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Control of grapevine wood fungi in commercial nurseries

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Summary. Previous surveys conducted in commercial nurseries found that different wood fungi, namely *Cylindrocarpon* spp., Botryosphaeriaceae, *Phomopsis viticola* and *Phaeomoniella chlamydospora* infect grapevine cuttings. Two field trials were carried out to evaluate the effectiveness of cyprodinil + fludioxonil, pyraclostrobin + metiram, fludioxonil and cyprodinil to prevent or reduce natural infections caused by such fungi. Rootstock and scion cuttings were soaked in fungicidal suspensions for 50 min prior to grafting. After callusing, the grafted cuttings were planted in two commercial field nurseries with and without a previous history of grapevine cultivation. After nine months in the nursery, the plants were uprooted and analysed for the incidence and severity of the wood fungi. Plants uprooted from the field without a previous history of grapevine cultivation were generally less strongly infected by wood fungi. Under this condition, only the mixture cyprodinil + fludioxonil simultaneously reduced the incidence of *Cylindrocarpon* and Botryosphaeriaceae fungi, as well as the severity of *Cylindrocarpon* infections. Treatments did not produce significant differences in the incidence and severity of *P. viticola*, and *Pa. chlamydospora*. For plants grown in the field with a grapevine history, all fungicides except cyprodinil significantly reduced the incidence and severity of *Cylindrocarpon* fungi. Also, the incidence and severity of Botryosphaeriaceae pathogens were significantly decreased both by cyprodinil + fludioxonil and by cyprodinil. No significant differences were noticed for *P. viticola* incidence and severity, and *Pa. chlamydospora* was not detected again. These results suggest that the practice of soaking grapevine cuttings in selected fungicides prior to grafting significantly reduces *Cylindrocarpon* spp. and Botryosphaeriaceae infections, thus improving the quality of planting material.

Key words: *Cylindrocarpon*, black foot disease, Botryosphaeriaceae, chemical control.

Introduction

Over the last decade young vine declines have dramatically increased all over the world, mainly on account of the poor-quality of the planting material, and poor nursery and planting practices (Waite and Morton, 2007). The huge amount of vines experiencing decline and death at a young age frequently necessitates the uprooting of sizeable portions of vineyards, causing substantial economic losses. Among the factors contributing to the poor-quality of the planting material, and subsequent failure of young vines, are the early infection of nursery materials with fungi (Oliveira *et al*., 1998; Scheck *et al*., 1998; Morton, 2000; Rego *et al*., 2000). *Cylindrocarpon* spp. (Rego *et al*., 2000; Fourie and Halleen, 2001; Halleen *et al*., 2006a, 2006b), Botryosphaeriaceae fungi (Phillips, 2002; van Niekerk *et al*., 2006), *Phaeomoniella* (*Pa.*) *chlamydospora* and *Phaeoacremonium* spp. (Mostert *et al*., 2006) are some of the most widespread fungi infecting propagation material. As a consequence, vineyards established with material infected with these fungi suffer from a high percentage of declining plants with slow growth, reduced vigour, retarded sprouting, shortened internodes, and sparse and chlorotic foliage. Infected vines internally exhibit dark-brown to black wood and black streaking, mainly at the basal

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end of the rootstock when *Cylindrocarpon* or *Pa. chlamydospora* are involved (Rego *et al*., 2000; Halleen *et al*., 2006b), or at the tissues adjacent to the wound when other fungi, such as Botryosphaeriaceae, are implicated (van Niekerk *et al*., 2006).

In Portugal, the decline of young vines from fungi has been mainly attributed to *Cylindrocarpon* spp., *Pa. chlamydospora* and Botryosphaeriaceae fungi, acting alone or more frequently in combination (Oliveira *et al*., 2004). Previous inspections in commercial nurseries showed that the quality of the canes used for producing cuttings was frequently insufficient to ensure high-quality plants (Rego *et al*., 2001). The analysis of canes of mother-plants of rootstocks and scions prior to grafting showed that they were mainly infected with the air-borne fungi Botryosphaeriaceae and *Phomopsis viticola* (Rego *et al*., 2001). In contrast, a different scenario was observed when rooted cuttings were analysed. Here, after rooting in open field nurseries, the plants were mostly colonised by *Cylindrocarpon* (black foot disease) and to a lesser extent by *Pa. chlamydospora* (Petri disease) (Rego *et al*., 2000). Both these fungi were isolated at much greater frequencies from the basal end of the rootstock, and the incidence was not related to the susceptibility of the rootstock cultivars, suggesting that *Cylindrocarpon* infections are mainly caused by soil-borne inoculum (Rego *et al*., 2001; Oliveira *et al*., 2004; Rego, 2004). Similar findings have also been reported by other researchers (Fourie and Halleen, 2001; Halleen *et al*., 2003; Gubler *et al.,* 2004; Halleen *et al*., 2007).

In recent years, there have been promising results on the management of Petri disease (Crous *et al*., 2001; Fourie and Halleen, 2001; Jaspers, 2001; Laukart *et al*., 2001; Rooney and Gubler, 2001; Fourie and Halleen, 2004a, 2004b, 2006a) and black foot disease (Fourie and Halleen*,* 2001; Gubler *et al.*, 2004; Petit, 2005; Rego *et al*., 2006a; Halleen *et al*., 2007), using chemical, biological and physical control. Of the control measures, hot water treatment (HWT) of dormant nursery vines has been recommended to control black foot and Petri disease pathogens in grapevine nurseries (Halleen *et al*., 2007). Nevertheless*,* HWT (50ºC/30 min) only has a short-term effect (Crous *et al*., 2001) and has also been implicated in grapevine failure, due to damage caused to sensitive grapevine cultivars and rootstocks (Waite and Morton, 2007). Because of the side effects of HWT, particularly when not applied correctly, the most trouble-free method to control grapevine wood pathogens in nursery is still spraying with fungicides.

Promising results in laboratory and greenhouse experiments, on the control of the most important grapevine wood fungi, were recently obtained using conventional fungicides or biopesticides (Rego *et al*., 2006a; Nascimento *et al*., 2007). However, under field conditions, these approaches were not promising enough to be singly recommended for large-scale application (Halleen *et al*., 2007).

The present study was carried out under field conditions in commercial nurseries to determine the effectiveness of selected fungicides against the most widely co-isolated wood fungi from nursery vines.

Materials and methods

Field trials were conducted in commercial nurseries in the "Bombarral", Estremadura region, where the majority of Portugal's grapevine nurseries are located. Two different sites were selected: one without a grapevine nursery history and the other with a grapevine nursery history. The first site had been under pine-forest cover for decades, and the soil was converted to a grapevine nursery after the trees were felled. Grapevines were then planted there for the first time. The second site had a long history of being used as a grapevine nursery. A crop-rotation system was followed where grapevine was planted every first and second year, and this was followed by three years of different crops. This site was selected because *Cylindrocarpon* fungi had been detected in nursery plants in previous seasons.

Both trials were carried out with cuttings obtained from 1103 Paulsen certified rootstock mother blocks. These blocks were also selected because they are often found to contain grapevine wood fungi. The grapevine cultivar Aragonez was always used as scion. In general, both the scion and the rootstock are thought to be very susceptible to grapevine wood fungi in Portugal.

Commercial formulations of four fungicides: cyprodinil+fludioxonil (Switch®)*,* pyraclostrobin+metiram (Cabrio Top®)*,* fludioxonil (Geoxe®) and cyprodinil (Chorus®), were tested in all experiments (Table 1). Aqueous suspensions of fungicides were prepared according to the recommended field rates for other grapevine diseases or similar fungi (Table 1).

Prior to grafting, dormant rootstock and scion cuttings were dipped for 50 min in a fungicide suspension. Control cuttings were immersed in water. After this, the cuttings were hand-grafted at the same nursery following standard practices. The grafted cuttings were stacked in separate callusing boxes containing a mixture of moistened peat moss and perlite for 3 weeks at 30ºC and 100% relative humidity. After that period, the grafted cuttings were randomly planted in the nursery field following standard nursery practices. Each trial was a completely randomised design with five treatments of 20 grafted cuttings per treatment. During the rooting period, plants were checked for other diseases or pests. Pesticides, irrigation and nutrition were given and other practices were followed according to nursery guidelines.

After nine months, the vines were uprooted and isolations were made from necrotic tissue located 5 cm above the basal end of the rootstock, as described by Rego et al. (2006a). Fungi isolation was done by placing 12 wood fragments from each plant onto potato dextrose agar (PDA) medium amended with chloramphenicol (250 mg l^{-1}) and incubated at 24ºC, in darkness for 4 weeks. Fungal growth was regularly monitored and, where necessary, hyphal tips were transferred to Petri dishes containing PDA for later identification. The main fungal species were identified on the basis of their morphological and cultural characters. For each trial, the incidence and severity of the fungi present in naturally infected grapevine plants were determined on 100 vines and 1200 fragments of colonised wood. The incidence of the fungi was determined as the mean percentage of grapevine plants that were infected with each fungus, and the severity as the mean percentage of infection of each fungus in each diseased plant.

Data collected from each trial were analysed separately using Statistica Version 6.0 (StatSoft Inc., Tulsa, OK, USA). Incidence data were subjected to Chi-Square statistical analysis (χ^2 test) ($P \leq 0.05$) and severity data to analysis of variance (ANOVA). Treatment means were compared using Tukey's test ($P \leq 0.05$). Percentages were transformed to arcsine-square root values before analysis.

Results

The effects of four selected fungicides, cyprodinil + fludioxonil, pyraclostrobin + metiram, fludioxonil, and cyprodinil, on the incidence and severity of grapevine wood fungi, were evaluated on naturally infected grapevine plants Aragonez/1103 Paulsen grown in two different commercial nursery soils, with and without a grapevine nursery history.

After nine months of rooting in the open-field nurseries, a high number of plants developed symptoms, which included poor growth, shortened internodes and a low number of roots. When the plants were cut open for isolation, internal necrosis was visible, particularly at the basal end of the rootstocks (data not shown).

In both trials, *Cylindrocarpon* spp., Botryosphaeriaceae and *Phomopsis viticola* were the most frequently isolated fungi. Unexpectedly, *Pa. chlamydospora* and/or *Phaeoacremonium* spp. were never detected.

The experiment conducted in the soil without a nursery history revealed a high percentage of naturally infected grapevine plants in the untreated control, with some vines co-infected with two or three fungi. Botryosphaeriaceae fungi (55%) and *Cylindrocarpon* spp. (40%) were predominant, followed by *P. viticola,* which was isolated from 15% of the plants (Table 2). Under these conditions, only the mixture cyprodinil + fludioxonil significantly ($P \leq$ 0.05) reduced the incidence of both *Cylindrocarpon* and Botryosphaeriaceae fungi. This treatment also significantly reduced *Cylindrocarpon* spp. severity. No significant differences were seen among the remaining treatments and the control, regarding the incidence and severity of these pathogens. Concerning *P. viticola*, no significant differences were found between treated and untreated plants (Table 2).

In the field with a nursery history, and despite the crop rotation that was practised, a very high proportion of plants (75%) was infected with *Cylindrocarpon* fungi, as compared with the untreated control (Table 3). Under such high disease pressure, all fungicides (cyprodinil + fludioxonil, pyraclostrobin + metiram, fludioxonil) except cyprodinil significantly reduced the incidence and severity of *Cylindrocarpon* spp. Also, the mixture cyprodinil + fludioxonil and cyprodinil alone significantly decreased the incidence and severity of Botryosphaeriaceae fungi. No significant differences were found in the incidence and severity of *P. viticola* (Table 3). The incidence and the severity of Botryosphaeriaceae fungi and *P. viticola* were comparable to those recorded in the other field trial.

The overall assessment of the fungicide treatments of rootstock and scion cuttings before grafting revealed that the mixed fungicides cyprodinil + fludioxonil and pyraclostrobin + metiram signifi-

Table 1. Details of fungicides tested to control grapevine wood fungi in commercial nurseries.

Fungicide	Trade name	Manufacturer	Concentration (%) a. i.	Field rate
Cyprodinil	Chorus	Syngenta	50% cyprodinil	1.00 g l^{-1}
Fludioxonil	Geoxe	Syngenta	50% fludioxonil	1.00 g l^{-1}
Cyprodinil +fludioxonil	Switch 62.5WG	Syngenta	37.5% cyprodinil + 25 % fludioxonil	1.00 g l^{-1}
Pyraclostrobin + metiram	Cabrio Top	BASF	55% metiram + 5% pyraclostrobin	1.50 g l^{-1}

Table 2. Effects of selected fungicides on the incidence and severity of *Cylindrocarpon,* Botryosphaeriaceae and *Phomopsis* in naturally infected grapevine plants grown in a nursery field without a previous history of grapevine cultivation.

Treatment	Incidence (%)[a]			Severity (%)[b]		
	Cylindrocarpon	Botryosphaeriaceae fungi	*Phomopsis*	*Cylindrocarpon*	Botryosphaeriaceae fungi	*Phomopsis*
Cyprodinil + fludioxonil	10.00 a	10.00 a	5.00 a	0.83 a	2.08 a	0.42 a
Pyraclostrobin + metiram	20.00 ab	35.00 ab	5.00 a	4.17 ab	4.17 ab	0.83 a
Fludioxonil	25.00 ab	50.00 ab	10.00 a	4.58 ab	10.83 b	1.25 a
Cyprodinil	40.00 b	35.00 ab	5.00 a	5.42 ab	4.17 ab	0.83 a
Water (control)	40.00 b	55.00 b	15.00 a	11.62 b	7.92 ab	3.33 a

[a] In each column data followed by the same letter did not differ significantly ($P \leq 0.05$) according to the $\chi 2$ test; each value is the mean of 20 repetitions of the plant data.
[b] In each column data followed by the same letter did not differ significantly ($P \leq 0.05$) according to Tukey`s test; each value is the mean of 20 replicates of the plant data.

Table 3. Effects of fungicides on the incidence and severity of *Cylindrocarpon,* Botryosphaeriaceae and *Phomopsis* in naturally infected grapevine plants grown in a nursery field with a previous history of grapevine cultivation.

Treatment	Incidence (%)[a]			Severity (%)[b]		
	Cylindrocarpon	Botryosphaeriaceae fungi	*Phomopsis*	*Cylindrocarpon*	Botryosphaeriaceae fungi	*Phomopsis*
Cyprodinil + fludioxonil	40.00 a	20.00 b	0.00 a	4.17 a	1.67 a	0.00 a
Pyraclostrobin + metiram	30.00 a	35.00 bc	5.00 a	4.17 a	4.17 ab	1.76 a
Fludioxonil	25.00 a	40.00 bc	10.00 a	2.92 a	4.58 ab	2.50 a
Cyprodinil	65.00 b	15.00 a	5.00 a	10.42 b	0.83 a	0.52 a
Water (control)	75.00 b	50.00 c	10.00 a	10.42 b	13.33 b	2.08 a

[a] In each column data followed by the same letter did not differ significantly ($P \leq 0.05$) according to the $\chi 2$ test; each value is the mean of 20 repetitions of the plant data.
[b] In each column data followed by the same letter did not differ significantly ($P \leq 0.05$) according to Tukey`s test; each value is the mean of 20 replicates of the plant data.

Table 4. Effects of fungicides on the total number of infected grapevine plants grown in nursery fields with and without a previous history of grapevine cultivation.

Treatment	Total number of infected plants (%)[a]	
	Field without grapevine history	Field with grapevine history
Cyprodinil + fludioxonil	25a[b]	50 a
Pyraclostrobin + metiram	45a	50 a
Fludioxonil	65 ab	65 ab
Cyprodinil	60 ab	69 ab
Water (control)	70 b	85 b

[a] A plant was considered infected if either *Cylindrocarpon* spp., or Botryosphaeriaceae, or *Phomopsis viticola* was detected.
[b] In each column, data followed by the same letter did not differ significantly ($P \leq 0.05$) according to the $\chi 2$ test; each value is the mean of 20 replicates of the plant data.

cantly decreased the incidence of grapevine wood fungi as a whole, in both nurseries (Table 4).

Discussion

The present study confirmed that *Cylindrocarpon* spp., along with Botryosphaeriaceae fungi, is a major threat to the grapevine nursery industry. Previous surveys carried out in Portugal showed that *Cylindrocarpon* spp. and *Pa. chlamydospora* were the most frequently isolated fungi from nursery vines and consequently the main cause of young grapevine decline. Botryosphaeriaceae fungi, along with other fungi, were concomitantly isolated from those materials, but their role as primary wood pathogens was not thoroughly investigated (Rego *et al.*, 2000). Subsequently, we saw that infections caused by the Botryosphaeriaceae started earlier in the canes of rootstock mother plants (Rego *et al.*, 2001; Pinto *et al.*, 2005). Consequently, rootstock cuttings were already infected prior to grafting. Botryosphaeriaceae fungi are frequently isolated from *Vitis vinifera* cultivars (Phillips, 2002; Rego *et al.*, 2006b) and therefore the scion cuttings are regularly infected.

In order to prevent young grapevine decline, a number of management strategies have been recommended to produce high-quality planting material free of serious pathogens (Waite and Morton, 2007). For fungal diseases, most of these experiments in commercial nurseries have focused on two diseases, Petri disease, caused by *Pa. chlamydospora* and *Phaeoacremonium* spp. (Fourie and Halleen, 2004b), and black foot disease, caused by *Cylindrocarpon* spp. and *Campylocarpon* spp. (Halleen *et al.*, 2007). In contrast, for Botryosphaeriaceae diseases of grapevine, control measures at the nursery stage have not been studied in detail (van Niekerk *et al.*, 2006). From the different control methods, comprising chemical, biological and physical treatments, HWT has been widely recommended as part of an integrated strategy for the management of both black foot and Petri diseases. However, in a recent review, Waite and Morton (2007) stated that HWT causes stresses in some sensitive cultivars, and is also responsible for the failure of treated plants.

The aim of the present study was to investigate the effect of some fungicides, cyprodinil + fludioxonil, pyraclostrobin + metiram, fludioxonil and cyprodinil, on wood fungi in naturally infected grapevines Aragonez/1103P, grown in two commercial nursery soils, with and without a previous nursery history. Prior to grafting, the dormant rootstock and scion cuttings were soaked in a fungicidal suspension for 50 min using a single method, without risks and easy to implement as a routine practice in the nursery. This treatment could replace HWT in cases where sensitive cultivars need treatment prior to grafting. Our approach examined the effects of fungicides on the rootstock, and the scion material, and on the prevention or reduction of the full range of wood infecting pathogens. Initial studies by Fourie and Halleen (2004b), who investigated the control of the pathogens causing Petri disease, only treated rootstock material prior to grafting. One of their treatments investigated the effect of HWT on dormant grapevines without any prior treatment. The first approach therefore excluded all infections that might come from the scion material. The second approach allowed for infections during almost all the nursery stages. However, rootstock and scion material was treated prior to cold storage, grafting and planting in subsequent studies (Fourie and Halleen, 2006). In general these repeated treatments reduced pathogen incidence. Unfortunately the incidence of *Cylindrocarpon* spp. was too low to draw any meaningful conclusions regarding the treatments. Treatment of only the basal ends of grafted vines prior to planting, specifically aimed at preventing *Cylindrocarpon* infections in the nursery, also did not consistently prevent these infections (Halleen *et al*., 2007). HWT of dormant vines was the only treatment that eradicated *Cylindrocarpon* infections.

The mixture cyprodinil + fludioxonil significantly reduced both the incidence and the severity of *Cylindrocarpon* spp. under high and very high inoculum pressure. The incidence of Botryosphaeriaceae fungi was also significantly reduced. The mixture did not eradicate these pathogens from the cuttings, but it markedly reduced their incidence and severity compared with the untreated control. The surface and most probably endophyte wood pathogens carried by these cuttings must have been reduced, thus improving the health of the nursery vines, by preventing further contamination during grafting, callusing and storage. Since *Pa. chlamydospora* was not detected in the study, the effect of the fungicide treatments against this pathogen could not be determined. However, previous laboratory findings have shown the efficacy of cyprodinil + fludioxonil in inhibiting the mycelial growth and spore germination of *Pa. chlamydospora* (Jaspers, 2001). This was also confirmed in greenhouse trials with potted grapevines, in which it significantly reduced the incidence of *Pa. chlamydospora* (Nascimento *et al*., 2007). It is therefore probable that cyprodinil + fludioxonil has a similar effect on *Pa. chlamydospora* in field nurseries.

The results obtained from treatment with pyraclostrobin + metiram are also interesting. Although this mixture only significantly reduced the incidence and severity of *Cylindrocarpon* spp. in the field with a previous nursery history, overall it was just as effective as cyprodinil + fludioxonil.

The study also found two different situations as regards nursery soil. One of the field experiments was established in a pine soil and where grapevine had never previously been grown. Control plants uprooted from this field revealed a high incidence of *Cylindrocarpon* spp. (40%), when they were expected to be almost free from this soilborne pathogen. This finding was unpredicted, because these fungi are rarely isolated from rootstock cuttings prior to rooting (Rego *et al*., 2001; Fourie and Halleen, 2002; Halleen *et al*. 2003), thus suggesting that the *Cylindrocarpon* spp. populations from pine soils can infect grapevine. The second field experiment was carried out in a typical grapevine nursery soil, where grapevine had been planted consecutively for two years, followed by three years of rotation with other crops (e.g. potato, cabbage, carrot, garlic, and leek) and/or gramineous plants. The findings of the study made it clear that *Cylindrocarpon* was significantly influenced by the repeated use of the nursery soil even when other crops were grown on it in a rotation programme. In this field a very high number of plants (75%) were infected with *Cylindrocarpon* spp., most probably from soilborne inoculum, thus demonstrating the importance of not replanting in the same nursery for prolonged periods of time. Legislation now in force in Portugal, allows only one year of continuous grapevine planting in grapevine nurseries, and this rule will probably contribute to a decrease in soilborne inoculum levels. The high incidence of *Cylindrocarpon* spp. in plants grown in the field with a long history of grapevine cultivation, as compared with other findings in Portugal (Rego *et al*., 2000), support the evidence that a build-up of these soilborne pathogens has occurred. Similar findings have been reported by other researchers in South Africa (Halleen *et al*., 2003, 2006b). Even under a rotation system, the long-term survival of *Cylindrocarpon* spp. in the soil and the cross-infection potential of these pathogens should be investigated.

In conclusion, the study indicated that some fungicides inhibited fungal infection and colonisation within vine plants, before and during rooting. In this regard, the overall reduction in fungal presence caused by cyprodinil+fludioxonil and pyraclostrobin+metiram clearly demonstrated that these fungicides applied in a dormant nursery were an alternative or a complement to other strategies. Most probably, the results could be improved still more by treating the cuttings before planting, thus providing additional protection to the basal end of the rootstock during the rooting process. To ensure plant health, it is also essential to use soils without a recent history of grapevine cultivation.

Acknowledgements

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