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Fungi associated with healthy grapevine cuttings in nurseries, with special reference to pathogens involved in the decline of young vines

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

Additional keywords: Cylindrocarpon destructans, *Phaeoacremonium aleophilum*.

Introduction

Over the last few years, a drastic reduction has been noted in the survival rate of vine cuttings due to a decline disease present in nurseries, as well as in young vineyards, in the Western Cape Province of South Africa (Ferreira 1998). The low, average-take percentages (40–60%) of young vines can be attributed to several factors, including fungal, bacterial and viral diseases, insect and nematode pests, abiotic factors, as well as nutritional deficiencies and toxicities (Ferreira 1999). Petri disease, caused by *Phaeomoniella* (*Pa*) *chlamydospora* (=*Phaeoacremonium* (*Pm*) *chlamydosporum*), as well as several species of *Phaeoacremonium*, has been implicated as a major contributor to the decline of young vines in South Africa (Ferreira *et al.* 1994; Ferreira 1998; Fourie *et al.* 2000*a*, 2000*b*; Fourie and Halleen 2001; Groenewald *et al.* 2001). Other than *Pa. chlamydospora*, several species of *Phaeoacremonium*, including *Pm. aleophilum* and *Pm. rubrigenum*, have been isolated from diseased vines in South Africa (Groenewald *et al.* 2001). The situation may be even more complex than presently accepted, however, as several additional species such as *Pm. inflatipes* (Mugnai *et al.* 1999), *Pm. angustius* (Chicau *et al.* 2000), *Pm. parasiticum* (Gatica *et al.* 2001) and *Pm. mortoniae* (Groenewald *et al.* 2001) have also been associated with Petri disease in other parts of the world.

Furthermore, *Cylindrocarpon* spp., which cause black foot disease of grapevine (Maluta and Larignon 1991), have also been found to be associated with the decline of young vines in South Africa (Fourie *et al.* 2000*b*; Fourie and Halleen 2001). Species of *Cylindrocarpon* are common soil inhabitants, occurring as saprobes or weak pathogens, often associated with roots of herbaceous woody plants (Brayford 1993). Two species, *C. destructans* and *C. obtusisporum*, have been reported as the causal agents of black foot disease of grapevines. The first record of *C. destructans* on

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grapevines was made in France in 1961 (Maluta and Larignon 1991). Since then it has been isolated from diseased vines in Tasmania (Sweetingham 1983), Italy (Grasso 1984) and Portugal (Rego *et al.* 2000). In Sicily (Grasso and Magnano di San Lio 1975) and California (Scheck *et al.* 1998), the causal agent of this disease was again identified as *C. obtusisporum*.

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

Methods

Plant samples

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

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

Data analysis

The relative importance values (RI) of species isolated were computed (Ludwig and Reynolds 1988). After standardisation of the RI values within each sample by assigning the most frequent species the value of 100%, the other RI values were computed as percentages of it. For ordination analysis, a simple correspondence analysis was performed using the data referring only to those fungi with a standardised RI value of at least 1%. The data were pooled by site and time of isolation, as it was felt this would be the best way of characterising the occurrence of *Cylindrocarpon*, *Phaeoacremonium* and *Phaeomoniella* in the course of the growth of the plants. Simple correspondence analysis was performed on the reduced matrix of the raw data with the package XLSTAT ver. 4.3 (Kovach Computing Services, Anglesey, Wales, UK).

Results

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

Cylindrocarpon was by far the most frequently isolated taxon (RI = 47.5%). Other known grapevine pathogens included *Phaeoacremonium* spp. (RI = 9.8%), *Pa. chlamydospora* (RI = 5.7%), *Botryosphaeria* spp. (RI = 5.4%), *Rhizoctonia solani* (RI = 5.4%) and *Phomopsis viticola* (RI = 2.1%). Although species of *Fusarium* were also isolated in relatively high numbers (Table 1), a previous study conducted on South African grapevines by Marais (1979) suggested that they were of less importance in this disease complex, and hence they were excluded from further consideration. The frequencies at which the three selected taxa, *Cylindrocarpon*, *Phaeoacremonium* and *Phaeomoniella* were isolated were generally very similar for

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

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

[A]Raw frequencies are given for three nurseries and four isolation dates. The figures are the total number of isolates from a given nursery and isolation date, e.g. L0, V0 and J0 indicate isolates from callused cuttings prior to planting in nurseries L, V and J, respectively; L3, V3 and J3 indicate isolates from rooted cuttings 3 months after planting in nurseries L, V and J, respectively. Only those fungi with a relative importance value of more than 1% have been considered.

all three nurseries and four isolation dates (Table 1). *Cylindrocarpon* spp. were isolated only twice from callused cuttings prior to planting in the nurseries, but were more frequently isolated from rooted grapevine cuttings 3, 6 and 9 months after planting in the nurseries. Species of *Phaeoacremonium* and *Phaeomoniella*, however, were isolated both from callused cuttings prior to planting, as well as from rooted cuttings 3, 6 and 9 months after planting (Table 1). *Cylindrocarpon* spp. were isolated from more than 50% of all plants at the final sampling date.

Further analysis to characterise the occurrence of the three selected taxa, *Cylindrocarpon*, *Phaeoacremonium* and *Phaeomoniella* in the nursery plants over time, is presented in Table 2. *Cylindrocarpon* spp. were mostly isolated from the roots, followed by isolations from the rootstocks (Table 2). Although they rarely occurred in the graft unions and scions, they were more frequently isolated from the roots and rootstocks as the season progressed. On the other hand, *Pa. chlamydospora* was most frequently isolated from the rootstocks and graft unions, followed by isolations from the scions (Table 2), and rarely occurred in the roots. The isolations from the roots were made later during the season, after 6 and 9 months. The frequency of *Pa. chlamydospora* isolations did not fluctuate much during the growing season. *Phaeoacremonium* spp. were most frequently isolated from the graft unions followed by isolations from the rootstocks (Table 2), and rarely occurred in the scions and roots. The frequency of *Phaeoacremonium* spp. also did not fluctuate much during the growing season.

Discussion

The only previous comparable study in South African grapevine nurseries was conducted by Marais (1980), who chiefly isolated from soil and roots of dead, dying or stunted vines. Marais (1980) found that species of *Pythium* and *Phytophthora* were the most frequently isolated pathogens, with *Phytophthora cinnamomi* being the most virulent root rot pathogen. No mention was made of *Cylindrocarpon* and species of *Fusarium* also appeared not to play any major role in nursery disease. An earlier study reported, however, that low numbers of *Cylindrocarpon* spp. were isolated from roots and rhizospheres of grapevines showing decline symptoms in several commercial vineyards (Marais 1979).

Of the dominant fungal genera isolated in the present study, *Phaeomoniella chlamydospora* and species of *Cylindrocarpon* and *Phaeoacremonium* have in recent years been positively linked to the decline of young vines (Scheck *et al.* 1998; Fourie *et al.* 2000*b*; Rego *et al.* 2000). The present study demonstrates clearly that species of *Cylindrocarpon*, which cause black foot disease, rarely occurred in propagation material being sold to commercial nurseries. Species of this genus were isolated at higher

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

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

percentages later during the season. Less than 1% of the plants were infected with *Cylindrocarpon* spp. prior to planting in nurseries (October), whereas 50% or more of the plants were infected in the nurseries by the end of the season (June). Infection of the roots occurred first, followed by infection of the basal end of the rootstock in some plants. At the time of planting, the basal ends (especially the pith area) of most of the cuttings are partly or even fully exposed for infection by soilborne pathogens, since callus tissue does not normally cover the entire area. The young callus roots often break during the planting process, resulting in small wounds susceptible to infection by these pathogens. The occurrence of *Cylindrocarpon* spp. in the graft union might be explained by the nursery practice of covering graft unions with soil for a period of approximately 5 weeks to prevent drying of the callus tissue.

In contrast to these data for the species associated with black foot disease, primary pathogens associated with Petri disease such as *Pa. chlamydospora* and *Phaeoacremonium* spp. were already present in the healthy rootstock propagation material as endophytes. It was also recently shown that these pathogens could infect plants via pruning wounds on the mother vines (Larignon and Dubos 2000). Plants were infected, therefore, even before the cuttings were sold to nurseries for grafting. *Pa. chlamydospora* and *Phaeoacremonium* spp. were isolated most frequently from the rootstock and graft union. The high frequency of these pathogens occurring in the graft unions might be explained by the availability of sufficient weakened plant tissue as a result of the grafting process, or aerial contamination during the grafting process. These fungi rarely occurred in the roots, which suggests they are not primary soilborne pathogens. Bertelli *et al.* (1998) hypothesised about the soilborne nature of *Pa. chlamydospora*, since the fungus produces chlamydospores in artificial media (Crous *et al.* 1996). Petri disease is commonly attributed to *Pa. chlamydospora* and several species of *Phaeoacremonium* (Mugnai *et al.* 1999). In South Africa, however, Groenewald *et al.* (2001) found *Pm. aleophilum* to be the most frequently isolated *Phaeoacremonium* species from vines. Several other, as yet unidentified, species of *Phaeoacremonium* have also subsequently been isolated from local vines (Mostert and Crous, unpublished data), and their potential role in this disease remains to be further elucidated.

Several species of *Botryosphaeria* were isolated in the present study. *B. obtusa*, *B. ribis* and *B. dothidea* are known to occur in grapevines in South Africa (Crous *et al.* 2000). *B. obtusa* is the causal agent of black dead arm disease in France (Larignon and Dubos 2001), and has also been linked to various other symptoms including black to brown streaks, brown-red wood, altered pith, brown necrosis, white decay, brown-red margin of decayed wood and even healthy wood (Mugnai *et al.* 1999). It has also been associated with vine decline and dieback symptoms in Australian vineyards (Castillo-Pando *et al.* 2001). Isolates from the *B. ribis / B. dothidea* species complex are associated with Macrophoma rot, occurring on blighted stems, and have been reported to cause stem blighting and stem cankers on numerous hosts (Milholland 1994). A recent molecular study (Zhou *et al.* 2001) has, however, shown that these taxa must be seen as two separate species. *B. dothidea* is also associated with black dead arm in France (Larignon and Dubos 2001) as well

as excoriose in Portugal (Phillips 1998, 2000). The precise roles of the different species found in grapevines are not yet clearly understood, but it is possible that they are primary pathogens of stressed wood in some cases and secondary invaders of wood infected by other fungi in other cases (Pascoe 1998).

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

In summary, our results suggest that the low percentage survival of vine plants observed in recent years might be due to infected propagation material, as well as to new infections established in nurseries. Further studies should thus investigate various culture and molecular techniques to screen plants (grafted nursery plants as well as mother vines used as propagation material) that are visually disease-free, thereby confirming their status as healthy plants. In South Africa, the same nursery soil has been used for decades. Standard nursery practice of a 2-year rotation system, whereby cuttings are planted every second year, alternated with a cover crop, might have led to a build-up of soilborne pathogens such as species of *Cylindrocarpon* that appeared insignificant in earlier studies (Marais 1979, 1980). The duration of this rotation period should, therefore, be investigated to establish the effect on pathogen populations. A method to protect the basal end of the cuttings at the time of planting, such as dipping the basal end in a suitable fungicide or biological control agent, should also be investigated.

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