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# Pathogenicity testing of lesser-known vascular fungi of grapevines

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**Abstract.** Several hyphomycetes were recently isolated from asymptomatic or symptomatic vascular tissues of grapevines showing Petri disease symptoms in South Africa. In most cases, their status as pathogens was unknown and pathogenicity studies were, therefore, conducted to determine their potential as decline pathogens. The fungi included *Acremonium* cf. *charticola*, *Acremonium* cf. *ochraceum*, *Cadophora luteo-olivacea*, *Phialemonium* cf. *curvatum*, *Pleurostomophora richardsiae*, *Phaeoacremonium* (*Pm.*) *krajdenii*, *Pm. parasiticum*, *Pm. subulatum*, *Pm. venezuelense* and *Pm. viticola*. Of these, *Pm. parasiticum* and *Pm. viticola* have been associated with Petri disease symptoms, although their pathogenicity has not been tested. *Phaeomoniella* (*Pa.*) *chlamydospora* and *Pm. aleophilum*, known to be involved in Petri disease and esca, were included as positive controls. Pathogenicity studies were conducted in glasshouse experiments where grapevine rootstocks were artificially inoculated, as well as in the field. Data obtained after 3 months from a glasshouse trial were difficult to interpret, due to the small lesions and similarity in disease expression among different species. However, *Pa. chlamydospora* produced the largest lesions and was by far the most aggressive pathogen. To supplement the glasshouse trial, grapevine trunks and pruning wounds of *Vitis vinifera* cv. Periquita were artificially inoculated in the field. Field trials, rated after 14 months, confirmed *Pa. chlamydospora* to be the most aggressive pathogen, since it produced the largest trunk and pruning wound lesions. Furthermore, it was re-isolated more frequently than any of the other fungi, especially from the pruning wounds. All the fungi were able to infect, colonise and produce lesions statistically different to those caused by the water control and the non-pathogen in the field trial.

## Introduction

Petri disease causes decline and dieback of grapevines (*Vitis* spp.). This disease has often been associated with young vines, causing losses in newly planted vineyards (Ferreira *et al*. 1994; Bertelli *et al*. 1998; Ferreira 1998; Scheck *et al*. 1998; Mugnai *et al*. 1999; Pascoe and Cottral 2000). Petri disease, caused by *Phaeomoniella* (*Pa.*) *chlamydospora* (= *Phaeoacremonium chlamydosporum*), as well as several species of *Phaeoacremonium* (*Pm.*), has been implicated as a major contributor to the decline of young vines (Mugnai *et al*. 1999; Pascoe and Cottral 2000; Groenewald *et al*. 2001). Species of *Phaeoacremonium*, which have been isolated from grapevines, include *Pm. aleophilum*, *Pm. angustius*, *Pm. australiense*, *Pm. austroafricanum*, *Pm. inflatipes*, *Pm. iranianum*, *Pm. krajdenii*, *Pm. mortoniae*, *Pm. parasiticum*, *Pm. scolyti*, *Pm. subulatum*, *Pm. venezuelense* and *Pm. viticola* (Crous *et al*. 1996; Dupont *et al*. 2000; Groenewald *et al*. 2001; Mostert *et al*. 2005, 2006). Although several isolates have formerly been identified from grapevines as '*Pm. rubrigenum*' (Groenewald *et al*. 2001), recent research has concluded that they represent a species complex, namely *Pm. scolyti*, *Pm. griseorubrum* and *Pm. alvesii*, and that *Pm. rubrigenum* has thus far only been confirmed from human infections (Mostert *et al*. 2005).

Over the past few years, a drastic reduction has been noted in the survival rate of vine cuttings in nurseries and young vineyards in the Western Cape Province of South Africa (Ferreira 1998; Fourie *et al*. 2000*a*, 2000*b*; Fourie and Halleen 2001). Various pathogens have been linked to this phenomenon, including those associated with Petri disease (Halleen *et al*. 2003). The *Phaeoacremonium* species isolated from grapevines in South Africa include *Pm. aleophilum*, *Pm. austroafricanum*, *Pm. parasiticum*, *Pm. krajdenii*, *Pm. scolyti*, *Pm. subulatum*, *Pm. venezuelense* and *Pm. viticola* (Crous *et al*. 1996; Groenewald *et al*. 2001; Mostert *et al*. 2005, 2006).

Several studies have been conducted to understand the role that the different fungi associated with the typical disease symptoms play in Petri disease. Various pathogenicity studies on grapevines have been conducted with *Pa. chlamydospora*, *Pm. aleophilum* (Adalat *et al*. 2000; Larignon and Dubos 2000; Eskalen and Gubler 2001; Sparapano *et al*. 2001) and *Pm.* '*inflatipes*' (= *Pm. aleophilum*) (Adalat *et al*. 2000; Eskalen and Gubler 2001). Several species have been shown to be phytotoxic (Bruno and Sparapano 2005), though

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pathogenicity tests have not been conducted. The toxigenicity of *Pm. angustius*, *Pm. inflatipes*, *Pm. parasiticum*, *Pm. rubrigenum* and *Pm. viticola* was determined with culture filtrates on detached grapevine leaves, which revealed a phytotoxic reaction in all cases. Several *Cadophora* species have been isolated from grapevines showing decline symptoms (Overton *et al*. 2005*a*). The pathogen status of *Cadophora* on grapevines remains uncertain. It has, however, been reported that *Cadophora malorum* in association with *Pm. aleophilum* and *Pm. parasiticum*, caused decline symptoms in kiwi fruit trees (Di Marco *et al*. 2004).

Fungi not previously associated with Petri disease in South Africa, though isolated from vascular tissues of vines, were *Acremonium* (*A.*) cf. *charticola*, *A.* cf. *ochraceum*, *Cadophora* (*C.*) *luteo-olivacea*, *Phialemonium* (*Phl.*) cf. *curvatum* and *Pleurostomophora* (*Pl.*) *richardsiae*. Five species of *Phaeoacremonium* (*Pm. krajdenii*, *Pm. parasiticum*, *Pm. venezuelense*, *Pm. subulatum* and *Pm. viticola*) were also isolated from grapevines. These fungal isolates were obtained from grapevines showing decline symptoms, or from apparently healthy plants in commercial nurseries during the growing season, before being sold to farmers. The present study was undertaken to determine the potential pathogenicity of the *Phialophora*-like, *Acremonium* and *Phaeoacremonium* species suspected of being involved in the decline of young vines. A species of *Lophiostoma*, often isolated from vascular tissues of grapevines, was included as a non-pathogen negative control. Pathogenicity tests were conducted in the glasshouse (rated after 3 months) where grapevine rootstocks were artificially inoculated, as well as in the field (rated after 14 months) where grapevine trunks and pruning wounds were inoculated.

## Methods

### *Isolates and identification*

Isolates were obtained from nursery plants (Halleen *et al*. 2003), as well as older grapevines displaying typical decline symptoms (Mostert *et al*. 2006). Isolates are maintained in the culture collections of the Department of Plant Pathology (University of Stellenbosch) and the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS). Isolates were identified to species level based on morphological and cultural characteristics (Schol-Schwarz 1970; Gams 1971, 2000; Domsch *et al*. 1980; Gams and McGinnis 1983; Crous *et al*. 1996; Crous and Gams 2000; De Hoog *et al*. 2000; Dupont *et al*. 2000; Harrington and McNew 2003; Dhanasekaran *et al*. 2004; Mostert *et al*. 2006), as well as DNA sequences.

### *DNA isolation and sequencing*

Genomic DNA was extracted using the isolation protocol of Lee and Taylor (1990). To confirm the species identity, the 5.8 S nuclear rRNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) were amplified using primers ITS1 and ITS4 (White *et al*. 1990). Polymerase chain reaction (PCR) amplification, purification and sequencing were done as described in Mostert *et al*. (2003). Raw sequence data were analysed using EditView v.1.0.1 (http://www.appliedbiosystems.com, verified 28 February 2007) and sequences compared to those in GenBank. The identities of the two *Acremonium* species, *C. luteo-olivacea* and *Phl.* cf. *curvatum,* were confirmed by BLAST searching their DNA sequences to those in the CBS databases. Sequences were deposited at GenBank for *Pl. richardsiae* (EF042106), *C. luteo-olivacea* (EF042108), *Phl.* cf. *curvatum* (EF042105), *A.* cf. *ochraceum* (EF042104), *A.* cf. *charticola* (EF042103) and *Lophiostoma* sp. (EF042107).

### *Pathogenicity studies*

#### *Glasshouse experiment*

Pathogenicity studies were conducted with grapevine nursery plants (*Vitis vinifera* cv. Shiraz/101–14 Mgt) maintained in a glasshouse. Dormant plants were planted in 20 cm polyurethane pots filled with a sterilised potting mixture (pine bark). Plants were left in the glasshouse (23–26°C) for 3 weeks to allow shoot development before inoculations were made. All the plants were pruned to three shoots. Wounds (3 mm deep) were made in the rootstock between the graft union and soil surface using a 4 mm cork borer. Twelve fungal species were used, with one isolate per species (Table 1). Colonised mycelium plugs were inserted as inoculum into the wounds and sealed with Parafilm. Mycelium plugs were obtained from the periphery of actively growing fungal colonies cultivated on 2% malt extract agar (MEA) (Gams *et al*. 1998). Uncolonised MEA plugs were used for control inoculations. Ten plants were inoculated with each of the 12 fungal isolates. Ten plants were used for control inoculations. Pots were arranged in a completely randomised design and watered twice weekly.

#### *Evaluation of glasshouse pathogenicity studies*

Plants were collected from the glasshouse after 3 months and immediately taken to the laboratory. Vines were split lengthwise through the inoculation hole to reveal the xylem and pith regions for measurement of the lesions. The two pieces of each vine were surface sterilised (30 s in 70% ethanol, 5 min in 0.35% sodium hypochlorite and 30 s in 70% ethanol) before isolations were made. Eight pieces of tissue (~0.5 × 2 mm) were removed from the edge of each lesion and placed in Petri dishes containing 2% potato-dextrose agar (Biolab, Midrand, Johannesburg) amended with chloromycetin (250 mg/L) (PDA-C) to reduce bacterial growth. Dishes were incubated at ± 25°C for 4 weeks before fungal identifications were made to determine whether the inoculated fungus could be re-isolated.

#### *Field experiment*

Ten isolates were used for field inoculations (Table 1). Control plants were treated with sterile water. Cultures were grown on MEA for 7 weeks before inoculations. Conidia were dislodged in sterile water and the final concentration was adjusted to $10^6$ conidia/mL.

#### *Trunk inoculations*

A 15-year-old *Vitis vinifera* cv. Periquita vineyard was selected at ARC Infruitec-Nietvoorbij (Nietvoorbij Campus, Stellenbosch, South Africa). The grapevines were cleaned of the outer bark at the area of inoculation and sprayed with 70% ethanol. A hole, 2 mm in diameter and 10 mm deep, was bored into the trunk of each vine and injected with a 500-µL spore

**Table 1. Grapevine cultivar, vine age, isolation details and reference numbers of isolates used**

| | Glasshouse (G) and/or Field (F) trial | Strain reference number | Scion/rootstock | Plant age (years) | Plant zone isolated from | Symptom | Area in South Africa | Farm |
|---|---|---|---|---|---|---|---|---|
| *Acremonium* cf. *charticola* | G | CBS 115996, STE-U 5434, L.M. 89 | Hanepoot/ 101–14 Mgt | 31 | Cordon | Yellow sector | Paarl | Zandrift |
| *Acremonium* cf. *ochraceum* | G | CBS 109930, STE-U 4487, L.M. 11 | Chenin blanc/ 99 Richter | 13 | Cordon | Brown sector | Paarl | Zandrift |
| *Cadophora luteo-olivacea* | G, F | CBS 109924, STE-U 4480, F. 78 | Sultana/Ramsey | – | Graft union of nursery grapevine | Asymptomatic nursery grapevine | Wellington | Voor Groenberg |
| *Lophiostoma* sp. | F | CBS 109932, STE-U 4489, L.M. 22 | Colombar/ 99 Richter | 20 | Basal end of trunk | Unknown | Paarl | Zandrift |
| *Phaeoacremonium aleophilum* | G | CBS 110828, STE-U 4652, L.M. 12 | Hanepoot/ 101–14 Mgt | 31 | Cordon | Brown sector | Paarl | Zandrift |
| *Phaeoacremonium krajdenii* | G, F | CBS 110118, STE-U 4647, L.M. 1 | Unknown | – | Roots | Unknown | Wellington | Ernita |
| *Phaeoacremonium parasiticum* | G, F | CBS 117178, STE-U 4650, F. 104 | Sultana/Ramsey | – | Graft union of nursery grapevine | Asymptomatic nursery grapevine | Malmesbury | Jakkalsfontein |
| *Phaeoacremonium subulatum* | G, F | CBS 113584, STE-U 4655, L.M. 16 | Chenin blanc/ 99 Richter | 13 | Trunk | Black ring | Paarl | Zandrift |
| *Phaeoacremonium venezuelense* | G, F | CBS 110119, STE-U 4648, L.M. 15 | Chenin blanc/ 99 Richter | 13 | Trunk | Brown sector | Paarl | Zandrift |
| *Phaeoacremonium viticola* | G, F | CBS 113065, STE-U 4653, L.M. 13 | Chenin blanc/ 99 Richter | 13 | Cordon | Brown sector | Paarl | Zandrift |
| *Phaeomoniella chlamydospora* | G, F | CBS 117179, L.M. 95 | Colombar/ 99 Richter | 20 | Cordon | Black spot | Paarl | Zandrift |
| *Phialemonium* cf. *curvatum* | G, F | CBS 115998, STE-U 5436, F. 27 | Sultana/Ramsey | – | Graft union of nursery grapevine | Asymptomatic nursery grapevine | Wellington | Voor Groenberg |
| *Pleurostomophora richardsiae* | G, F | CBS 117177, STE-U 4657, L.M. 31 | Chenin blanc/ 99 Richter | 13 | Basal end of trunk | Unknown | Paarl | Zandrift |

suspension. The wound was sealed with petroleum jelly and wrapped with 'cling wrap'. Ten trunks were inoculated with each fungus.

### *Pruning wound inoculations*

Grapevines were spur-pruned to two buds. Fifty pruning wounds were inoculated with a 100-µL spore suspension of each of the fungal isolates. Both the trunk and pruning wound inoculations were made on 18 July 2002. The inoculated plants were allowed to grow under natural environmental conditions. Evaluations were conducted during September 2003 by means of destructive sampling.

The inoculated plants were collected from the vineyard and immediately taken to the laboratory. Vines were split lengthwise through the inoculation hole and pruning wound to reveal the xylem and pith regions for lesion measurement. The two pieces of each vine were surface sterilised (30 s in 70% ethanol, 5 min in 0.35% sodium hypochlorite and 30 s in 70% ethanol) before isolations were made. Eight pieces of tissue (~0.5 × 2 mm) were removed from the edge of each lesion in the pruning wounds (12 from trunks) and placed in Petri dishes containing PDA-C. Dishes were incubated at ± 25°C for 4 weeks before fungal identifications were made to determine whether the inoculated fungus could be re-isolated.

### *Statistical analysis*

#### *Glasshouse experiment*

A complete randomised experimental design was used with 12 fungal species plus a control with 10 random replications of each. An experimental unit was a single vine planted in a polyurethane pot. Data were subjected to a one-way analysis of variance using SAS v. 8.2 (SAS 1999). The Shapiro–Wilk test was performed to test for non-normality (Shapiro and Wilk 1965). Student's *t*-least significant difference (l.s.d.) was calculated at the 5% level to compare treatment means.

#### *Field experiment*

Data were subjected to a one-way analysis of variance using SAS v. 8.2 (SAS 1999). The Shapiro–Wilk test was performed to test for non-normality (Shapiro and Wilk 1965). Student's *t*-l.s.d. was calculated at the 5% level to compare treatment means.

## Results

### *Fungal identification*

The fungal species identified and used in the pathogenicity studies are listed in Table 1. In the case of *A.* cf. *ochraceum* (Fig. 1*a*), *A.* cf. *charticola* (Fig. 1*b*) and *Phl.* cf. *curvatum* (Fig. 1*c*), a high DNA sequence similarity was obtained to representative strains of these species. However, some nucleotide differences were present, which could suggest that the grapevine isolates represent cryptic, undescribed species (R. S. Summerbell, pers. comm.). The strain of *C. luteo-olivacea* (formerly known as *Phialophora luteo-olivacea*) (Fig. 1*d*) had sequence similarity of 99.6% with the ex-type strain (CBS 141.41). A culture commonly isolated from grapevines had the closest similarity (89.6%) with *Lophiostoma corticola* (AF383957) and was therefore named *Lophiostoma* sp. The strain of *Pl. richardsiae* (formerly *Phialophora richardsiae*) was confirmed by a 99.4% sequence similarity with the ex-type strain (CBS 270.33).

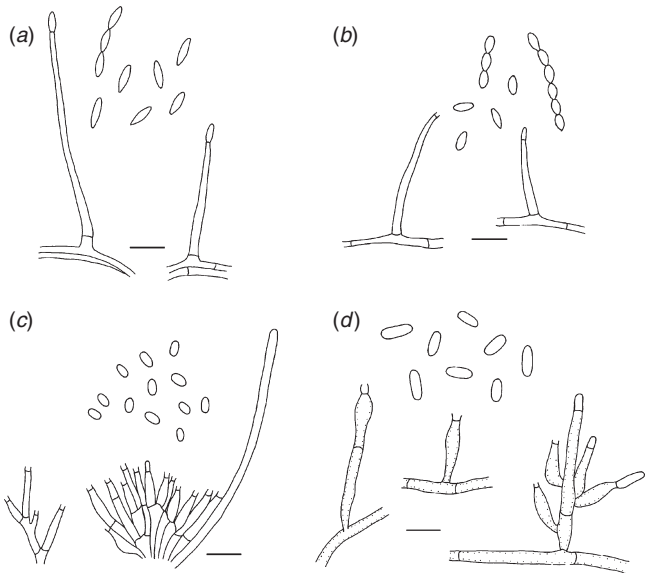


**Fig. 1.** Micromorphological structures of lesser-known vascular fungi isolated from grapevines. (*a*) Conidiophores, phialides and conidia of *Acremonium* cf. *ochraceum* (CBS 109930); (*b*) *Acremonium* cf. *charticola* (CBS 115996); (*c*) *Phialemonium* cf. *curvatum* (CBS 115998); (*d*) *Cadophora luteo-olivacea* (CBS 109929). Scale bars = 10 µm.

### *Glasshouse trial*

The mean lesion length resulting from inoculation by the 12 isolates and control are given in Table 2. The longest lesions (mean = 17.045 mm) were obtained from plants inoculated with *Pa. chlamydospora.* The success of re-isolation, given as a percentage of the plants inoculated with a specific fungus, is also shown in Table 2. Only *A.* cf. *charticola* and *Pm. subulatum* could not be re-isolated, indicating that additional trials would be required.

According to Student's *t*-l.s.d. test, fungal species evaluated in this trial were categorised as pathogenic, non-pathogenic or intermediately pathogenic (Table 2). *Phaeomoniella chlamydospora* formed the largest lesion mean and was considered to be pathogenic. All the species that did not differ significantly from the negative control, i.e. *Pm. aleophilum*, *Pm. viticola*, *Pm. krajdenii*, *C. luteo-olivacea* and *Acremonium* spp. were classified as non-pathogenic. The remaining species were classified in an intermediate group. The intermediate group's reaction can be seen as a weak pathogenic reaction. The fungi that have frequently been isolated from diseased vines and that gave a weak host response, indicate that they might not be able to cause disease on their own, but only in synergism with the other fungi of this complex. As expected, the known grapevine pathogens *Pm. aleophilum*, *Pm. parasiticum* and *Pa. chlamydospora* were re-isolated from the majority of inoculated plants.

**Table 2. Results obtained from a pathogenicity study conducted with potted grapevine plants (*Vitis vinifera* cv. Shiraz 101–14 Mgt) artificially inoculated with fungi**
Means followed by the same letter are not significantly different ($P = 0.05$)

| Fungus | Isolate number | Re-isolation (%) | Mean lesion length (mm) |
|---|---|---|---|
| *Phaeomoniella chlamydospora* | CBS 117179 | 70 | 17.05a |
| *Phaeoacremonium venezuelense* | CBS 110119 | 100 | 12.38b |
| *Pleurostomophora richardsiae* | CBS 117177 | 90 | 11.88bc |
| *Phaeoacremonium parasiticum* | CBS 117178 | 90 | 11.43bcd |
| *Phialemonium* cf. *curvatum* | CBS 115998 | 60 | 11.24bcde |
| *Phaeoacremonium subulatum* | CBS 113584 | 0 | 10.87bcdef |
| *Phaeoacremonium aleophilum* | CBS 110828 | 90 | 9.62cdefg |
| *Phaeoacremonium viticola* | CBS 113065 | 60 | 9.51cdefg |
| *Phaeoacremonium krajdenii* | CBS 110118 | 100 | 9.37defg |
| *Cadophora luteo-olivacea* | CBS 109924 | 40 | 8.89efg |
| *Acremonium* cf. *charticola* | CBS 115996 | 0 | 8.75fg |
| Control | – | – | 8.20g |
| *Acremonium* cf. *ochraceum* | CBS 109930 | 40 | 8.14g |
| l.s.d. ($P = 0.05$) | – | – | 2.475 |

### *Field trial*

Several of the strains used in the glasshouse trial were excluded from the field trial. *Acremonium* cf. *charticola* and *A.* cf. *ochraceum* were eliminated from the field trial because of their low re-isolation percentages and small lesions caused in the glasshouse trial. *Phaeoacremonium aleophilum* was also excluded, since pruning wound and trunk inoculations of field grapevines have previously been conducted by Sparapano *et al*. (2001).

### *Trunk inoculations*

*Phaeomoniella chlamydospora* caused the largest lesions (449.20 mm), although it did not differ significantly from five other treatments (Table 3). However, *Pa. chlamydospora* was re-isolated more frequently from inoculated plants than any of the other fungi. The *Lophiostoma* sp. was the only fungus that did not differ from the control treatment and could therefore be regarded as a non-pathogen. It could also not be re-isolated from any of the trunks inoculated with this fungus. All the other fungi caused extensive lesions differing significantly from the control and non-pathogen treatments and could therefore be regarded as true wood colonisers and pathogens.

### *Pruning wound inoculations*

*Phaeomoniella chlamydospora* caused the largest lesions in the pruning wounds (45.15 mm), although it did not differ significantly from *C. luteo-olivacea* (Table 4). However, *Pa. chlamydospora* was re-isolated more frequently from inoculated plants than any of the other fungi. The *Lophiostoma* sp. was again the only fungus that did not differ from the control treatment and could, therefore, be regarded as a non-pathogen. The fungus could also not be re-isolated from any of the inoculated pruning wounds. All the other fungi caused extensive lesions differing significantly from the control and non-pathogen treatments, and clearly demonstrated their capability as colonisers of pruning wounds, and could, therefore, be regarded as pathogens.

**Table 3. Results obtained from a pathogenicity study where grapevine trunks (*Vitis vinifera* cv. Periquita) were artificially inoculated with a specific fungal spore suspension**
Means followed by the same letter do not differ significantly ($P < 0.05$)

| Treatment | Isolate number | Re-isolation (%) | Mean lesion length (mm) |
|---|---|---|---|
| *Phaeomoniella chlamydospora* | CBS 117179 | 90 | 449.2a |
| *Cadophora luteo-olivacea* | CBS 109924 | 40 | 438.5a |
| *Phaeoacremonium krajdenii* | CBS 110118 | 50 | 430.4ab |
| *Phaeoacremonium viticola* | CBS 113065 | 40 | 409.9abc |
| *Phaeoacremonium venezuelense* | CBS 110119 | 60 | 401.5abc |
| *Phialemonium* cf. *curvatum* | CBS 115998 | 40 | 376.7bcd |
| *Phaeoacremonium subulatum* | CBS 113584 | 10 | 361.8cd |
| *Pleurostomophora richardsiae* | CBS 117177 | 70 | 339.8de |
| *Phaeoacremonium parasiticum* | CBS 117178 | 60 | 307.3e |
| *Lophiostoma* sp. | CBS 109932 | 0 | 50.8f |
| Control (water) | – | – | 33.6f |
| l.s.d. ($P = 0.05$) | – | – | 53.91 |

**Table 4. Results obtained from a pathogenicity study where pruning wounds (*Vitis vinifera* cv. Periquita) were artificially inoculated with a specific fungal spore suspension**

Means followed by the same letter do not differ significantly ($P < 0.05$)

| Treatment | Isolate number | Re-isolation (%) | Mean lesion length (mm) |
|---|---|---|---|
| *Phaeomoniella chlamydospora* | CBS 117179 | 94 | 45.15a |
| *Cadophora luteo-olivacea* | CBS 109924 | 36 | 40.51ab |
| *Phaeoacremonium viticola* | CBS 113065 | 56 | 38.47b |
| *Pleurostomophora richardsiae* | CBS 117177 | 48 | 37.66b |
| *Phaeoacremonium krajdenii* | CBS 110118 | 36 | 36.31b |
| *Phaeoacremonium parasiticum* | CBS 117178 | 44 | 35.94b |
| *Phaeoacremonium venezuelense* | CBS 110119 | 34 | 35.93b |
| *Phaeoacremonium subulatum* | CBS 113584 | 48 | 29.32c |
| *Phialemonium* cf. *curvatum* | CBS 115998 | 36 | 28.23c |
| *Lophiostoma* sp. | CBS 109932 | 0 | 7.90d |
| Control (water) | – | – | 5.12d |
| l.s.d. ($P = 0.05$) | – | – | 6.256 |

## Discussion

The trunk inoculations and pruning wound results of the field trial showed that the fungal species *C. luteo-olivacea*, *Phl.* cf. *curvatum*, *Pl. richardsiae*, *Pm. parasiticum*, *Pm. viticola*, *Pm. krajdenii*, *Pm. venezuelense* and *Pm. subulatum* caused vascular discolouration similar to that seen in Petri diseased grapevines. Even though the inoculum levels used were much higher than in the case of natural infections, these results indicate that these fungi should be considered as vascular pathogens of grapevines.

The glasshouse trial was evaluated after a 3-month period and this short period could have contributed to the difficulty in distinguishing between the isolates. Measurement problems were also encountered during evaluation, since most of the plants were severely affected by vascular streaking caused by factors other than the inoculations. According to Stamp (2001), wood discolouration in the basal end of the rootstock might also be attributed to overly large basal rootstock disbudding sites. Recent studies have shown that many of these fungi are associated with stress-related diseases (Ferreira *et al*. 1999). Therefore, pathogenicity studies would be very difficult to conduct. It was recommended that pathogenicity studies be conducted under field conditions with a longer period of time.

Three of the species tested were isolated from asymptomatic nursery material, *C. luteo-olivacae*, *Pm. parasiticum* and *Phl.* cf. *curvatum*. It would seem that these fungi could contribute to decline in nurseries or young vineyards. However, the relative importance of the different fungi needs to be confirmed with the incidence of fungal isolations from diseased grapevines of different ages. *Phaeoacremonium aleophilum*, *Pm. parasiticum* and *Pm. viticola* have often been isolated from diseased vines. *Phaeoacremonium parasiticum* was frequently associated with *Pm. aleophilum*, *Pa. chlamydospora*, *Fomitiporia* sp. and *Botryodiplodia* sp. from 'hoja de malvon' symptoms (similar to esca) of grapevines in Argentina (Gatica *et al*. 2001; Dupont *et al*. 2002). *Phaeoacremonium viticola* has been isolated in a relatively low frequency along with *Pm. aleophilum* and *Pa. chlamydospora* from esca necrosis in France (Dupont *et al*. 2000). The *Lophiostoma* species has often been isolated from vascular tissue of diseased and healthy grapevines (data not shown). The results showed that the *Lophiostoma* sp. caused only pale discolourations, similar to the control and could not be re-isolated. This shows that *Lophiostoma* sp. is not a pathogen of grapevines. Since *Lophiostoma* sp. has often been isolated from healthy grapevines, it can be seen as an endophyte. Various unidentified sterile mycelium isolates, resembling the *Lophiostoma* sp., were obtained from a grapevine endophyte study (Mostert *et al*. 2000).

Although *Pm. aleophilum* was re-isolated from 90% of inoculated plants in the glasshouse trial, lesions were not significantly greater than that of the control. Adalat *et al*. (2000) obtained similar results when *Pm. aleophilum* was injected or applied to the wounded surface of spurs of grapevine cultivars Pinot Noir and Chardonnay. These authors further proved that *Pm. aleophilum* significantly affected plant height, number of internodes, total number of roots and leaf dry weight when single bud cuttings were planted in inoculated sand, but concluded that *Pm. aleophilum* was not such an aggressive coloniser of grapevine pruning wounds as *Pa. chlamydospora*. Despite its ability to penetrate pruning wounds, Larignon and Dubos (2000) suggested that this might not be the way *Pm. aleophilum* penetrates grapevines in France, mainly because of the absence of aerial inoculum during winter pruning. However, Gubler *et al*. (2001) concluded that in Californian vineyards, *Pm. aleophilum* was capable of infecting pruning wounds due to the presence of airborne inoculum during winter and spring (Eskalen and Gubler 2001), and significantly reduced growth in shoots emerging from infected spurs (Gubler *et al*. 2001). Therefore, it is clear that the results obtained from pathogenicity studies might be influenced by various factors, including cultivar and the method of inoculation used.

If one considers the fact that most of the spurs of the pruning wound inoculations were no more than 5 cm long (spurs were pruned to two buds), it is clear that these fungi might even be able to colonise grapevine tissue beyond the spur into the cordon in a relatively short period of time where they

might cause severe damage. In this regard, the contribution of *Pa. chlamydospora* in the esca complex is well documented (Mugnai *et al*. 1999) and pruning wounds have been identified as a major site of entry for this pathogen (Larignon and Dubos 2000). In similar studies, Adalat *et al*. (2000) observed black vascular discolouration extending 8.5 cm in Pinot Noir spurs inoculated with *Pa. chlamydospora* and 3.8 cm in the case of Chardonnay (6 months after inoculation).

In the past, species of *Acremonium* have been isolated from asymptomatic grapevine nursery material (Halleen *et al*. 2003), but have also been associated with fungi isolated from plants displaying symptoms of Petri disease (present study). The fact that *A.* cf. *charticola* and *A.* cf. *ochraceum* were re-isolated 0% and 40% (compared with the re-isolation percentage of the other fungi in the trial), respectively, together with the small lesions, suggest that these fungi do not play a role in the Petri disease complex. However, this needs to be confirmed with trials over a longer period of time. An *Acremonium* species was found to co-occur with *Phialophora gregata*, causing brown stem rot of soybean plants (Mengistu and Grau 1986). Pathological studies by these authors showed that *Acremonium* isolates caused low to moderate degrees of vascular and pith discolouration of soybean plants. It did not, however, cause any foliar symptoms, and only resulted in a slight reduction in plant height.

*Cadophora luteo-olivaceae*, *Phialemonium* cf. *curvatum* and *Phaeoacremonium parasiticum* were isolated from asymptomatic nursery grapevines. *Phaeomoniella chlamydospora* has also been isolated from apparently healthy rooted grapevines (Halleen *et al*. 2003), as have been found elsewhere (Bertelli *et al*. 1998). The presence of these fungi should be of great concern to the South African grapevine industry, since they have proven to be of pathogenic importance to grapevine cultivation. Standards of The Plant Improvement Act (Act 53 of 1976) as specified by the Vine Improvement Association of South Africa in its current form would, therefore, not be able to accommodate any of these infections, due to the absence of visible symptoms at the time of grafting and at the stage when 'apparently healthy, disease free' grapevines are sold to farmers. The fact that some of these infections occur at a stage before planting in the nursery, suggests that they spread via infected propagation material or as a consequence of contamination during the grafting process. Investigations into the occurrence of Petri disease fungi in canes of rootstock mother vines, have shown that *Pa. chlamydospora* and *Phaeoacremonium* spp. were present, although at very low levels (Fourie and Halleen 2002; Edwards *et al*. 2003). Unfortunately, it was not evident whether *Pa. chlamydospora* invaded mother plants via unprotected pruning wounds, although this appeared to be the case for *Botryosphaeria* and *Phomopsis* species (Fourie and Halleen 2004).

These findings highlight the need for pruning wound protection to protect rootstock mother plants from these infections. It is also strongly recommended that more sensitive techniques be developed for the detection of these pathogens, especially during the very early stages of the propagation process. The use of real-time PCR detection of *Pa. chlamydospora* and *Phaeoacremonium* spp. with species- and genus-specific primers, respectively, has proven to be sensitive and reliable in the detection of these fungi in artificially inoculated and naturally infected grapevines (Overton *et al*. 2005*b*). Species-specific primers have also been developed for all of the known *Phaeoacremonium* species which will aid in the correct identification of the species involved in Petri disease (Mostert *et al*. 2006). *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. were frequently isolated from the graft unions which might suggest that contamination occurred during the grafting process (Halleen *et al*. 2003). The presence of *Pa. chlamydospora* in the hydration tanks, on grafting tools and in callusing media was confirmed with species-specific PCR (Whiteman *et al*. 2002, 2004; Retief *et al*. 2006). Recommendations of rootstock drenches in benomyl or *Trichoderma* formulations integrated with hot water treatment, should be considered for the proactive management of Petri disease pathogens (Fourie and Halleen 2004).

Larignon and Dubos (1997) concluded that esca symptoms of grapevines are caused by a succession of fungi. Wood colonising fungi detoxify inhibitory compounds after which decay fungi, i.e. *Fomitiporia mediterranea* (syn. *F. punctata*) can more easily occupy and grow in the wood (Mugnai *et al*. 1999). It has been shown that *Pa. chlamydospora* and *Pm. aleophilum* could grow *in vitro* on medium with phenolic compounds usually produced in the reaction zone of the plant, whereas *F. mediterranea* could not (Mugnai *et al*. 1999). Subsequent field inoculation studies by Sparapano *et al*. (2001) have shown that individual inoculation, as well as co-inoculation of these three species in various combinations, resulted in symptom expression. This suggests that the succession of fungi is a possible event, but not the base of the process leading to foliar symptom development. Furthermore, the role of the lesser known vascular fungi and their possible synergy in Petri disease and esca as early colonisers, still needs to be determined.

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