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Occurrence of *Phaeomoniella chlamydospora* on grapevine planting material in Sardinia and its control with combined hot water and cyproconazole treatments

Salvatorica SERRA [1], Maria Angela MANNONI [1], Virna LIGIOS[1] and Pier Paolo FIORI [2]

[1] Dipartimento di Protezione delle Piante, Università, Via De Nicola 9, 07100 Sassari, Italy
[2] Dipartimento per la Ricerca nell'Arboricoltura, AGRIS-Sardegna (Agricultural Research Agency of Sardinia), via Mameli n. 126/d, 09123 Cagliari, Italy

Summary. The occurrence of *Phaeomoniella chlamydospora* was investigated during vine propagation in an Italian nursery, and combined hot water (HWT) and cyproconazole treatments were carried out to limit the spread of the fungus in nursery vines. In the three-year period 2005–2007, cutting and graft samples (scion cv. Sangiovese, rootstock cv. 140Ru in 2005 and 1103P in 2006–2007) were taken during propagation at several infection risk stages, and the occurrence of *P. chlamydospora* was assessed by nested PCR. In 2005 and 2006, cuttings from esca-symptomatic grapevines (scion cv. Sauvignon blanc, rootstock cv. 140Ru in 2005 and 1103P in 2006) were treated at different stages of the propagation process. In 2007, artificially infected 1103P cuttings were treated with HWT and cyproconazole after being inoculated with *P. chlamydospora*, and the fungus was detected by isolation on agar medium. The effect of HWT on the growth of 1103P, 779P, Sangiovese and Cabernet franc cuttings was also assessed. Despite extended wood discoloration, *P. chlamydospora* was scarce in nursery vines during the three-year period. The contamination of planting material may have resulted from already infected mother plants (0.0 to 6.7% of infected cuttings) or may have started during the propagation, particularly after grafting (0.0 to 23.3% of infected grafts). Canes from esca-diseased mother plants were always infected, but the incidence of infection of the cuttings varied widely (about 30% in 2005, from 1.9 to 4.1% in the other years). However, no final conclusions could be drawn about the stage or stages at which the nursery vines mainly became infected, because the infection frequencies detected in the propagation process were too low and irregular. As regards control of *P. chlamydospora*, HWT of cuttings performed before or after cold storage influenced plant growth, depending on both the cultivar and the growing conditions, but HWT was harmful to callused graftlings. Natural contamination of nursery material in 2005 and 2006 was insufficient to allow the effectiveness of treatments to be assessed. In 2007, HWT and cyproconzole alone did not reduce the percentage of infection in artificially inoculated cuttings. Only cyproconazole immediately followed by HWT significantly reduced the number of infected cuttings, but this procedure did not eradicate the pathogen.

Key words: Petri disease, esca disease, epidemiology, nursery plants.

Introduction

The fungus *Phaeomoniella chlamydospora* is one of the main causal organism of Petri disease and esca of grapevine, which are becoming more common in grape-growing areas all over the world (Mugnai *et al*., 1999). Several species of *Phaeoacremonium* are also associated with these diseases, but *P. chlamydospora* is generally considered the most important, especially with regard to Petri disease (Mostert *et al*., 2006).

Phaeomoniella chlamydospora can spread as an air-borne inoculum (Larignon and Dubos, 2000; Eskalen and Gubler, 2001) and can invade the wood through wounds (Mugnai *et al*., 1999; Larignon and Dubos 2000; Eskalen *et al*., 2007; Serra *et al*., 2008). Once inside the wood, it estab-

Corresponding author: S. Serra
Fax: +39 079 229316
E-mail: salvase@uniss.it

lishes itself in or around the vessels (Feliciano and Gubler, 2001; Troccoli *et al*., 2001) and can spread through the sap to new canes that may be intended for propagation (Edwards *et al*., 2004; Whiteman *et al*., 2007). Recent studies have found that nursery vines ready for sale can already be infected with *P. chlamydospora* (Bertelli *et al*., 1998; Rego *et al*., 2000; Laukart *et al*., 2001; Halleen *et al*., 2003; Aroca *et al*., 2006; Zanzotto *et al*., 2007).

Several studies have examined how nursery plants are infected. *P. chlamydospora* usually occurred at low frequencies (0.0–12%) in mother plant canes (Larignon and Dubos, 2000; Rego *et al*., 2001; Fourie and Halleen, 2002; Giménez-Jaime *et al*., 2006; Whiteman *et al*. 2007; Zanzotto *et al*., 2007; Pollastro *et al*., 2009; Aroca *et al*., 2010). Only rarely was *P. chlamydospora* incidence in canes higher (20.5%, Retief *et al*., 2006). Nursery vines may also be contaminated during the propagation process. A large number of wounds are caused during that process and *P. chlamydospora* inoculum has been detected on cutting tools and grafting machines, in hydration tanks, callusing media and nursery soil (Whiteman *et al*. 2004; Retief *et al*., 2006; Edwards *et al*. 2007; Pollastro *et al*., 2009; Aroca *et al*., 2010). It is also possible that the greater incidence of *P. chlamydospora* in nursery vines is not only due to new infections that are initiated during the propagation process, but also to the gradual colonization of the wood starting from infection in the canes (Fourie and Halleen, 2004).

To limit the spread of *P. chlamydospora* through the propagation material, the control strategy most studied has been to treat dormant cuttings and nursery plants with hot water (Waite and Morton, 2007). The vine wood is usually dipped in a water bath at 50°C for 30 min. However, the effectiveness of this treatment has been inconsistent (Crous *et al*., 2001; Laukart *et al*., 2001; Rooney and Gubler, 2001; Fourie e Hallen, 2004; Habib *et al*., 2008; Gramaje *et al*., 2009a). Hot water treatment (HWT) can however adversely affect plant growth. According to Waite and May (2005), the effect of HWT on plant viability and quality depends on the cultivar, the growing conditions of the mother vines, the type of nursery material used (cuttings or uprooted plants), cold storage, pre-soaking, and especially on the growing conditions after callusing. Despite these drawbacks, HWT is common in grapevine nurseries outside Europe (Waite and Morton, 2007), but it is not favoured in Italy.

Another control strategy involves the chemical treatment of the nursery material. Benomyl, captan, chitosan, cyprodinil+fludioxonil, dodecyldimethylammonium, fenarimol, phosphonates, prochloraz Mn and pyraclostrobin all reduce *P. chlamydospora* incidence in nursery material and young vines (Di Marco *et al*., 2000; Laukart *et al*., 2001; Fourie and Halleen, 2004, 2006; Nascimento *et al*., 2007; Gramaje *et al*., 2009b; Vigues *et al*., 2010). Benomyl was the most common and effective active ingredient, but it has been banned in Europe since 2003. Even though chemical treatment was less effective than HWT, it could be useful when the fungicide is added to the hydration tanks, provided that the water is changed frequently (Fourie and Halleen, 2004).

Unfortunately, even when pathogen free vines are planted, the opportunities for new infections to arise in the vineyard are numerous because every year grapevines are subjected to pruning cuts of varying intensity depending on the training system. The wounds made on one-year-old canes to establish the bearing units are a real Achilles' heel, as these wounds remain susceptible to infection for several months (Eskalen *et al*., 2007; Serra *et al*., 2008). Despite frequent opportunities for healthy vines to become infected in the vineyard, it is nevertheless important to minimize the spread of wood fungi in the nursery.

The aims of this study were to determine at which stages in the vine propagation process the risk of infection was high in an Italian nursery, and to evaluate the effectiveness of combined hot water and cyproconazole treatments against *P. chlamydospora* in nursery material. Cyproconazole has never been used to treat nursery materials, but it has proved effective in reducing foliar symptoms on retrained grapevines affected with esca (Calzarano *et al*., 2004).

Materials and methods

The trials were carried out in the three-year period 2005–2007 in a Sardinian nursery (Sassari, Italy). Since some growers prefer to plant rooted rootstocks and to do the field grafting themselves, treatment trials were carried out on both grafts and rootstocks.

Production of nursery vines

The standard propagation process in the nursery was as follows. Canes were harvested from mother plants in winter (December and January). Rootstock cuttings (40–45 cm) and 1-m cane segments of a scion cultivar were prepared and placed in cold storage (4°C, 90% RH) until grafting or planting. Shortly before grafting, usually in early spring, the rootstock cuttings were disbudded and the scion cultivar cane segments were cut to obtain one-bud scions. Cuttings and scions were soaked in water with thiophanate methyl (about 1 g a.i. L^{-1}) for 2–5 h and were then grafted with a omega-cut machine. The grafts were dipped in a molten (80–85°C) wax formulation containing 0.003% of 2,5–dichlorobenzoic acid and 0.1% of 8–hydroxyquinoline sulphate, and then packed upright in callusing boxes with moist sawdust. Boxes were moved to a hot-room (25–30°C, 80% RH) for about 20 days for the callusing process. Successfully callused graftlings were trimmed and dipped again in a different molten wax formulation with added pigments to protect callused grafts in arid and/or sunbaked areas, prior to being transplanted to the nursery field. Ungrafted rootstock cuttings were soaked as above before planting in late winter or early spring.

Upon planting (spacing 0.06×1.30 m) graftlings and cuttings were covered with soil to prevent dehydration. The soil cover was removed following bud burst. Growing plants were irrigated and fertilized and foliar diseases were controlled with regular fungicide sprays. In December, the nursery plants were uprooted, graded and put on the market.

All of the trials were carried out following this procedure, except where stated otherwise.

Assessment of infection risk in the propagation process

Canes sufficient to provide about 450 grafts were collect from mother blocks in the nursery. Rootstock canes consisted of cv. 140 Ruggeri (140Ru) in 2005, and cv. 1103 Paulsen (1103P) in 2006 and 2007; scion canes consisted of cv. Sangiovese. The canes of each cultivar were harvested from the same mother blocks during the three years.

Up to 50 cuttings or grafts were collected at each of a number of stages in propagation. At the end of the propagation process the remaining grafts were planted in the nursery field. Seven types of samples were obtained:

1) cuttings obtained directly from canes with disinfected pruning shears (infections in the mother plants);

2) cuttings obtained following standard nursery practices (infections through cuts);

3) grafts obtained with disinfected blades and callused in sterilized sawdust after hydration and disbudding following standard nursery practices (infections carried by water);

4) grafts obtained following standard nursery practices but callused in sterilized sawdust (infections through cuts);

5) grafts callused following standard nursery practices but trimmed with disinfected pruning shears (infections carried by sawdust);

6) graftlings trimmed following standard nursery practices (infections through cuts);

7) graftlings obtained following standard nursery practices and planted in the nursery field.

At the end of 2006 about 40 uprooted-vines from the nursery field were trimmed, dipped in molten wax and placed in cold storage (4°C) for about 4 months. They were replanted near new graftlings in 2007 (sample 8).

Cuttings and grafts from samples 1–6 were potted singly in sterile soil and grown in a shade house; cuttings were hydrated in sterile water and the end outside the soil was protected with sealant. Pruning and grafting tools were disinfected with bleach (7% active chlorine) and then rinsed in sterile water, while sawdust and soil were sterilized by autoclaving at 121°C for 50 min. Sterilized soil was used not less than one week after sterilization. Rootstock cuttings grown in pots were cut as usual (40–45 cm) in 2005, while in 2006 and 2007 two-bud cuttings were obtained. Sangiovese cuttings were always two buds long.

Effectiveness of HWT and cyproconazole

Hot water and cyproconazole were tested both singly and in combination. HWT was performed in the laboratory by dipping nursery material in a circulating hot water bath at 50°C (heating immersion circulator Julabo, Seelbach, Germany). After 30 min the nursery material was immediately cooled in water to about 18–20°C. Cyproconazole was applied by soaking cuttings in 20–30

litres of a cyproconazole suspension (0.1 g a.i. L^{-1}) for at least 12 h.

Canes representing rootstock cv. 140Ru in 2005 and 1103P in 2006 and scion cv. Sauvignon blanc in both years, were harvested from vineyards affected with esca, particularly from vines that had previously shown foliar symptoms, to obtain about 350 cuttings and 200 grafts. Treatments were carried out at different stages to obtain six types of samples:

1) cuttings obtained directly from canes with disinfected pruning shears (scion and rootstock);
2) cuttings treated with:
   A, cyproconazole before, and hot water after, cold storage (rootstock);
   B, hot water and cyproconazole after cold storage (rootstock);
   C, hot water and cyproconazole before cold storage (scion and rootstock);
3) cuttings treated as 2A and 2B above and planted in the nursery field (rootstock);
4) untreated cuttings planted in the nursery field (rootstock).

Some of the rootstocks and scions treated before cold storage (sample 2C) were grafted producing about 100 grafts which were hot-water treated after callusing. About 50 grafts were obtained that had not received any treatment. In 2007, cuttings were taken, using disinfected pruning shears (sample 1), only from canes collected in the Sauvignon blanc vineyard, and these were not treated. Two types of sample were obtained: 1A, canes from mother plants showing foliar symptoms in the summer of 2006; 1B, canes from plants showing symptoms in the 2002–2005 period, but not in the summer of 2006.

Up to 50 cuttings from each of samples 1, 1A, 1B, 2A, 2B and 2C were grown in pots as above. Rootstock cuttings grown in pots had a standard length (40–45 cm) in 2005, while in 2006 they were two buds long. Sauvignon blanc cuttings were always two buds long.

Given the low infection level of untreated material in 2005 and 2006, cv. 1103P cuttings were artificially inoculated in 2007. A *P. chlamydospora* strain isolated from a Sardinian grapevine was grown in a liquid medium containing 2% malt-extract. After 10 days of incubation at 25°C, the culture liquid was filtered through a double layer of cheesecloth and centrifuged at 950 *g* for 10 min. The pellet was diluted to obtain a conidial suspension of about 2×10^7 conidia mL^{-1}. Cuttings were soaked in the conidial suspension overnight and drained before being used in two trials.

In the first trial, 50 two-bud cutting samples were treated as follows: 1) HWT; 2) cyproconazole; 3) HWT+cyproconazole; 4) cyproconazole+HWT; 5) not treated.

Cuttings were grown in pots as above.

In the second trial, 800 40–45-cm-long cuttings were grafted with both Sangiovese and Moscato scions (400 grafts per cultivar). Grafts were callused, grown in the nursery field according to standard practices and hot-water treated after uprooting.

In 2007, the influence of HWT on the viability of nursery vines was further evaluated on the rootstock cultivars 1103P and 779 Paulsen (779P) and on the scion varieties Sangiovese and Cabernet franc. In this trial the occurrence of *P. chlamydospora* was not investigated. Two hundred cutting samples from each rootstock variety and 100 cutting samples from each scion variety were hot water treated as follows: 1) before cold storage; 2) after cold storage; 3) not treated.

Standard cuttings (40–45 cm long) were planted in the nursery field.

Examination of nursery vines and *P. chlamydospora* detection

At the end of one growing season, uprooted cuttings and uprooted grafts grown in pots or in the nursery field were visually inspected, recording root and shoot development (scored on a four-point scale: 1, poor; 2, fair; 3, good; 4, optimal), weight and length of viable vines.

Up to 30 viable vines per sample were stripped of the bark and split open longitudinally to check for the occurrence and the length of dark streaks inside the wood (xylem vessels turning brown or black). All viable vines obtained from diseased mother plants in 2006 and 2007 were thus cut and inspected. Usually only plants with the shoot originating in a scion (grafts) or an apical bud (cuttings) were considered, but when their number in samples was low, plants with shoots originating in intermediate or basal buds were also examined. Tiny wood fragments were collected from 1 cm above the rootstock base, from 2 cm below the shoot or the graft point, from the graft point, from

the shoot and, in long cuttings or grafts, also from one or two points along the rootstock. All samples were examined separately. Wood fragments were freeze-dried at -20°C prior to DNA extraction.

DNA extraction and nested PCR

About 50 mg of freeze-dried wood from each sample was placed in 2 mL microcentrifuge tubes each having one 5 mm steel bead. The samples were ground in a Mixer Mill (Retsch Gmbh & Co. KG, Germany) for two rounds, 2.5 minutes at 30 Hz each. The DNA was extracted following Doyle and Dickson (1987), then suspended in 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4°C until the nested PCR.

Nested PCR was performed using the primer pairs $OPA13_{844}D$ for the first-round and $OPA13_{844}F$ for the second-round, developed by Pollastro *et al*. (Dipartimento di Protezione delle Piante e Microbiologia Applicata, University of Bari, Italy, unpublished). The first-round PCR reactions were performed in a volume of 50 µL containing: 1 µL of extracted DNA (template), 5 µL of 10× buffer, 2 mM of $MgCl_2$, 200 µM of dNTPs (each), 0.5 µM of each forward and reverse primer $OPA13_{844}D$, 2 Units of Taq DNA polymerase (EuroClone, Celbio, Milano, Italy). A negative control with sterile deionised water and a positive control with *P. chlamydospora* DNA from wood tissue was included. The second round was carried out with primers $OPA13_{844}F$ (forward and reverse) by adding 1 µL of the first round product to 49 µl of the mixture described above in a new tube. The cycling conditions in the PCR termalcycler (One Personal, EuroClone) were: (first amplification phase) 95°C 3 minutes (1 cycle); 95°C 30 sec; 57°C 30 sec; 72°C 1 min (30 cycles); 72°C 5 min (1 cycle); (second amplification phase) 95°C 3 min (1 cycle); 95°C 30 sec; 60°C 30 sec; 72°C 1 min (35 cycles); 72°C 5 min (1 cycle).

Amplification products were analysed by electrophoresis in 1.5% agarose gel containing 0.5 µg mL^{-1} ethidium bromide with TAE running buffer (40 mM Tris-acetate pH 8.0 and 1 mM EDTA) at 5 V cm^{-1}. Gel images were acquired with a Gel Doc 2000 Apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The product size was estimated by comparing it with the SharpMass 100–DNA ladder (EuroClone) and Quantity One software (Bio-Rad Laboratories).

Isolation on agar medium

Phaeomoniella chlamydospora in artificially inoculated cuttings was detected on agar medium as described by Serra *et al*. (2008). One wood section (1–2 mm thick, covering the entire cross-sectional area) was cut out 1 cm above the cutting base, 1 cm below the shoot and from the middle of the cutting. *P. chlamydospora* was identified by the macroscopic features of the colonies and by observation of the reproductive structures using an optical microscope.

Data analysis

Vines were taken to be infected if at least one wood sample was positive to PCR or gave rise to a colony of *P. chlamydospora*. Data were expressed as a percentage of infected vines per stage or treatment.

Only data from treated material were subjected to statistical analysis. Arcsin-square root transformed percentages of infected plants, weight and shoot length were analysed by one way ANOVA. Means were separated using Tukey's HSD test. Root and shoot development were analysed by the Kruskal-Wallis non-parametric test. Strike rates were analysed by the χ^2 test of independent groups. All statistical analyses were performed with Statgraphics plus, 2000 (Standard ed., version 5, Manugistic Inc., Rochville, MD, USA).

Results

Phaeomoniella chlamydospora was detected in 0.96, 0.69 and 1.32% of non-inoculated wood samples in 2005, 2006 and 2007 respectively, analysed by nested-PCR (the number of samples in these three years was 2594, 1891 and 1215 respectively). In 2005, a considerable number of wood samples collected from unviable vines was analysed by nested-PCR, but *P. chlamydospora* was never detected in them (data not shown). The frequency of *P. chlamydospora* in artificially inoculated wood samples was 54.3% (out of 449 samples analysed by isolation on agar medium).

Differences in both viability and vegetative growth among vines from different samples were not related to *P. chlamydospora* occurrence, but to the cultivar, the treatment, and most of all to the growing conditions as described below. Only 13.3% of naturally infected cuttings and 0.7% of

artificially infected cuttings sprouted from intermediate or basal buds and only 18.8% of naturally infected grafts sprouted from rootstock. By contrast, all artificially infected grafts failed. In the tables only the percentages of vines with their shoot originating in the scion or the apical bud (strike rates), were shown, except for Table 6.

All vines, both grafted and ownrooted, exhibited dark streaks, starting from wounds (the ends of the cutting, the graft, or disbudding points), often extending the full length of the cuttings or the graft-cuttings. Dark streaks were never detected in the shoots. No relationship was found between streak length and *P. chlamydospora* incidence.

Infection risk in the propagation process

2005 trial

In 2005, rates of healed grafts after callusing were low in some samples (healing rates varying from 5 to 70%). Even nursery grafts on 140Ru collected from the same mother plants block showed poor callus development at both the graft and the bottom points, as is usual with this rootstock. Apparently healed grafts grown either in pots or in the field produced a low number of properly developed vines (strike rates 0.0–36.0%, Table 1); in some samples, a good number of vines sprouted from the rootstock (from 0.0 to about 54.3%). Moreover, the length of rootstock cuttings was suitable for growing in the field, but not for growing in pots. The portion of the cuttings outside the soil dried out and did not sprout, particularly in sample 2: only 14% of cuttings yielded properly developed vines, while 16.0% sprouted from the basal buds.

Phaeomoniella chlamydospora was detected in a small percentage (3.3%) only in Sangiovese cuttings obtained directly from mother plants (sample 1, Table 1).

2006 trial

In the second year, the rootstock was changed because of meagre strike and healing rates of cv. 140Ru in 2005. Cultivar 1103P showed a better healing rates of the grafts (>80% in all samples) than cv. 140Ru and all the cutting samples grown in pots showed good strike rates (>90%, Table 1), which were also supported by the shorter length of the cuttings (two buds long). Over 80% of grafts grown in pots yielded properly developed vines, except in sample 4; the strike rate of the grafts grown in the field (sample 7) fell within the nursery standard.

Phaeomoniella chlamydospora occurred more frequently than in 2005: in 3.3% of the 1103P cuttings obtained directly from mother plants (sample 1), in 10% of grafts trimmed with non-disinfected pruning shears (sample 6) and in 3.3% of grafts obtained following standard nursery practices (Table 1).

2007 trial

In 2007, no fungicides were added to the hydration tank. Callusing occurred at room temperature due to air-conditioning failure. The lower temperatures lengthened the time required for callusing and lowered the success rate (healing rates were between 40 and 70%); they also reduced root growth, so that roots were not trimmed and sample 6 was eliminated, the corresponding grafts being pooled in sample 5. Only grafts grown in the field (sample 7) had a lower strike rate than in 2006 (Table 1).

Phaeomoniella chlamydospora was detected in sample 1, obtained from Sangiovese mother plants (6.7%), as in 2005, but not in canes from 1103P mother plants (Table 1). More grafts were infected than in previous years, particularly grafts obtained following standard nursery practices and planted in the nursery field (23.3%). Only grafts callused in non-sterilized sawdust and two-year-old plants (uprooted at the end of 2006 and replanted in 2007, near new grafts) were free from *P. chlamydospora*.

Effectiveness of HWT and cyproconazole

Hot-water treatment and cyproconazole significantly influenced the strike rates of non-inoculated cuttings depending on the cultivar, year and growing conditions (Table 2).

2005 trial

Hot water and cyproconazole-treated rootstocks grown in pots (samples 2A, 2B and 2C) were less viable than untreated rootstocks (sample 1), but there were no statistical differences in the scions (Table 3). Rootstock cuttings sprouted from basal or intermediate buds at fairly high percentages (52.0, 36.7 and 36.0% in treated samples 2A, 2B and 2C respectively), but the percentage was only 16.0% in the untreated sample 1. Differences

Table 1. Strike rates and *Phaeomoniella chlamydospora* (Pch) infection of cutting and graft grapevine samples collected during the propagation process at several stages in 2005–2007.

Sample/ propagation stage	2005				2006				2007			
	No. cuttings or grafts	Strike rate %[a]	No. examined vines[b]	Pch-infected %	No. cuttings or grafts	Strike rate %[a]	No. examined vines[b]	Pch-infected %	No. cuttings or grafts	Strike rate %[a]	No. examined vines[b]	Pch-infected %
Cuttings												
1 Rootstock[c]/cane collection	50	88.0	30	0.0	51	96.1	30	3.3	54	100.0	30	0.0
1 Scion[c]/cane collection	47	85.1	30	3.3	51	94.1	30	0.0	54	90.7	30	6.7
2 Rootstock[c]/cane cutting	50	14.0	15	0.0	51	96.1	30	0.0	50	92.0	30	0.0
2 Scion[c]/cane cutting	50	86.0	30	0.0	51	96.1	30	0.0	50	80.0	30	0.0
Grafts												
3/Rootstock and scion hydration	2	0.0	-	-	42	81.0	30	0.0	48	75.5	30	3.3
4/Grafting	5	20.0	2	0.0	42	64.3	30	0.0	48	87.5	30	6.7
5/Callusing	25	36.0	18	0.0	42	85.7	30	0.0	68	80.9	30	0.0
6/Trimming	35	22.9	21	0.0	41	82.9	30	10.0	-	-	-	-
7/Grafts in field nursery	110	7.3	24	0.0	170	66.5	30	3.3	107	43.0	30	23.3
8/Two–years old vines	-	-	-	-	-	-	-	-	43	81.4	30	0.0

[a] Only vines with the shoot originating in a scion (grafts) or an apical bud (cuttings).
[b] Vines examined to detect Pch by nested PCR after one growing season.
[c] Rootstock cv. 140Ru in 2005 and cv. 1103P in 2006 and 2007; scion cv. Sangiovese.

between treatments in rootstock cuttings grown in the nursery field were significant, with better strike rates for cuttings treated with cyproconazole prior to cold storage and with hot water following cold storage (sample 3A). The strike rate of cuttings treated with hot water and cyproconazole after cold storage (sample 3B) was low, but similar to that of the untreated cuttings (sample 4). The grafts that were hot-water treated after callusing all failed.

Approximately 30% of the untreated rootstock and scion cuttings obtained directly from diseased mother plants (sample 1) were infected with *P. chlamydospora* (Table 3). No infection was detected in either treated or untreated cuttings (sample 4).

2006 trial

In this year, the treatments significantly influenced the strike rates of both rootstocks and scions grown in pots, and untreated samples developed better than the treated samples (Table 3). Only the strike rate of rootstock cuttings exposed to both HWT and cyproconazole before cold storage (sample 2C) did not differ significantly (χ^2=3.4; P=0.065) from the untreated cuttings (sample 1). Rootstock cuttings grown in the nursery field had low strike rates with no significant differences between treated and untreated cuttings. Nevertheless, in the untreated sample 4, 30.4% of plants sprouted from basal or intermediate buds against 8.2% in sample 3A and 3.2% in samples 3B, both treated. None of the grafts that were hot-water treated after callusing were viable.

The percentages of untreated infected cuttings obtained directly from diseased mother plants (4.1 and 2.0% in rootstocks and scions respectively, sample 1) were consistently lower than they had been in 2005 (Table 3). *P. chlamydospora* was not detected in any treated samples, but 12.5% of untreated cuttings were infected (sample 4).

2007 trial

Neither treatment with hot-water and cyproconazole nor *P. chlamydospora* adversely influenced the strike rates of 1103P cuttings (Table 4). Artificial inoculation was successful, with 96.5% of untreated cuttings becoming infected. Unfortunately, the infection percentages of treated cuttings were also high. Only in treatment 4 (cuttings treated with cyproconazole and soon after with hot water) was the percentage of infected cuttings significantly lower than that of the untreated cuttings.

The healing rates of the cultivars Sangiovese and Moscato grafted on inoculated 1103P rootstock were 60.5 and 74.5% respectively. More than 500 graftlings were planted in the nursery field, but they all failed; only a small percentage (about 13%) sprouted from the rootstock. As a consequence, HWT could not be carried out after uprooting.

Hot-water treatment before or after cold stor-

Table 2. χ^2 test of independent groups for strike rates of treated or untreated rootstock and scion cuttings grown in different conditions in 2005 and 2006.

Growing conditions	2005		2006	
	χ^2	P^a	χ^2	P^a
Rootstock grown in pot	70.19	<0.0001	15.11	0.002
Scion grown in pot	1.28	0.258	16.28	<0.0001
Rootstock grown in the nursery field	17.04	<0.0001	5.56	0.062

[a] χ^2 test is significant at $P \leq 0.05$.

Table 3. Influence of hot water treatment (HWT) and cyproconazole (CYP) on the strike rate of rootstock and scion cuttings grown in different conditions and on *Phaeomoniella chlamydospora* (Pch) in 2005 and 2006.

Sample/propagation stage		Treatment	2005		2006	
			Strike rate %[a]	Pch-infected %[b]	Strike rate %[a]	Pch-infected %[b]
Rootstock[c] grown in pots	1/cane collection	None	72.0 s	33.3	94.1 s	4.1
	2A/cane cutting	CYP before and HWT after cold storage	14.0	0.0	71.2	0.0
	2B/cane cutting	HWT and CYP after cold storage	0.0	0.0	64.8	0.0
	2C/cane cutting	HWT and CYP before cold storage	30.0	0.0	82.4	0.0
Scion[c] grown in pot	1/cane collection	None	78.3 ns	29.0	100.0 s	2.0
	2C/cane cutting	HWT and CYP before cold storage	68.0	0.0	72.0	0.0
Rootstock[c] grown in the nursery field	3A/cane cutting	(see sample 2A)	26.0 s	0.0	8.2 ns	0.0
	3B/cane cutting	(see sample 2B)	7.1	0.0	1.1	0.0
	4/cuttings	None	6.7	0.0	4.3	12.5

[a] See Table 1.
[b] Pch was detected by nested PCR after one growing season.
[c] Rootstock cv. 140Ru in 2005 and 1103P in 2006; scion cv. Sauvignon blanc.
s, Significant differences between treatments (see Table 2).
ns, No significant differences between treatments (see Table 2).

Table 4. Strike rate and *Phaeomoniella chlamydospora* (Pch) infection of artificially infected 1103P cuttings treated with hot water (HWT) and cyproconazole (CYP) in 2007.

Treatment	No. cuttings	Strike rate %[a]	No. examined vines[b]	Pch-infected %
1. HWT	50	90.0 ns	30	80.0 a[c]
2. CYP	50	96.0	30	86.7 a
3. HWT+CYP	50	84.0	30	80.0 a
4. CYP+HWT	50	90.0	30	26.7 b
5. Not treated	50	90.0	30	96.7 a

[a] See Table 1.
[b] Vines examined to detect Pch by isolation on agar medium after one growing season.
[c] Values followed by the same letter did not differ statistically according to Tukey's HDS test at $P \leq 0.05$ (F ratio=10.74; P=0.0012).
ns, No significant differences between treatments (χ^2=4.00; P=0.406).

age significantly influenced plant growth, but not all the growth parameters and all the cultivars. In some cases the hot-water treated cuttings grew better than the untreated cuttings (Tables 5 and 6). As regards cv. 779P, HWT significantly affected all the growth parameters of this cultivar, but not the strike rates. Generally, cuttings treated after cold storage had the best growth. 1103P cuttings differed significantly in all parameters except root growth and weight. With this rootstock, the cuttings grew best when they were treated prior to cold storage. As regards the scion cultivars, HWT after cold storage generally had an adverse effect on growth parameters, but differences with the other treatments were not always significant.

The incidence of infected Sauvignon blanc cuttings obtained directly from diseased mother plants was similar to that in 2006 (about 2.0%, Table 7). There were no differences between cuttings from mother plants with foliar symptoms in the summer of 2006 and cuttings from mother plants with symptoms in the period from 2002 to 2005.

Discussion

In all trials, the propagation material was examined after one vegetative season to improve *P. chlamydospora* detection. During the growth of cuttings and grafts, the pathogen, if present, would spread through the vascular tissues inside the plant, increasing the likelihood of detection.

Growth and strike rates differed depending on the growing conditions, the cultivar and the treatment, but not on the health of the vine. Generally, growth of infected vines was the same as that of the healthy vines in the same sample. The strike rate of grafts (sample 7) was lower in 2007 than in 2006; this may have been due to a higher percentage of infection in 2007. Nevertheless, *P. chlamydospora* was never detected in unviable vines and only 18.8% of infected grafts sprouted from the rootstock. On the other hand, callusing at room temperature in 2007 may have hampered the healing of grafts and then contributed to the lower strike rate.

Dark streaks occurred in all samples and years. This symptom is often, but not always, associated with *P. chlamydospora* in nursery vines (Bertelli *et al*., 1998; Laukart *et al*., 2001; Rooney and Gubler, 2001; Stamp, 2001; Aroca *et al*., 2006; Whiteman *et al*., 2007; Zanzotto *et al*., 2007; Pollastro *et al*., 2009). Wood discoloration not associated with pathogenic micro-organisms is frequent in nursery vines and has been linked to cuts and hydration made during propagation (Frisullo *et al*., 1992; Triolo *et al*., 1993). This symptom arises from cicatrisation caused by degeneration of the xylem vessels and related parenchyma cells due to oxidation, gummosis and tyloses, and it does not occur in the wood that forms after the cuttings are planted. In our trials dark streaks started from the wounds, and since *P. chlamydospora* was scarce in most samples and years, the streaks may have been predominantly due to cicatrisation. We measured the length of the streaks in lengthwise-section. In all probability, the thickness and intensity of the discoloration in cross-section, and hence its spread to wood that formed after planting, would be a more suitable data to connect wood discoloration to *P. chlamydospora* occurrence (Habib *et al*., 2008).

As regards the risk of infection during propagation, canes from mother plants sometimes were infected, but the infection percentages varied among years and were independent of the age of the vineyards, the incidence of diseased vines or the occurrence of foliar symptoms. Rootstock mother blocks chosen to assess the infection risk were planted in 2002 and never showed any decline, while the cv. Sangiovese mother block was planted in 1993 and it had few vines with esca symptoms. By contrast, the rootstock and scion vineyards chosen for the control trials were planted in 1991 and 1989 respectively, and they had a high incidence (over 50%) of esca affected and missing grapevines. Nevertheless, the incidence of infected cuttings from symptomatic plants was fairly high only in 2005. In the other years, a greater number of vines had to be examined, and even so only a few infected cuttings were detected.

One reason for this may be that the canes were collected from different rootstock mother blocks in two of the three years of the survey. On the other hand, Sauvignon blanc canes came from the same location in the same vineyard in all three years, and this suggests that environmental factors influenced the incidence of *P. chlamydospora* in these canes. This fungus invades the xylem vessels, and its spread from the trunk and the cordon to new shoots is facilitated by the sap (Edwards *et*

Table 5. Statistical analysis[a] for growth parameters of vines obtained from 779P, 1103P, Sangiovese and Cabernet franc cuttings treated with hot water before or after cold storage, or untreated in 2007.

Cultivar	Strike rate %[b]		Root development		Shoot development		Weight		Shoot length	
	χ^2	*P*	KW[c]	*P*	KW[c]	*P*	F ratio	*P*	F ratio	*P*
779P	1.57	0.47	10.28	0.0059	10.29	0.0058	10.48	<0.0001	24.24	<0.0001
1103P	40.7	<0.0001	3.98	0.1362	7.15	0.0280	0.95	0.3870	35.28	<0.0001
Sangiovese	0.26	0.88	8.61	0.0134	5.73	0.0570	3.21	0.0430	8.28	0.0004
Cabernet franc	8.1	0.017	4.14	0.1264	13.61	0.0011	13.39	<0.0001	35.53	<0.0001

[a] Tests are significant at $P \leq 0.05$.
[b] See Table 1.
[c] KW, Kruskal-Wallis.

Table 6. Growth parameters of vines obtained from 779P, 1103P, Sangiovese and Cabernet franc cuttings treated with hot water before (1) or after (2) cold storage, or untreated (3) in 2007.

Cultivar	Treatment	Number of cuttings	Strike rate %[a]	Root development[b]	Shoot development[b]	Weight g	Shoot length cm
779	1	200	81.5 ns	2.09 s	2.07 s	69.4 a[c]	36.5 a[c]
	2	200	78.0	2.48	2.47	87.8 b	42.3 b
	3	200	76.5	2.20	2.26	74.2 a	45.6 c
1103P	1	200	88.5 s	2.37 ns	2.31 s	76.5 a[c]	44.4 c[c]
	2	200	61.5	2.20	2.03	75.3 a	40.0 b
	3	200	67.0	2.44	2.33	70.8 a	33.5 a
Sangiovese	1	100	64.0 ns	2.05 s	2.10 ns	65.8 a[c]	37.9 a[c]
	2	100	61.0	1.44	2.25	58.3 a	36.3 a
	3	100	64.0	1.85	2.67	68.2 a	47.6 b
Cabernet franc	1	100	85.0 s	2.26 ns	2.52 s	119.5 c[c]	35.5 b[c]
	2	100	70.0	1.91	1.91	86.0 a	26.7 a
	3	100	83.0	2.10	2.52	103.3 b	36.1 b

[a] See Table 1.
[b] Root and shoot development was visually scored on a four-point scale: 1, poor; 2, fair; 3, good; 4, optimal.
[c] Values followed by the same letter along the column do not differ statistically according to Tukey's HDS test at $P \leq 0.05$ (see Table 5).
s, Significant differences between treatments (see Table 5).
ns, No significant differences between treatments (see Table 5).

Table 7. Strike rate and *P. chlamydospora* (Pch) infection of Sauvignon blanc cuttings collected from esca symptomatic vines in 2007.

Symptom expression	No. cuttings	Strike rate %[a]	No. examined vines[b]	Pch-infected %
Symptomatic vines in the summer of 2006	54	94.4	51	2.0
Symptomatic vines in the 2002–2005 period	54	98.1	52	1.9

[a] See Table 1.
[b] Vines examined to detect Pch by nested PCR after one growing season.

al., 2004). An intense xylem flow, due to the supply of water in the soil, may favour the movement of *P. chlamydospora* through the vessels. In the area where the Sauvignon blanc vineyard is located, the rainfall in the growing period (April–October) of 2004, 2005 and 2006 was 276.8, 247.8 and 179.6 mm respectively. The higher rainfall in 2004 may explain the higher incidence of *P. chlamydospora* in the canes collected in the following year. However, rainfall was also high in 2005 and yet the fungus only had a low incidence in 2006. Most likely, other factors besides rainfall influenced the occurrence of *P. chlamydospora* in the canes; this matter requires further study.

Cutting the canes into pieces to obtain the cuttings did not seem to increase the risk of infection in spite of the wounds caused, since cuttings obtained with standard practices were never infected. In 2005, no infections were detected after the disbudding and hydration stages (samples 3–7), but only a few vines were examined in some samples. In the other years, the infection rates varied from zero to 10.0% until the trimming stage (samples 3–6), and infected samples were different in 2006 and 2007. Only grafts grown in the nursery field (sample 7) were infected in both years, but they had quite different rates of infection (3.3% in 2006 and 23.3% in 2007). The main reason for this may have been that in 2007, unlike 2006, cuttings and scions were soaked in water without fungicide before grafting. In 2005 and 2006, the amount of thiophanate methyl in the soaking bath was about three times the level indicated for foliar spray against *Botrytis cinerea* on grapevines, but it was four times less than the level recommended for soaking carnation bulbs against rot by *Botrytis* and *Fusarium* species. For *P. chlamydospora*, the EC50 of thiophanate methyl varies from 0.31 to 0.88 mg L^{-1} on mycelial growth and from 3.43 to 16 mg L^{-1} on conidial germination (Jaspers, 2001; Gramaje *et al*., 2009b), well below the concentration of 1 g a.i. L^{-1} in the soaking suspension used here. Nevertheless, it has not be demonstrated that thiophanate methyl actually caused the lower levels of *P. chlamydospora* in 2005 and 2006: the soaking suspension remained unchanged for many days, and *P. chlamydospora* was also detected in fairly substantial percentages (10%) in 2006. To explain these different rates of infection in the nursery field we must also consider the plot of land where the grafts were planted, which changed each year, because infection may also spread through contaminated soil (Gaforio *et al*., 2005; Whitemann *et al*., 2005). Despite this, none of the two-year-old vines (uprooted in 2006 and planted again in 2007, near new grafts) were infected.

Hot water and cyproconazole may adversely affect the viability of treated cuttings and grafts. Such adverse effect would mostly be due to HWT. DMI fungicides are known to inhibit shoot extension and cause stunted growth, sinces they affect gibberellin metabolism (Shive and Sisler, 1976), but in our trials the main adverse effect was budburst failure. However, HWT was not clearly shown to have a negative effect here. Only cut-

tings grown in pots were less viable than untreated cuttings in 2005 and 2006, while both treated and untreated cuttings grown in the nursery field showed low strike rates in those years. In 2007 treated and untreated cuttings grown in pots did not differ substantially. In the trial using different rootstock and scion cultivars, there were differences between cuttings treated before and after cold storage, and between treated and untreated cuttings, and these difference depended on the cultivar and on the growth parameters. Generally, HWT after cold storage adversely affected growth in the cuttings of 3 out of 4 cultivars, but it did not impair all the growth parameters in all the cultivars. Climatic and cultural stresses during plant growth almost certainly impaired the viability of vines as much as, or more than, HWT (Waite and May 2005). For example, in 2006 the rootstock cuttings were not planted until late in the nursery field, in May, shortly before a period of hot and dry weather that may well have caused wood dehydration and bud-burst failure. Other factors such as properly matured wood (wood with sufficient carbohydrate reserves), the thickness of the cuttings and the cultivar used also had a role. In 2005 and 2006, when strike rates were low, canes were collected from esca-diseased grapevines. Cane thickness and wood maturation in symptomatic plants was sometimes poor and irregular, and this may have made the cuttings from these plants more susceptible to HWT. On the other hand, HWT was definitely deleterious to callused graftlings, confirming its harmfulness on non-dormant wood (Waite and Morton, 2007).

As regards the effectiveness of HWT and cyproconazole against *P. chlamydospora*, the results of trials carried out in 2005 and 2006 showed that the combined approach was effective on naturally infected material, but the infection level on untreated plants at the end of the propagation process was too low for a reliable evaluation to be made. In 2007, the combination of cyproconazole plus HWT was the only approach that significantly reduced the percentage of artificially infected cuttings, but solely when it was applied in this order. When HWT was given before cyproconazole, the combination was much less effective, probably because the hydration level of the cuttings was too high. Cyproconazole is a systemic fungicide, but only in green leaves. In dormant cuttings the cyproconazole suspension is absorbed by capillary action and through the bark (Di Marco, unpublished data). As a consequence, if the cuttings are well hydrated, the amount of active ingredient absorbed is very low. When HWT was given before cyproconazole, the cuttings were first dipped in hot water, hydrating them, and then immediately cooled in the cyproconazole suspension. In the actual trials however, neither hot water nor cyproconazole alone was sufficient to control *P. chlamydospora*.

The conflicting results that have been obtained with HWT in controlling *P. chlamydospora* have above all been due to the different experimental conditions in which HWT was applied, the most important of which are: whether the infection was natural or artificially induced, and whether *P. chlamydospora* was detected in dormant wood soon after HWT or in plants after growing. Our results were consistent with Rooney and Gubler (2001) who studied inoculated cuttings and concluded that the action of HWT was only fungistatic. Moreover, Habib *et al*. (2008) found that shortly after the HWT of naturally infected uprooted grafts, the level of *P. chlamydospora* was very low, but that after one growing season it was again comparable with the levels recorded before HWT (Habib *et al*., unpublished data).

In 2007, it was not possible to apply HWT to inoculated grafts after uprooting because none of these grafts were viable despite an adequate healing rate after callusing. Reed *et al*. (2005) found that 10^4 cfu mL^{-1} of *P. chlamydospora* inoculated into a graft wound was sufficient to significantly weaken the resulting grafts, while Graham *et al*. (2007) found that if grafts were inoculated with 10^6 cfu mL^{-1}, more than 90% of vines died or had to be rejected. Thus, it can be presumed that the heavy infection of the rootstock (the concentration of the inoculated suspension was 2×10^7 conidia mL^{-1}), combined with callusing at room temperature, prevented the complete healing of the grafts and caused the death of the plants in our trial. This assumption was supported by the results of the infection risk assessment carried out in the same year: 47% of cv. Sangiovese grafted on non-inoculated 1103P and planted near the inoculated grafts turned out to be viable. Further experiments are needed to confirm the effectiveness of combined HWT and chemical fungicides

using lower concentrations of the conidial suspension.

In conclusion, planting material may have been infected because their mother plants were already infected or the infection may have started during the propagation process, particularly in the stages following grafting, as already found in other studies (Halleen *et al*., 2003; Giménez-Jaime *et al*., 2006; Retief *et al*., 2006; Pollastro *et al*., 2009). However, it was not possible to attribute a major role to any of these factors because the infection frequencies detected in the propagation process were too low and irregular. Unfortunately, this is not always the case in Italian nurseries, where not only *P. chlamydospora*, but also *Phaeoacremonium* spp. can occur at high level (Bertelli 1998; Zanzotto *et al*., 2007; Pichierri *et al*., 2009). The combined cyproconazole and hot water treatment seemed to corroborate the statement of Fourie and Halleen (2004) that an integrated approach is needed to improve *P. chlamydospora* control in the nursery; however the carrying out of the treatments, particularly their order, should be studied in more detail.

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