry rot due to *P. viticola* lesions on the bunch peduncle have been described by Du Plessis (1950) as seldom serious. Berries were therefore excluded. Plant tissues were sampled from six sections across the length of the shoot. The first two sections were from the previous year's spur and the third to sixth sections from the new shoot.

Two samples (5 mm long) were respectively sampled over the length of the internodes, leaf petiole as well as tendril. The nodes were cut into four sections to distinguish between the ends and the sides of the sample. Five pieces (5 x 5 mm^2) were sampled from the leaves: at the leaf base and apex, on the main vein, side vein, and between a side vein and the leaf margin (Fig. 1).

After preliminary trials, tissue pieces were sterilised using the following protocol: 30 sec in 70% ethanol, 2 min in NaOCl (1%) and 15 sec in 70% ethanol. Tissue samples

were plated on potato dextrose agar (PDA, Biolab) amended with streptomycin (0.15 µg/ml)) and incubated at 25°C. Two pieces from the internode, leaf petiole, tendril and rachis were respectively plated on each Petri dish. Each node (four pieces) and leaf (five pieces) were plated on separate dishes. Fungal growth from plated tissue pieces was monitored daily. Emerging fungal colonies were hyphal-tipped and transferred to PDA slants for further identification.

Data analyses

The relative frequencies were calculated for each species from every plant part and time interval. The relative importance values (RI) of endophyte species isolated were computed (Ludwig & 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. To assess the distribution of the fungi in the plant and throughout the season, a correspondence analysis (CA) was used to visualize the patterns of correlation between fungal species, tissues, and time. Simple CA was performed on a reduced matrix that contained all fungal species with standardised RI values (over all samples) of at least 5%, using the package SimCA 2.1 (Greenacre, 1992).

Pathogenicity

Pathogenicity tests were conducted on green shoots of the cultivar Riesling to distinguish between 'true' endophytic and pathogenic *Phomopsis* spp. (Clay, 1991; Freeman & Rodriguez, 1993; Webber & Gibbs, 1984). Eight isolates, representing two distinct morphological groups were used. Three isolates of taxon 1 (only three were isolated), and five isolates of taxon 2 (which proved common) were used. Green shoots were pruned from vines and placed into Erlenmeyer flasks, and maintained at 25°C under a 12 hr day/night light regime, and watered daily. Shoots were wounded using a 3 mm diam. cork borer. Colonised mycelium plugs from 2-week-old cultures were used as inoculum and the wounds sealed with Parafilm. Uncolonised agar plugs were used to inoculate control shoots. Each isolate including the control were replicated three times on separate plants. After 7 d the lesion length, width and shoot diameter were determined. A one-way analysis of variance (ANOVA) was carried out on the data to determine whether a

difference in lesion size correlated with the two morphological groups identified in this study. Lesion lengths were calculated by subtracting the size of the wound formed on the control shoots from the lesion formed on the inoculated shoots. The fungal isolates were re-isolated from the periphery of each respective lesion.

RESULTS

Endophyte study

Two different morphological types of *P. viticola* were isolated, correlating with the Australian system, which distinguished four taxa in the *P. viticola* complex (Merrin & al., 1995). Of all the *P. viticola* isolates obtained, 94% were morphologically similar to the Australian taxon 2, while 6% correlated with taxon 1.

Taxa 1 and 2 of the *P. viticola* complex occurred endophytically in vines. The low frequency of isolation suggests, however, that they were not dominant endophytes. Out of 1705 isolates obtained, only 3% belonged to the *Phomopsis* species complex. When only the nodes (plant parts where *P. viticola* usually causes disease) were taken into consideration, the relative frequency of isolation was 3.2% and the RI 10%. An analysis of the relative importance of the different species isolated showed that *P. viticola* taxon 2 accounted for a RI of 8%.

The most important endophytes isolated from vine are listed in descending order according to their RI values in Table 1. All the fungi isolated with their respective raw frequencies for time and plant tissue can be seen in Table 2.1 and 2.2. In general, the *Alternaria* Nees spp. and a *Sphaeropsis* Sacc. sp. appeared to be the dominant endophytes occurring in grapevine plants in the present study.

The distribution of the *P. viticola* complex was scattered. A statistical analysis of the frequency data using the Chi-square test was therefore not advisable as it did not yield any reliable statistics, and for this reason any confirmatory statistical analysis was omitted. Inspection of Figs 2 and 3, on the other hand, reveals that *P. viticola* was predominantly found in the nodes and internodes. Of the 51 *P. viticola* isolates obtained, 48 were isolated from the nodes and internodes, two from the leaf petiole, and one from a

leaf. Only three of the isolates were obtained from above the fifth nodes of the shoots. Eighteen isolates were obtained from the bud sides of the nodes, and fifteen from the opposite side. No clear trend was observed for the frequency of *P. viticola* isolations with regard to tissue age (Fig. 3). However, this study showed that 53% of the *P. viticola* isolates were obtained from the two nodes and internodes of the previous year's spurs, and 39% of the isolates from the first two nodes and internodes of the new shoots.

The distribution of endophytes in the host plant over the growing season was analysed with a simple correspondence analysis (Fig. 4). The percentage of inertia explained by the first three components was approximately 72%, which indicated a good fit of the model to the data. The samples taken at an advanced stage of development (times 5-8) were mostly colonized by the two *Alternaria* spp. and by fungi known to be saprobic inhabitants of plant parts [*Pleospora herbarum* (Pers. ex Fr.) Rabenh. and *Nigrospora oryzae* (Berk. & Broome) Petch]. On the other hand, the first axis separated most of the node and internode samples from all the others with both tissue types preferentially inhabited by *P. viticola*, *Epicoccum nigrum* Link and the two sterile mycelia. This confirmed the observations that *P. viticola* preferentially colonized the node and internode tissues. N7 and N8 were located close to the other samples, indicating that *Alternaria* spp. may actually have some competitive advantages during and after harvest over species such as *P. viticola*, the two sterile mycelia and *E. nigrum*.

Pathogenicity tests

The two taxa could not be distinguished from one another on the basis of lesion length alone (Table 3) ($P = 0.2716$). Shoot diameter did not influence these measurements ($P = 0.1105$). However, isolates of taxon 1 were characterised by causing light brown, small lesions, while those of taxon 2 were generally larger and black in colour (Fig. 5). Although the black discolouring of lesions was a prominent feature of taxon 2, obvious differences in virulence were also detected between isolates. These data correlated well with that observed in the field, where over the last two years no isolates of taxon 1 were obtained from diseased vines. In all cases the *Phomopsis* isolates could be re-isolated from lesions, while the control inoculations remained healthy.

DISCUSSION

Although *P. viticola* was isolated endophytically from *Vitis vinifera*, it proved to be uncommon. Two taxa were isolated from the *P. viticola* complex, namely taxa 1 and 2, thereby confirming that more than one *Phomopsis* sp. occurred on grapevines in South Africa. Taxon 1 was isolated infrequently and has yet to be associated with the disease in the field. This indicated that taxon 1 could be seen as a true endophyte. Data from isolations made from diseased vine material during 1997 - 1998 showed taxon 2 to be dominant in vineyards in the Western Cape Province. Inoculations into healthy, young vine tissue showed taxon 2 to be a pathogen, and thus suggested that it was a latent pathogen rather than an endophyte. As both *Phomopsis* spp. occurred together in vines, it should be established whether the presence of taxon 1 could render vines more resistant to infections by taxon 2, as reported by Matta (1971), where non-pathogenic strains of fungi made plants more resistant to pathogenic fungi.

Taxon 1 has been regularly isolated from dead buds, suggesting that this fungus can cause bud mortality (Brant *et al.*, 1999). This hypothesis was, however, not tested in the present study. Pathogenicity tests were restricted to wound inoculations onto green shoots.

Although these two taxa differ morphologically, studies are underway to establish the genetic basis of the difference between the non-pathogenic endophytic taxon 1 and the pathogenic taxon 2. In other endophytic fungi, where isolates of the same species were either pathogenic or not, it was reported that a simple mutation at one genetic locus could switch endophytes to pathogens (Freeman *et al.*, 1993). In this study, however, isolates of taxon 1 and 2 were morphologically distinguishable, and therefore I believe them to be different species.

The spraying of penconazole could possibly have influenced the occurrence of *P. viticola*, since it is known that triazoles such as flusilazole also inhibit this pathogen (Fareta *et al.*, 1987). However, penconazole would not play a role after flowering (stage 19), as it is sprayed in the early growth season only. This could possibly explain the lower number of *P. viticola* isolates obtained at stage 05.

In general, *Alternaria* spp. and *Sphaeropsis* sp. appeared to be the most dominant endophytes occurring in grapevine plants in this study. It has been reported that known epiphytes are able to live endophytically within plant tissue (Petrini, 1986). *Alternaria alternata* (Fr.) Keissler was found to be the cause of decay of cold-stored table grapes (Swart & Holz, 1991). Futhermore, Swart & De Kock (1994) found that Alternaria fruit rot was caused by opportunistic forms of this fungus, which were isolated endophytically from asymptomatic bunches. Although isolations were not made from berries in the present study, the endophytic *A. alternata* found on the vine shoots and bunch peduncle might be a source of inoculum for infections of grape berries, and should be considered further. *Botryosphaeria* Ces. & De Not. spp., which have anamorphs in *Fusicoccum* Corda, *Diplodia* Fr., *Sphaeropsis* and *Lasiodiplodia* Ellis & Everh., are well-known pathogens of grapevines in South Africa (Pearson *et al.*, 1994). Whether the dominant *Sphaeropsis* sp. isolated here is a pathogen of vines has yet to be determined.

Positive correlation between the age of the plant material and the number of endophytic fungi colonizing the tissues has been reported (Petrini & Carroll, 1981; Fisher & Anson, 1986). Although the total number of endophytes increased with time in the present study, the distribution of *P. viticola* over the growing season seemed to be independent from tissue age. No trends could be established due to the small sample size.

Phomopsis viticola was found to be tissue specific to the lower nodes and internodes, correlating with the basal three to six internodes, where the disease symptoms are usually observed (Pearson *et al.*, 1994). These results suggested, therefore, that this area had to be targeted for disease control. No clear difference was observed between the number of *P. viticola* isolates obtained from the bud side and the non-bud side of the node. This indicated that *P. viticola* not only resides asymptomatically in the bud, but in the whole node.

This study showed that the majority of *P. viticola* isolates were obtained from the two nodes and internodes of the previous year's spurs. It therefore indicated that viable *P. viticola* isolates resided in apparently healthy pruned spurs, which could act as an inoculum source for new growth. To control disease inoculum in apparently healthy

pruned spurs, eradicant fungicides can be applied during dormancy. This, however, is not common practice because of the toxicity of these fungicides (Chairman *et al.*, 1982). Alternative, safer fungicides have shown potential as winter treatments of dormant canes (Castillo-Pando *et al.*, 1997).

It is evident from this study that *Phomopsis viticola* taxon 2 could occur latently within healthy vine tissue. Further research now needs to be focused on latent infections, and the influence of contact and systemic fungicides thereon.

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Table 1. Relative importance (RI) of the most important endophyte species isolated from grapevines.

Taxon	**RI (%) (overall)**
Alternaria alternata complex	100
Sphaeropsis sp.	40
Epicoccum nigrum	27
Sterile black mycelium	26
Pleospora herbarum	18
Alternaria tenuissima complex	17
Sterile brown mycelium	17
Phomopsis viticola taxon 2	8

Table 2.1 Fungi most frequently isolated from vines from dormancy (08.09.97) until flowering (29.10.97). The raw frequencies are given. A space represents no fungus isolated. Only those fungi with a relative importance of more than 1% have been considered [a].

Fungi isolated	Stage 01 (08.09.97)						Stage 05 (18.09.97)						Stage 12 (29.09.97)						Stage 19 (29.10.97)					
	N[b]	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp
Alternaria alternata complex	46	13					14	13					19	2	2				17	4	13	1		
Alternaria tenuissima (Kunze ex Pers.) Wiltshire complex	4	1											4	1					2	4	5			
Alternaria infectoria E.G. Simmons complex	2																							
Alternaria sp.							1						1	1										
Chaetomium Kunze sp.	1												1	1	2				9		1	2	1	
Cladosporium cladosporioides (Fresen.) G.A. de Vries																								
Epicoccum nigrum	13						9	1					15	6					3	1				
Gliocladium roseum Bainier	1						2	1					2	3	1	1			3					
Nigrospora oryzae	1												1						1		1	1		
Phoma Sacc. sp.						1															2			

Continued

Table 2.1 (continued)

Fungi isolated	Stage 01 (08.09.97)						Stage 05 (18.09.97)						Stage 12 (29.09.97)						Stage 19 (29.10.97)					
	N[b]	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp
Phomopsis viticola taxon 2	7	2					3						4	3					6	2				
*Pleospora herba*rum																			2		3			
Sphaeropsis sp.							22	5					14	5					26	5				
Sporormiella minimoides (Cain) S.I. Ahmad & Cain																					3			
Sterile black mycelium	45	8					31	11					12	6					2					
Sterile brown mycelium	14	6					7						14	1					8		3			
Sterile pink mycelium																			1		2			
Sterile white mycelium	1												1		1				1					
Sterile yellow mycelium													1	1					1					
Verticillium Nees sp.	3						2						4											

[a] Isolates with standardised relative importance less than 1% include: *Acremonium* Link sp., *Ascochyta* Lib. sp., *Ascotricha* Berk. sp.1, *Ascotricha* sp.2, *Bipolaris cynodontis* (Marignoni) Shoemaker, *Coniochaeta* (Sacc.) Cooke sp., *Coniothyrium* Corda sp., *Curvularia clavata* B.L. Jain, *Drecshlera* S. Ito sp., *Fusarium acuminatum* Ellis & Everh., *Fusarium chlamydosporum* Wollenw. & Reinking, *Fusarium oxysporum* Schltdl. emend. Snyder & H.N. Hansen, *Fusarium poae* (Peck) Wollenw., *Fusarium* Link sp., *Fusiccocum parvum* Pennycook & Samuels, *Gelasinospora* Dowding sp., *Geniculosporium* Chesters & Greenh. anamorph of *Hypoxylon serpens* (Pers.: Fr.) J. Kickx f., *Microsphaeropsis olivacea* (Bonord.) Höhn., *Nigrospora sphaerica* (Sacc.) E.W. Mason, *Phomopsis viticola* taxon 1, *Seimatosporium* Corda sp., *Sordaria lappae* Potebnia, *Trichoderma* Pers. sp., *Ulocladium botrytis* Preuss, unidentified coelomycete and *Veronaea* Cif. & Montemart sp..

[b] Codes for the plant tissues: N = nodes, I = internodes, L = leaf, Lp = leaf petiole, T = tendril and Bp = bunch peduncle.

Table 2.2 Fungi most frequently isolated from vines from pea-size stage (25.11.97) until after harvest (26.02.98). The raw frequencies are given. A space represents no fungus isolated. Only those fungi with a relative importance of more than 1% have been considered [a].

Fungi isolated	Stage 31 (25.11.97)						Stage 35 (19.01.98)						Stage 38 (11.02.98)						Stage 41 (26.02.98)					
	N[b]	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp
Alternaria alternata complex	46	6	37	2	3	1	50	9	38	5	2	4	68	4	28	3	1	2	63	6	50	4	2	7
Alternaria tenuissima complex	6	2	5	1			10		14	2	1	1	5	2	8				17		4		2	
Alternaria infectoria complex			1				1					1	2		1			2	1		1			
Alternaria sp.	2		1				1						2		4				1		1			
Chaetomium sp.	2	2	4	1			1																	
Cladosporium cladosporioides	1	1																1	2	1	1			
Epicoccum nigrum	33	1	3				23	1	5	2		1	14	2	2				21	1	1		1	
Gliocladium roseum																								
Nigrospora oryzae	2		9		1		4	1	8										1					
Phoma sp.							1				1			1					1					1
Phomopsis viticola taxon 2	2	3	1				4	1					6	4								1		

Continued

Table 2.2 (continued)

Fungi isolated	Stage 31 (25.11.97)						Stage 35 (19.01.98)						Stage 38 (11.02.98)						Stage 41 (26.02.98)					
	N[b]	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp	N	I	L	Lp	T	Bp
Pleospora herbarum	6	4	11	2			9	4	12	2	1	1	3	1	10	1	2	2	12	5	11		1	2
Sphaeropsis sp.	16	1	1				51	25		1			25	13					16	7				2
Sporormiella minimoides		1						1		1			3		2	2		2		1	6			
Sterile black mycelium	8	2	1				5	1					4	2	2				7	7				
Sterile brown mycelium	9	5	8	1			3	2	6	1			2	1	2			1	4		1			
Sterile pink mycelium				1						1					1									
Sterile white mycelium	1	1	1				1		2										1		1			
Sterile yellow mycelium		1	1				3																	
Verticillium sp.																								

[a] Isolates with standardised relative importance less than 1% include: *Acremonium* sp., *Ascochyta* sp., *Ascotricha* sp.1, *Ascotricha* sp.2, *Bipolaris cynodontis*, *Coniochaeta* sp., *Coniothyrium* sp., *Curvularia clavata*, *Drecshlera* sp., *Fusarium acuminatum*, *Fusarium chlamydosporum*, *Fusarium oxysporum*, *Fusarium poae*, *Fusarium* sp., *Fusiccocum parvum*, *Gelasinospora* sp., *Geniculosporium* anamorph of *Hypoxylon serpens*, *Microsphaeropsis olivacea*, *Nigrospora sphaerica*, *Phomopsis viticola* taxon 1, *Seimatosporium* sp., *Sordaria lappae*, *Trichoderma* sp., *Ulocladium botrytis*, unidentified coelomycete and *Veronaea* sp..

[b] Codes for the plant tissues: N = nodes, I = internodes, L = leaf, Lp = leaf petiole, T = tendril and Bp = bunch peduncle.

Table 3. Lesion lengths of taxa 1 and 2 on green Riesling shoots as measured after 7 d of inoculation.

Taxon	Number of isolates	Average lesion length (mm)
1	2	3.92 (± 2)*
2	5	10.6 (± 7)

*Standard deviation

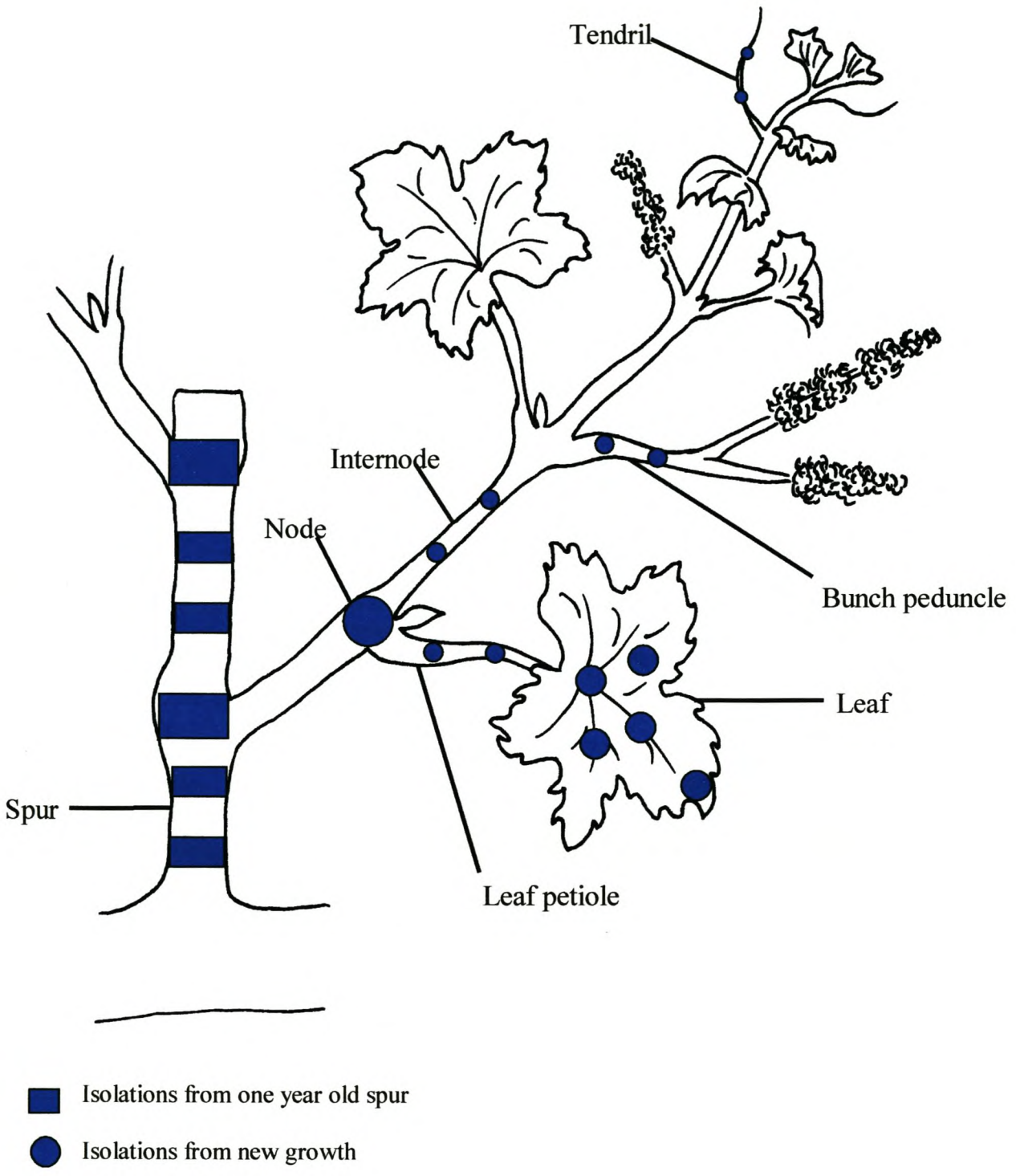

Fig. 1. Diagrammatic illustration of the specific plant tissues analyzed.

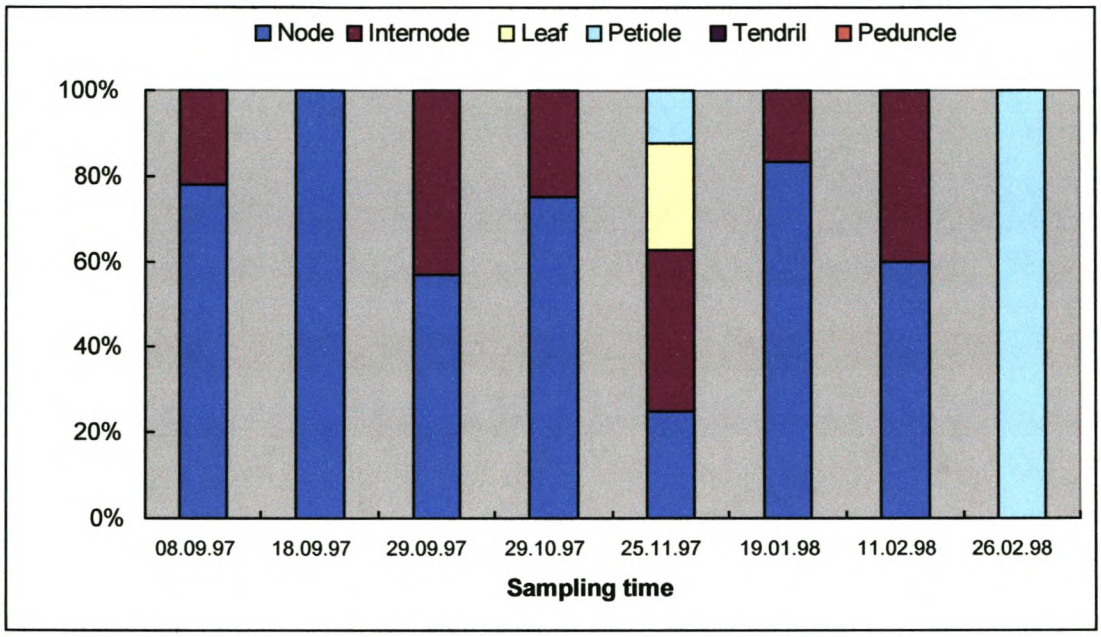

Fig. 2. Isolation of *Phomopsis viticola* taxa 1 and 2 from the different plant parts.

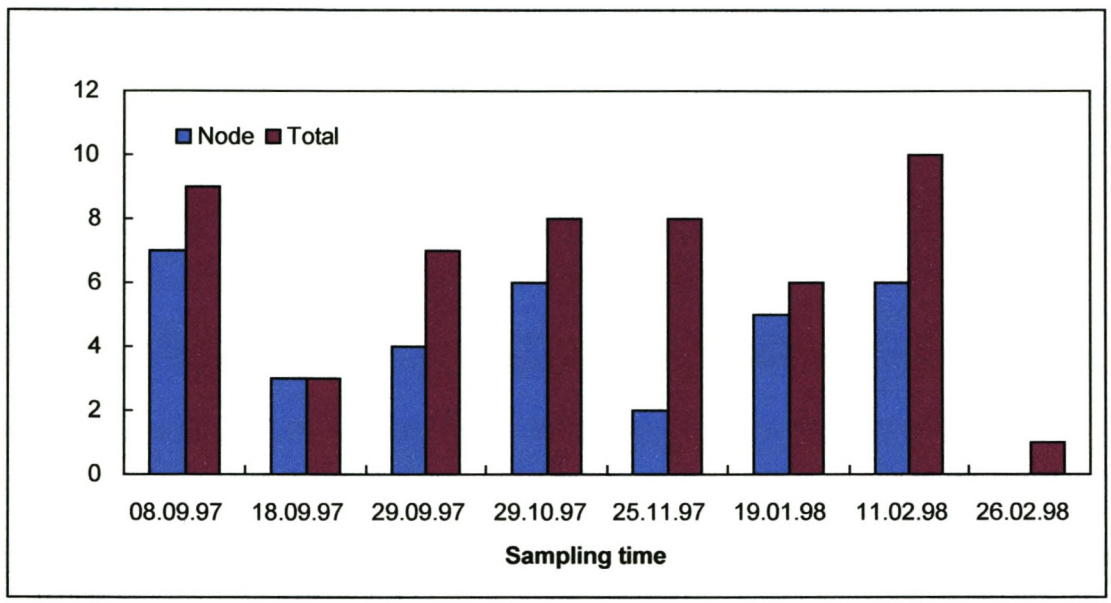

Fig. 3. Frequency of isolation of *P. viticola* taxon 2 from the nodes as compared with the total number of isolations.

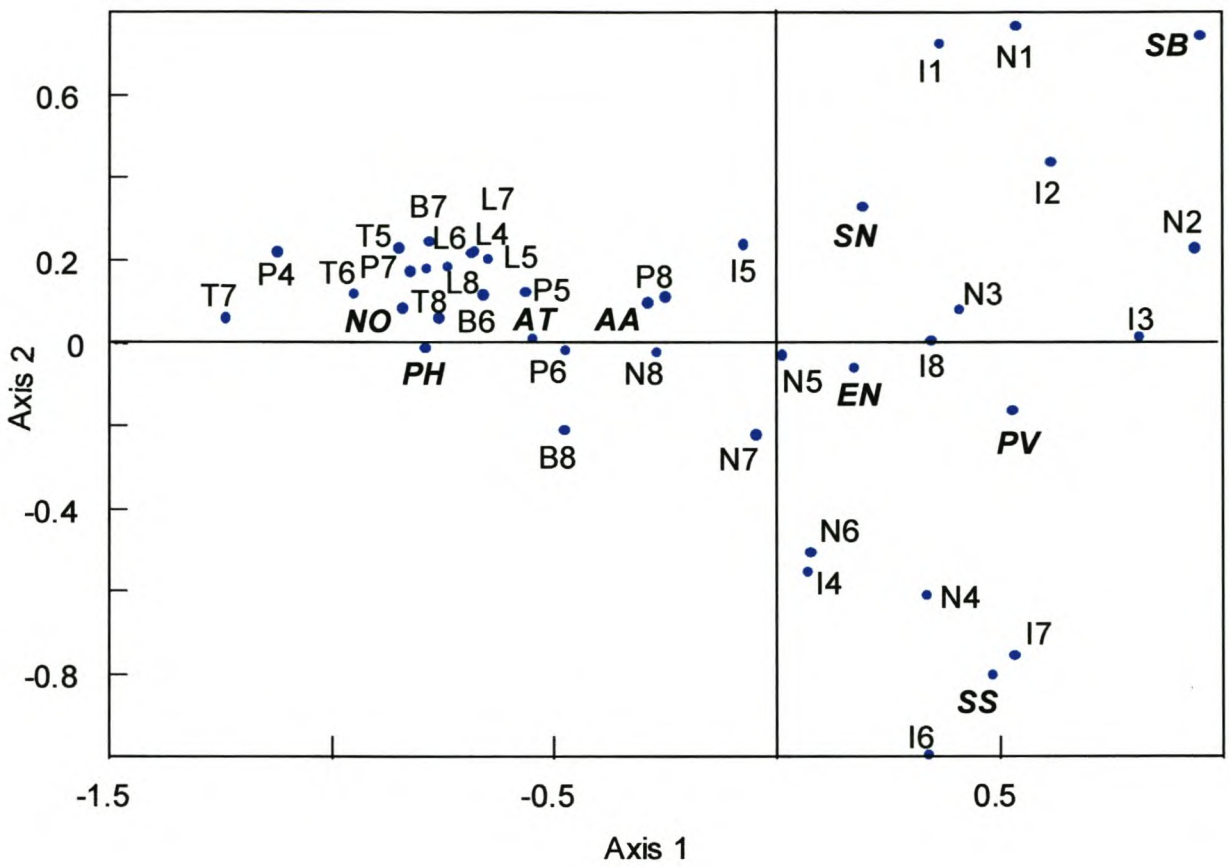

Fig. 4. Results of the simple correspondence analysis showing the distribution of endophytes over the growing season in the host plant. It was performed on the matrix containing the most important fungi (RI ≥ 5%) isolated from vine plants at different sampling times. Abbreviations used in the graphical display of the analysis are as follows: *Alternaria alternata* complex = AA, *Sphaeropsis* sp. = SS, *Epicoccum nigrum* = EN, Sterile black = SB, *Pleospora herbarum* = PH, *Alternaria tenuissima* complex = AT, Sterile brown mycelium = SN, *Phomopsis viticola* taxon 2 = PV and *Nigrospora khuskia-oryzae* = NO. As for the samples, they have been abbreviated as follows: N = Node; I = Internode; L = Leaf; P = Petiole; T = Tendril and B = Bunch peduncle. The sampling times have been numbered from 1 to 8, thus, e.g.: N1 = Node, at sampling time 1.

Fig. 5. Lesions formed on grapevine shoots 7 d after inoculation with mycelial plugs colonised by *Phomopsis viticola* taxa 1 and 2.

3. *PHOMOPSIS* SPP. OCCURRING ON GRAPEVINES WITH SPECIFIC REFERENCE TO SOUTH AFRICA: MORPHOLOGICAL, CULTURAL, MOLECULAR AND PATHOLOGICAL CHARACTERIZATION

ABSTRACT

Sixty one *Phomopsis viticola* isolates collected from 58 vineyards in the different viticultural regions of the Western Cape province of South Africa were characterized. Isolates obtained from Australia, Italy, Portugal, Turkey and the U.S.A. were also included. Species delimitation was primarily based on alpha conidium and conidiophore morphology, cultural characteristics, pathogenicity to *Vitis*, and the formation of the teleomorph *in vitro*. The identity of each taxon was confirmed by means of phylogenetic analyses of the nuclear ribosomal DNA internal transcribed spacers (5.8S, ITS1 and ITS2) and the 5' end partial sequence of the mitochondrial small subunit (mtSSU). Three different taxa, and a *Phomopsis* species commonly associated with shoot blight of peaches in the U.S.A., *P. amygdali*, were identified among the South African isolates. Examination of the Australian culture designated as taxon 4 was found to be a species of *Libertella*, thus excluding it from the *P. viticola*-complex. An Italian isolate was found to represent a species of *Phomopsis* not previously known from grapevines, and this was subsequently described as taxon 5. *P. amygdali* was isolated once only and appeared to be of lesser importance in this disease complex. Furthermore, taxa 1 *(D. perjuncta)* and 3 were also rarely encountered and proved to be non-pathogenic, indicating their non-functional role in Phomopsis cane and leaf spot disease. Taxon 2 (*Phomopsis viticola*) was common and widely distributed throughout diseased vineyards. This taxon was associated with the typical cane and leaf spot disease symptoms and proved to be highly virulent. Morphologically taxon 2 corresponded best with *P. viticola*, which was also neotypified in this study.

INTRODUCTION

Phomopsis cane and leaf spot disease of grapevine (*Vitis vinifera* L.), caused by *Phomopsis viticola* (Sacc.) Sacc., can lead to losses of up to 50% of the normal yield

(Pine, 1958; Berrysmith, 1962; Pscheidt & Pearson, 1989). Losses can occur from shoots breaking off near their base, stunting of vines, loss of vigor, reduced bunch set and infected fruit developing Phomopsis rot (Punithalingam, 1979; Chairman *et al.*, 1982; Nicholas *et al.*, 1994; Pearson & Goheen, 1994).

Phomopsis cane and leaf spot occurs in most countries where vines are grown (Punithalingam, 1979). In South Africa this disease was first noticed in 1935 (Du Plessis, 1938), and was reported to cause serious disease problems (Synnott, 1958). It has since occurred sporadically in the Helderberg, Firgrove, Somerset West, Rawsonville and Slanghoek areas in the Western Cape province (Marais, 1981).

Phomopsis viticola was originally described as *Phoma viticola* Sacc. from canes of *V. vinifera* collected in France (Saccardo, 1880). In 1885 Cooke described a later homonym from Britain as *Phoma viniferae* Cooke (Cooke, 1885). *Phomopsis cordifolia* Brunaud, described from vines in Italy, resembled *P. viticola* in having similar alpha conidium dimensions, and was thus considered synonymous (Uecker & Johnson, 1991). Saccardo (1915) established a new combination for *Phoma viticola* as *Phomopsis viticola* (Sacc.) Sacc., and for the first time cited a specimen. This specimen was not the French material on which the name *Phoma viticola* was originally based, but was from *Vitis aestivalis* Michx. in Albany, New York, U.S.A., collected by H.D. House (No. 149) (Saccardo, 1915). In addition to these names, several other *Phomopsis* species were also described from grapevines (Table 1). In the latter descriptions, the dimensions of the α-conidia were longer and narrower than those described by Saccardo (1915).

Reddick (1909) collected canes of *Vitis labrusca* L. in the U.S.A., and found a fungus similar to *P. viticola*, which he named *Fusicoccum viticolum* Reddick. Even though symptoms caused by *P. viticola* are similar to those caused by *Botryosphaeria dothidea* (Moug. : Fr.) Ces. & de Not. (anamorph: *Fusicoccum aesculi* Corda) (Phillips, 1998), it is evident from the illustrations that *F. viticolum* was indeed a species of *Phomopsis*, which consequently led to the new combination, and later homonym, *Phomopsis viticola* (Reddick) Goid. (Goidànich, 1937). Goidànich never examined Reddick's material, which I have also failed to locate in the present study (BPI, NY, CUP, K, IMI, B, PAD). To further confuse the matter, the specimen lodged by

Goidànich in 1938 (CBS 252.38) was found to be representative of *Coniella granati* (Sacc.) Petrak & Syd. (Merrin *et al.*, 1995).

A comparative study (Pine, 1958) done on isolates of Phomopsis cane and leaf spot disease from Canada, South Africa, Italy and New York showed no difference in their cultural and morphological characteristics. In his subsequent treatment of the pathogen, Punithalingam (1979) established a relatively uniform concept of *P. viticola*, and also placed several of the former names in synonymy. However, a recent Australian study distinguished four taxa from grapevines based on alpha conidium morphology, pycnidium formation, colour and structure, cirrhus colour and mycelial growth rate (Merrin *et al.*, 1995). These groupings were also supported by host inoculation, conidium germination and growth study data, as well as pectic enzyme profiles. On the basis of conidium dimensions, Merrin *et al.* (1995) suggested that their taxon 1 correlated with Saccardo's description of *Phomopsis viticola*, and that taxon 2 could resemble *Fusicoccum viticolum* (Merrin *et al.*, 1995). In a re-examination of these species, however, Phillips (2000) concluded that the name *Diaporthe perjuncta* Niessl was available for taxon 1, while taxon 2 resembled *P. viticola* (Sacc.) Sacc. Other than the four taxa already associated with the *P. viticola*-complex of grapevines, an additional two species were also recently described from this host. *P. longiparaphysata* Uecker & Ker-Chung (Uecker & Ker-Chung, 1992) was primarily distinguished based on its prominent paraphyses, while *P. vitimegaspora* Ker-Chung & Lii-Sin (Ker-Chung & Lii-Sin, 1998) was characterized by large alpha conidia and different disease symptoms.

The research reported in the present study was aimed at clarifying the taxonomy of the various *Phomopsis* spp. associated with grapevines, and circumscribing *Phomopsis viticola*, the causal organism of Phomopsis cane and leaf spot disease.

MATERIALS AND METHODS

Isolates

Shoots and leaves showing typical Phomopsis cane and leaf spot symptoms were collected from wine and table grape vineyards in the Western and Nothern Cape provinces' viticultural regions. Most of the collections were made from autumn to

spring of 1997. Vine shoots and leaves were surface sterilized in 70% ethanol for 20 sec, 1% sodium hypochlorite for 2 min, and 70% ethanol for 20 sec. Dissected leaf and shoot pieces were incubated in Petri dishes with sterilized, moist tissue paper to enhance pycnidial formation. Isolations were also made directly from lens-shaped lesions that appeared on shoots, as well as the dark brown leaf spots with yellow halos. Pycnidia and tissue pieces were plated directly onto water agar (Biolab Diagnostics (Pty) Ltd, Midrand, South Africa) plates amended with 1ml/L streptomycin (WAS), and incubated at 22 °C under near-ultraviolet light. After 14 d single-conidium isolates were prepared by making dilution plates of exuding spore masses onto WAS plates. Four single germinating conidia were retained from each collection. Stock cultures (STE-U) were maintained at 5°C in McCartney bottles containing 2% malt extract agar (MEA, Biolab) sterile water, or sterile paraffin oil, respectively.

Two isolates collected during an earlier endophyte study (part 2) were also included. Additional isolates were those identified as *P. viticola* from vines in Australia, Italy, Portugal, Turkey and the U.S.A., together with an ex-type strain of *P. vitimegaspora* Kuo & Leu from vines in Taiwan (Ker-Chung *et al.*, 1998). Isolates of *Phomopsis* spp. from diverse hosts such as rose (*Rosa* sp.), plum (*Prunus* sp.), protea (*Protea* sp.) and pear (*Pyrus* sp.) were also included (Table 2).

Sexual compatibility

A subset of 26 isolates were chosen from the various morphological groups, and plated onto water agar plates, each containing 4 cm long pieces of double autoclaved vine shoots (WAV). Using the technique as described by Schoch *et al.* (1999), isolates were mated in all possible combinations to induce the teleomorph. Plates were incubated for 3 wk at 25°C, after which they were placed under near-ultraviolet light at 10°C. Plates were scanned weekly over a 5 mo period for the presence of perithecia. After 10 wk, perithecia were squashed on glass slides, and mounted in water as well as lactophenol, respectively, to observe the morphology of the asci, ascospores and paraphyses.

Cardinal temperature requirements for growth

Recent studies have shown that specific temperature requirements for growth could be used to allocate isolates to the various taxa recognized in the *P. viticola*-complex (Merrin *et al.*, 1995). Twenty-seven isolates were plated on 220x220 mm^2 MEA plates, and incubated for 7 d in the dark at eight different temperatures from 5-40°C in 5° intervals. Three plates were used per isolate for each temperature, and the experiment was repeated once. Linear mycelial growth was determined by obtaining two perpendicular measurements for each plate, and calculating the mean of six measurements for every isolate at each temperature.

Stem inoculations

The pathogenicity was measured by means of wound inoculations into green grapevine shoots. Seventeen South African grapevine *Phomopsis* isolates were chosen, namely 14 of taxon 2, and one each of taxa 1, 3 and *P. amygdali*, respectively. Three *Phomopsis* isolates obtained from other hosts such as protea, plum and pear, were also tested for host specificity to grapevine. Green shoots (1-2 cm diam) were pruned from healthy Chenin blanc vines, and placed in 500 ml flasks (1 shoot/flask), each containing 300 ml of a nutrient emended water solution (Hewitt, 1952). Flasks with shoots were maintained in the laboratory at 22°C with a 12 hr fluorescent white light/dark regime. Shoots were swabbed with 70% ethanol, and wounded (at 1 cm shoot diam) using a 3 mm diam cork borer to remove the outer cortex. Colonized mycelial plugs from 2-wk-old cultures (MEA) were inserted as inoculum into the wounds, and sealed with Parafilm. Uncolonized MEA plugs were used for control inoculations. Each isolate, including the control, was replicated three times on separate shoots, with one inoculation per shoot. Flasks were supplemented with fresh nutrient emended water every second day. Pathogenicity was determined by measuring the lesion length 7 d after inoculation. Lesion lengths were calculated by subtracting the size of the wound formed on the control shoot from the lesion formed on the inoculated shoots. An analysis of variance was conducted on the results to establish significant differences between the taxa compared. Additional pathogenicity tests were also conducted to test the cross pathogenicity of *P. viticola* and the grapevine *P. amygdali* isolate to peach and

grapevines. Colonized mycelium plugs of the two species were used to inoculate potted plants of *Prunus persica* L. cv Kakemas, and *Vitis vinifera* cv Riesling. Each isolate was inoculated on three plants of both hosts. Uncolonized MEA plugs were used for control inoculations. Disease symptoms were evaluated 12 d after inoculation by measuring lesion lengths. Re-isolations were made from the lesion margins.

Sequence comparisons

Twenty-nine isolates representing the different taxa were selected for sequence comparisons (Table 2). Additional sequences of *Phomopsis* species from plum, pear and peach (Uddin & Stevenson, 1998) were retrieved from GenBank (U 94898, U 91617 and U 86406) and included in the analysis. DNA extraction was performed using the isolation protocol described by Lee and Taylor (1990). Two genomic areas were sequenced. The ITS1 and ITS2 internal transcribed spacers as well as the 5.8S ribosomal RNA gene were amplified using primers ITS1 and ITS4. The small subunit of the mitochondrial ribosomal RNA genes (mtSSU) was amplified using primers MS1 and MS2. Genomic locations and primer sequences are presented in White *et al.* (1990). The PCR products were purified using Wizard PCR Preps (Promega Corporation, Madison, Wisconsin). Both strands of the PCR product were sequenced using the ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut). A Dye Terminator Cycle Sequencing Ready Reaction Kit containing AmpliTaq DNA Polymerase (Perkin-Elmer) was used for sequencing reactions. Fragments of sequencing reactions were finally purified using Centri-Sep Spin columns (Princeton Separations, Adelphia, New Jersey) and loaded onto the sequencing gel. Sequences were aligned using Sequence Navigator, from which a consensus sequence was created. Sequences obtained from this study and GenBank retrievals were aligned with Clustal W (Thompson *et al.*, 1994). The final alignment was optimized manually. Alignment gaps were coded as missing data in the analysis. The sequences of *Cryphonectria parasitica* (Murrill) Barr were used as outgroups both in the ITS data, and the data composed of ITS and mtSSU (GenBank AF 172658 and AF 029891). Phylogenetic analyses were performed with PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b2a (Swofford, 1999). Maximum parsimony analysis was performed using heuristic search option with 1000 random

sequence input orders for exact solution. The unconstrained topologies of the equally parsimonious trees were compared using the Kishino-Hasegawa test. The best topology was selected as the most parsimonious tree topology and evaluated with 1000 bootstrap replications to test the clade stability of the tree. The decay indices were also calculated using AutoDecay (Erikkson, 1998) to further test the robustness of the branches of the tree. Other measures including tree length, consistency index, retention index, rescaled consistency index and homoplasy index (CI, RI, RC and HI) were also calculated. The ITS data included 32 sequences. Because of ambiguities in alignment (data not shown, but available from Lizel Mostert, Deparment of Plant Pathology, University of Stellenbosch), short sequence segments in ITS1 (49-81) and ITS2 (362-379) were excluded from the analysis. A partition homogeneity test in PAUP (Swofford, 1999) was conducted for the ITS and mtSSU sequences of 14 taxa to examine the possibility of a joint analysis of the two data sets. The DNA sequences obtained were lodged at GenBank and given the following accession numbers: ITS sequences, AF 23043 – AF 230770 and mtSSU, AF 230771 – AF 230784.

Morphological comparisons

The South African and representative overseas isolates were compared morphologically on divided plates containing WAV on the one side, and potato dextrose agar (PDA, Biolab) on the other. The plates were incubated at 25°C under near-ultraviolet light. Colony colours were rated (surface and reverse) according to colour charts of Rayner (1970). The colony texture, margin, elevation and zonal growth (PDA), and the pycnidial density and colour of the exuding conidial cirrhi (WAV) (Wechtl, 1990) were rated after 14 d. Plates were monitored daily to record the time of pycnidial formation on WAV. Pycnidia were characterized according to their distribution, shape, colour, presence or absence of aerial hyphae, being erumpent or immersed, colour of the conidial cirrhus, number of ostioles, pycnidial dimensions and wall anatomy (Wechtl, 1990). Sporulating pycnidia on WAV were mounted on slides with lactophenol, and examined at 1000X magnification. Several characters were noted, including the presence or absence and general morphology of alpha, beta and gamma conidia; conidiophore aggregation, shape, size, septation and branching; conidiogenous cell morphology, the

presence of collarettes, periclinal thickening and paraphyses. Sporulating perithecia were mounted in water and lactophenol, and the morphology of their asci, paraphyses and ascospores noted. Thirty measurements were taken of morphological structures, averages determined, and the minimum and maximum ranges given in parentheses. In addition, the 95% confidence intervals were also determined for α-conidia. Vertical sections through fruiting bodies were obtained using a Leica CM1100 freezing microtome. Sections (10 µm) were mounted in lactic acid for examination.

RESULTS

Isolates

Sixty one *Phomopsis* isolates were obtained from diseased grapevine material from 58 vineyards located from Lutzville to Swellendam. No disease was found in the hot, dry climate of the Oudtshoorn and Orange River vineyard production regions. Taxon 2 was predominantly isolated. These isolates were mostly from lesions on nodes, indicating the node and bud region to be important sites in which the fungus could survive during winter. Upon further investigatin of the taxon 1 isolates obtained endophytically in part 2, they proved to be representative of taxa 1 and 3.

Sexual compatibility

After 51 d only three isolates (STE-U 2655, 2676 and 2677) formed the teleomorph. Only the Australian isolate (STE-U 2676) was of ascospore origin. The teleomorph was self-fertile, confirming this fungus to be homothallic (Phillips, 2000).

Stem inoculations

Phomopsis viticola (taxon 2) and *P. amygdali* formed distinct dark brown lesions that were similar in length. Taxa 1 and 3 formed no pronounced lesions. Only a dark brown rind developed around the wound. The analysis of variance conducted showed that lesion size of taxa 1 and 3 were significantly different than taxon 2 ($P = 0.0064$). Grapevine isolates of taxa 1 and 3 formed lesions less than 2 mm in length, whereas those of *P. viticola* (taxon 2) were up to 20 mm in length. The pear *Phomopsis* sp. produced a lesion comparable to that of taxon 2 (*P. viticola*), while the protea and plum

isolates formed no lesions on the grapevine shoots. *P. amygdali* formed lesions of up to 16 mm in length. The difference in lesion appearance of taxa 1 and 3 in relation with part 2 was most probably due to the shoots being 4 mo older.

Peach trees inoculated with *P. viticola* (taxon 2) and the control formed no lesions. However, the *P. amygdali* isolate produced prominent brown lesions on the inoculated peach trees, as well as on grapevines. In each case where lesions were formed, the fungus was successfully re-isolated.

Sequence comparisons.

Maximum parsimony analysis of the ITS data gave 39 equally most parsimonious trees (MPT) with 54 parsimony informative characters in the alignment. In accordance with the result of the Kishino-Hasegawa likelihood test (data not shown), the best tree topology of the 39 MPTs was selected as the phylogenetic tree topology and evaluated with 1000 bootstrap replications and decay indices for clade stability. The result of the partition homogeneity test ($P = 0.32$, where $P \geq 0.05$ was significantly incongruent) indicated that the two data sets could be combined. Maximum parsimony analysis of the combined data sets resulted in 24 MPTs with 49 parsimony informative characters in the alignment. The best tree topology of the 24 MPTs, which was indicated by the Kishino-Hasegawa likelihood test (data not shown), was selected as the phylogenetic tree topology and evaluated with 1000 bootstrap replications and decay indices for the clade stability. The final phylogenetic trees (Figs 1 & 2) were compatible with accepted morphological delimitation of taxa associated in the *P. viticola*-complex. Representative isolates of taxon 2 clustered together with a strong bootstrap. Clade 3 was not well supported by bootstrap and contained isolates of taxon 3 as well as *Phomopsis* species form pear and protea. Clade 1 included ascospore isolates of *Diaporthe perjuncta* (taxon 1) as well as those originally obtained from conidia (STE-U 2677 and STE-U 2655). One of the South African isolates (STE-U 2632) showed a high similarity with a sequence of the peach shoot blight pathogen, *Phomopsis amygdali*. The South African isolates from plum and pear grouped distant from other isolates from these hosts (GenBank U 94898 and U 91617), suggesting that they represent another species. Taxon 4 (Merrin *et al.*, 1995) grouped distant from the genus *Phomopsis*, and was therefore

exluded from the analyses. A *Phomopsis* sp. from *Vitis* (STE-U 2674) and one from rose (STE-U 2680) did not support the ITS phylogeny and grouped separately. The Italian isolate (STE-U 2674) from grapevine was subsequently designated as taxon 5.

Morphological comparisons

Analyses of the morphological characteristics of all the isolates studied indicated that the most commonly isolated taxon was taxon 2. Only one respective isolate of taxa 1, 3 and *P. amygdali* were isolated, and none of taxa 4 or 5. The cultural and morphological characteristics that could be used to distinguish the different taxa included colony growth patterns and alpha conidium dimensions and shape. Secondary characters included pycnidium shape and distribution, alpha conidium guttulation, beta conidium formation, conidiophore branching and septation. The presence of collarettes on conidiogenous cells did not prove to be reliable.

Taxon 1

Diaporthe perjuncta Niessl, Hedwigia 17: 44. 1878. Figs 3-12

Anamorph. Phomopsis sp.

Perithecia globose, solitary, scattered to aggregated, subepidermal, 210–500 µm wide, 250–350 µm tall. Wall consisting of two regions of *textura angularis*: outer region dark brown, 3–4 cells thick, 15–65 µm wide; inner region pale brown, 3–4 cells thick, 12–32 µm wide. Perithecial neck of *textura prismatica*, with outer region dark brown, 10–20 µm wide, inner region hyaline, 5–10 µm wide. Ostiole red-brown, widening when spores start exuding, 90–150 µm wide. Necks long, without external hyphae, red-brown at the tip, becoming dark brown towards the perithecium, constricted in the middle of the neck, 870–1500 × 70–100 µm ($\bar{x}$ = 1128 × 83 µm). *Asci* unitunicate, cylindrical-clavate, with refractive apical ring, 8-spored, biseriate, 55–61 × 5–8.5 µm ($\bar{x}$ = 57 × 6.5 µm). *Paraphyses* septate, unbranched, tapering towards apex with a rounded tip, extending above the asci, 48–109 µm long, 4–7 µm wide at bottom and 2–3 µm at the apex. *Ascospores* hyaline, smooth, fusoid, widest at the septum, tapering towards both ends,

slightly curved or straight, medianly septate, slightly constricted at the septum with 1 to 2 large guttules, (8–)11.5–13(–15) × (2.5–)3–3.5(–4.0) µm ($\bar{x}$ = 12 × 3.5 µm); hyaline appendages punctiform, restricted to the tips of ascospores, 1–2 µm long. Sterile pycnidia formed after 17 d at 25 C in the dark. *Conidia*: alpha conidia biguttulate to eguttulate, fusoid with obtuse ends, 5–5.5(–6) × 1.5–2 µm (*in vivo*). Beta conidia and gamma conidia absent. Description based on STE-U 2655.

Notes. The morphology of the *Diaporthe* sp. from South Africa (STE-U 2655) corresponded well with that of the Australian isolate (STE-U 2676). Differences in the morphology of the perithecial necks and ascopores were, however, observed between these isolates and one from Portugal (STE-U 2677). STE-U 2655 and STE-U 2676 had single, solitary perithecia, red-brown at the tip with sparse external hyphae covering perithecial necks, and fusoid ascospores that were widest in the middle. In contrast, STE-U 2677 had aggregated perithecia, yellow-brown at the tip, extensive hyphae covering perithecial necks, and fusoid-ellipsoidal ascospores with obtuse ends, being widest in the middle of the apical cell. In spite of these morphological differences, however, these isolates could not be distinguished based on the phylogeny of their DNA sequence data. Phillips (2000) also concluded that even though some isolates lacked mucous ascospore appendages, they still represented the same taxon. Several *Diaporthe* Nitscke teleomorphs have also been associated with grapevines. The first *Diaporthe* species described from this host was *D. viticola* Nitscke, reported from Germany and Maine (Nitschke, 1870) and *D. silvestris* Sacc. & Berl. on *V. vinifera* in Italy (Saccardo & Berlese, 1885). Although teleomorphs of *Phomopsis* reside in the genus *Diaporthe* (Wehmeyer, 1933), Shear (1911) described *Cryptosporella viticola* Shear as the teleomorph of *Fusicoccum viticolum* Reddick. This material could not be located, however, and the status of this teleomorph thus remains uncertain.

Recently, the teleomorph of the Australian *Phomopsis* taxon 1 (Merrin *et al.* 1995) was collected in Australia and attributed to *Diaporthe viticola* (Scheper *et al.*, 2000). A *Diaporthe* sp. was subsequently also found on vines in Portugal (Phillips, 2000). Following the concepts of Wehmeyer (1933), Phillips (2000) determined that based on the limited entostroma, ascospores that were wider than 2.5 µm, and perithecia

with single, erumpent ostioles, the name *Diaporthe perjuncta* Niessl would be more suitable than *D. viticola* for isolates of taxon 1 collected in Australia and Portugal (Phillips, 2000).

Cultures. Colonies woolly, predominantly white, with greyish sepia tufts (15""i) and rosy buff (13"d) undertone. Reverse, buff (19"d) to rosy buff (15"d).

Cardinal temperature requirements. Colonies obtained a maximum growth of 67-93 mm diam at 20 °C after 7 d in the dark. No growth occurred below 10 °C or above 35 °C.

Hosts. Vitis vinifera, but presumed wide host range.

Distribution. Australia, Portugal and South Africa.

Cultures examined. AUSTRALIA. SOUTH AUSTRALIA: Coonawarra, Mildara. Shiraz grapevine, Jul. 1995, *R.W.A. Schepers* (RS85 = STE-U 2676). PORTUGAL. Oeiras. Galego Dourado grapevine, Jan. 1998, *A.J.L. Phillips* (P/CA/15/20/1 = STE-U 2677). SOUTH AFRICA. WESTERN CAPE: Stellenbosch. Riesling grapevine, Nov. 1997, *L. Mostert*, (STE-U 2655) (specimen PREM 56458).

Taxon 2

Phomopsis viticola (Sacc.) Sacc., Ann. Mycol. 13: 118. 1915. Figs 13-29

Phoma viticola Sacc., Michelia 2: 92. 1880.

Fusicoccum viticolum Reddick, Cornell Univ. Agr. Expt Sta. Bull. 263: 331-332. 1909.

Phomopsis viticola (Reddick) Goid., Atti R. Accad. Naz. Lincei 26: 107-112. 1937.

Phomopsis viticola Sacc. var. *ampelopsidis* Grove, Bull. Misc. Inf. (Kew) 4: 183-184 (1919).

Phomopsis ampelina (Berk. & Curt.) Grove, Bull. Misc. Inf. (Kew) 4: 184. 1919.

Teleomorph. Unknown *Diaporthe* sp.

Pycnidia eustromatic, subepidermal, brown to black, scattered or aggregated, globose, flask-like to conical, outer surface smooth, convoluted to unilocular, singly ostiolate, up to 430 µm wide and 190–300 µm tall, including short necks which rarely occur. Pycnidial wall consisting out of two regions of *textura angularis*; the outer region brown, 2–3 cells thick, 5–7 µm wide, inner region brown, 3–4 cells thick, 7–15 µm wide, with the outside cells compressed. Pycnidia sporulate within 4–10 d on WAV. *Conidial mass* globose or in cirrhi, white, pale-yellow to yellow, but predominantly pale yellow. *Conidiophores*: alpha conidiophores cylindrical, some filiform, rarely septate and branched, 5–35 × 1–3 µm ($\bar{x}$ = 25 × 2 µm); beta conidiophores ampulliform to subcylindrical, rarely branched, 10–34 × 1–2 µm ($\bar{x}$ = 26 × 1.5 µm). *Conidiogenous cells*: alpha conidiogenous cells subcylindrical, tapering towards the apex, collarettes and periclinal thickening present, 3–19 × 1–2.0 µm ($\bar{x}$ = 10 × 1.5 µm); beta conidiogenous cells subcylindrical, tapering towards the apex, collarette and periclinal thickening present, 7–14 × 1–2 µm ($\bar{x}$ = 11–1.5 µm). *Conidia*: alpha conidia commonly found, fusoid-ellipsoidal, apex acutely rounded, base obtuse to subtruncate, multiguttulate with guttules grouped at the polar ends, rarely biguttulate, (7–)9.5–10.5(–13) × (1.5–)2–3(–3.5) µm ($\bar{x}$ = 10 × 2.5 µm); beta conidia less common than alpha conidia, straight, curved or hamate, 20–25 × 0.5–1 µm ($\bar{x}$ = 23–1 µm); gamma conidia rarely found, fusoid to subcylindrical, apex acutely rounded, base subtruncate, multiguttulate, 12–18 x 1.5–2 µm ($\bar{x}$ = 15 x 2 µm). Description based on STE-U 2637.

NEOTYPE. FRANCE. BORDEAUX: Naujan-et-Postiac. Cabernet Sauvignon grapevine, May 1998, *P. Larignon*, specimen PREM 56460, culture ex-type PV F98-1 = STE-U 2660.

Notes. The alpha conidium shape was uniform for most isolates of *P. viticola*. Three isolates of *P. viticola* (STE-U 2641, STE-U 2649 and STE-U 2642) had dense, small pycnidia which were mainly depressed. STE-U 2641 and STE-U 2649 were also culturally different in that they had numerous dark brown pycnidia that formed across the surface, and olivaceous (21"m) mycelium. These isolates also grew slower at 25 °C, and caused smaller lesions on inoculated stems. These differences did not prove to be

significant, however, since they clustered with strong bootstrap support to other isolates of *P. viticola* (Figs. 1, 2).

As mentioned earlier, *P. viticola* was originally described from vines collected in France. In the original description of *Phoma viticola*, no type specimen was designated. When the latter was redisposed to *Phomopsis*, Saccardo (1915) cited an American specimen (H.D. House No. 149, PAD). This specimen is charcaterized as follows: pycnidia occurring on stems, immersed, solitary, black, depressed, uniloculate; wall stromatic, composed of several layers of pseudoparenchymatic cells, with several dark brown, thick-walled, chlamydospore-like cells around the pycnidium on the host surface. Conidiogenous cells hyaline, simple, ampulliform, with minute periclinal thickening, without visible collarettes, 5-10 x 5-8 µm. Very few conidia were observed. Alpha conidia ellipsoidal, widest in the middle or slightly above, apex rounded to acute, base flat, mono of biguttulate, 6-10 x 2.5-4 µm (slide heated in lactophenol (Fig. 28). The latter specimen is depauperate, however, and should therefore not been selected as type. Because no other type material could be located, a neotype had to be chosen. The French material designated as neotype (PREM 56460) here corresponds with the House specimen in morphology, and also clustered with other isolates of *P. viticola* in the phylogenetic analyses.

Grove (1919) distinguished *P. ampelina* (Berk. & Curt.) Grove (K 58408) from *P. viticola* by its external appearance on the host. However, conidia were ellipsoid-fusoid, 8-12 x 2.5-3.5 µm, thus similar to that of *P. viticola* (Fig. 29). No material could be located of *P. viticola* Sacc. var. *ampelopsidis* Grove, and therefore the synonymy proposed by Punithalingam (1979) could not be confirmed. Punithalingam (1979) also regarded *P. ampelopsidis* Petrak to be synonymous with *P. viticola*. An examination of material of this fungus (PR 7579, BPI 358265) suggests, however, that it is distinct. Conidiophores were long and slender, tightly aggregated, 0-3-septate, 15-25 x 2.5-3.5 µm, with conidiogenous cells that were 8-15 x 2.5-3 µm. Alpha conidia were generally smaller than that of *P. viticola*, namely (6-)7-9(-12) x 2-1.5(-3) µm (Fig. 30). Of the taxa in the *P. viticola*-complex, *P. ampelopsidis* most closely resembles taxon 3.

Colonies. Colony colour was predominantly buff (19"d) to honey (19"b) with smoke grey (21""f) and citrine green (23") patches. Reverse, buff (19"d) to greyish sepia (15""i) with some darker iron grey patches (23"""k). Colonies slightly raised, with a felty texture and prominent growth rings.

Cardinal temperature requirements. Colonies obtained maximum growth of 16-52 mm diam at 25 °C after 7 d in the dark. No growth occurred below 10 °C or above 35 °C. Four isolates reached their maximum growth at 20 °C (STE- U 2669, STE-U 2638, STE-U 2642, STE-U 2649) and one at 30 °C (STE-U 2666).

Host. Vitis aestivalis Michx., *V. vinifera.*

Distribution. Widely distributed with host.

Cultures examined. AUSTRALIA. TASMANIA: Cloverhill Vineyard. Chardonnay vines, Jul. 1996, *R.W.A. Schepers* (RS91 = STE-U 2662); Marion's Vineyard. Chardonnay vines, Jul. 1996, *R.W.A. Schepers* (RS 110 = STE-U 2663); SOUTH AUSTRALIA: Padthaway. *Vitis vinifera*, *N.G. Nair* (VRU 0083 = STE-U 2665). ITALY. *V. vinifera*, *A. Zazzerini* (CBS 267.8 = STE-U 2671). FRANCE. BORDEAUX: Naujan-et-Postiac. Cabernet Sauvignon grapevine, May 1998, *P. Larignon* (PV F98-1 = STE-U 2660) (specimen PREM 56460). PORTUGAL. Oeiras. Galego Dourado grapevine, Des. 1997, *A.J.L. Phillips* (P/CA/20/28/1/1 = STE-U 2666); Galego Dourado grapevine, Jan. 1998, *A.J.L. Phillips* (P/CA/51/34/1 = STE-U 2667); Santo Tirso. Loureiro grapevine, Feb. 1998, *A.J.L. Phillips* (P/BU/2/2 = STE-U 2669, P/BU/5/4 = STE-U 2670). SOUTH AFRICA. WESTERN CAPE: Rawsonville, Excelsior. Chenin blanc grapevine, Mar. 1997, *L. Mostert* (STE-U 2633); Vredendal, Uitsig. Muscat d'Alexandrie grapevine, Apr. 1997, *L. Mostert* (STE-U 2634); Bonnievale, Middelvlei. *V. vinifera*, Apr. 1997, *L. Mostert* (STE-U 2635); Slanghoek, Twee heuwels. Colombar grapevine, Apr. 1997, *L. Mostert* (STE-U 2636); Philadelphia, Joostfontein. Chenin blanc grapevine, May 1997, *L. Mostert* (STE-U 2637); Darling, The Granneries. Chenin blanc grapevine, May 1997, *L. Mostert* (STE-U 2638); Somerset West, Eendrag. Carignan grapevine, May 1997, *L. Mostert* (STE-U 2639); Stellenbosch, Groenerivier. Weiser Riesling grapevine, May 1997, *L. Mostert* (STE-U 2640); Paarl, St. Peter's Roches. Red Gobe grapevine, Jun. 1997, *L. Mostert* (STE-U 2641) (specimen PREM 56462); Stellenbosch, Vorentoe.

Cinsaut grapevine, Jul. 1997, *L. Mostert* (STE-U 2642) (specimen PREM 56461); Hermanus, Old Lands Stud & Vineyard. Sauvignon blanc grapevine, Oct. 1997, *L. Mostert* (STE-U 2643); Franschhoek, Deu Donne. Chenin blanc grapevine, Oct. 1997, *L. Mostert* (STE-U 2644); Lutzville, Omega. Emerald Riesling grapevine, Oct. 1997, *L. Mostert* (STE-U 2645); Riebeek-Kasteel, Dagbreek. Red Globe grapevine, Oct. 1997, *L. Mostert* (STE-U 2646); Riebeek-Kasteel, Dagbreek, Dan Ben Hannah grapevine, Oct. 1997, *L. Mostert* (STE-U 2647); Porterville, De Tuine. Red Globe grapevine, Oct. 1997, *L. Mostert* (STE-U 2648); Worcester, Diepkloof. Raisno blanc grapevine, Oct. 1997, *L. Mostert* (STE-U 2649); Worcester, So Verby. Colombar grapevine, Oct. 1997, *L. Mostert* (STE-U 2650); Malmesbury, Elsana. Chenin blanc grapevine, Nov. 1997, *L. Mostert* (STE-U 2651); Southern Cape: Swellendam, Olivedale. Frans grapevine, Nov. 1997, *L. Mostert* (STE-U 2652); Western Cape: Botriver, Beaumont. Chenin blanc grapevine, Nov. 1997, *L. Mostert* (STE-U 2653). TURKEY. Unknown. *V. vinifera*, Ali Anbaroglu (CBS 323.77 = STE-U 2672). U.S.A. CALIFORNIA: Tokay vines, 1979, *J.D. Cucuzza* (ATCC 48153 = STE-U 2673).

Specimens examined: U.S.A. NEW YORK STATE: *Vitis aestivalis*, Mar. 1914, *H.D. House* No. 149, (PAD 1268) (*P. viticola*). GERMANY: Mähr-Weisskurchenand. *Ampelopsidis quinquefolia*, 3 Aug. 1920, *F. Petrak* No. 1439, (PR 7579, BPI 358265) (*P. ampelopsidis*). U.S.A.: PENNSYLVANIA: *Vitis* sp., Herb. Berk No. 4094, (K 58408) [*P. ampelina* (Berk & Curt.) Grove].

Taxon 3

Phomopsis sp. Figs 31-41

Pycnidia eustromatic, dark brown to black, almost superficial, separate, sparse, ampulliform, conical to finger-like, covered with hyphae, convoluted, up to 400 µm wide and 570–810 µm tall, including prominent neck. Pycnidial wall consists of two regions; outer region thick-walled, dark brown, 3–4 cells thick, 10–30 µm wide, becoming pale brown inwardly with inner region of *textura globulosa* to *angularis*, 3–4 cells thick, 20–40 µm wide. Pycnidia formed after 17–27 d with only a few remaining sterile. *Conidial*

mass mostly formed cirrhi or globose, yellow-white to white. *Conidiophores*: alpha conidiophores branched, septate, 10–38 × 1–2 µm ($\bar{x}$ = 22 × 1.5 µm); beta conidiophores septate, branched, 13–38 × 1–2.5 µm ($\bar{x}$ = 25 × 1.5 µm). *Conidiogenous cells*: alpha conidiogenous cells filiform, tapering towards the apex, collarette and periclinal thickening present, 5–18 × 1–2 µm ($\bar{x}$ = 12 × 1.5 µm); beta conidiogenous cells subcylindrical, tapering towards the apex, collarette and periclinal thickening present 10–25 × 1 µm ($\bar{x}$ = 14 × 1 µm). Alpha and beta conidiogenous cells occurred in the same pycnidium, but with beta conidia and conidiophores being more prominent. *Conidia*: alpha conidia fusoid with obtuse apices, mostly biguttulate, (4–)6.5–7(–10) × (1.5–)2(–2.5) µm ($\bar{x}$ = 7 × 2 µm); beta conidia straight or curved, 19–25 × 0.5–1 µm ($\bar{x}$ = 21 × 1µm), more abundant than alpha conidia. Gamma conidia rarely found, fusoid to subcylindrical with acute apices, multiguttulate 10–17 × 1–2 ($\bar{x}$ = 13 × 1 µm). Description based on STE-U 2654.

Notes. Alpha conidia from pear had acute apices, whereas alpha conidia of STE-U 2655 and STE-U had oblong apices. STE-U 2668 and STE-U 2654 also formed multiguttulate alpha conidia while the other isolates formed biguttulate alpha conidia. STE-U 2676 formed beta conidia only. STE-U 2664 had distinctly long beta conidiophores, (15–)25.5(–38) µm. The isolate from Portugal, STE-U 2668, had paraphyses and its spore mass was crystalline. STE-U 2661 and STE-U 2668 had pycnidia that were small and densely distributed with white spore masses.

Cultures. Colonies were woolly, predominantly white on the surface with citrine green (23"), hazel brown (17"') and greyish sepia (15""i) patches. Reverse, olivaceous buff (17"'d) to greyish sepia (15""i). Mycelium raised with no growth zones.

Cardinal temperature requirements. Colonies obtained a maximum diameter of 49-67 mm at 25 °C after 7 d in the dark. No growth occurred below 10 °C or above 35 °C.

Host. *Protea* sp., *Prunus* sp., *Pyrus* sp., *Vitis vinifera*.

Distribution. Australia, Portugal, South Africa.

Cultures examined. AUSTRALIA. SOUTH AUSTRALIA: Adelaide Plains, Two Wells. Grenache vines, Aug. 1996, *R.W.A. Schepers* (RS 114 = STE-U 2661); VICTORIA: Yarra Valley, Chardonnay vines, *D.L. Whisson* (DAR 69458 = STE-U 2664). PORTUGAL. Montemor-o-Novo. Aragonez 229 grapevine, April 1998, *A.J.L. Phillips* (CAP 78 = STE-U 2668). SOUTH AFRICA. WESTERN CAPE: Stellenbosch. Riesling grapevine, Nov. 1997, *L. Mostert*, (STE-U 2654) (specimen PREM 56457); *Pyrus* sp., Sept. 1997, *L. Basson* (STE-U 2656); *Protea* sp., Oct. 1997, *S. Denman* (STE-U 2659); Stellenbosch. *Prunus* sp., Nov. 1992, *S. Denman* (STE-U 2657); Grabouw. *Rosa* sp., Oct. 1998, *S. Denman* (STE-U 2658).

Taxon 4

Libertella sp. Figs 42-45

Pycnidia stromatic, sparse, subepidermal, inconspicuous, multilocular, convoluted, up to 700 µm wide. Pycnidial wall consisting out of two regions of *textura angularis*; outer region of pycnidial wall pale brown, 4–5 cells thick, 3–15 µm wide, inner region 3–4 cells thick, 5–15 µm wide; hyphal growth present around pycnidia. Pycnidia formed after 11 d. *Conidial mass* mostly in cirrhi on vines, pale yellow to orange-pink. *Conidiophores* subcylindrical, branched, 14–40 × 1.5–2 µm ($\bar{x}$ = 21 × 1 µm). *Conidiogenous cells* subcylindrical, tapering to a minute apex, proliferating sympodially, 10–20 × 1–2.0 µm ($\bar{x}$ = 13 × 1.5 µm). *Paraphyses* prominent, subcylindrical, tapering slightly towards an obtuse apex, branched, septate, arising from the hymenium, 21–45 × 1–2 µm ($\bar{x}$ = 31.5 × 1.5 µm). *Conidia* curved or hamate, non-septate, (14–)16–18(–20.0) × 1 µm ($\bar{x}$ = 17 × 1µm).

Notes. Taxon 4 of the *Phomopsis viticola*-complex is charcaterised by isolates that form beta conidia only (Merrin *et al.* 1995). An examination of the strain typifying this taxon showed, however, that is would be better accommodated in *Libertella* Desm. than *Phomopsis*. The main difference resides in the mode of conidiogenesis. The sympodial proliferation observed here is well-known for members of *Libertella* (Glawe & Rogers, 1985). Furthermore, the non-germinating scolecospores and orange cirrhi also

resemble other Libertella species that have *Eutypa* Tul. & C. Tul. teleomorphs. The presence of paraphyses and the much shorter conidia further distinguish this fungus from *Eutypa lata* (Pers. : Fr.) Tul. & C. Tul. (anamorph *Libertella blepharis* A.L. Smith), which causes Eutypa dieback on grapevines. However, differences in conidium size and cultural characteristics have been found among *Eutypa* isolates form grapevine (Glawe & Rogers, 1982), suggesting that more than one species of *Eutypa* is present on this host.

Cultures. Colonies were woolly, white with buff (19"f) undertones. Reverse buff (19"f) with a few greyish sepia (19""I) patches. After 20 d abundant dull green (27''m) mycelial growth patches were observed. Colonies were erumpent without growth zones.

Cardinal temperature requirements. Colonies obtained maximum growth of 97 mm diam at 25 °C after 7 d in the dark. No growth occurred below 10 °C or above 35 °C.

Distribution. Australia.

Culture examined. AUSTRALIA. New South Wales: Hunters Valley. Chardonnay grapevine, 1994, *S.J. Merrin* (DAR 69484 = STE-U 3313).

Taxon 5

Phomopsis sp. Fig. 46

Pycnidia eustromatic, dark brown to black, almost superficial, separate, sparse, circular, covered with hyphae, convoluted up to 550 µm wide and 370 µm tall, including short necks. Pycnidial wall consisting of two regions of *textura angularis*; outer region brown, 4–5 cells thick, 30–40 µm wide; inner region light brown to hyaline, 3–4 cells thick, 10–15 µm wide. Pycnidia sporulated within 17 d on WAV. *Conidial mass* globose or in exuding cirrhi, pale yellow to white. *Conidiophores*: alpha conidiophores cylindrical, septate, 15–46 × 1–3 µm ($\bar{x}$ = 31 × 2 µm); beta conidiophores branched, septate, subcylindrical 15–32 × 1.5–2 µm ($\bar{x}$ = 22 × 2 µm). *Conidiogenous cells*: alpha conidiogenous cells cylindrical, filliform, collarette and periclinal thickening present, 10–31 × 1–2 µm ($\bar{x}$ = 21 × 1.5 µm); beta conidiogenous cells subcylindrical tapering towards

the apex, collarette and periclinal thickening present, 8–12 × 1 µm ($\bar{x}$ = 10 µm). *Conidia*: alpha conidia fusoid, apices narrowly acute, biguttulate, (8–)10–11(–13) × (1.5–)2(–2.5) µm ($\bar{x}$ = 10 × 2 µm); beta conidia curved, hamate,13–21 × 1 µm ($\bar{x}$ = 15 x 1 µm). No gamma conidia were observed.

Cultures. Colony growth felty with woolly tufts, buff (19''f) with greyish sepia (15''''i) patches. Reverse buff (19''f) with greyish sepia (15''''i) and fuscous black (7''''k) patches. Colony erumpent without growth zones.

Cardinal temperature requirements. Colonies obtained a maximum growth of 60-69 mm diam at 25 °C after 7 d in the dark. No growth occurred below 10 °C or above 35 °C.

Host. Vitis vinifera.

Distribution. Italy.

Culture examined. ITALY. *Vitis* sp., 1992, *F. Gobbo* (IMI 352882 = STE-U 2674).

Phomopsis amygdali (Del.) Tuset & Portilla, Can. J. Bot. 67: 1280. 1989.

Figs 47-53

Fusicoccum amygdali Del., Bull. Trimest. Soc. Mycol. Fr. XXI, 3: 184. 1905.

Phomopsis amygdalina Canonaco, Riv. Patol. Veg. XXVI: 157. 1936.

Pycnidia eustromatic, subepidermal, dark brown to black, separate, sparsely distributed, ampulliform to finger-like, hairy, multiloculate and convoluted, 700–800 µm wide and 400–600 µm tall, including necks, which were seldom observed. Pycnidial wall consisting of two regions of *textura angularis*; outer region prominent, dark brown, 3-4 cells thick, 10–40 µm wide; inner region brown, 4-5 cells thick, 20–40 µm. Extensive hyphal growth occurred around conidiomata. Pycnidia started to sporulate within 10 d on WAV. *Conidial mass* globose, mostly white, rarely yellow. *Conidiophores*: alpha conidiophores subcylindrical, 6–25 × 1–2 µm ($\bar{x}$ = 15 × 1.5 µm); beta conidiophores filiform, septate, branched, 6–75 × 1–2 µm ($\bar{x}$ = 33 × 1.5 µm). *Conidiogenous cells*:

alpha conidiogenous cells seldomly branched, collarette and periclinal thickening present, 3–15 × 1–2 µm ($\bar{x}$ = 10 × 1.5 µm); beta conidiogenous cells filiform, tapering towards the apex, collarette present, periclinal thickening prominent, 3–15 × 1–2 µm ($\bar{x}$ = 11 × 1.5 µm). *Conidia*: alpha conidia fusoid, apices acute, eguttulate, (4.5–)5–6(–8) × 1–2 µm (5.5 × 1.5 µm); beta conidia straight, slightly curved, 12–20 × 0.5–1 µm ($\bar{x}$ = 16 × 1 µm). Alpha and beta conidia can occur in equal proportions in the same pycnidium, or with either dominating. Gamma conidia rarely found, fusoid to subcylindrical, eggutulate, 8–11 × 1 µm ($\bar{x}$ = 9 × 1 µm).

Notes. Alpha conidium and colony morphology of the *Vitis* isolate match that reported by Farr *et al.* (1999) for fruit tree isolates. Tuset *et al.* (1989) description differed in that the alpha conidia were biguttulate, whereas our isolate lacked guttules. However, Delacroix (1905) noted in his description of *Fusicoccum amygdali* that guttulation varied from none to two. Apart form these morphological differences, however, the phylogenetic analyses showed that the South African isolate clustered with 100% boostrap support with *P. amygdali* from peach trees. Although cross pathogenicity studies also supported this isolate as being pathogenic to peach trees, this disease has not yet been recorded on the latter host in South Africa.

Cultures. Colony growth woolly, predominantly pale olivaceous grey (21""d) on surface, with lighter shades to white tufts and a few patches of greyish sepia (15""i) and olivaceous buff (21'"b). Reverse olivaceous grey (21""i) with patches of iron grey (24""k). Felty to woolly texture, colony raised with no growth zones.

Cardinal temperature requirements. Colonies obtained maximum growth of 105 mm diam at 25 °C after 7 d in the dark. No growth occurred below 10 °C or above 35 °C.

Host. *Prunus armeniaca* L., *P. dulcis* (Mill.) A.A. Webb., *P. persica* (L.) Batsch, *Vitis vinifera*.

Distribution. Italy, South Africa, U.S.A.

Culture examined. SOUTH AFRICA. WESTERN CAPE: Constantia. Pinotage grapevine, Mar. 1997, *L. Mostert*, (STE-U 2632) (specimen PREM 56460).

DISCUSSION

In this study the *Phomopsis* isolates associated with cane and leaf spot disease of grapevines were grouped into six taxa. Various studies have previously determined that the delimitation of species of *Phomopsis* based on morphological criteria is unreliable because of character plasticity (Hahn, 1930; Van der Aa *et al.*, 1990; Rehner & Uecker, 1994; Uddin *et al.*, 1997). Varying emphasis has subsequently been placed on different morphological structures. Phillips (2000) considered conidiophore size, septation and branching to be important additional characters. Conidiophore structure has, however, been neglected in most descriptions of *Phomopsis* species, and was also not included in Wechtl's (1990) synoptic key of *Phomopsis* species. Farr *et al.* (1999) also reported conidiophores of *Phomopsis* to be variable. Although some variation was observed *in vitro* for conidiophore structure in the present study, general trends in size and aggregation could be observed that did indicate differences between taxa.

The production of beta conidia was variable as reported by (Uecker, 1988), and influenced by factors such as dextrose and nitrogen concentration, temperatures above 30 C, carbon dioxide saturated atmosphere and the presence of *Bacillus subtilis* (Nitimargi, 1935; Pine, 1957; Punithalingam, 1979). Alpha conidium shape, guttulation and dimensions were used as distinguishing features for the delimitation of taxa 1 and 2 on grapevines (Merrin *et al.*, 1995). However, shape and guttulation have been observed to change with age (Wechtl, 1990). These characters proved to be relatively stable for the different taxa when using standardised media and growth conditions. Based on these findings, it became apparent that it would be more dependable to combine morphological data with cultural, pathogenicity, physiological and molecular characteristics (Hahn, 1930; Shivas *et al.*, 1991; Anderson, 1992; Merrin *et al.*, 1995).

The nucleotide sequences of the 5.8S rRNA gene and ITS1 and ITS2 flanking regions have been successfully used to study the phylogenetic relationships of species in many diverse genera (Carbone & Kohn, 1993; Nakasone & Sytsma, 1993; Zambino & Szabo, 1993; Yao *et al.*, 1999). Rehner and Uecker (1994) used this genomic area to elucidate the host specificity of several different *Phomopsis* species. The genetic uniqueness of three pathogenic *Phomopsis* isolates from peach, plum and pear was also

confirmed by comparing their ITS1 and ITS2 regions (Uddin *et al.*, 1998). However, based on similar data, Farr *et al.* (1999) were unable to determine the broader relationships among different *Phomopsis* species, and suggested that a more conserved rDNA or protein coding gene may prove to be more informative. In the present study sequence data from this region was combined with that obtained from the 5' end partial sequence of the mtSSU. Although these data could be used to address the questions posed in the present study, it is possible that other, more informative sites would be able to provide a finer division of morphologically variable isolates presently still treated under the same epithet.

The cultural, morphological and pathological differences between *P. viticola* and *D. perjuncta* (taxon 1), *P. amygdali* and taxon 3 were confirmed by the separate groupings obtained in the phylogenetic analyses. Two *Phomopsis* isolates, STE-U 2674 (from grapevines) and STE-U 2680 (from roses) did not cluster with any other species, suggesting them to be different. The morphological differences observed in the alpha conidium and cultural growth pattern of the grapevine isolate suggested it to be distinct. Because no name in the present study could be attributed to this species, it was subsequently designated as taxon 5.

Results from pathogenicity studies indicated that taxa 1 and 3 were non-pathogenic, whereas taxon 2 was pathogenic on Chenin blanc shoots. In comparison with the inoculation studies done on Riesling, differences occurred in lesion lengths. These differences should be viewed in relation to the difference in age of the green shoots used and cultivar, even though both these cultivars are highly susceptible (part 1). It would therefore be important to use Chenin blanc shoots to test for the absence of lesion formation when identifying the different taxa within in the *Phomopsis viticola* complex.

Although host plants have been used in the past as a key feature in the identification of species of *Phomopsis* (Brayford, 1990; Wechtl, 1990), results obtained in recent studies (Rehner & Uecker, (1994) suggested that *Phomopsis* species could infect more than one host, or that host switching occurred frequently during speciation. These observations were also confirmed by results obtained in the present study. It is, therefore, necessary to establish whether *Phomopsis* spp. have a wide host range,

allowing other agricultural crops and plants grown nearby to be a source of inoculum. Host specificity data would also seriously influence estimates of the number of *Phomopsis* species that exist, since fewer species would be needed where the host range is known of morphologically similar species (Uecker, 1988).

Species of *Phomopsis* from pear and protea clustered with grapevine isolates of taxon 3. Cross pathogenicity studies with the pear (STE-U 2656) and protea (STE-U 2659) isolates onto grapevines were, however, inconclusive. The other *Phomopsis* sequence from pear (U 91717) included in the phylogenetic analysis grouped with a plum isolate, and both isolates were found by Uddin *et al.* (1997) to cause cankers when inoculated onto peach shoots. Stem inoculations confirmed that the grapevine *P. amygdali* isolate could be a serious pathogen of peach. Uddin and Stevenson (1998) showed that the *Phomopsis* species from peach had a wide host range, being able to infect plum, pear and apple shoots. These trees could thus also act as hosts for inoculum of *P. amygdali*. Pathogenicity studies on plum and pear trees would, however, still be required to confirm this. Some species of *Phomopsis* do appear to be host specific, as reported by Rehner *et al.* (1994) for the *Phomopsis* species isolated from blueberry (*Vaccinium*). A similar senario appears to be true for *P. viticola* (taxon 2) (Fig. 1) on grapevines.

From the pathogenicity study it was evident that taxon 2 was the pathogenic species and taxa 1 and 3 non-pathogenic. Although taxon 1 did cause some discolouration of green shoots in the pathogenicity tests in part 2 of this study, this can be attributed to the different age of shoots used. Furthermore, taxa 1 and 3 were also not associated with typical cane and leaf spot disease symptoms.

In conclusion, based on material collected in Australia, Merrin *et al.* (1995) characterized four taxa in the *Phomopsis viticola*-complex of grapevines. Taxon 2 (*P. viticola*) was found to be the causal organism of Phomopsis cane and leaf spot disease, and was also neotypified in this study. Three additional species, *D. perjuncta* (taxon 1), *P. amygdali* and taxon 3 were also found to be present in South Africa. Furthermore, a distinct isolate from Italy was described as taxon 5 in the *P. viticola*-complex. Although taxon 4 was not found in South Africa, an Australian culture representing this taxon was

found to be a species of *Libertella*, and should therefore be excluded from this complex. *P. amygdali*, which is associated with Phomopsis shoot blight of peach in the U.S.A. was also newly reported from South Africa, where it occurs on grapevines. Other than adding valuable information to the *P. viticola*-complex on grapevines, this study has once again reiterated the importance of integrating molecular techniques with the identification of *Phomopsis* spp., and underlined the effects such identifications could have on agriculture and export.

KEY TO TAXA IN THE *PHOMOPSIS VITICOLA*-COMPLEX ON GRAPEVINES

1. Colonies slow growing, felty; conidiophores seldomly branched; alpha conidia fusoid-ellipsoidal, (7–)9.5–10.5(–13) ($\bar{x}$ = 10 µm) long..... *P. viticola*

1. Colonies fast growing, woolly; conidiophores prominently branched; alpha conidia fusoid..... 2

2. Alpha conidia with narrowly acute apices, (8–)10–11(–13) ($\bar{x}$ = 10 µm) long..... taxon 5

2. Alpha conidia tapering to broadly acute or rounded apices..... 3

3. Causing lesions on peach and grapevine shoots; alpha conidia (4.5–)5–6(–8) ($\bar{x}$ = 5.5 µm) long..... *P. amygdali*

3. Not causing lesions when inoculated on hosts above..... 4

4. Teleomorph produced in culture; alpha conidia 5–5.5(–6) x 1.5–2 µm ($\bar{x}$ = 5.5 µm) long..... *D. perjuncta*

4. Teleomorph not produced in culture; alpha conidia (4–)6.5–7(–10) x (1.5–)2(–2.5) µm ($\bar{x}$ = 7 µm) long..... taxon 3

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Table 1. Chronological description of *Phomopsis* and related species from grapevines.

Species	Alpha conidium morphology			Reference
	Length x width (µm)	Shape	Guttulation	
Phoma viticola Sacc.	7 x 4 µm	Ellipsoid	Eguttulate	Saccardo (1880)
Phoma viniferae Cooke	7 x 4 µm	-	Eguttulate	Cooke (1885)
Fusicoccum viticolum Reddick	6.3-11.2 x 1.7-2.8 µm	Fusoid	Multi- to biguttulate	Reddick (1909)
Phomopsis cordifolia Brunaud	7-9 x 2.5-3 µm			Brunaud (1912)
Phomopsis viticola (Sacc.) Sacc.	Same as for *Phoma viticola* Sacc.			Saccardo (1915)
Phomopsis ampelopsidis Petrak	6-11 x 2-3 µm	Bacillus to fusoid	Bi- to triguttulate	Petrak (1916)
Phomopsis viticola (Sacc.) Grove	7-10 x 2-2.5 µm	Ellipsoid-fusoid	-	Grove (1917)
Phomopsis viticola (Sacc.) Sacc. var. *ampelopsidis* Grove	8-9 x 2 µm	Ellipsoid-fusoid	Biguttulate	Grove (1919)
Phomopsis viticola (Sacc.) Sacc.	8-9 x 2 µm	Ellipsoid-fusoid	Biguttulate	Saccardo (1931)
Phomopsis longiparaphysata Uecker & Ker-Chung	(5-)6-7(-11) x 2-2.5(-3.5) µm	Fusoid-ellipsoid	Biguttulate to multiguttulate	Uecker and Ker-Chung (1992)
Phomopsis viticola taxon 1	(3.8-)4.8-7.2(-9) x (1-)1.4-2.2(-2.9) µm	Ellipsoid to oblong	Biguttulate	Merrin *et al.* (1995)

Continued

Table 1. (continued)

Species	Alpha conidium morphology			Reference
	Length x width (µm)	Shape	Guttulation	
Phomopsis viticola taxon 2	(-7)8.0-11.8(-14) x (1.6-)2.0-3.2(-3.9) µm	Fusoid-ellipsoid	Multiguttulate	Merrin *et al.* (1995)
Phomopsis viticola taxon 3	(5.5-)6.2-8.8(-9.2) x (1.3-)1.5-2.2(-2.4) µm			Merrin *et al.* (1995)
Phomopsis viticola taxon 4	No alpha conidia			Merrin *et al.* (1995)
Phomopsis vitimegaspora Ker-Chung & Lii-Sin	(10-)13-8(-22) x (3-)4-5(-6) µm	Fusoid-ellipsoid	Multiguttulate	Ker-Chung and Lii-Sin (1998)

Table 2. *Phomopsis* species included in the sequence analysis.

Accession no.	Host	Origin	Taxon	Area sequenced
STE-U 2655	*Vitis vinifera*	South Africa	*D. perjuncta* (Taxon 1)	I[1], M[2]
STE-U 2676	*Vitis vinifera*	Australia	*D. perjuncta* (Taxon 1)	I, M
STE-U 2677	*Vitis vinifera*	Portugal	*D. perjuncta* (Taxon 1)	I, M
STE-U 2638	*Vitis vinifera*	South Africa	*P. viticola* (Taxon 2)	I
STE-U 2641	*Vitis vinifera*	South Africa	*P. viticola* (Taxon 2)	I
STE-U 2642	*Vitis vinifera*	South Africa	*P. viticola* (Taxon 2)	I, M
STE-U 2646	*Vitis vinifera*	South Africa	*P. viticola* (Taxon 2)	I
STE-U 2648	*Vitis vinifera*	South Africa	*P. viticola* (Taxon 2)	I
STE-U 2660	*Vitis vinifera*	France	*P. viticola* (Taxon 2)	I
STE-U 2662	*Vitis vinifera*	Australia	*P. viticola* (Taxon 2)	I, M
STE-U 2666	*Vitis vinifera*	Portugal	*P. viticola* (Taxon 2)	I, M
STE-U 2669	*Vitis vinifera*	Portugal	*P. viticola* (Taxon 2)	I
STE-U 2671	*Vitis vinifera*	Italy	*P. viticola* (Taxon 2)	I
STE-U 2672	*Vitis vinifera*	Turkey	*P. viticola* (Taxon 2)	I
STE-U 2673	*Vitis vinifera*	America	*P. viticola* (Taxon 2)	I
STE-U 2679	*Vitis vinifera*	America	*P. viticola* (Taxon 2)	I

Continued

Table 2. (continued)

Accession no.	Host	Origin	Taxon	Area sequenced
STE-U 2654	*Vitis vinifera*	South Africa	Taxon 3	I, M
STE-U 2656	*Pyrus* sp.	South Africa	Taxon 3	I, M
STE-U 2659	*Protea* sp.	South Africa	Taxon 3	I, M
STE-U 2664	*Vitis vinifera*	Australia	Taxon 3	I, M
STE-U 2661	*Vitis vinifera*	Australia	Taxon 3	I, M
STE-U 2668	*Vitis vinifera*	Portugal	Taxon 3	I, M
STE-U 2632	*Vitis vinifera*	South Africa	*P. amygdali*	I, M
STE-U 2675	*Vitis. Vinifera*	Taiwan	*Phomopsis vitimegaspora*	I
STE-U 2657	*Prunus* sp.	South Africa	*D. ambigua*	I
STE-U 3390	*Prunus* sp.	South Africa	*D. ambigua*	I
STE-U 2674	*Vitis vinifera*	Italy	Taxon 5	I
STE-U 2680	*Rosa* sp.	South Africa	*Phomopsis* sp.	I, M

[1]I = 5.8S and flanking ITS1 and ITS2 rDNA.

[2]M = Mitochondrial small subunit rDNA.

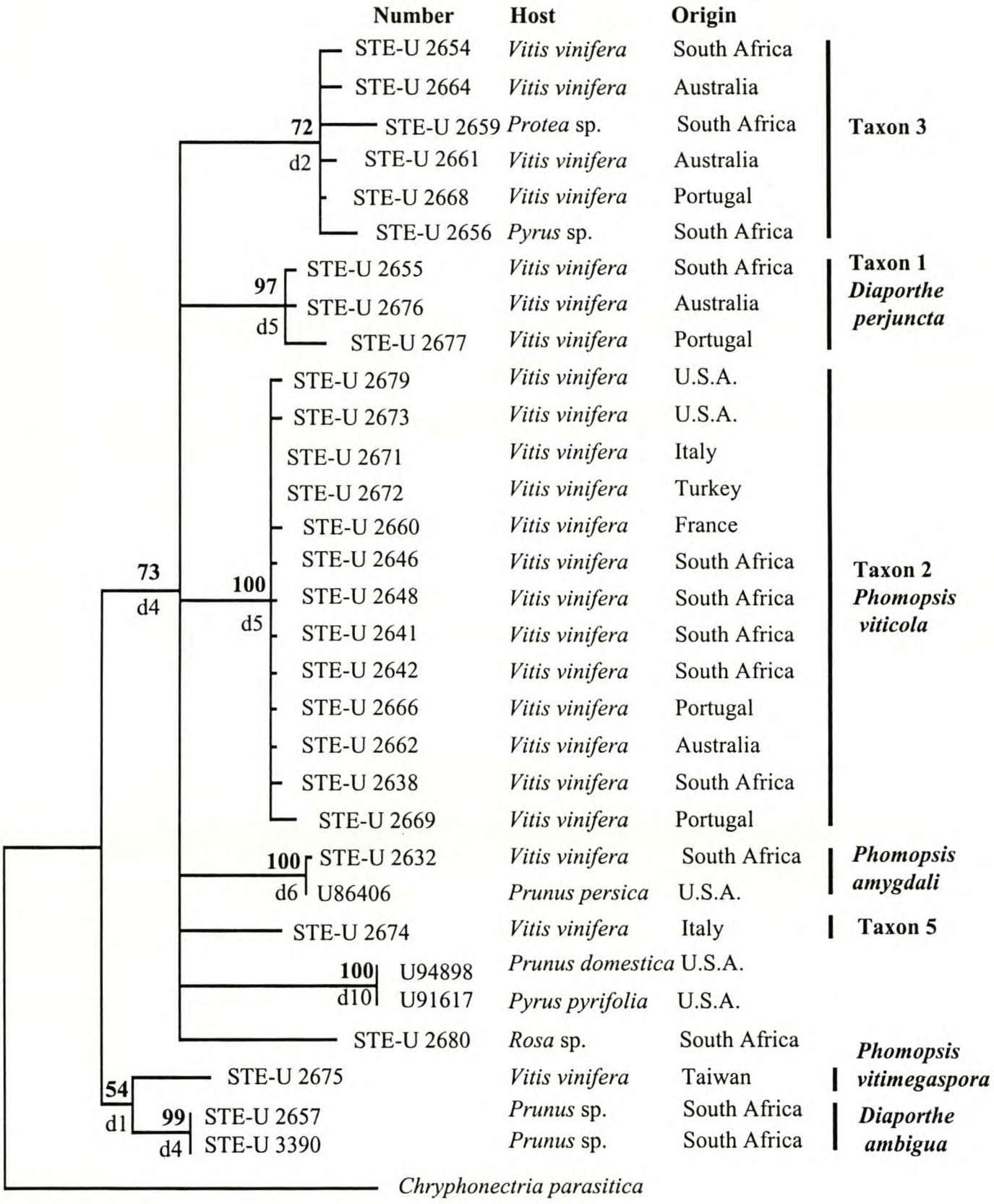


Fig. 1. One of 39 MPTs resulting from maximum parsimony analysis (heuristic search option) of aligned sequences of the 5.8S rRNA gene, flanking ITS1 and ITS2 regions (length = 356 steps, CI = 0.629, RI = 0.749, RC = 0.471 and HI = 0.371). Bootstrap values above 52% are shown as well as decay indices. The sequence of *Cryphonectria parasitica* was used as outgroup.

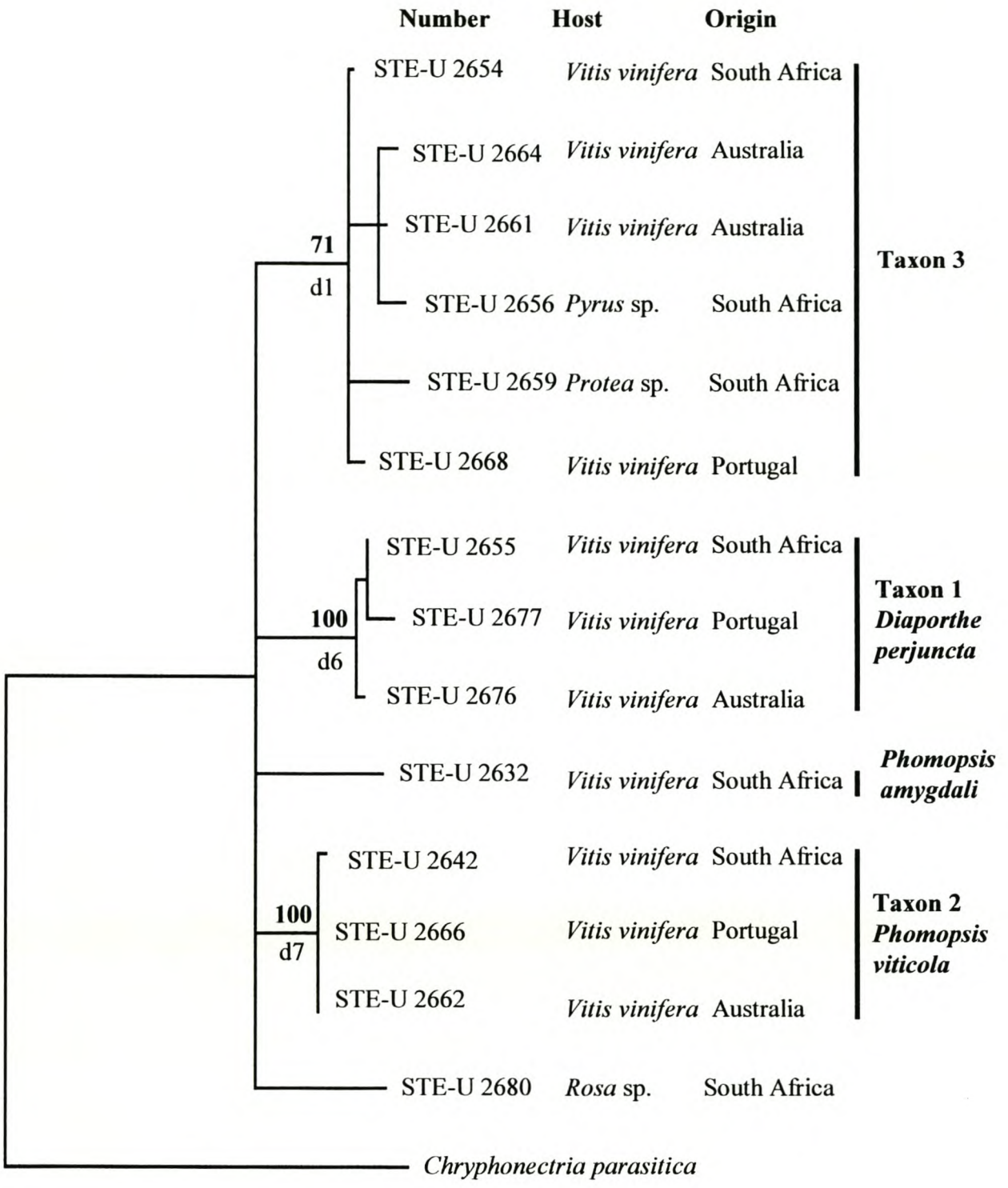
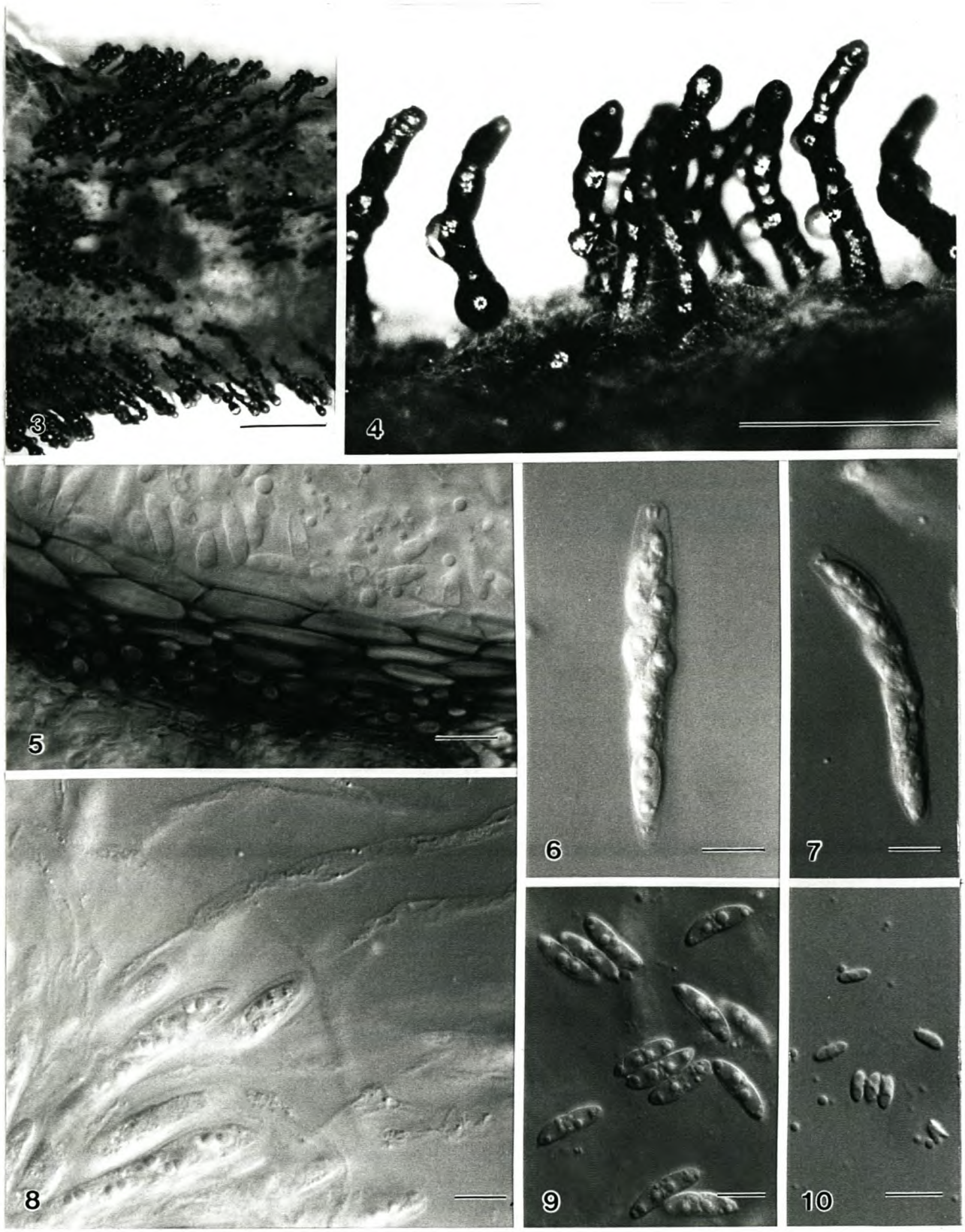
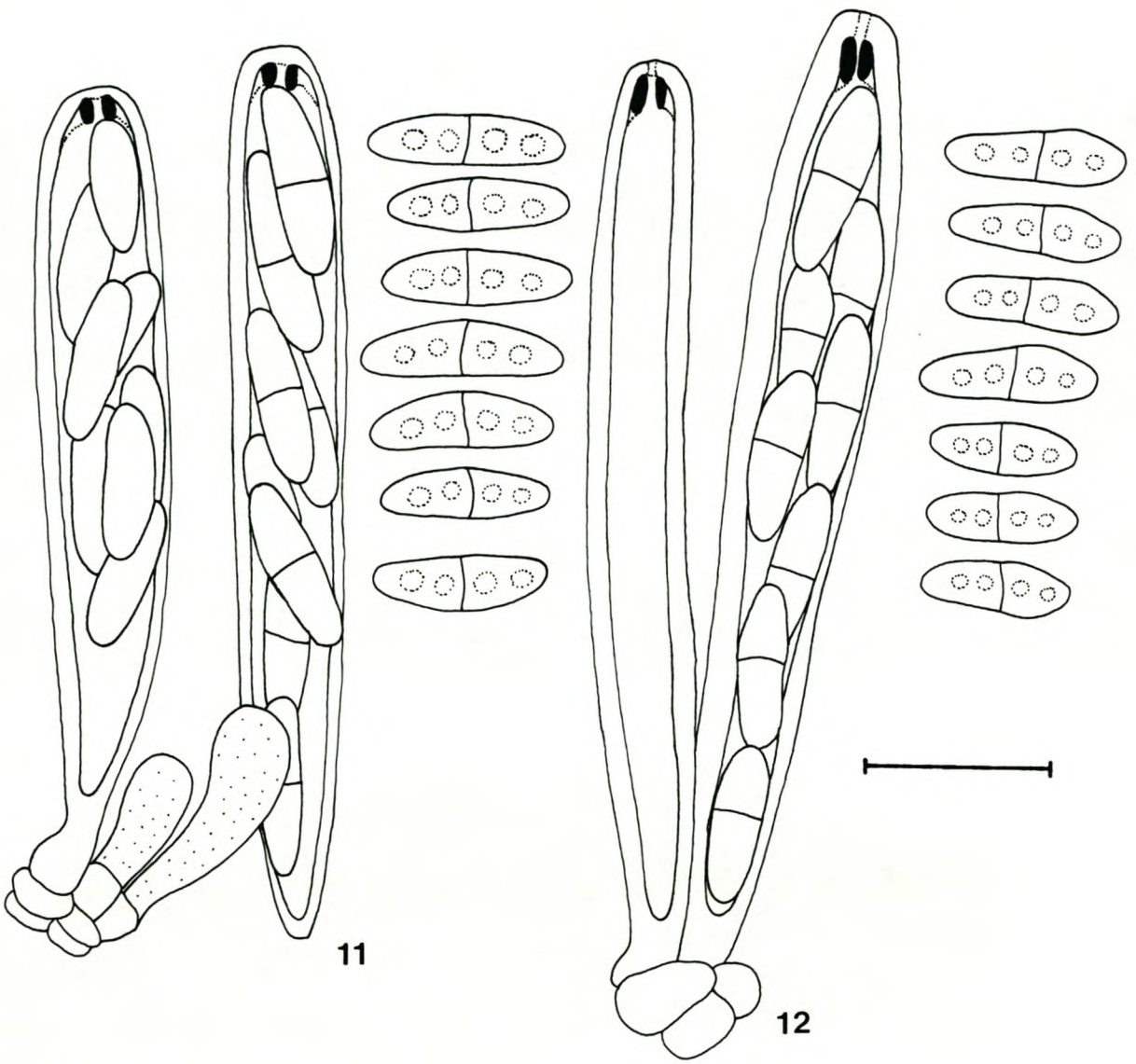


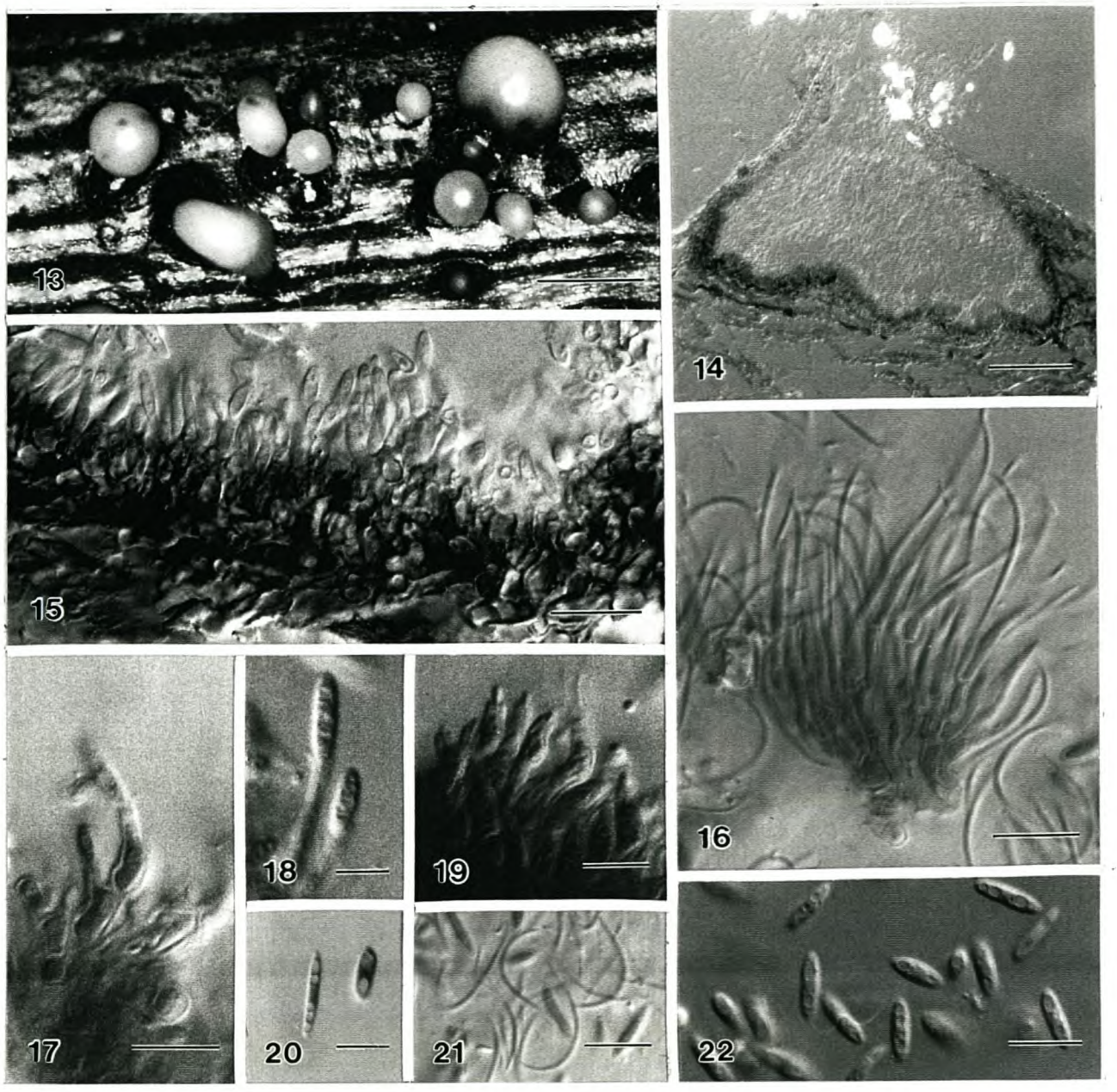
Fig. 2. One of 24 MPTs resulting from maximum parsimony analysis (heuristic search option) of aligned sequences of the 5.8S rRNA gene and flanking ITS1 and ITS2 and the mtSSU regions (length = 347 steps, CI = 0.790, RI = 0.723, RC = 0.571 and HI = 0.210). Bootstrap values above 52% are shown as well as decay indices. The sequence of *Cryphonectria parasitica* was used as outgroup.



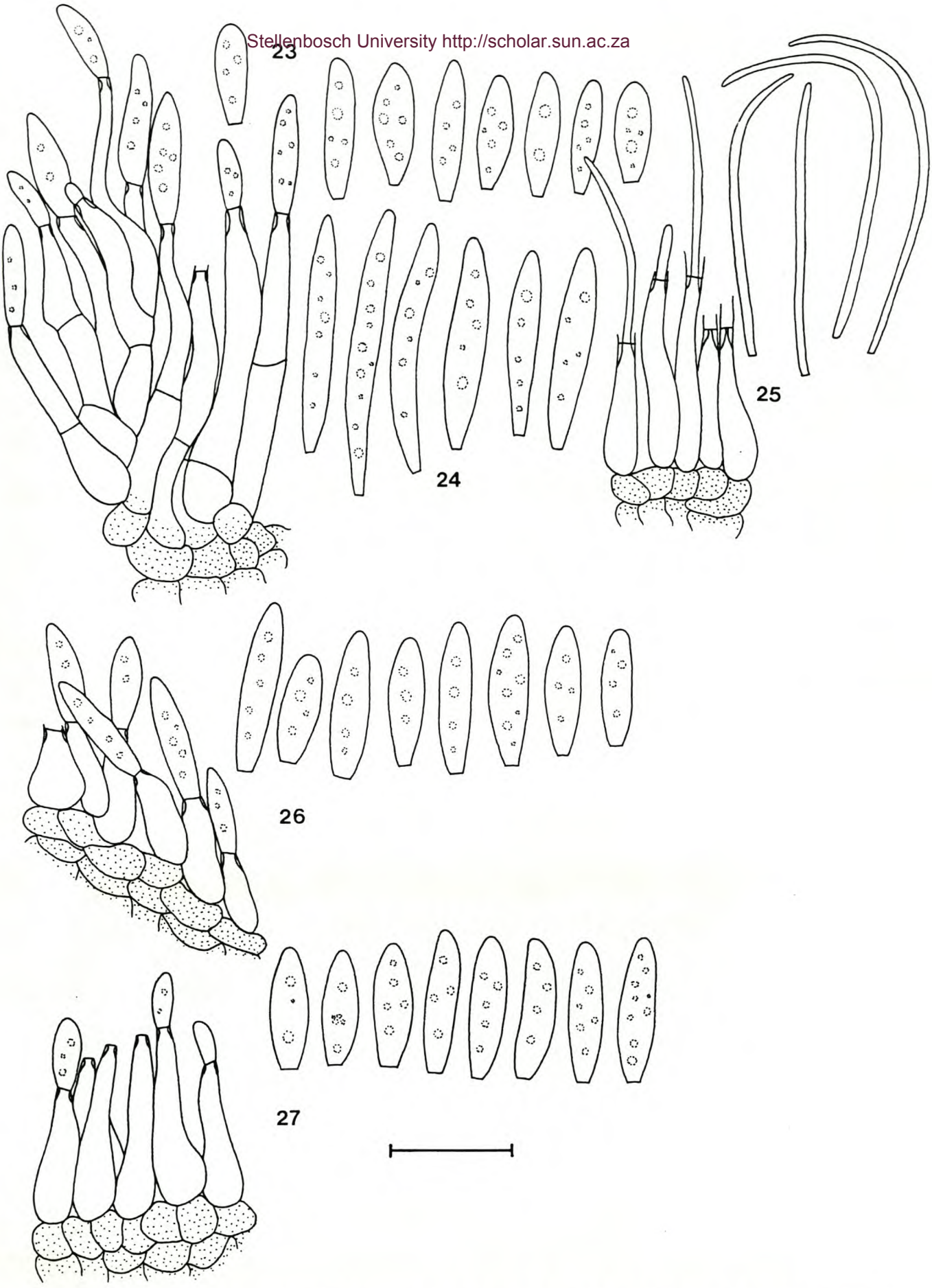
Figs 3-10. *Diaporthe perjuncta* (taxon 1) with *Phomopsis* anamorph (STE-U 2655). **Figs 3, 4.** Perithecia on vine canes. Bars = 1000 µm. **Fig. 5.** Longitudinal section through perithecial wall. **Figs 6, 7.** Asci. **Fig. 8.** Asci with paraphyses. **Fig. 9.** Ascospores. **Fig. 10.** Alpha conidia. Bars = 10 µm.



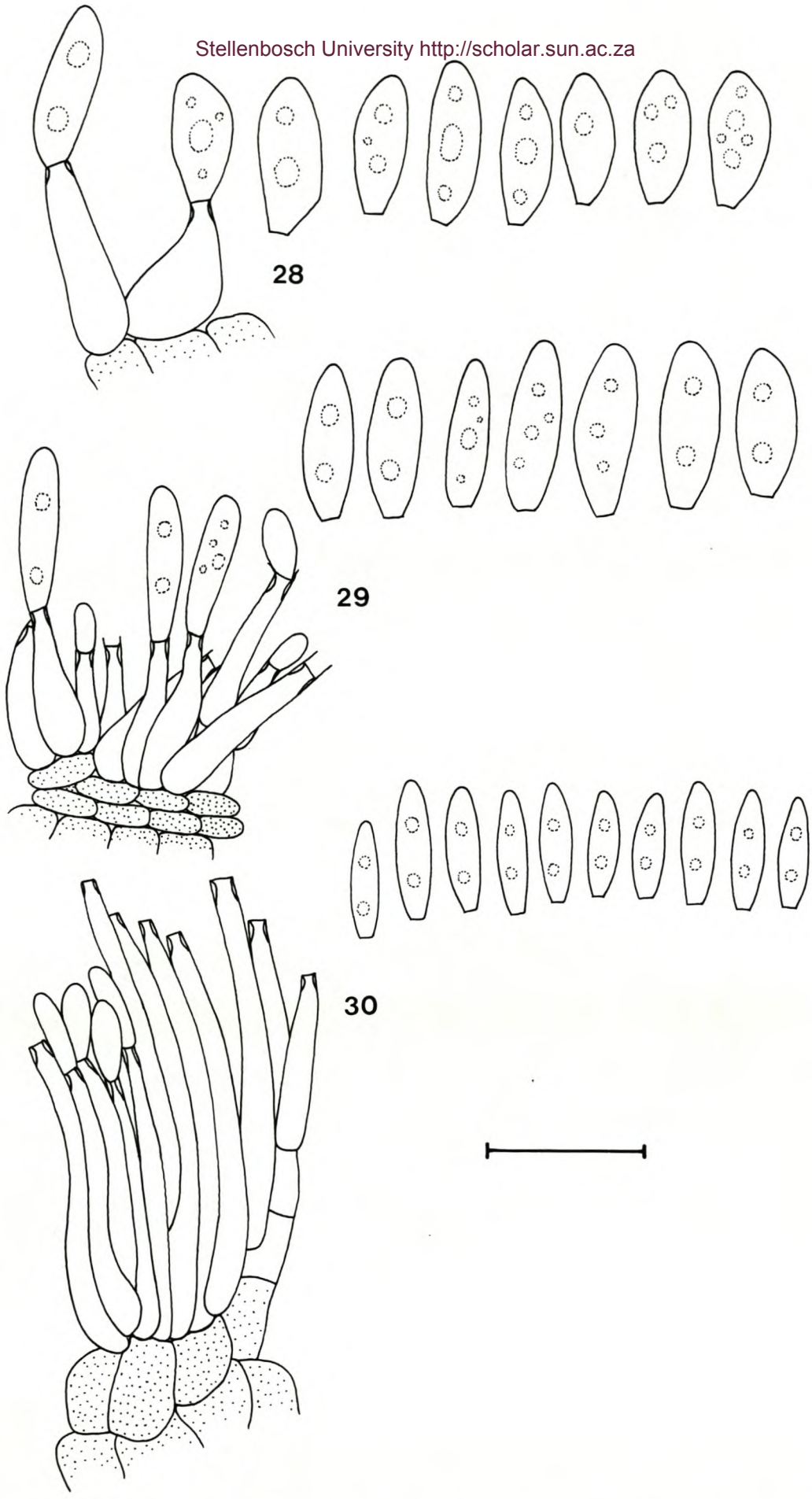
Figs 11, 12. Asci and ascospores of *Diaporthe perjuncta* from a South African (STE-U 2655) and Portuguese (STE-U 2677) collection, respectively. Bar = 10 µm.



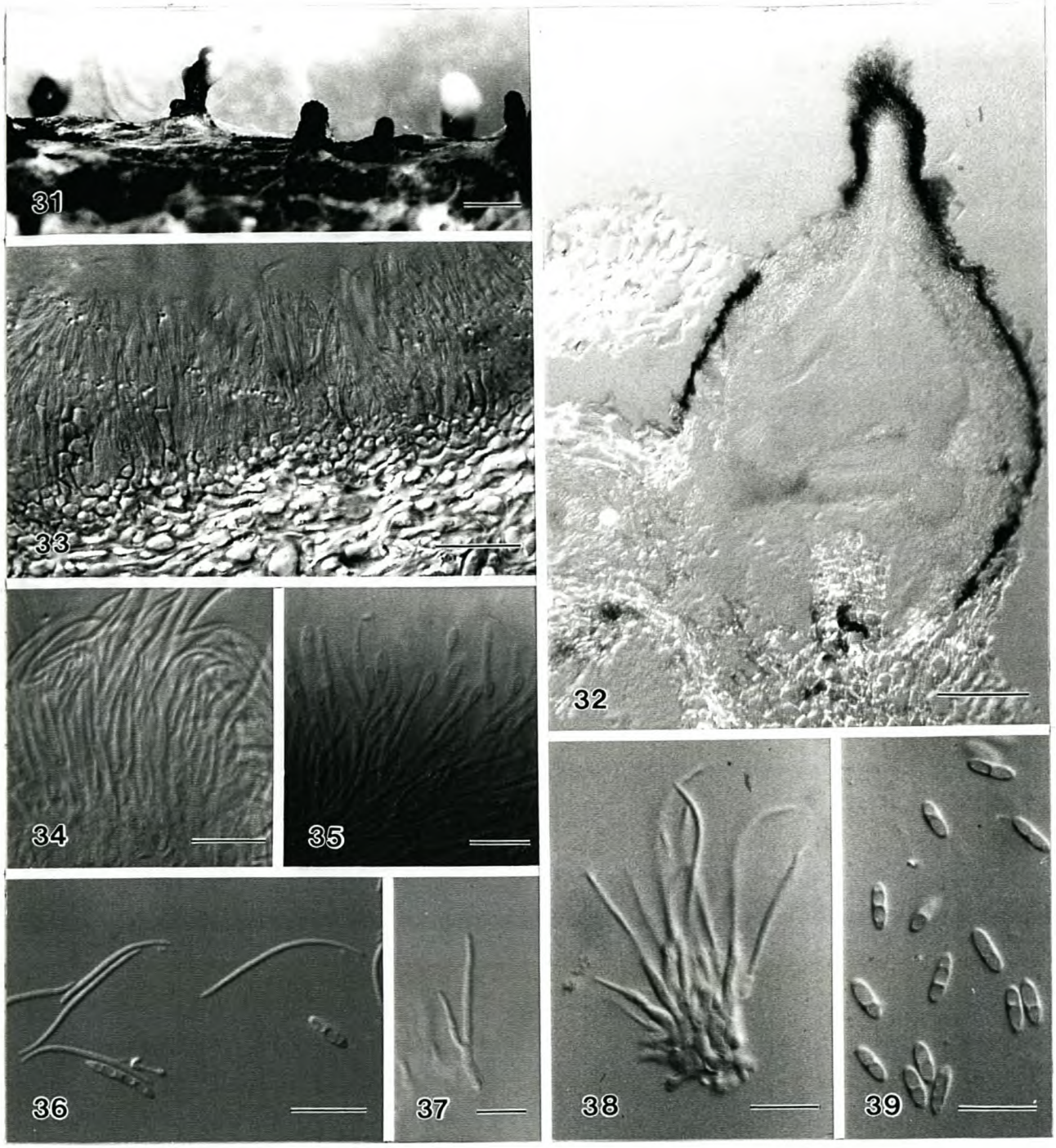
Figs 13-22. *Phomopsis viticola* (taxon 2) (STE-U 2660). **Fig. 13.** Pycnidia on vine cane. Bar = 1000 µm. **Fig. 14.** Longitudinal section through pycnidium. Bar = 100 µm. **Fig. 15.** Section through pycnidium wall. **Fig. 16.** Beta conidiophores and conidiogenous cells. **Fig. 17.** Branched alpha conidiophore. **Figs 18, 19.** Alpha conidiogenous cells with collarettes. **Fig. 20.** Alpha and gamma conidia. **Fig. 21.** Alpha and beta conidia. **Fig. 22.** Multiguttulate alpha conidia. Bars = 10 µm.



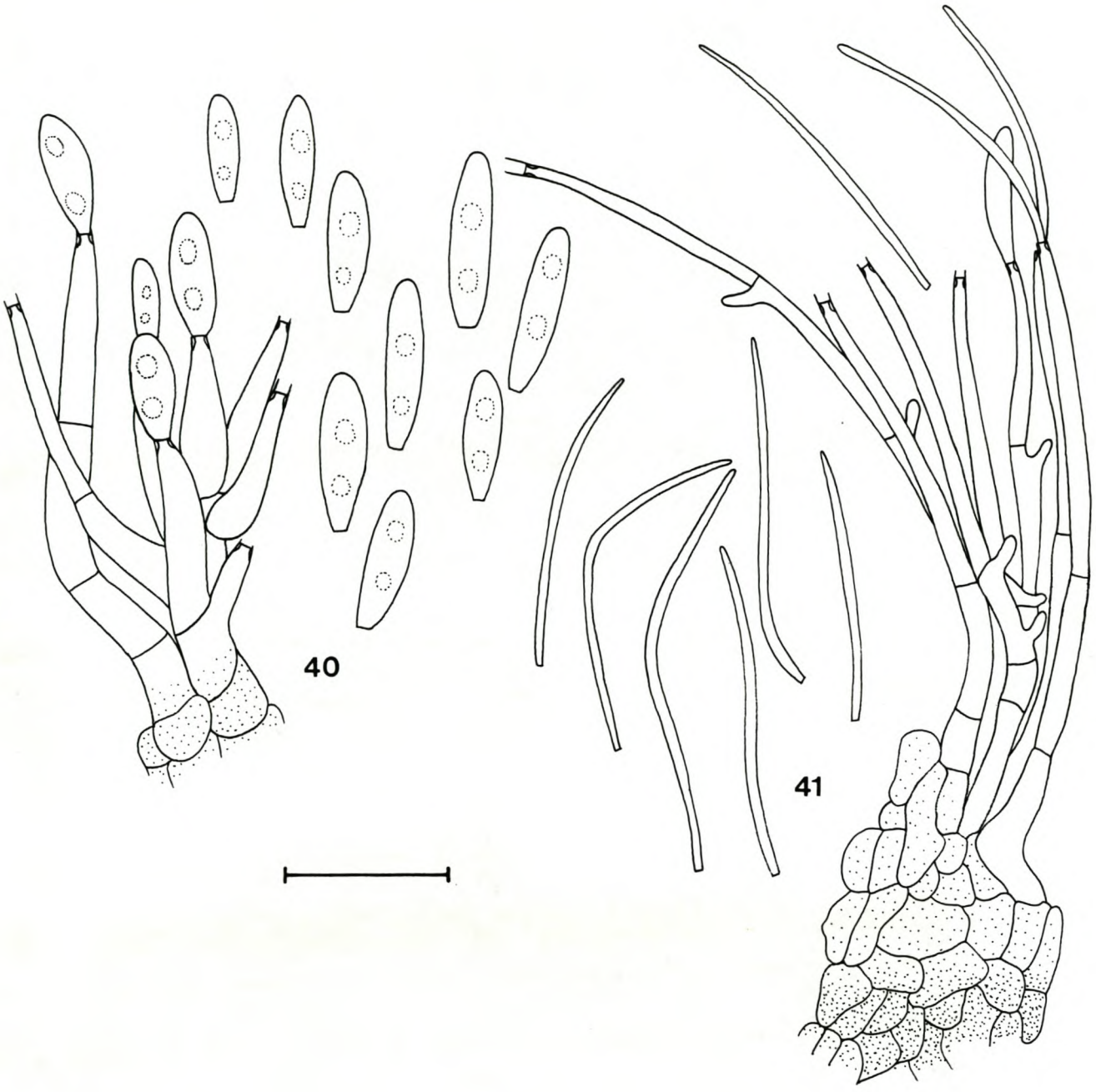
Figs 23-27. *Phomopsis viticola* (taxon 2). **Fig. 23.** Alpha conidiophores, conidiogenous cells and conidia. **Fig. 24.** Gamma conidia. **Fig. 25.** Beta conidiogenous cells and conidia (STE-U 2660). **Fig. 26.** Alpha conidiogenous cells and conidia (STE-U 2673). **Fig. 27.** Alpha conidiogenous cells and conidia (STE-U 2642). Bar = 10 µm.



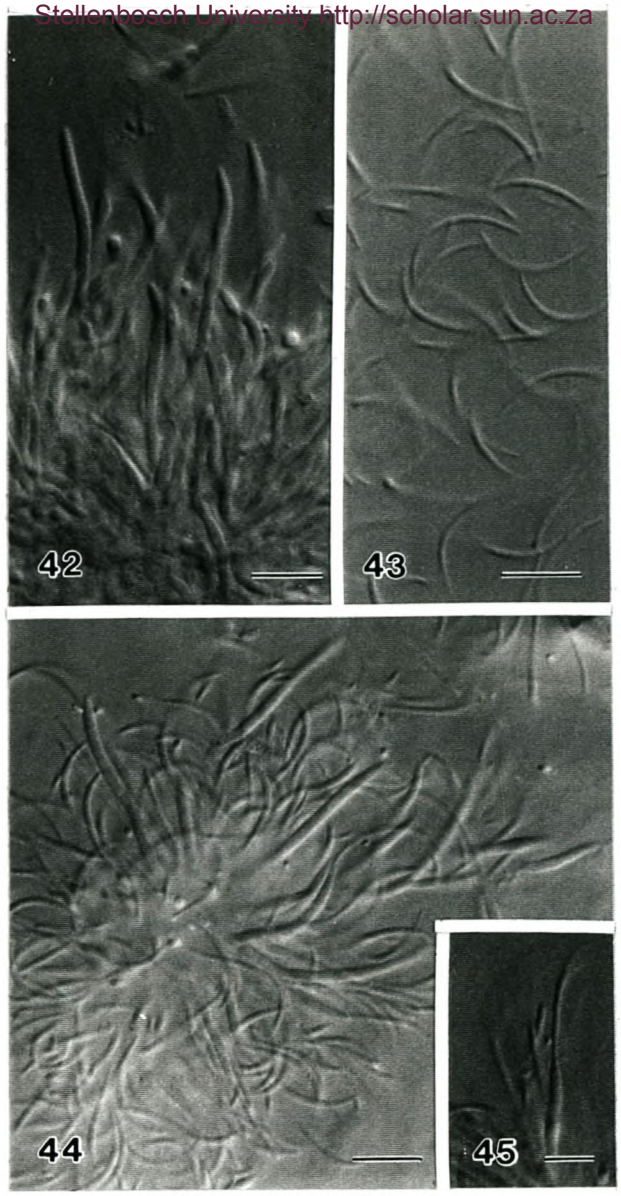
Figs 28-30. Alpha conidia and conidiogenous cells of *Phomopsis* spp. **Fig. 28.** *P. viticola* (H.D. House No. 149, PAD). **Fig. 29.** *P. viticola* (type of *P. ampelina*, K 58408). **Fig. 30.** *Phomopsis ampelopsidis* (BPI 358265). Bar = 10 µm.



Figs 31-39. *Phomopsis* sp. (taxon 3) (STE-U 2654). **Fig. 31.** Pycnidia on vine cane. Bar = 1000 µm. **Fig. 32.** Longitudinal section through pycnidium. Bar = 100 µm. **Fig. 33.** Section through pycnidial wall. **Fig. 34.** Branched beta conidiophores. **Fig. 35.** Alpha conidiophores. **Fig. 36.** Alpha, beta and gamma conidia. **Fig. 37.** Branched alpha conidiophore. **Fig. 38.** Beta conidiogenous cells. **Fig. 39.** Alpha conidia. Bars = 10 µm.



Figs 40, 41. *Phomopsis* sp. (taxon 3) (STE-U 2654). **Fig. 40.** Alpha conidiophores and conidia. **Fig. 41.** Alpha and beta conidiophores and beta conidia. Bar = 10 µm.



Figs 42-45. *Libertella* sp. (taxon 4) (STE-U 3313). **Fig. 42.** Paraphyses. **Fig. 43.** Conidia. **Fig. 44.** Paraphyses and conidia. Bars = 10 µm. **Fig. 45.** Conidiogenous cells. Bar = 5 µm.

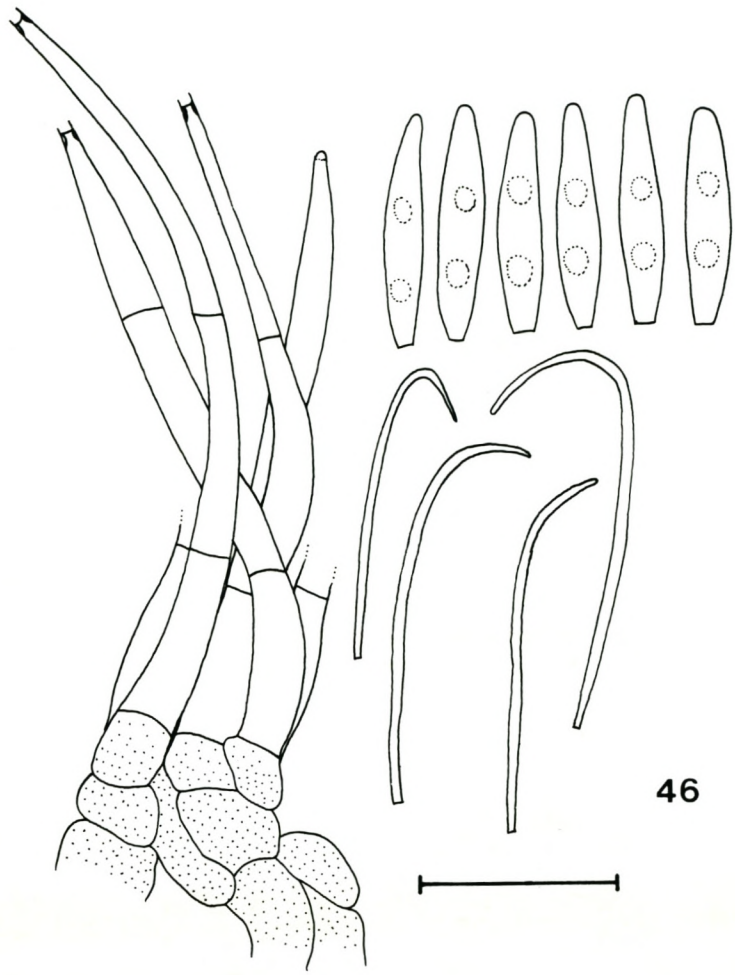
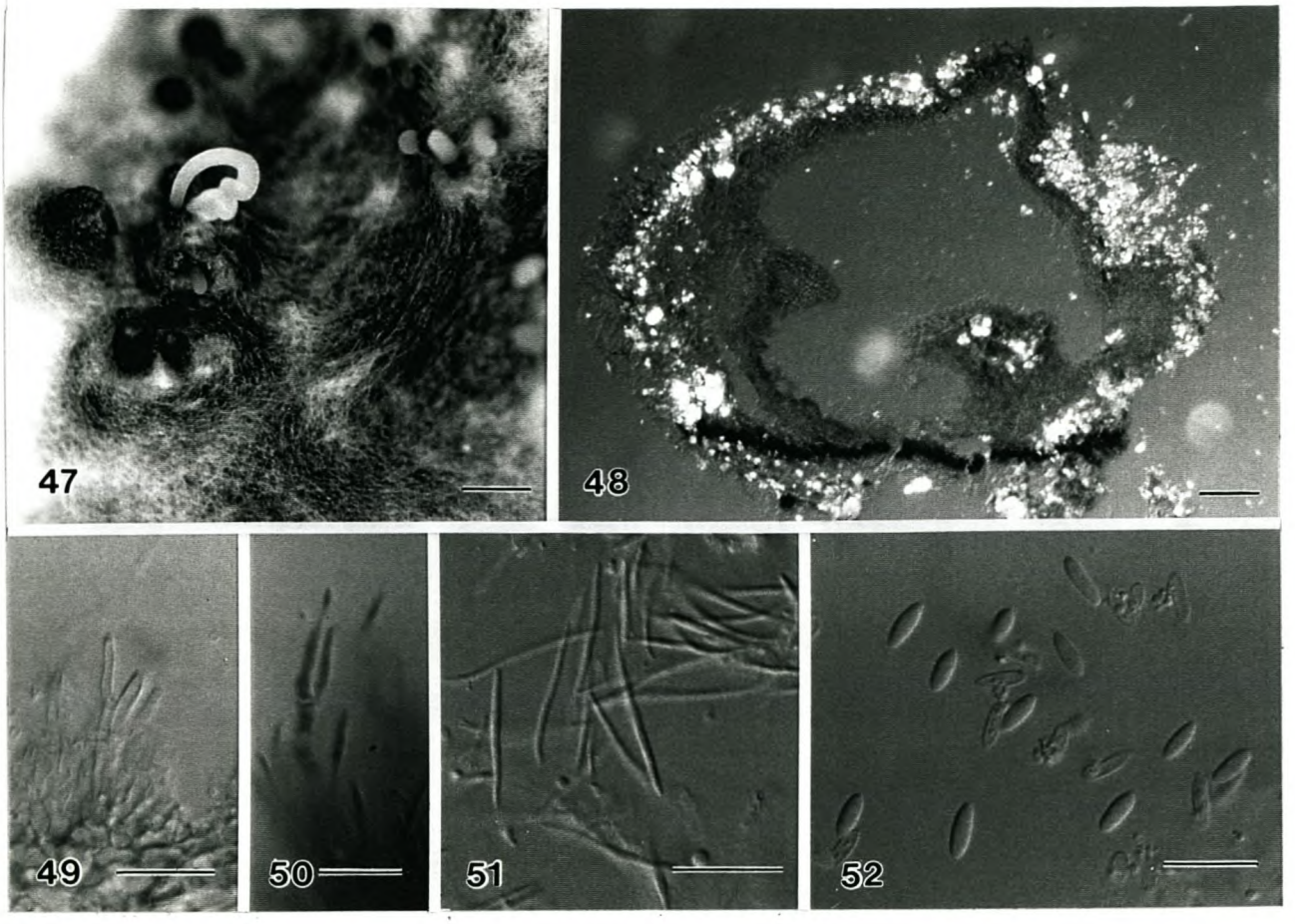


Fig. 46. Conidiophores, alpha and beta conidia of *Phomopsis* sp. (taxon 5) (STE-U 2674). Bar = 10 μm.



Figs 47-52. *Phomopsis amygdali* (STE-U 2632). **Fig. 47.** Pycnidia on vine cane. Bar = 1000 µm. **Fig. 48.** Longitudinal section through pycnidium. Bar = 100 µm. **Figs 49, 50.** Branched alpha conidiophores. **Fig. 51.** Beta conidia. **Fig. 52.** Alpha conidia. Bars = 10 µm.

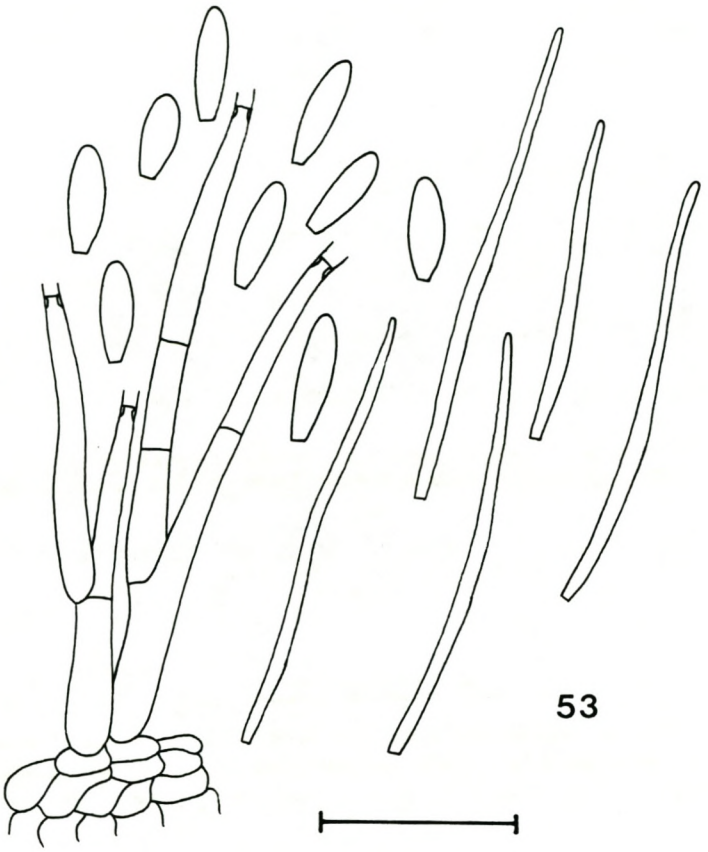


Fig. 53. Branched alpha conidiophores, alpha and beta conidia of *Phomopsis amygdali* (STE-U 2632). Bar = 10 µm.

4. *IN VITRO* EFFECTIVENESS OF FUNGICIDES TO *PHOMOPSIS VITICOLA*

ABSTRACT

Phomopsis viticola is the cause of Phomopsis cane and leaf spot disease of grapevines. Two different taxa within the *P. viticola* complex were isolated from grapevines in the Western Cape. Taxon 2 was associated with the typical Phomopsis cane and leaf spot symptoms. In contrast, taxon 1 was mostly isolated as an endophyte from grapevines and was not associated with the disease. The effectiveness of nine fungicides (azoxystrobin, flusilazole, folpet, fosetyl-Al + mancozeb, kresoxim-methyl, mancozeb, penconazole, spiroxamine and trifloxystrobin) to isolates of *P. viticola* were determined *in vitro* using the mycelial growth test. Additionally, azoxystrobin, folpet, kresoxim-methyl, mancozeb, propineb and trifloxystrobin were tested for their ability to inhibit spore germination *in vitro*. Ten isolates of taxon 2 and three of taxon 1 were used in the mycelium inhibition tests, and five isolates of taxon 2 in the spore germination tests. The effective concentration at which mycelial growth was inhibited by 50% and at which 50% of the spores (EC_{50} value) were inhibited from germinating, was calculated for each isolate/fungicide combination. In the mycelium growth test flusilazole, penconazole and trifloxystrobin gave significantly better inhibition at lower concentrations than folpet and fosetyl-Al + mancozeb. Azoxystrobin, flusilazole, kresoxim-methyl, penconazole, spiroxamine and trifloxystrobin inhibited mycelial growth equally well. There was also no significant difference between the mean EC_{50} values obtained for azoxystrobin, kresoxim-methyl and mancozeb. Flusilazole and penconazole inhibited mycelial growth at the lowest mean EC_{50} values obtained. Kresoxim-methyl and trifloxystrobin inhibited spore germination at significantly lower concentrations than folpet or mancozeb. Folpet required the highest concentration to inhibit 50% germination and was significantly different from mancozeb and propineb. There were also no significant differences between mancozeb, propineb and azoxystrobin, even though mancozeb and propineb required higher concentrations to inhibit 50% germination. The mean EC_{50} values of the strobilurin fungicides were not significantly different from one another. The strobilurin fungicides have not yet been registered against *P. viticola*. These results indicate that

they inhibited the mycelial growth and spore germination of *P. viticola*. Further trials need to be conducted to verify these findings under field conditions.

INTRODUCTION

Phomopsis cane and leaf spot of grapevine, caused by the fungus *Phomopsis viticola* (Sacc.) Sacc., can lead to significant yield losses. Serious disease outbreaks with yield losses of up to 50% have been reported in several countries (Pine, 1958; Berrysmith, 1962; Pscheidt & Pearson, 1989). Losses can occur from shoots breaking off near the base where lesions have developed, stunting of grapevines, loss of vigour, reduced bunch set and post-harvest fruit rot (Punithalingam, 1979; Chairman *et al.*, 1982; Nicholas *et al.*, 1994; Pearson & Goheen, 1994). However, the actual economic losses incurred as a result of *P. viticola* are minor in most years (Chairman *et al.*, 1982) because the disease occurs sporadically and causes a slow dieback of the vines.

Four taxa, of which taxa 1 and 2 were dominant, have been associated with Phomopsis cane and leaf spot in Australia (Merrin *et al.*, 1995). Taxon 1 did not cause symptoms on young growth while taxon 2 caused dark brown lesions on young shoots and petioles (Merrin *et al.*, 1995). These two taxa have also been isolated from South African grapevines. Taxon 2 was mainly associated with the typical cane and leaf spot symptoms, while taxon 1 proved to be a non-pathogenic endophyte (part 2).

Phomopsis cane and leaf spot disease occurs sporadically in South Africa. Swart and De Kock (1994) reported that the occurrence of *Phomopsis* on table grapevines had increased over the years. The disease appears to be more common in cooler and coastal areas, spreading during wet weather in spring (Shea, 1961; Nicholas *et al.*, 1994). Serious disease incidences have been recorded from vineyards in regions such as Helderberg, Firgrove, Somerset West, Rawsonville and Slanghoek (Marais, 1981) justifying producers controlling the disease with chemical means.

Chemical control of *Phomopsis* includes the application of eradicant fungicides during dormancy, and protective fungicides on new plant growth early in the growing season. *Phomopsis* is generally controlled through the application of protectant fungicides at 1-3 cm shoot length, and again at 6-12 cm shoot length (Pine, 1957). When

weather conditions favour the disease, an application of contact fungicides every 2 wk commencing at bud burst should provide satisfactory protection (Emmet *et al.*, 1992). Up to five sprays might be needed of which the first two are considered to be the most important. The fungicides used for the protective control of *P. viticola* are aimed at protection of young plant material during critical periods, namely when spores are released after prolonged periods of rain in the spring. A number of fungicides have been registered against *P. viticola* (Punithalingam, 1979). The following fungicides are registered against this disease in South Africa: copper oxychloride, copper oxychloride/sulphur, copper sulphate/lime, folpet, fosetyl-Al + mancozeb, mancozeb, propineb and sulphur (Nel *et al.*, 1999). In the past fosetyl-Al + mancozeb, folpet and mancozeb were commonly used to control this disease (Swart *et al.*, 1994). Various *in vitro* studies have been carried out overseas to test the performance of different fungicides for the control of *P. viticola* (Dula & Kaptas, 1982; Fareta *et al.*, 1987; Macek & Zgur, 1989; Kuropatwa, 1994). A new fungicide, strobilurin, has since come onto the market. The strobilurin fungicides have been registered against *Phomopsis* elsewhere (Anonymous, 1998), though not yet in South Africa.

An integrated approach to grapevine disease control would minimize the use of fungicides. Even though downy mildew and *Phomopsis* could be simultaneously controlled (Swart *et al.*, 1994), is little known of the effects of powdery mildew fungicides on *Phomopsis*.

The aim of this study was therefore to assess the *in vitro* effectiveness of *P. viticola* to the strobilurin fungicides, and to compare it with the contact fungicides currently used to control *P. viticola* as well as three powdery mildew fungicides. Isolates representing taxa 1 and 2 of the *P. viticola* complex were tested.

MATERIAL AND METHODS

Isolates

Isolates were obtained from symptomatic grapevine shoots and leaves from different viticultural regions in the Western Cape province. Three isolates of taxon 1 and ten isolates of taxon 2 were used in the mycelial growth tests (Table 1). Five of the taxon 2

isolates were selected for the spore germination tests. All isolates are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U).

Fungicides

A range of systemic, contact and quasi-systemic fungicides were tested (Table 2). The contact fungicides included folpet, mancozeb, and propineb. Azoxystrobin, kresoxim-methyl and trifloxistrobin are three different chemical variations of strobilurin and have a quasi-systemic mode of uptake in the plant (Ypema & Gold, 1999). However, Nel *et al.* (1999) grouped azoxystrobin and trifloxystrobin with systemic formulations and kresoxim-methyl with non-systemic formulations. The systemic fungicides tested include penconazole, flusilazole and spiroxamine. Even though fosetyl-Al is a systemic fungicide, the mixture of fosetyl-Al + mancozeb will be treated as a contact fungicide, since only mancozeb had an effect on *Phomopsis*.

Mycelial growth test

The effects of the following fungicides were tested on mycelial growth of *P. viticola*: azoxystrobin, flusilazole, folpet, fosetyl-Al + mancozeb, kresoxim-methyl, mancozeb, penconazole, spiroxamine, and trifloxystrobin. The fungicides were tested at the following concentrations: 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 µg a.i./ml. Bottles containing prepared malt extract agar (MEA, Biolab, Johannesburg) (40g/L) was kept at 50°C and fungicides were added from stock solutions. MEA was used for the control, and contained no fungicide.

Plates were inoculated within 24 h after they were poured. Three plugs (5 mm diam.) were cut from the margins of actively growing colonies and used to inoculate each plate. Mycelial plugs were inverted, and arranged in equal distances from each other. Mycelial growth was recorded after 4 d by determining the perpendicular diameters. The experiment was repeated once.

Spore germination test

A pilot study was conducted to test whether the fungicides used in the mycelial growth study could be used for spore germination tests. From this preliminary study, it was evident that flusilazole, penconazole and spiroxamine did not inhibit spore germination. These fungicides were therefore excluded from the spore germination tests. Azoxystrobin, folpet, kresoxim-methyl, mancozeb, propineb and trifloxystrobin were consequently tested *in vitro* for their ability to inhibit spore germination. Kresoxim-methyl and trifloxystrobin were tested at the following concentrations 0.00005, 0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05 µg a.i./ml. Azoxystrobin was tested at 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5 and 1.0 µg a.i./ml. Folpet, mancozeb and propineb were tested at 0.005, 0.01, 0.05, 0.1, 0.2, 0.5, 1.0 and 5.0 µg a.i./ml. Prepared water agar (WA) (12g/L) was kept at 50°C and dilutions were made with fungicide stock solutions. MEA was used for the control, and contained no fungicide.

Isolates were cultured on sterile pieces of grapevine cane on WA. A spore suspension was made by adding cane pieces with sporulating pycnidia to sterile water which contained 0.01% Tween 80. The spore suspension was diluted to obtain a concentration of 1 x 10^5 spores/ml. Within 24 h of being poured, plates were inoculated with 800 µl of spore suspension. The spore suspensions were evenly dispersed over the plate with a sterile glass hockey stick. Plates were allowed to dry in the laminar flow cabinet for 20 min and germination determined 24 h after inoculation. Thirty spores were counted in three fields (40x magnification) per plate. Spores were considered to have germinated if the length of the germ tube was equal to or greater than the lenght of the spore. The experiment was repeated once.

Statistical analyses

Mycelial growth test

Isolate growth was determined by calculating the mean of two colony diameters of three replicate colonies. The effective concentration at which mycelial growth was inhibited

50% (EC_{50}) was calculated with inhibition as a proportion to the control. The percentage inhibition was plotted against concentration for each fungicide/isolate combination. The most suitable regression was fitted to each data set and the EC_{50} values calculated. Analyses of variance were conducted on the EC_{50} values to determine significant differences within isolates of taxa 1 and 2. These EC_{50} values were tested for normality using the Shapiro-Wilk statistic. Due to the lack of normality, the data were square root transformed prior to analysis of variance. Student's t-test was carried out to determine whether there was a significant difference between the two taxa. Since no significant difference between the taxa occurred, it was decided to use only data of taxon 2 for further analyses. This decision was further substantiated by the fact that taxon 2, rather than taxon 1, is the cause of Phomopsis cane and leaf spot disease. An analysis of variance was carried out to establish the significant differences of inhibitory effects of the various fungicides. The mean EC_{50} values were calculated for each fungicide, and pair-wise student t-tests were conducted on the EC_{50} values.

Spore germination test

The effective concentration at which the germination of spores were inhibited by 50% (EC_{50}) was calculated. The percentage inhibition was plotted against the log of the concentrations tested for each fungicide/isolate combination. The EC_{50} values were calculated from the most suitable regression fitted to each curve. An analysis of variance was conducted on the EC_{50} values to determine whether there were significant differences between the isolates and fungicides tested. The mean EC_{50} values were calculated for each fungicide, and pair-wise student t-tests were conducted on the EC_{50} values.

RESULTS

Mycelial growth test

In separate analyses of the variance on the EC_{50} values, no significant differences were found within isolates of taxon 1 ($P = 0.5615$) or taxon 2 ($P = 0.0704$). The t-test done on the two taxa indicated that there was also no significant difference between these two taxa ($P = 0.2535$). Significant differences were found among the fungicides (Table 3).

Flusilazole, penconazole and trifloxystrobin gave significantly more inhibition at lower concentrations than folpet and fosetyl-Al + mancozeb. Azoxystrobin, flusilazole, kresoxim-methyl, penconazole, spiroxamine and trifloxystrobin inhibited mycelial growth equally well (Fig. 1). There was also no significant difference between azoxystrobin, kresoxim-methyl and mancozeb. Flusilazole and penconazole inhibited mycelial growth at the lowest concentrations. The coefficient of variance given in Table 5 for both tests performed indicated that the mycelial growth tests were more accurate than the spore germination tests.

Inhibition of spore germination

No fungicide/isolate interaction occurred and no significant differences were detected among the isolates (Table 6). There was, however, significant differences among the fungicides (P = 0.0001). Kresoxim-methyl and trifloxystrobin gave significantly more inhibition of germination at lower concentrations than folpet or mancozeb (Fig. 2). Folpet required the highest concentration among the fungicides tested to inhibit 50% of the spore germination. There were also no differences between the concentrations of mancozeb, propineb and azoxystrobin needed to inhibit spore germination. Higher concentrations of azoxystrobin and propineb were required than kresoxim-methyl and trifloxystrobin to inhibit 50% germination. These differences, however, were not statistically significant.

DISCUSSION

The findings that the different fungicides were equally effective *in vitro* to isolates of taxa 1 and 2 in the mycelial growth tests, indicated that taxon 1, which was isolated as an endophyte (part 2), would also be controlled by the fungicides tested.

Results obtained in this study further showed that the strobilurins inhibited 50% of mycelial growth and spore germination at lower concentrations than possible for the contact fungicides. These differences were, however, not significant in all cases tested. Lower concentrations of strobilurin fungicides were needed to inhibit the spore germination than were needed to inhibit mycelial growth. This indicated that these compounds would be effective protectant fungicides. Strobilurins have been reported to

inhibit spore germination of *P. viticola* when used prophylactically (Anonymous, 1998). Strobilurins could, therefore, be applied preventatively early in the season when the environmental conditions are conducive to infection. No significant differences were found between the different strobilurins in their ability to inhibit spore germination, indicating that azoxystrobin, kresoxim-methyl and trifloxystrobin had similar efficacy *in vitro*. The strobilurin data also represent base line sensitivity of *P. viticola* isolates towards these fungicides. Since strobilurins have a single-site mode of action, it would be important to monitor the possible fungicide resistance that may develop due to the continuous use of these fungicides. These fungicides, however, hold various advantages as they are active against a wide range of pathogens, quasi-systemic, easily absorbed by the plant, environmentally safe and have no cross reactivity with other fungicides currently on the market (Ypema & Gold, 1999).

Some of the contact fungicides required similar concentrations than the strobilurin fungicides to inhibit mycelial growth and spore germination. Mancozeb was comparable to both kresoxym-methyl and azoxystrobin regarding its ability to inhibit mycelial growth. Mancozeb and propineb were comparable with that of azoxystrobin in the inhibition of spore germination. However, generally the strobilurin fungicides were more effective at lower concentrations than the contact fungicides. Even though less of strobilurin fungicides will be required, would it still remain more expensive to apply. The dithiocarbamate contact fungicides also hold the advantage of having a multi-site mode of action, and could be applied more often than the strobilurins.

Although folpet inhibited mycelial growth and spore germination at the highest concentrations tested, field studies conducted by Swart *et. al.* (1994) showed that folpet mostly gave better control of *Phomopsis* than mancozeb and fosetyl-Al + mancozeb. This confirms the importance of field testing to validate *in vitro* results.

Penconazole and flusilazole fall into the triazole group inhibiting demethylation (DMI) in the biosynthesis of sterols. Spiroxamine's mode of action is similar to morpholines, inhibiting sterol biosynthesis (Esters, 1997). These fungicides can be referred to as sterol biosynthesis inhibiting fungicides (SBI). SBI fungicides inhibited the mycelial growth of *P. viticola* at low concentrations. Previous studies also found that

flusilazole inhibited the mycelial growth of *P. viticola* at the lowest concentrations tested (Fareta *et al.*, 1987; Kuropatwa, 1994). Penconazole, flusilazole and spiroxamine are recommended for powdery mildew control. Powdery mildew control starts early in the growth season and, if necessary, continues till after harvest with non-systemic fungicides. The results in the present study indicated that these fungicides could also provide disease control of *Phomopsis* when applied early in the growth season by inhibiting mycelial growth of endophytic *Phomopsis* isolates. *Phomopsis* usually becomes inactive near the flowering stage. However cool, wet weather conditions could reactivate this fungus (Pearson *et al.*, 1994), under which conditions these fungicides could again provide disease control.

The critical period of *Phomopsis* disease control is during the early growth stages in spring through the application of spore inhibiting fungicides to prevent the spread of the disease. In vineyards with a history of *Phomopsis*, regular disease inspection is essential and protective fungicides need to be applied after prolonged periods of rain. The results of this *in vitro* study showed that strobilurin fungicides inhibited spore germination and mycelial growth of *P. viticola* and would most likely also do so in the field. The field environment is different from that experienced *in vitro*, and field trials would therefore be required to determine the efficacy of strobilurin fungicides *in vivo*.

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Table 1. Origin of *Phomopsis viticola* isolates in the Western Cape province and taxon type used in screening against various fungicides in mycelial growth and spore germination tests.

Accession no.	Taxon	Location	Test performed
STE-U 2654	1	Stellenbosch	M[1]
STE-U 2655	1	Stellenbosch	M
STE-U 2632	1	Constantia	M
STE-U 3128	2	Stellenbosch	M, S[2]
STE-U 3129	2	Botriver	M
STE-U 2641	2	Paarl	M, S
STE-U 2648	2	Porterville	M
STE-U 3130	2	Slanghoek	M
STE-U 3131	2	Bonnievale	M, S
STE-U 3132	2	Vredendal	M
STE-U 3133	2	Lutzville	M, S
STE-U 3134	2	Paarl	M, S
STE-U 3135	2	Montaqu	M

[1]Mycelial growth.

[2]Spore germination.

Table 2. Description of fungicides used for *in vitro* tests against isolates of *Phomopsis viticola*.

Active ingredient	Trade name and company	Recommended disease application on grapes in South Africa*	Formulation	Active ingredient (g)	Highest recommended spray dosage/ 100L H_2O and equivalent a.i. in µg/ml#
Azoxystrobin	Quadris, Zeneca	*Uncinula necator* (Schwein.) Burrill *Plasmopara viticola* (Berk. & M.A. Curt.) Berl. & de Toni	WP	500 g/kg	35 g (175 µg/m*l*)
Flusilazole	Olymp, Du Pont	*Uncinula necator*	EC[1]	100 g/*l*	50 m*l* (50 µg/m*l*)
Folpet	Folpan, Makhteshim-Agan	*Phomopsis viticola* *Plasmopara viticola* *Elsinoë ampelina* (de Bary) Shear *Botrytis cinerea* Pers.	SC[2]	500 g/*l*	200 m*l* (1000 µg/m*l*)
Fosetyl-Al+ mancozeb	Mikal M, Rhône-Poulenc	*Phomopsis viticola* *Plasmopara viticola*	WP	440/260 g/kg	350 g (910µ g/m*l* for mancozeb)
Kresoxim-methyl	Stroby, BASF	*Uncinula necator* *Plasmopara viticola*	WP	500 g/kg	15 g (75 µg/m*l*)

Continued

Table 2. (continued)

Active ingredient	Trade name and company	Disease fungicide is registered on grapes in South Africa*	Formulation	Active ingredient (g)	Highest recommended spray dosage/ 100L H_2O and equivalent a.i. in µg/ml#
Penconazole	Topaz, Novartis	*Uncinula necator*	EC	100 g/*l*	22.5 m*l* (22.5 µg/m*l*)
Propineb	Antracol, Bayer	*Phomopsis viticola* *Plasmopara viticola* *Elsinoë ampelina*	WP[3]	700 g/kg	200 g (1400 µg/m*l*)
Mancozeb	Dithane, Sanachem	*Phomopsis viticola* *Plasmopara viticola*	WP	800 g/kg	200 g (1600 µg/m*l*)
Spiroxamine	Prosper, Bayer	*Uncinula necator*	EC	500 g/*l*	60 m*l* (300 µg/m*l*)
Trifloxystrobin	Flint, Novartis	*Uncinula necator* *Plasmopara viticola*	WP	500 g/kg	15 g (75 µg/m*l*)

* According to Nel *et al.* (1999).

\# Recommended dosage for powdery mildew taken for fungicides not registered against *Phomopsis*.

[1] EC = emulsifiable concentrate.

[2] SC = soluble concentrate.

[3] WP = wettable powder.

Table 3. Analysis of variance for significant differences between calculated EC_{50} values of Taxon 2 isolates for the various fungicides tested.

Analyses of variance				
Source	**Degrees of freedom**	**Mean square**	***F* value[a]**	***P* value[b]**
Experiment	1	1.526	1.42	0.2787
Fungicide	8	5.440	5.02	0.0232
Error	6	1.083		

[a]*F* value = Variance.

[b]*P* value = Probability of $P \leq 0.05$ considered as significant.

Table 4. Comparison between the accuracy of EC_{50} values obtained for the mycelial growth and spore germination tests as indicated by the coefficient of variation.

	Mycelial inhibition	**Spore germination**
Mean EC_{50} values	1.423	0.1742
Standard deviation	1.041	0.2140
Coefficient of variation CV %	74	123

Table 5. Analysis of variance for significant differences of calculated EC_{50} values for inhibition of spore germination.

Analyses of variance				
Source	**Degrees of freedom**	**Mean square**	***F* value[a]**	***P* value[b]**
Repeat	1	0.1530	3.34	0.0781
Fungicide	5	0.3584	7.82	0.0001
Isolate	4	0.02057	0.45	0.7725
Fungicide/Isolate	20	0.01261	0.28	0.9980
Error	29	0.04585		

[a]*F* value = Variance.

[b]*P* value = Probability of $P \leq 0.05$ considered as significant.

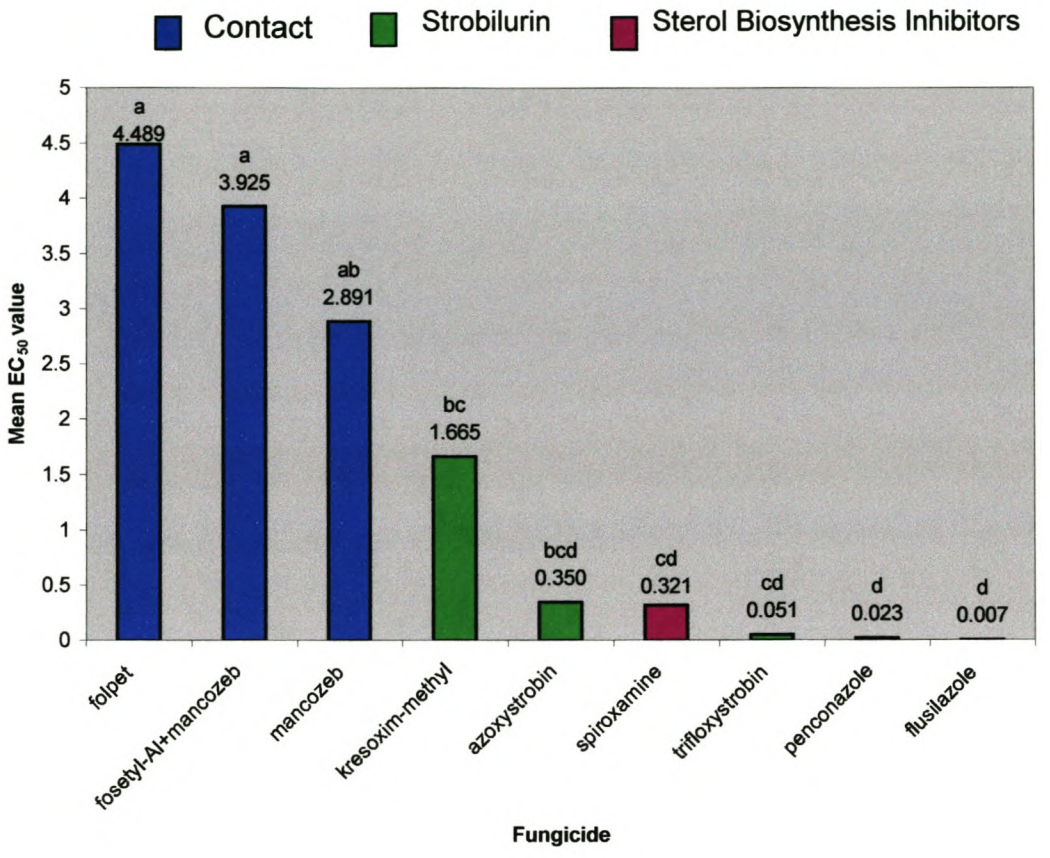

Fig. 1. Mean EC_{50} values (µg/ml) of the different fungicides inhibiting mycelial growth. Bars with the same letters do not differ significantly ($P = 0.05$).

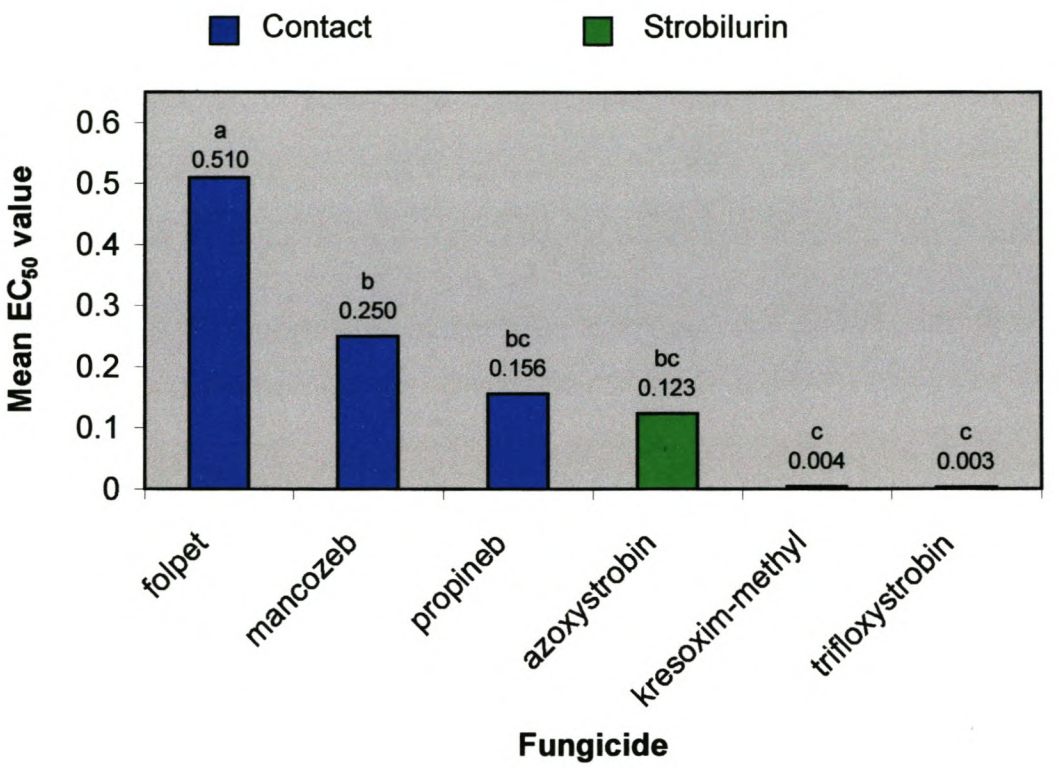

Fig. 2. Mean EC_{50} values (µg/ml) of the different fungicides inhibiting spore germination. Bars with the same letters do not differ significantly ($P = 0.05$).