Detection of Grapevine Fungal Trunk Pathogens on Pruning Shears and Evaluation of Their Potential for Spread of Infection

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Abstract

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Four vineyards visibly affected by trunk diseases were surveyed at pruning time in 2012 and 2013 in Spain, to determine whether pruning tools are capable of spreading grapevine trunk diseases from vine to vine. In each vineyard, pruning shears were regularly rinsed with sterile water, collecting liquid samples for analysis. Molecular detection of grapevine fungal trunk pathogens (GFTPs) was performed by nested polymerase chain reaction using specific primers to detect Botryosphaeriaceae spp. *Eutypa lata*, *Cadophora luteo-olivacea*, *Phaeoacremonium* spp., and *Phaeomoniella chlamydospora*. All of these GFTPs, with the exception of *E. lata*, were detected in samples from the four vineyards, *C. luteo-olivacea* and *Phaeoacremonium* spp. being the most prevalent. Co-occurrence of two, three, or four different GFTPs from the same sample were found, the simultaneous detection of *C. luteo-olivacea* and *Phaeoacremonium*

spp. being the most prevalent. In addition, fungal isolation from liquid samples in semiselective culture medium for *C. luteo-olivacea*, *Phaeoacremonium* spp., and *P. chlamydospora* was also performed but only *C. luteo-olivacea* was recovered from samples collected in three of four vineyards evaluated. Pruning shears artificially infested with suspensions of conidia or mycelial fragments of *C. luteo-olivacea*, *Diplodia seriata*, *E. lata*, *Phaeoacremonium aleophilum*, and *Phaeomoniella chlamydospora* were used to prune 1-year-old grapevine cuttings of '110 Richter' rootstock. Successful fungal reisolation from the cuttings 4 months after pruning confirmed that infested pruning shears were able to infect them through pruning wounds. These results improve knowledge about the epidemiology of GFTPs and demonstrate the potential of inoculum present on pruning shears to infect grapevines.

Grapevine fungal trunk pathogens (GFTPs) cause a wide range of symptoms on grapevines, including stunted growth, reduced vigor, retarded or absent sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, wilting, dieback, and eventual death (Bertsch et al. 2013; Luque et al. 2009; Mostert et al. 2006; Mugnai et al. 1999). The consequences include loss of productivity, increased management costs, and reduced longevity of vineyards, compromising the stable production in viticulture and the wine industry in grape-producing areas worldwide (Gramaje and Armengol 2011; Mostert et al. 2006; Mugnai et al. 1999; Úrbez-Torres 2011).

Inoculum of esca, petri disease, and Botryosphaeria and Eutypa dieback of grapevine (*Vitis vinifera* L.) can be spread by wind, rain splash, and arthropod vectors. Infection of vines by GFTPs primarily occurs when fungal spores (sexual or asexual) enter through pruning wounds during the dormant season (Baskarathevan et al. 2013; Díaz and Latorre 2013; Mostert et al. 2006; Moyo et al. 2014; Serra et al. 2008; Trouillas and Gubler 2010; Úrbez-Torres and Gubler 2011). Several studies have indicated that inoculum of *Cadophora luteo-olivacea* (J. F. H. Beyma) T. C. Harr. & McNew; species of *Phaeoacremonium* W. Gams, Crous & M. J. Wingf.; *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & Mugnai) Crous & W. Gams; species included in the Botryosphaeriaceae Theiss. & Syd. and Diatrypaceae Nitschke families; and ligninolytic basidiomycete species such as *Fomitiporia mediterranea* M. Fisch. are present in vineyards for long periods of time in both the dormant and growing seasons, especially when weather conditions are favorable for spore release and dispersal, being available for infection of susceptible wounds (Eskalen et al. 2007; Lecomte and Bailey 2011; Munkvold and Marois 1995; Petzoldt et al. 1981; Surico et al. 2000b; Úrbez-Torres and Gubler 2011; Van Niekerk et al. 2011).

The susceptibility of pruning wounds to GFTPs has been already investigated and this knowledge is a critical factor in better understanding the epidemiology of these diseases (Úrbez-Torres 2011). Grapevines have the highest risk of infection during the pruning period, from late fall to early spring, because of the high number of wounds made on a single grapevine (Eskalen et al. 2007; Rolshausen et al. 2010). In general, most studies have shown that pruning in late winter or early spring reduces wound susceptibility to trunk pathogens compared with early pruning in late autumn or early winter (Chapuis et al. 1998; Munkvold and Marois 1995; Petzoldt et al. 1981; Rolshausen et al. 2010; Serra et al. 2008; Weber et al. 2007). In this sense, several studies performed in South Africa confirmed that pruning wounds made early in the dormant season are much more susceptible and stay susceptible to infection by petri disease or Botryosphaeria and Eutypa dieback pathogens for much longer periods (7 to 9 weeks) than pruning wounds made during the mid and late dormancy periods, in which the susceptibility decreases only to 1 to 2 weeks (Halleen et al. 2010; Mostert et al. 2006; Van Niekerk et al. 2011). In contrast, a study carried out in California by Eskalen et al. (2007) showed that pruning wounds remained susceptible to *Phaeoacremonium aleophilum* W. Gams, Crous, M. J. Wingf. & Mugnai and *Phaeomoniella chlamydospora* for up to 4 months, even though infection incidence decreased after the second month. Recently, Luque et al. (2014) found that, in the El Penedés region (northeastern Spain), the rate of natural infection of pruning wounds by petri disease and Botryosphaeria and Eutypa dieback pathogens was lower following early pruning (autumn) than following late pruning (winter). Thus, it is generally accepted that susceptibility is dependent on the age of the pruning wounds and the time of pruning.

The spatial spread of esca disease was analyzed by Surico et al. (2000b), who suggested that, in the vineyards examined, airborne spores could spread from distant or internal inoculum sources. Further studies showed that the incidence of esca disease varied from year to year, perhaps in relation to the amount of rainfall or air temperature, and the tendency to aggregation of symptomatic plants being the common pattern of spread observed (Pollastro et al. 2000, 2009; Redondo et al. 2001; Surico et al. 2000a), with the exception of Sofia et al. (2006), who observed a random distribution of esca-symptomatic

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plants. Other authors indicated that the spread of esca is more probable along the rows, suggesting that pruning tools can carry fungal inoculum, which could play an important role in the spread of infection (Mugnai et al. 1999; Zanzotto et al. 2013). This assumption puts us in a scenario similar to that of grapevine nurseries, where several authors have already demonstrated that propagation tools are potential inoculum sources of petri disease and Botryosphaeria dieback pathogens (Aroca et al. 2010; Billones-Baaijens et al. 2013 a,b; Gramaje et al. 2011). In fact, these pathogens have been detected in scissors used for cutting buds and in omega-cut grafting machines used during the grapevine nursery propagation process, by means of nested polymerase chain reaction (PCR) or fungal isolation by culturing on semi-selective medium.

Nevertheless, to date, the potential role of pruning tools on the spread of infection of grapevines by GFTPs in vineyards at pruning is still uncertain. Thus, the objectives of this study were to (i) detect GFTPs in shears used for pruning and (ii) explore the potential of artificially infested shears to infect pruning wounds.

Materials and Methods

Detection of GFTPs from pruning shears. *Field sites and sampling.* Three vineyards ('Tempranillo'/'110 Richter' rootstock) located in Albacete Province (Central Spain) and one vineyard ('Cabernet Sauvignon'/'110 Richter rootstock) located in Barcelona Province (northeastern Spain), were used for this study. The vineyards located in Albacete Province were planted in 2007 (vineyards A and B) and 1999 (vineyard C) and pruned in February to March 2012. The vineyard located in Barcelona Province was planted in 1986 (vineyard D) and pruned in February 2013. All of these vineyards were symptomatic for trunk diseases, and the presence of petri disease and Botryosphaeria dieback pathogens had been confirmed by isolation in previous years. Pruning in each vineyard was carried out by workers following standard pruning practices. The vines were cordoned and only 1-year-old wood was cut. Before sampling, pruning shears were disinfested by immersion for 30 s in a 1.5% sodium hypochlorite solution and washed twice with sterile distilled water (SDW). Then, 10 grapevines were pruned and the pruning shears were immediately washed with a solution of 0.2% Tween-20 in SDW. Approximately 150 ml of the washing liquid was recovered in a plastic sterile tube. Pruning shears were disinfested again, as described above, between each group of 10 pruned plants. In all, 20 liquid samples were randomly collected from 200 vines in each vineyard.

All liquid samples were divided in two aliquots of 100 ml and 10 ml for subsequent analyses. The detection of *C. luteo-olivacea*, *Phaeoacremonium* spp., and *P. chlamydospora* was studied by using two different techniques: (i) nested PCR using specific primers and (ii) fungal isolation by culturing on semiselective medium. However, the detection of Botryosphaeriaceae spp. and *Eutypa lata* (Pers.) Tul. & C. Tul. was performed only by nested PCR, due to the lack of suitable methods for isolation of these fungi from liquid samples by filtering and culturing on semiselective medium.

Molecular detection by nested PCR. Each liquid sample (100 ml) was centrifuged for 15 min at 232 relative centrifugal force (rcf);

supernatant was discarded. The resulting liquid sample pellets were mixed and centrifuged again for 5 min at 930 rcf in order to obtain a sample pellet, which was stored at -20°C . Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-Tek, Norcross, GA). Before following the DNA extraction kit manufacturer's instructions, pellet samples were shaken using small steel balls in a vial filled with 600 μl of DNA extraction buffer (P1) for 10 min at 50 Hz using TissueLyser LT (Qiagen, Hilden, Germany). The homogenized samples (600 μl) were then transferred to new vials to complete the DNA extraction. Extracted DNA was kept at -20°C until needed for PCR amplifications.

A nested PCR was performed to achieve more sensitivity in detecting GFTPs. The first PCR reaction was carried out with internal transcribed spacer (ITS) primers ITS1F/ITS4 (Gardes and Bruns 1993) containing 1 $\times$ PCR buffer, 1.25 mM MgCl_2 , 80 μM each dNTP, 0.2 μM each primer, 0.7 U of *Taq* polymerase, and 1 μl of template DNA (20 ng μl^{-1}). The PCR reaction mix was adjusted to a final volume of 25 μl with water. The program consisted of an initial step of 3 min at 94°C , followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 45 s. A final extension was performed at 72°C for 10 min. PCR product was diluted with water 1:100 and 1 μl was used as template DNA for the secondary PCR, which was carried out with specific primers BOT100F/BOT472R (Ridgway et al. 2011), Clu-FOR/Clu-REV (Spadaro et al. 2011), Lata 3/Lata 2-1 (Lecomte et al. 2000), Pm1/Pm2 (Aroca and Raposo 2007), and Pch1/PCh2 (Tegli et al. 2000), to detect Botryosphaeriaceae spp., *C. luteo-olivacea*, *E. lata*, *Phaeoacremonium* spp., and *P. chlamydospora*, respectively. Each reaction contained 1 $\times$ PCR buffer, 2.0 mM MgCl_2 , 80 μM each dNTP, 0.2 μM each primer, 0.7 U of *Taq* polymerase, and 1 μl of template DNA. The PCR reaction mix was adjusted to a final volume of 25 μl with water. Five different cycling programs were used for each specific primer pairs (Table 1). Nested-PCR product was visualized on 1.5% agarose gels stained with ethidium bromide (Green and Sambrook 2012). A negative control (no DNA) and a positive control of Botryosphaeriaceae spp., *C. luteo-olivacea*, *E. lata*, *Phaeoacremonium* spp., and *P. chlamydospora* DNA was included in each test.

Fungal isolation in semiselective culture medium. Raw liquid samples (10 ml) were forcibly passed through syringe filters (5- and 0.45- μm pore size; Sartorius Stedium Biotech, Goettingen, Germany) attached in tandem to sterile 20-ml syringes (BD Biosciences, Madrid), as described by Aroca et al. (2010). The 5- μm filters trapped debris and large fungal spores. The 0.45- μm filters trapped smaller spores, including those of *C. luteo-olivacea*, *Phaeoacremonium* spp., and *P. chlamydospora*, which mostly have spore sizes smaller than 5 μm (Crous and Gams 2000; Gramaje et al. 2011; Mostert et al. 2006). The filters were rinsed with 4 ml of sterile water to backwash any trapped spores. The solutions were collected in microfuge tubes and 1-ml aliquots were then spread onto a semiselective culture medium composed of 2% malt extract agar (MEA; Oxoid Ltd., Basingstoke, Hants, England) amended with streptomycin sulfate (1.5 g liter $^{-1}$), folpet (0.02 g liter $^{-1}$), and ampicillin (0.015 g liter $^{-1}$) (Tello et al. 2009). Plates were air dried in a laminar flow hood with lids

Table 1. Nested polymerase chain reaction (PCR) cycling programs used for the detection of Botryosphaeriaceae spp., *Cadophora luteo-olivacea*, *Eutypa lata*, *Phaeoacremonium* spp., and *Phaeomoniella chlamydospora* from liquid samples by using specific primers for each species

Nested-PCR cycling program (T ^a -Time)								
Fungi	Specific primers ^z	Expected PCR product size (bp)	Initial denaturation	Amplification				
				Number of cycles	Denature	Anneal	Elongation	Final extension
Botryosphaeriaceae spp.	BOT100F/BOT472R	372	95°C, 5 min	30	94°C, 30 s	58°C, 30 s	72°C, 1 min	72°C, 10 min
<i>C. luteo-olivacea</i>	Clu-FOR/Clm-REV	494	95°C, 2 min	35	95°C, 15 s	58°C, 15 s	72°C, 1 min	72°C, 7 min
<i>E. lata</i>	Lata 3/Lata 2-1	281	94°C, 2 min	35	94°C, 30 s	65°C, 30 s	72°C, 45s	72°C, 10 min
<i>Phaeoacremonium</i> spp.	Pm1/Pm2	415	94°C, 5 min	40	94°C, 30 s	52°C, 30 s	72°C, 50 s	72°C, 7 min
<i>P. chlamydospora</i>	Pch1/PCh2	360	94°C, 5 min	35	94°C, 20 s	62°C, 30 s	72°C, 40 s	...

^a Botryosphaeriaceae spp. (Ridgway et al. 2011), *C. luteo-olivacea* (Spadaro et al. 2011), *E. lata* (Lecomte et al. 2000), *Phaeoacremonium* spp. (Aroca and Raposo 2007), and *P. chlamydospora* (Tegli et al. 2000).

partially lifted for 30 min. Plates were incubated for 1 month at 25°C in the dark while being observed daily for mycelial growth. All colonies were transferred to potato dextrose agar (PDA; Biokar-Diagnostics, Zac de Ther, France) supplemented with streptomycin sulfate (Sigma-Aldrich, St. Louis) at 0.5 g liter⁻¹. Isolates were single-spored prior to morphological and molecular identification with the serial dilution method (Dhingra and Sinclair 1995).

Fungal colonies were identified by morphological characteristics such as conidiophore morphology, size of phialides and conidia, and cultural characteristics (radial growth after 8 days at 25°C, colony colors, and pigment production) on oatmeal agar (OA; 60 g of oatmeal and 12.5 g of agar; Difco, France), MEA, and PDA (Gramaje et al. 2011). In addition, fungal identification was confirmed by molecular techniques. For DNA extraction, fungal mycelium and conidia from pure cultures grown on PDA for 2 to 3 weeks at 25°C in the dark were scraped and mechanically disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-Tek) following the manufacturer's instructions. DNA was visualized on 0.7% agarose gels stained with ethidium bromide (Green and Sambrook 2012) and stored at -20°C. Identification of *C. luteo-olivacea* was confirmed by sequencing the ITS1-5.8S-ITS2 region of ribosomal DNA (rDNA) amplified using the fungal universal primers ITS1F and ITS4 (Gardes and Bruns 1993). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany), and were sequenced in both directions by Macrogen Inc. (Seoul, Republic of Korea). The sequences obtained were then blasted in GenBank.

Potential of pruning shears artificially infested with GFTPs to infect grapevine pruning wounds. Fungal isolates and inoculum preparation. The following species (one isolate of each) were used in this study: *C. luteo-olivacea* (isolate Clo 47), *Diplodia seriata* De Not. (isolate Ds 3), *E. lata* (isolate El 1), *Phaeoacremonium aleophilum* (isolate Pal 20), and *Phaeomoniella chlamydospora* (isolate Pch 4), obtained from the culture collection of the IAM, UPV, Spain. Fungal isolates were grown on PDA and incubated for 1 to 3 weeks at 25°C in the dark.

A conidial suspension was prepared for *C. luteo-olivacea*, *Phaeoacremonium aleophilum*, and *Phaeomoniella chlamydospora* isolates from a 21-day culture on PDA by flooding the agar surface with 10 ml of SDW and scraping with a sterile bent-glass rod. The resulting spore suspension was filtered to remove mycelial fragments through two layers of cheesecloth into a 250-ml Erlenmeyer flask. The filtrate was diluted with SDW and, for each species, two different suspensions were adjusted to 10⁴ and 10⁶ conidia ml⁻¹ using a hemocytometer.

Inoculum of *D. seriata* and *E. lata* isolates consisted of mycelial fragments from liquid cultures and was prepared as described by Travadon et al. (2013). To produce the starter culture, 10 2-by-2-mm plugs from a 7-day culture on PDA were inoculated into a 250-ml Erlenmeyer flask containing 100 ml of potato dextrose broth (PDB; Difco Laboratories) and incubated at 25°C and 150 rpm (5 days for *E. lata* or 3 days for *D. seriata*). A blender cup (Oster 4655ESP) was used to homogenize 40 ml of the PDB culture (1 min, speed 3), and 2 ml of this homogenized starter culture was then inoculated to a 125-ml Erlenmeyer flask containing 40 ml of PDB. After incubation at 25°C and 150 rpm (5 days for *E. lata* or 3 days for *D. seriata*), the entire 40 ml of liquid culture was homogenized and the concentration of mycelial fragments, which were primarily <0.5 mm in length, was estimated with a hemocytometer. For each species, two different suspensions were adjusted with SDW to 10⁴ and 10⁶ fragments ml⁻¹ using a hemocytometer.

Pruning of grapevine cuttings with pruning shears artificially infested with GFTPs. Pruning wound susceptibility tests were conducted in 1-year-old grapevine cuttings of 110 Richter rootstock. In April 2012, dormant cuttings were cut into uniform lengths containing four to five buds, then hot-water treated (HWT) at 53°C for 30 min to eliminate potential infections by GFTPs (Gramaje et al. 2009). In order to enhance callusing and rooting, HWT cuttings were buried into sterilized peat moss in plastic boxes and placed in a callusing room at 25°C and 100% humidity for 4 weeks. After callusing and rooting, cuttings

were planted immediately in individual pots (20 cm in diameter) containing sterilized peat moss, placed in a temperature-controlled greenhouse, and watered every 3 days for bud sprout and shoot development.

In February 2013, the cuttings were pruned in the original cutting stem between the two upper internodes using pruning shears previously immersed for 2 s in the conidial or mycelia suspensions of each pathogen. The pruning shears were disinfested for 5 s in a 1.5% sodium hypochlorite solution, and washed twice with SDW before the experiment and again each time before immersion in a spore solution and pruning a cutting. Eight cuttings (replicates) per treatment combination of pathogen and conidial concentration were used. Additionally, eight cuttings were pruned between the two upper internodes using pruning shears previously immersed in SDW as negative controls. In all cases, wounds were immediately wrapped with parafilm. Cuttings were maintained in the greenhouse and arranged in a completely randomized design. The experiment was repeated.

Fungal reisolation from the pruning wounds was conducted 4 months after inoculation. In the greenhouse, we cut a piece between 1 and 5 cm below the wound from each cutting. In the laboratory, these 4-cm canes were surface disinfested for 1 min in a 1.5% sodium hypochlorite solution and washed twice with SDW. After air drying, the bark around the canes was removed and small fragments of necrotic tissue were cultured on MEA supplemented with streptomycin sulfate (Sigma-Aldrich) at 0.5 g liter⁻¹ in an attempt to recover the inoculated fungi and complete Koch's postulates.

C. luteo-olivacea was identified as described above. *P. chlamydospora* and *Phaeoacremonium aleophilum* were identified by conidiophore morphology, conidial size and shape, and cultural characteristics (radial growth after 8 days at 25°C, cardinal temperatures for growth, and colony colors) on OA, PDA, and MEA (Crous and Gams 2000). Molecular identification was carried out as previously described. Identification of *Phaeomoniella chlamydospora* isolates was confirmed by using specific primers Pch1-Pch2 (Tegli et al. 2000). PCR products were purified and sequenced as described before. The sequences obtained were then blasted in GenBank. Identification of *Phaeoacremonium aleophilum* isolates was confirmed by sequencing of the β -tubulin gene fragment using primers T1 and Bt2b (Dupont et al. 2000) and by comparison with the polyphasic, online identification system for *Phaeoacremonium* sp. recognition (<http://www.cbs.knaw.nl/phaeoacremonium/biolomics.aspx>) developed by Mostert et al. (2006). *D. seriata* was identified by colony and conidial morphology (Phillips et al. 2013). In order to enhance sporulation, cultures were placed with sterilized pine needles on 2% water agar (Biokar-Diagnostics) at 25°C with a 12-h day (Philips TDL18W/33) (Slippers et al. 2004). Isolates were examined weekly for formation of pycnidia and conidia. Conidial morphology (cell wall, shape, color, and presence or absence of septa) from pycnidia was recorded. DNA extractions were carried out as previously described. Identification of Botryosphaeriaceae species was confirmed by analysis of the elongation factor 1- α gene (EF) amplified using EF1-728F and EF1-986R primers (Carbone and Kohn 1999). PCR products were purified and sequenced as described before. The sequences obtained were then blasted in GenBank. *E. lata* was identified by colony morphology on PDA and MEA (Rappaz 1987). DNA extractions were carried out as previously described. Identification of *E. lata* was confirmed by sequencing the ITS region of rDNA amplified using the fungal universal primers ITS1F and ITS4 (Gardes and Bruns 1993). PCR products were purified and sequenced as described before. The sequences obtained were then blasted in GenBank.

Statistical analysis of the fungal reisolation results was done using one-way analysis of variance (ANOVA), with spore or mycelial fragment suspension concentrations as independent variable and the isolation (percent) as dependent variable. The Student's least significant difference test was used to compare the overall means of each spore or mycelial fragment suspension concentration at $P < 0.05$. Statistical analyses were performed using Statgraphics Plus 5.1 software (Manugistics Inc., Rockville, MD). For all fungal species tested, ANOVA analyses indicated that the isolation data between the two repetitions were similar ($P > 0.05$). Thus, in all cases, data from both experiments were combined.

Results

Detection of GFTPs from pruning shears. *Molecular detection by nested PCR.* Results of the molecular detection of GFTPs in samples taken from pruning shears at pruning are shown in Table 2. Botryosphaeriaceae spp., *C. luteo-olivacea*, *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* were detected; *C. luteo-olivacea* and *Phaeoacremonium* spp. being the species found most frequently in all vineyards evaluated. *E. lata* was not detected in any samples. The percentage of samples positive with any GFTPs ranged from 80% (vineyard B) to 100% (vineyard D).

Co-occurrence of two, three, or four different fungi in the same sample was found, with the simultaneous presence of two fungi being the most frequent (Table 2). The percentage of positive samples in which two different fungi were found simultaneously ranged from 52.6% (vineyard C) to 62.5% (vineyard B).

Fungal isolation in semiselective culture medium. Only *C. luteo-olivacea* was recovered in samples taken from shears during pruning by fungal isolation in semiselective culture medium. In all, eight isolates of *C. luteo-olivacea* were obtained from samples collected in vineyards A (three samples, one isolate per sample), B (two samples, one isolate per sample), and C (three samples, one isolate per sample). However, no isolates of GFTPs were recovered from samples collected in vineyard D.

Potential of pruning shears artificially infested with GFTPs to infect grapevine pruning wounds. Results of the frequency of positive cuttings and isolation of fungal trunk pathogens from grapevine cuttings are shown in Table 3. Isolation data of the two experiments from each fungal species evaluated were combined. Four months after inoculation, *C. luteo-olivacea*, *D. seriata*, *Phaeoacremonium aleophilum*, and *Phaeomoniella chlamydospora* were isolated from

internal wood tissues of grapevine cuttings of 110 Richter rootstock pruned with shears infested at the two concentrations tested, 10^4 and 10^6 conidia or fragments ml^{-1} , whereas *E. lata* was only isolated from cuttings pruned with shears infested at 10^6 fragments ml^{-1} . Fungal pathogens were not isolated from cuttings pruned using pruning shears previously immersed in SDW. Frequency of positive cuttings and mean percentage of isolation of all species tested were greater in cuttings pruned with shears immersed in the highest conidia or mycelial fragment suspension concentration compared with the lower concentration, with mean percentage of isolation being significantly different only for *C. luteo-olivacea* and *E. lata*.

Discussion

In this study, Botryosphaeriaceae spp., *C. luteo-olivacea*, *Phaeoacremonium* spp., and *P. chlamydospora* were detected from liquid samples collected from shears used to prune four Spanish vineyards by means of molecular detection by nested PCR and, in the case of *C. luteo-olivacea*, by fungal isolation in semiselective culture medium. To our knowledge, this is the first work evaluating the potential spread of infection of GFTPs with pruning shears.

In general, using PCR, *C. luteo-olivacea* and *Phaeoacremonium* spp. were the most frequently detected in all vineyards evaluated, whereas *P. chlamydospora* was detected at very low levels. These results are in accordance with those obtained by other authors, who indicated that the occurrence of *P. chlamydospora* in vineyards is often lower than *Phaeoacremonium* spp. (Aroca et al. 2010; Mostert et al. 2006). Botryosphaeriaceae spp. were detected at low frequency in samples collected from vineyards A, B, and C, whereas their detection increased markedly in samples collected from vineyard D, which was 13 and 21 years older than vineyards C, and A and B, respectively. However, *E. lata* was not detected on shears from any vineyard evaluated. This may be due to the low incidence of *E. lata* reported in Mediterranean regions of Spain (Armengol et al. 2001; Luque et al. 2009).

The simultaneous presence of two different species in the liquid samples was quite common, the co-occurrence of *C. luteo-olivacea* and *Phaeoacremonium* spp. being the most frequent, whereas the co-occurrence of three or four species was less frequent. The presence of multiple GFTPs may be due to diverse infection events through pruning wounds, in accordance with the current knowledge that esca, petri disease, and Botryosphaeria and Eutypa dieback can occur alone or together on the same plant (Bertsch et al. 2013; Luque et al. 2009; Mugnai et al. 1999). This result emphasizes the need to develop molecular techniques which could allow the detection of multiple GFTPs at the same time for rapid, specific, and sensitive detection and diagnosis of GFTPs in grapevine.

Table 2. Molecular detection by nested polymerase chain reaction of Botryosphaeriaceae spp. (Bot), *Cadophora luteo-olivacea* (Clo), *Phaeoacremonium* spp. (Phaeo), or *Phaeomoniella chlamydospora* (Pch) and their simultaneous presence in samples collected from shears used to prune vines in four different vineyards

Fungi detected	Samples per vineyard ^x			
	A	B	C	D
One				
Bot	3	4	5	17
Clo	14	12	15	17
Phaeo	17	12	17	10
Pch	0	2	2	3
Total ^y	18/20	16/20	19/20	20/20
Percent	90	80	95	100
Two				
Bot+Clo	0	2	0	7
Clo+Phaeo	10	6	8	3
Clo+Pch	0	1	0	0
Bot+Phaeo	0	0	1	1
Bot+Pch	0	0	0	0
Phaeo+Pch	0	1	1	0
Total ^z	10/18	10/16	10/19	11/20
Percent	55.6	62.5	52.6	55
Three				
Bot+Clo+Pch	0	0	0	1
Bot+Clo+Phaeo	3	2	4	4
Bot+Phaeo+Pch	0	0	0	0
Clo+Phaeo+Pch	0	0	1	0
Total ^z	3/18	2/16	5/19	5/20
Percent	16.6	12.5	26.3	25
Four				
Bot+Clo+Phaeo+Pch	0	0	0	2
Total ^z	0/18	0/16	0/19	2/20
Percent	0	0	0	10

^x Number of samples from which each fungus or its simultaneous presence was detected.

^y Number of total positive samples/number of total samples evaluated.

^z Number of samples from which two, three, or four fungi were detected simultaneously/number of total positive samples detected.

Table 3. Frequency of positive cuttings and isolation of *Cadophora luteo-olivacea*, *Diplodia seriata*, *Eutypa lata*, *Phaeoacremonium aleophilum*, and *Phaeomoniella chlamydospora* from grapevine cuttings of 110 Richter rootstock after pruning with shears preimmersed in suspensions of conidia or mycelial fragments at 10^4 and 10^6 conidia or fragments ml^{-1}

Fungal species	Frequency of positive cuttings ^x		Isolation (%) ^y	
	10^4	10^6	10^4	10^6
<i>C. luteo-olivacea</i>	2/16	6/16	3.6 a	23.2 b
<i>D. seriata</i>	5/16	7/16	21.4 a	23.2 a
<i>E. lata</i>	0/16	4/16	0 a	13.4 b
<i>Phaeoacremonium aleophilum</i>	6/16	7/16	19.6 a	28.6 a
<i>Phaeomoniella chlamydospora</i>	3/16	5/16	7.1 a	12.5 a
Control ^z	0/16	0/16	0 a	0 a

^x Number of infected cuttings/number of pruned cuttings with shears preimmersed in suspensions of conidia or mycelial fragments (8 cuttings per treatment combination and repetition, 16 cuttings in total).

^y Mean percentage of wood fragments from which fungal species were isolated (112 wood fragments for each combination; 7 fragments per cutting). Analysis of variance: means in a row followed by the same letter are not significantly different according to Student's least significant test at $P < 0.05$.

^z Pruning with shears preimmersed in sterile distilled water.

In a complimentary experiment culturing the liquid samples on semi-selective medium to evaluate the presence of viable propagules of GFTPs, *C. luteo-olivacea* was the only species isolated from pruning shears washings. This demonstrated that pruning tools may be an important source of inoculum for this species which, in recent years, has been increasingly reported in grapevine nurseries and young vineyards worldwide, causing a major concern in both grape growers and nurseries (Gramaje et al. 2014). This finding is complementary to that obtained by Gramaje et al. (2011), who isolated *C. luteo-olivacea* in semiselective culture medium from scissors and omega-cut grafting machines used during the grapevine nursery propagation process in Spain. *Phaeoacremonium* spp. and *P. chlamydospora* were not isolated by using this technique, perhaps due to a low inoculum level in the liquid samples, because the inoculum was composed of mycelium or infected wood fragments larger than 5 µm, which is the pore size of the syringe filters used, or to the fact that viable propagules were absent.

In this study, the susceptibility of pruning wounds to infection by pruning shears artificially infested with GFTPs was also evaluated. *C. luteo-olivacea*, *D. seriata*, *E. lata*, *Phaeoacremonium aleophilum*, and *Phaeomoniella chlamydospora* were isolated from the internal tissues of grapevine cuttings pruned with artificially infested pruning shears, and *Phaeoacremonium aleophilum* showed the highest incidence and isolation percentages at both concentrations tested. In this experiment, the use of artificial inoculum with fixed-spore or mycelial fragment concentrations ensured repeatability and eliminated any environmental and seasonal confounding associated with natural inoculum, which would have been present in an experiment performed in an established vineyard. This result indicates that fresh pruning wounds may be infected by petri diseases and Botryosphaeria and Eutypa dieback pathogens present on pruning shears, as suggested by Mugnai et al. (1999) and Zanzotto et al. (2013). Although our results are only preliminary, they demonstrated that it may be possible to spread some pathogens via pruning shears. This conclusion is based on the inoculum levels used in the pruning experiments with artificially infested shears, and further research is needed to quantify the amount, type, and viability of pathogen inoculum that is carried on shears after pruning, as well as to determine the inoculum level thresholds for successful infection of each GFTP.

Although airborne spores penetrating through pruning wounds may play the primary role in the infection of grapevines in a vineyard (Eskalen et al. 2007; Rolshausen et al. 2010), the use of contaminated pruning shears along the rows could be significant for the secondary spread of GFTPs. This supports the findings of Zanzotto et al. (2013), who demonstrated the spatial spread of esca disease along vine rows, which was assumed to be spread by pruning. In this way, it is advisable that viticulturists should disinfect all pruning tools frequently (Zanzotto et al. 2013). These cultural practices will be effective if they are integrated with other management strategies such as double pruning (Úrbez-Torres and Gubler 2011; Weber et al. 2007) and protecting pruning wounds with a paste or liquid formulation containing a broad-spectrum fungicide (Díaz and Latorre 2013; Rolshausen et al. 2010; Sosnowski et al. 2008) or biological control agents (Halleen et al. 2010; John et al. 2005; Munkvold and Marois 1993), together with removing and destroying all diseased wood to reduce inoculum sources of GFTPs in vineyards (Úrbez-Torres and Gubler 2011).

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