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RESEARCH PAPERS - 10TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

Response of four Portuguese grapevine cultivars to infection by *Phaeomoniella chlamydospora*

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Summary. Little is known of the response of Portuguese Dão wine appellation's most common grapevine cultivars to the causal agents of esca and Petri diseases, despite the high incidence of both diseases in the region and the consequent economic losses. *Phaeomoniella chlamydospora* has been considered one of the major causal agents of these diseases in that region. The present study evaluated the responses of four of the most propagated Dão's grapevine cultivars – Alfrocheiro, Aragonez, Jaen and Touriga Nacional – to infection by three different Portuguese isolates of *P. chlamydospora*. Field trials were conducted in 2012, 2013 and 2015. The cultivar Alfrocheiro was the most susceptible to *P. chlamydospora* while cv. Jaen was the least. Variation in parameters such as lesion length and pathogen recovery from infected spurs (within trial years) suggest relation of pathogenicity with weather data, particularly temperature. Differences in aggressiveness among isolates were also detected, with one, a non-native, being the most aggressive. These results provide valuable information for local winegrowers, identifying, for the first time, susceptibility differences among local cultivars to *P. chlamydospora*, and suggesting adjustments to recommended pruning strategies, specifically to leave long spurs and avoid late winter pruning, thus reducing grapevine trunk colonization by *P. chlamydospora*.

Key words: esca, field infection, cultivar susceptibility, *Vitis vinifera*.

Introduction

The Dão Protected Geographical Indication (PGI) is a very traditional and distinct Portuguese wine region, where, unlike other Portuguese wine appellations, local cultivars predominate. Due to their oenological and consequent economic interest, four grapevine cultivars dominate, including Alfrocheiro, Aragonez (Tempranillo), Jaen (Mencia), and Touriga Nacional. There is considerable interest in understanding the agronomic behaviour and susceptibility to certain diseases for these cultivars.

Previous research (Tomaz *et al.*, 1989; Sofia *et al.*, 2006, 2013) has shown esca as one of the most important grapevine trunk diseases (GTDs) in the Dão PGI. This disease, which leads to the decline and eventual death of affected plants, has been considered a complex of several syndromes - esca proper, esca, young esca, Petri disease, and brown wood streaking (Surico, 2009; Bertsch *et al.*, 2013). This complex is commonly associated with several fungal pathogens such as the anamorphic ascomycetes *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams and *Phaeoacremonium* spp. and the basidiomycete *Fomitiporia mediterranea* M. Fisch. (Mugnai *et al.*, 1999; Bertsch *et al.*, 2013). Esca proper, characteristic of mature grapevines (i.e. 7 years and older), involves the co-occurrence of Petri disease (young esca, a manifestation of esca in plants less than 6 years old) and esca on the same plant (Surico *et al.*, 2006; Surico, 2009; Bertsch *et al.*, 2013). *Phaeomoniella chlamydospora*

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and *Phaeoacremonium* spp. are the two most important pathogens related to brown wood streaking of host xylem vessels, Petri disease and young esca (Crous and Gams, 2000; Bruez *et al*., 2013), whereas esca is associated with several basidiomycetes, most commonly *F. mediterranea*. Recently, a simplification of these syndromes has been proposed: the designation "young esca" was replaced by "grapevine leaf stripe disease" (GLSD), while "esca" was used to designate grapevines showing symptoms in internal woody tissues of soft white rot, usually due to *F. mediterranea* (Fischer 2002, 2006; Bertsch *et al*. 2013), and esca proper, "Brown wood streaking", "Petri disease" and "grapevine leaf stripe disease" were grouped under the designation "phaeotracheomycotic complex", emphasizing the involvement of *P. chlamydospora* and/or *Phaeoacremonium minimum* in the three syndromes (Bertsch *et al*., 2013). *Phaeoacremonium* spp. are consistently considered to have less important roles in the esca complex than *P. chlamydospora* (Adalat *et al*., 2000; Halleen *et al*., 2007; Fischer and Kassemeyer, 2012; Markakis *et al*., 2017). Also, *P. chlamydospora* is the most frequently isolated species (Mugnai *et al*., 1999; Clearwater *et al*., 2000; Pascoe and Cottral, 2000; Whiteman *et al*., 2002), and considered the most important and aggressive fungal organism associated with Petri disease (Wallace *et al*., 2003; Ridgway *et al*., 2005; Halleen *et al*., 2007; Sofia *et al*., 2007; Zanzotto *et al*., 2008; Laveau *et al*., 2009; Pouzoulet *et al*., 2013). This fungus is consistently recovered from plants in the Dão appellation affected by the esca syndromes (Sofia *et al*., 2006; Sofia, 2007).

Considered previously as a solution for control of esca symptoms (Mugnai *et al*., 1999), sodium arsenite was banned from all European winegrowing countries at the beginning of the 21st century due to its high toxicity. Several active ingredients were reported as effective for reducing *in vitro* growth of *P. chlamydospora* and *Phaeoacremonium* spp., but no chemical treatment has been considered effective against any of the esca syndromes (Gramaje *et al*., 2009; Martín and Martín, 2013; Travadon *et al*., 2013).

When a grapevine shows symptoms of GTDs, normal practice recommends its removal and destruction (burned or composted), followed by replacement with a new plant. If the plant is considered viable, options are the removal of the diseased plant parts and the retraining of new canes to replace the ablated parts (Travadon *et al*., 2013; Fontaine *et al*., 2016). Protection against GTDs relies mainly on prophylactic measures (e.g., pruning wound disinfection) or remedial surgery, both of which are costly and work-demanding. Viticulture is under pressure for change. Environmental costs due to phytochemical protection, public health concerns, and stringent new legislation restraining the use of pesticides (e.g., Directive 2009/128/EC of the 21 October), all demand environmentally-friendly disease management practices. According to Agrios (2005) "… the use of resistant varieties is the least expensive, easiest, safest and one of the most effective means of controlling plant diseases in crops".

Artificial inoculation of standing grapevines is a potential tool for increasing knowledge of the susceptibility of different cultivars to esca (Feliciano *et al*., 2004). Regarding control of *P. chlamydospora*, employing less susceptible genotypes has, until now, received little attention. Some research, based on the external manifestation of disease symptoms, has assessed the susceptibility of some cultivars to esca-related fungi (Larignon *et al*., 2009; Bertsch *et al*., 2013). Evidence of cultivar susceptibility to esca has also emerged from observations carried out under controlled conditions (Feliciano *et al*., 2004; Martin *et al*., 2009). However, there are few reports based on field infections of standing grapevines for relative susceptibility to *P. chlamydospora*, and none for Portuguese cultivars.

The purpose of the present study was to evaluate the susceptibility responses of most popular Dão cultivars to infection by *P. chlamydospora*, using an infection method emulating natural contamination by this pathogen. To our knowledge, this is the first attempt to evaluate susceptibility under field conditions in typical Dão productive vineyards.

Material and methods

Plant material

Trials were carried out in the experimental facilities of CEVDão in Nelas [UTM coordinates (Datum WGS 84): 29 T 596837, 4486566], Portugal, on 15-year-old standing grapevines of the cultivars Aragonez, Alfrocheiro, Jaen and Touriga Nacional. These were all grafted on rootstock 110 Richter, trained in bilateral cordons, and spur-pruned. In the first two trials (during 2012 and 2013) 18 plants of each cultivar were used per treatment while in the third trial (in 2015) 26 plants were used per treatment. Before each trial, during the summer season, all plants were assessed

for esca symptom expression. Plants showing foliar or wood symptoms that could be related to GTDs were discarded, whereas asymptomatic plants were included in the trial. In each trial year, grapevine growth stages were recorded according to Baggiolini's scale (Baggiolini, 1952).

Fungus isolates

Three isolates of *P. chlamydospora* were compared for infection of the different grapevine cultivars. These were isolate 43 (GenBank KP886991), obtained from a 2-year-old "Petit Verdot" grapevine from Vidigueira, Alentejo, Southern Portugal; isolate 48 (GenBank KP886996) obtained from a Touriga Nacional grapevine from Arruda-dos-Vinhos, Central Portugal; and isolate 52 (GenBank KP887000) obtained from a 15-year-old Jaen grapevine from Nelas. Isolates were maintained in potato dextrose agar (PDA, Difco, Beckton, Dickinson and Co.) slants and transferred to Petri dishes containing PDA to promote colony growth. Cultures were incubated at 24°C (±1°C) in complete darkness for 15 d.

Conidium suspensions were obtained by removing, with a sterile cork borer, an agar plug approx. 5 mm from the border of the culture, which was then added to a 250 mL capacity Erlenmeyer flask containing potato dextrose broth (PDB, Difco, Beckton, Dickinson and Co.) and placed at 20°C, in darkness, in a reciprocal shaker (90 revolutions min^{-1}), for 21 d. One day before inoculation, formed lumps of mycelium were crushed with a sterile glass rod, and the solution was filtered through a sterile gauze pad. The resulting suspension was kept under agitation, with a magnetic stirrer. The suspension was adjusted to 1×10^5 conidia mL^{-1} after determining numbers using a haemocytometer. Suspensions were stored overnight at 10°C, until used.

Inoculation methods

Inoculations were performed during the late pruning seasons of the trial years, on freshly pruned canes of standing vines, respectively on 25 March 2012, 5 April 2013 and 20 March 2015. Pruning for immediate infection was only performed after the beginning of bleeding in all cultivars. For each cultivar, inoculations were applied to distinct groups of plants, with each of the three fungal isolates, and with a control group of plants inoculated with sterile PDB. Under the vineyard training system, canes are typically pruned to the two first buds, each forming a spur approx. 5–7 cm long. The spurs predestined for inoculation were left about 15 cm long but with two buds - the bottommost and uppermost - to allow development of potential necrosis and to avoid complete destruction of the fruiting unit, when on recovery of infected wood, the plants could be returned to production in the vineyard. All intermediate buds were cut off with disinfected pruning scissors. Spurs were inoculated immediately after pruning, in the afternoon, with fair weather, no wind and temperature above 10°C. A 40 µL droplet of conidium suspension, containing approximately 4×10^3 conidia, was carefully deposed onto each pruning wound, and allowed to soak for 10-15 min. The inoculation point was then covered with a cotton pad moistened with sterile distilled water and protected with a strip of plastic film (Parafilm® "M", Pechiney Plastic Packaging) to avoid natural infection and other contamination. All wound protections were left in place for 1 week and then removed, allowing the inoculated spurs to be exposed to the vineyard conditions.

Lesion length and pathogen recovery rates

To assess infection, approx. 9 months after inoculation, the inoculated spurs were each cut off 1 cm above the bottommost bud. They were then packed into freezer bags, and refrigerated at 5°C, until further use. All samples were processed within 72 h after collection. To avoid external contamination, for each sample, the bark was peeled off with a sterile knife, and the exposed wood was disinfected by spraying with a 70% ethanol solution. Thin slices of each sample were taken upwards with pruning scissors, beginning from the lowest end of the spur until black dot lesions were visible inside the wood. All slices were kept in the sequential order they were removed, for subsequent re-isolations. The distance from the infection point to the previously detected end of each lesion was measured with an electronic calliper. All samples were then longitudinally sectioned to verify the presence and continuity of the putative lesions.

Mycological analyses

From each sample, three subsamples were taken: in the length direction, one from the middle of the lesion (ML), one from the lesion end (BL) and one

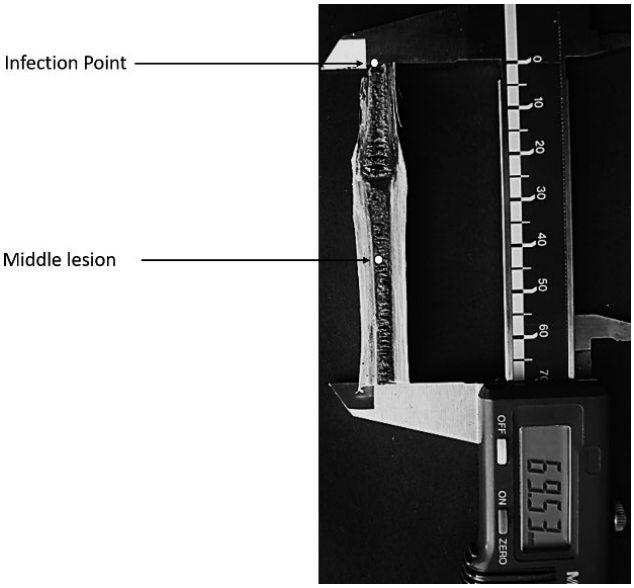

Figure 1. Details of infection assessment sampling from grapevine stems: white dots indicate inoculation points and middle lesion (ML) sampling position. Symptomless wood (SW) was removed 0.5 cm bellow the lesion end. Calliper reading shows lesion length (LL) in mm.

from symptomless wood collected approx. 0.5 cm below the lesion end (SW) (Figure 1). From the end of each subsample, two transversal cuts, approx. 1 mm apart, were made with disinfected pruning scissors. The resulting tissue pieces were surface disinfected for 1 min. by immersion in 2% sodium hypochlorite solution, rinsed with sterile distilled water and then allowed to air dry for 1 min. on a sterile filter paper pad. Each piece was then sectioned into four small fragments, which were plated onto a Petri plate containing PDA amended with 250 mg L^{-1} chloramphenicol (BioChemica, AppliChem), four pieces per PDA plate. Each plate was sealed and then incubates at 24°C (± 1°C), in darkness. Isolation plates were observed every 2 d until fungal growth permitted identification. Recovery proportions of *P. chlamydospora* were calculated as the percentage of fragments from which the pathogen was recovered out of the total number of samples.

Weather data

A "Campbell CR 510" automatic weather station, located on the trial site and property of Centre Portugal Regional Directorate of Agriculture and Fisheries provided daily temperature and rainfall data.

Statistical analyses

Assumptions for analyses of variance analyses were assessed with software R (www.r-project.org). When the assumptions were not accomplished, the influence of distinct levels of one factor was assessed using the Kruskal-Wallis non-parametric test. In this case, when statistically significant differences were found ($P < 0.05$), the comparisons between the distinct levels were made using the ranks.

Results

All three *P. chlamydospora* isolates produced lesions, characterised by black/brown streaking on longitudinal cuts of the inoculated grapevine spurs, and were recovered from those lesions. Control (uninoculated) plants had minor internal discolourations (lesion extension ranged from 9.5 to 12.3 mm), which were usually associated with desiccation of the internodes, and rarely showed black streaking on the longitudinal cuts. *Phaeomoniella chlamydospora* was never recovered from those lesions. Therefore, control values were not included in statistical analyses.

The susceptibility of the cultivars to infection by *P. chlamydospora*, and the aggressiveness of the isolates, were based on the assessment of lesion length and of fungal recovery from the three isolation levels (ML, BL or SW). These analyses were carried out using non-parametric methods, as assumptions for linear models were not fully accomplished for each factor (data not shown).

The results of inoculations with *P. chlamydospora* carried out on four cultivars in the 3 year trial showed that the mean percentages of recovery of *P. chlamydospora* were greater from cv. Alfrocheiro (47.0% in 2012, 35.1% in 2013, and 17.4% in 2015) than from the other three cultivars. Recovery from cvs Touriga Nacional and Aragonez were similar while recovery was least from cv. Jaen (26.3% in 2012, 16.1% in 2013, and 6.9% in 2015). The largest lesions were recorded in cv. Touriga Nacional (mean = 54.9 mm), but these were not significantly larger than those observed in cv. Alfrocheiro (mean = 50.1 mm). Cultivar Aragonez developed significantly smaller lesions (mean = 48.9 mm) than cv. Touriga Nacional, and cv. Jaen repeated this pattern (mean=41.7 mm) towards Aragonez.

Mean lesion lengths in cv. Alfrocheiro and Touriga Nacional were not significantly different (Table 1).

The distribution of recovery rates (Figure 2) showed that the percentage of *P. chlamydospora* colonies recovered from ML ranged in all cultivars from 0 to 100%, with lower pathogen recovery rates in BL and even lower rates in SW. Median values, indicating the recovery percentage that includes half of the sorted samples analysed, showed the same trends. In the cuts corresponding to ML, median values of about 50% for cv. Alfrocheiro indicated that 50% of the samples had up to 50% of recovery, whereas in cvs Aragonez and Touriga Nacional up to 50% of the samples had less than 20% recovery. In cv. Jaen, 50% of the samples analysed did recover *P. chlamydospora* colonies. Considering BL, the pattern was similar, with recovery percentage from cv. Alfrocheiro being much greater than from the other cultivars. In this case, no *P. chlamydospora* colonies were recovered from 50% of the samples of cvs Aragonez, Jaen or Touriga Nacional, which was opposite from recovery from cv. Alfrocheiro. In symptomless wood, the tendency was reinforced; for all cultivars, 50% of the samples did not allow recovery of *P. chlamydospora*, but still cv. Al-

Table 1. Mean proportions (%) of *Phaeomoniella chlamydospora* colonies recovered from middle of lesion, bottom of lesion or from symptomless wood, and mean lesion lengths, recorded for four grapevine cultivars inoculated with three *P. chlamydospora* isolates in three trial years.

Cultivar	***Phaeomoniella chlamydospora* recovered colonies (%)**			**Average lesion length (LL) (mm)**
	Middle of the lesion (ML)	**Bottom of the lesion (BL)**	**Symptomless wood (SW)**	
Alfrocheiro	47.0 a*	35.1 a	17.4 a	50.1 ab
Touriga Nacional	30.8 bc	21.2 b	8.0 b	54.9 a
Aragonez	33.8 b	18.4 bc	7.8 b	48.9 b
Jaen	26.3 c	16.1 c	6.9 b	41.7 c

* Different letters in each column indicate significant differences (α=0.05) based on ranks assessed by Kruskal-Wallis analyses.

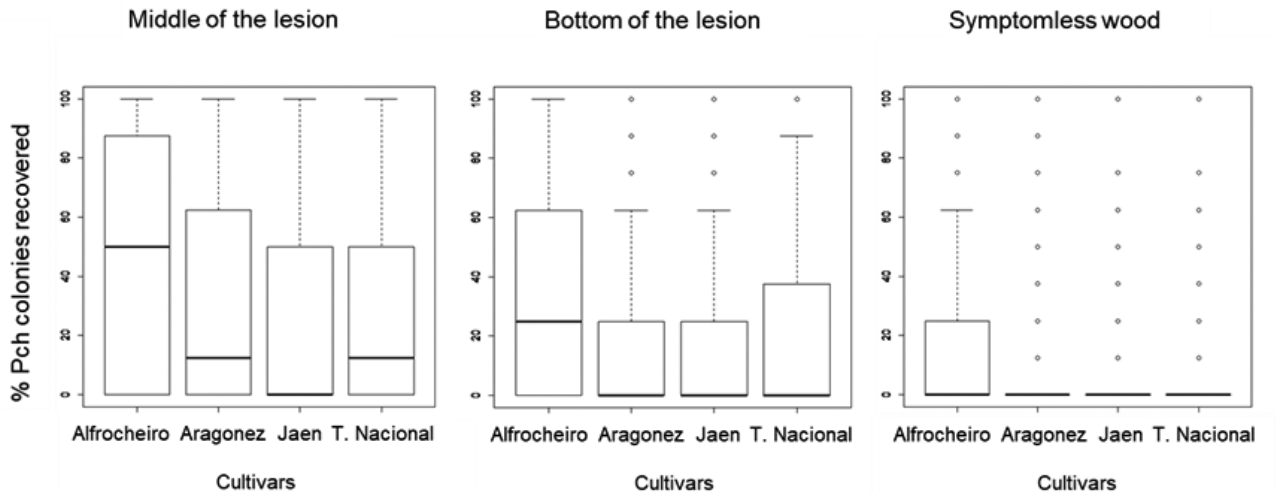

Figure 2. Boxplots of proportions (%) of *Phaeomoniella chlamydospora* colonies recovered from the middle of lesion, the bottom of the lesion or from symptomless wood, for the four grapevine cultivars. The median is represented by the solid line.

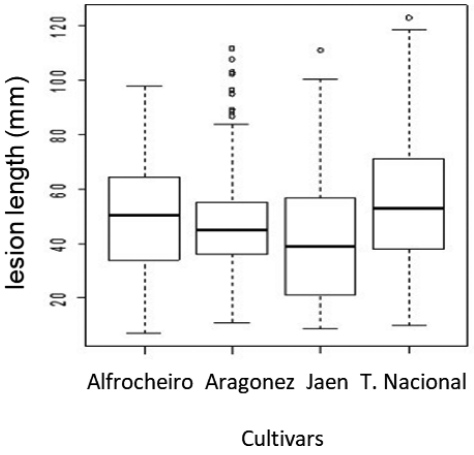

Figure 3. Boxplots of the average lesion lengths recorded for the four grapevine cultivars. The median is represented by the solid line.

frocheiro samples gave consistently greater recovery rates than the other cultivars.

A similar non-parametric analysis of lesion length data (Figure 3) corroborated the results presented in Table 1, with cvs Alfrocheiro and Touriga Nacional showing similar and greater mean values than lesions of cvs Aragonez or Jaen. The distribution of lesion lengths for Jaen suggested a trend to smaller lesions, with 50% of the samples harbouring lesions smaller than 40 mm and 75% of the samples with lesions smaller than 60 mm.

The present study employed three *P. chlamydospora* isolates. The proportion of *P. chlamydospora* colonies recovered from ML were significantly different between all isolates, while for BL isolations, only isolate 48 was significantly different from the other two, which performed similarly. Isolate 48 recovery rates were greater and significantly different from those for the other two isolates (Table 2).

The proportions of *P. chlamydospora* colonies recovered from SW were significantly greater for isolate 48 than for isolate 43 and isolate 52 gave intermediate re-isolation proportion. Mean lesion length was significantly greater for isolate 48 than for isolates 52 and 43 and were not significantly for these two isolates (Table 2).

The analysis of range and distribution indicated a decreasing trend of recovery from the middle lesion sections to the base lesion sections, and further to the symptomless wood sections (Figure 4). For the ML sections, isolate 43 was less aggressive than the two other isolates, with 50% of the samples not yielding any pathogen re-isolation. Isolates 48 and 52 behaved similarly. For BL sections, isolate 48 was distinguishable from isolate 52; isolate 52 was less aggressive than isolate 48 and similarly aggressive to isolate 43. In symptomless wood, almost no colonies were recovered from isolate 43 inoculations; again, isolates 48 and 52 behaved similarly and were more aggressive than isolate 43. The median lesion length was slightly greater for isolate 48 (Figure 5) than for the other two isolates. The distributions of lesion lengths indicated a small difference between isolate 48 and the other two isolates, which behaved similarly.

A joint analysis combining isolates and cultivars indicated an overall trend for isolate 48 to cause larger lesions on all the four cultivars. However, this trend did not occur on cv. Touriga Nacional, where the largest lesions resulted from isolate 43. Cvs Alfrocheiro and Jaen showed similar patterns, and in both cases, isolate 48 was more aggressive to this host than the

Table 2. Mean proportions (%) of *Phaeomoniella chlamydospora* recovered from the middle of lesion, bottom of the lesion or from symptomless wood, and mean lesion lengths, recorded after inoculations of four grapevine cultivars in three trial years.

Isolate	***Phaeomoniella chlamydospora* recovered colonies (%)**			**Average lesion length (LL) (mm)**
	Middle of the lesion (ML)	**Bottom of the lesion (BL)**	**Symptomless wood (SW)**	
43	27.2 c*	18.6 b	8.5 b	47.6 b
48	42.2 a	29.1 a	12.6 a	52.8 a
52	34.9 b	21.3 b	9.5 ab	46.4 b

* Different letters in each column indicate significant differences (α=0.05) based on ranks assessed by Kruskal-Wallis analyses.

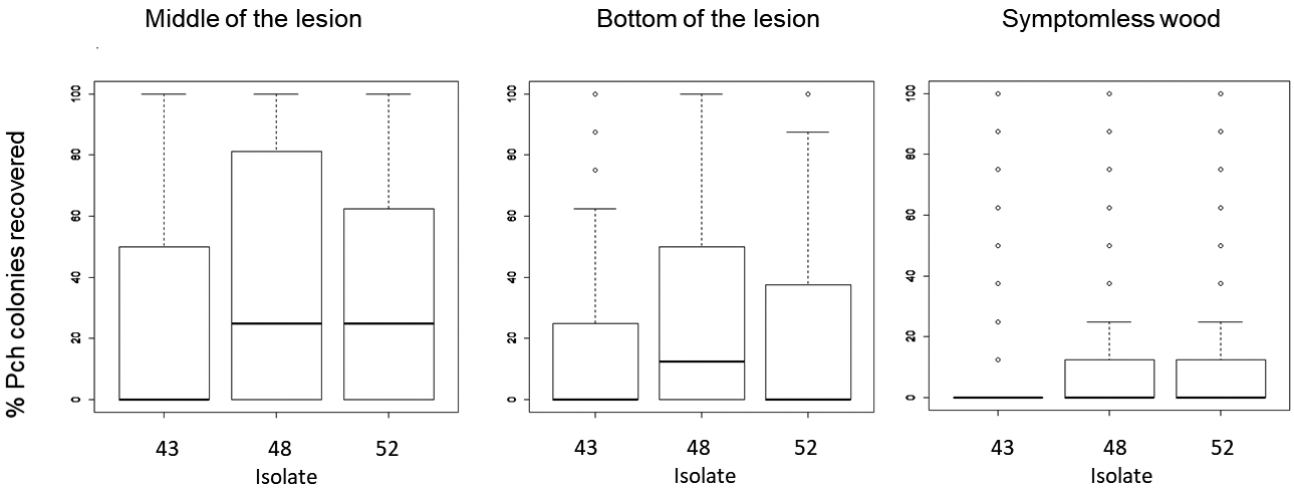

Figure 4. Boxplots of percentage of *Phaeomoniella chlamydospora* colonies recovered from the middle of lesions, the bottom of the lesions or from symptomless wood, for the three *P. chlamydospora* isolates. The median is represented by the solid line.

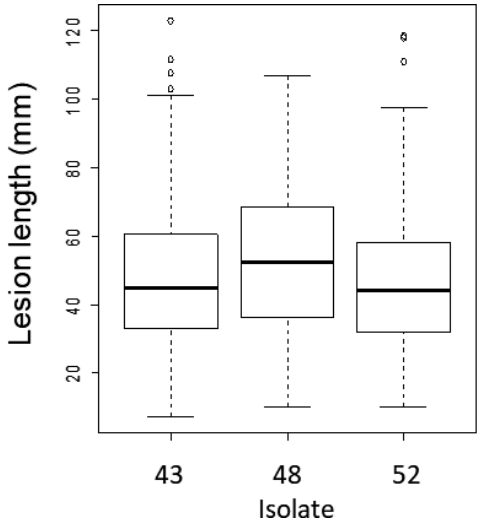

Figure 5. Boxplots of average lesion lengths recorded after inoculations of grapevines with three *Phaeomoniella chlamydospora* isolates. The median is represented by the solid line.

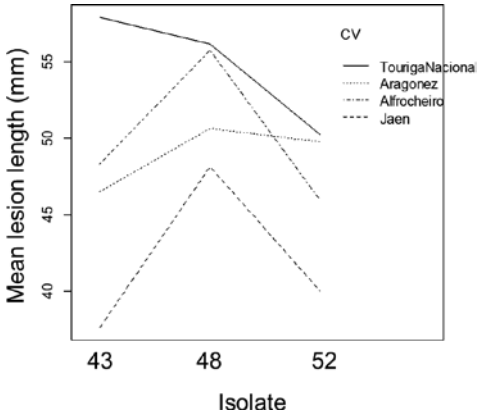

Figure 6. Mean lesion lengths (mm): trends for three isolates (43, 48 and 52) and four grapevine cultivars (Alfrocheiro, Aragonez, Jaen and Touriga Nacional).

other two isolates, that produced lesions of similar size. For cv. Aragonez, all three isolates were equally aggressive (Figure 6).

The inoculations applied in 2012 gave greater proportions of pathogen recovery than in the other two years, from the ML and BL stem isolations. For the SW, this trend was followed, but the year differences were not so strong, as the recovery percentage for 2013 was not significantly different from either 2012 or 2015 (Table 3).

Inoculations in 2012 gave the greatest amount of infection, while infection was least in 2015 and intermediate in 2013. This pattern also followed for mean lesion lengths, with those in 2012 and 2013 were larger than those obtained from the inoculations of 2015. The median lesion length was greatest in 2013, but in 2012 higher values were achieved occasionally (not sufficiently frequent to increase the median value). Close analysis of the ranges and distributions of the proportions of *P. chlamydospora* colonies re-

Table 3. Mean proportions (%) of *Phaeomoniella chlamydospora* colonies recovered from middle of lesion, bottom of the lesion, or from symptomless wood, and mean lesion lengths, from grapevines inoculated in 2012, 2013 and 2015 (all isolates, and cultivars).

Year	*Phaeomoniella chlamydospora* recovered colonies (%)			Average lesion length (LL) (mm)
	Middle of the lesion (ML)	Bottom of the lesion (BL)	Symptomless wood (SW)	
2012	47.6 a*	34.2 a	16.7 a	50.3 ab
2013	35.6 b	23.6 b	10.9 ab	52.5 a
2015	26.1 c	15.7 b	5.7 b	45.9 b

* Different letters in each column indicate significant differences (α=0.05) based on ranks assessed by Kruskal-Wallis analyses.

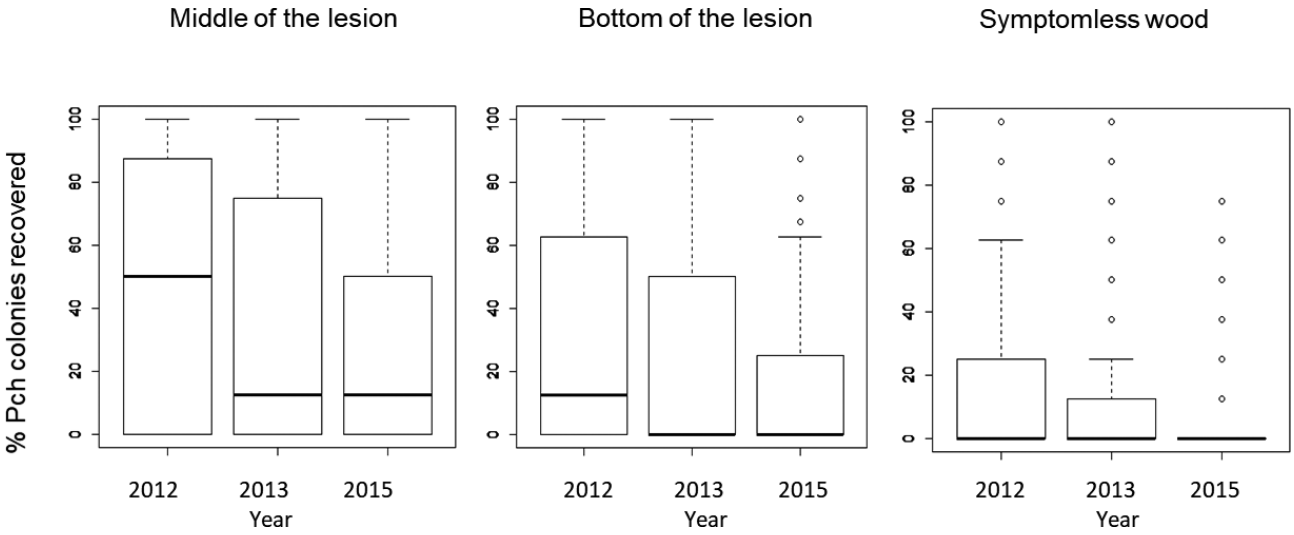

Figure 7. Boxplots of percentage of *Phaeomoniella chlamydospora* colonies recovered from the middle of lesion, the bottom of the lesion or from symptomless wood, for the three trial years 2012, 2013 or 2015. The median is represented by the solid line.

covered from the ML, BL, and SW (Figure 7) shoot tissue pieces corroborates these results. There was a decreasing trend of recovery proportions from the ML to BL samples, and further to SW sections. For the symptomatic tissues, recovery ranges and distributions for 2013 and 2015 were similar, indicating less efficient inoculations than in 2012. For ML sections, in 2013 and 2015, 50% of the samples delivered less than about 10% *P. chlamydospora* colonies. For the BL sections collected in 2013 and 2015, *P. chlamydospora* was not recovered from 50% of the samples. The lower infection from inoculations carried out in 2015 was emphasized from the analysis for the symptomless tissues, which showed that there was no recovery of *P. chlamydospora* recovery, indicating less effectiveness of the inoculations in that year (Figure 7).

The lesion length data were very similar for all three trial years (Figure 8), although there was a trend for the lesions to be smaller from inoculations carried out in 2015.

Statistical analysis combining inoculation year and isolates indicated that isolates 43 and 52 behaved similarly, producing smaller lesions in 2012 and 2015 and larger lesions in 2013. Isolate 48 behaved in an opposite manner (Figure 9).

Analysis combining trial years and cultivars indicated different responses for the cultivars in the different years. The lesions in cv. Touriga Nacional in

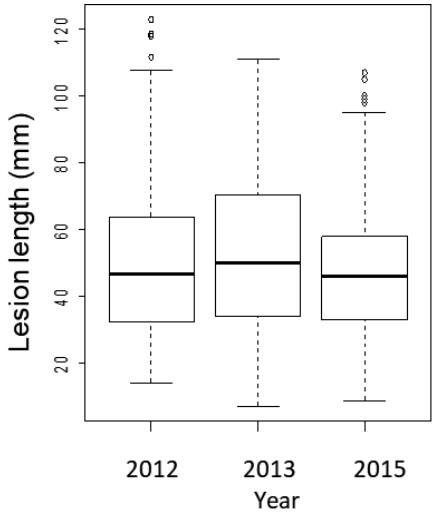

Figure 8. Boxplots of the average lesion lengths for the three trial years 2012, 2013 or 2015. The median is represented by the solid line.

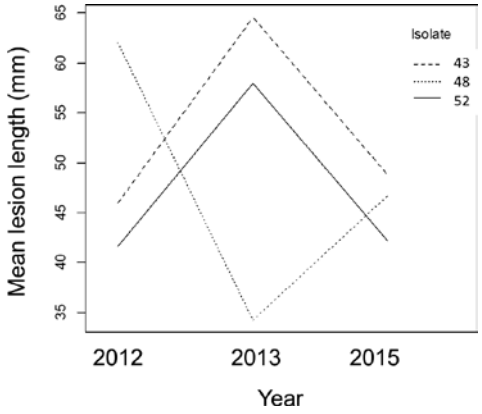

Figure 9. Mean lesion lengths (mm): trends between years (2012, 2013 or 2015) and *Phaeomoniella chlamydospora* isolates (43, 48 and 52).

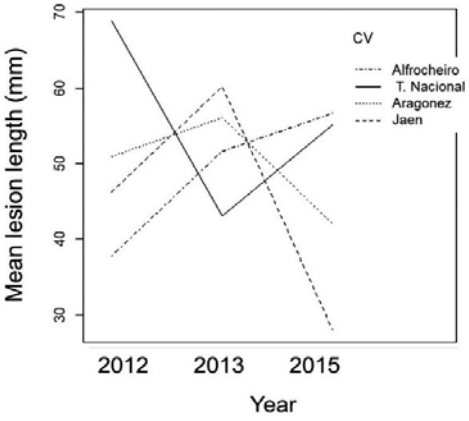

Figure 10. Mean lesion length (mm): trends between years (2012, 2013 or 2015) and grapevine cultivars (Alfrocheiro, Aragonez, Jaen and Touriga Nacional).

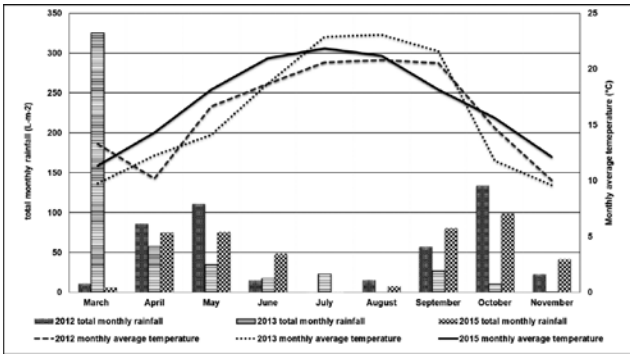

Figure 11. Mean monthly rainfall and temperatures for three trial years, 2012, 2013 and 2015.

2013 were smaller compared with those in 2012 or 2015 (Figure 10). The distinct pattern showed by each cultivar indicates that the meteorological conditions during the inoculation and colonization processes probably caused different host pathogen interactions. Amount and distribution of rainfall (Figure 11) were similar in 2012 and 2015 (respectively, 481 and 459 Lm^{-2}), while in 2013, accumulated rainfall was almost these amounts (894 Lm^{-2}), and most rain fell in the first 3 months of 2013. In 2012, an exceptional period of mild temperatures occurred during March, until 2 weeks after inoculation. Maximum temperatures reached more than 25°C during a week after inoculation, with the minimum temperature never being below 10°C for the same period. In the 2 weeks after inoculation, average temperatures remained between 12 and 19°C, and were never below 10°C. In 2013, the inoculations were delayed due to heavy rain at the beginning of April, maximum temperatures after inoculation stayed below 16°C during the following week, and minimum temperature for the same period was 0 to 8°C. Average temperature for the 2 weeks period after inoculation was 7 to 14°C, and only at the end of this post-inoculation period. In 2015, temperatures in the first week after inoculation decreased, with

maximum temperature going from 20 to 10°C, and minimum temperature from 10 to 2°C. Average temperatures during the 2 weeks after inoculation in 2015 never rose above 11°C, usually never being above 8°C.

Discussion

This study is the first to comprehensively assess the *in situ* susceptibility of the four most commonly grown Dão appellation grapevine cultivars to *P. chlamydospora*. The assessments of field inoculations revealed different levels amongst the cultivars in susceptibility to colonization by *P. chlamydospora*. Susceptibility of the cultivars assessed from lesion lengths and rates of pathogen recovery from those lesions. Cv. Alfrocheiro gave the greatest recovery of *P. chlamydospora*, and also exhibited the greatest lesion sizes, while the least *P. chlamydospora* recovery and smallest lesions were recorded from cv. Jaen. The cvs Touriga Nacional and Aragonez/Tempranillo demonstrated an intermediate behaviour, with slightly larger lesions and greater recovery proportions for cv. Touriga Nacional. These results demonstrate that cv. Jaen was the least susceptible of the four assessed, cv. Alfrocheiro as the most susceptible. The other two cultivars showed intermediate susceptibility.

Martin *et al*. (2009) considered cv. Aragonez more susceptible and more predisposed to infection by *P. chlamydospora* than cvs Touriga Nacional and Cabernet Sauvignon, which is not in accordance with our results. However, there were differences in the experimental conditions of these two studies. The age of the plants, trial conditions, trial durations and virulence of the isolates could explain these differences. The present results also contradict long-established empirical observations by Portuguese winegrowers, that cv. Aragonez is more prone to esca than other cultivars. This opinion has possibly developed because cv. Aragonez is ubiquitous in Portugal, while cvs. Touriga Nacional, Alfrocheiro and Jaen are less widely grown in Portuguese vineyards (OIV, 2017b).

Resistance of grapevine cultivars to infection by *P. chlamydospora* is not yet completely understood. One reason for different tolerance to *P. chlamydospora* among cultivars may be as hypothesized by Pouzoulet *et al.* (2017), who suggested that grapevine cultivars may differ in resistance to *P. chlamydospora* infection because they had different xylem vessel diameters. These authors proposed that greater amounts of low diameter vessels could impair *P. chlamydospora* progression and spread of pathogen toxins, by efficiently isolating infected sections of xylem vessels through deposition of gels and formation of tyloses. Cv. Merlot is anatomically characterized by narrow diameter xylem vessels and is usually considered less susceptible to esca (Christen *et al*., 2007), while the Thompson seedless grapevine variety, which harbours wide xylemic vessels, is regarded as very susceptible (Murolo and Romanazzi, 2014). Portugal has 341 regulated grapevine cultivars, of which more than 250 are indigenous (Fraga *et al*., 2016). Anatomical and morphological studies on these Portuguese cultivars, to describe their vessel configurations, could provide increased understanding of their relative susceptibilities to esca. This would allow winegrowers to take account of these characteristics when choosing cultivars for new vineyard plantings.

In our experiments, average lesion lengths following inoculations ranged from 41.7 mm to 54.9 mm, depending on the cultivar, yet *P. chlamydospora* was also recovered from symptomless wood, although in relatively low proportions. This was also observed by Landi *et al.* (2012). These lesion sizes, attained just 9 months after inoculation, demonstrate the ability of *P. chlamydospora* to reach the permanent structures (arms and trunks) of grapevines. This emphasizes the disease management strategy proposed by Elena and Luque (2016), that using increased length of the pruned spurs, may make it difficult for *P. chlamydospora* to invades grapevine arms and trunks.

Phaeomoniella chlamydospora recovery rates decreased from the middle portion of the lesions downwards, on all re-isolation samples obtained from all cultivars. This pattern was expected, as the fungus develops in xylem from inoculation points downwards (Pascoe and Cottral, 2000; Feliciano and Gubler, 2001; Serra *et al*., 2008). The advance of the infection became obvious when the ranges and distributions of recovery proportions from the different wood section locations were assessed.

All three *P. chlamydospora* isolates were able to infect, colonise and produce vascular discolourations or lesions in inoculated grapevines, but were not detected in uninoculated plants. The isolates were re-isolated from lesions and adjacent stem tissues. These results showed that the three isolates had different infection behaviours. Lesion lengths and fungal recovery rates indicated that isolate 48 was more aggressive than the other two isolates, while isolate 43 was less virulent than isolates 48 and 52. Differences in aggressiveness

between fungal strains are well known (Sneh, 1998; Boland, 2004), and have been reported among *P. chlamydospora* isolates by Santos *et al.* (2005) and Laveau *et al.* (2009). Although the reasons for these differences were not studied here, previous studies (Sofia *et al.*, 2015), focusing on the ITS sequences of *P. chlamydospora* isolates from distinct Portuguese regions including those considered here,,revealed two distinct phylogenetic groups in this pathogen. Isolate 43 was included in Group 2 while isolates 48 and 52 belonged to the Group 1. In a 2 year trial, pathogenicity tests were carried out on potted plants of cv. Touriga Nacional grafted onto 1103 Paulsen (data not published), using 22 *P. chlamydospora* isolates belonging to both groups. Isolates from Group 1 produced larger lesions and gave greater re-isolation frequencies than isolates from Group 2, indicating that Group 2 isolates were the more virulent. Another hypothesis to explain this difference could be effects of isolate age and subculturing frequency. Isolates 52 and 48 were 4 years younger than isolate 43. Reduction of virulence of fungal strains under continuous sub-culturing has been reported for *Eutypa lata*, the causal agent of Eutypa dieback (Laveau *et al.*, 2009) and for entomopathogenic fungi (Kary and Alizadeh, 2017). Further studies are required to fully explain the virulence differences we detected. Isolate 48, the most virulent of the three we assessed, was not obtained from the Dão appellation, which indicates no relationship between terroir and strain virulence. Comont *et al.* (2010) and Sofia et al. (2015) also demonstrated absence of significant geographic structuring of the *P. chlamydospora* populations, thus reinforcing the risks inherent in transmitting potentially infected grapevine material between wine-producing regions.

Mild temperatures verified in the periods after infection may explain the high rates of *P. chlamydospora* recovery that occurred after the 2012 inoculations, and the low recovery observed in 2015. Optimum temperature for *P. chlamydospora* growth is 25°C (Whiting *et al.*, 2001; Valtaud *et al.*, 2009). Optimum growth conditions occurred in the post inoculation period of 2012, while in 2015 the low temperatures recorded after inoculation were unlikely to favour high infection rates. Luque *et al.* (2014) expressed a similar opinion when advocating the advantages of forestalling pruning, suggesting cool dry conditions after early pruning could hamper fungal infection and pathogen development. They also proposed that seasonal variance in pruning wound susceptibility was not due to the time of the year, but to the favourable climatic conditions experienced after pruning (i.e. humid and warmer weather). This would favour conidium release and dispersion by the pathogen, as well as infection and colonisation of pruning wounds. Serra *et al.* (2008) also observed that regularly distributed rainfall promoted grapevine growth and the pathogen infection processes. Both of these were promoted during the 2012 trial in the present study.

In the Dão wine region, standard good vineyard management practices include advice for late pruning as the best option to avoid esca related fungi from infecting pruning wounds. Temperatures in February/March each year tend to rise, accompanied by increased relative humidity. Results obtained in 2012, together with the recorded temperature and rainfall, indicated that further research is required to support the benefits or otherwise of the standard pruning practices. Pruning recommendations should be flexible and adapted to local conditions, following the suggestions of Elena and Luque (2016), taking cognizance of the discrepancy in pruning wound susceptibility among geographic regions and the influence of local abiotic and biotic conditions. Pruning wound susceptibility should be studied on a local or regional basis, to better understand host–pathogen interactions within the infection processes, to define improved pruning protocols.

Meteorological features of the three years in which trials were carried out influenced grapevine development. While grapevine growth stages developed very similarly in 2012 and 2015, in 2013, due to heavy rain and low temperatures, bud burst was delayed by 2 weeks. These conditions caused extended vine bleeding after pruning. In 2013, bleeding started late and lasted for a longer period than in the other two trial years. This would likely hinder fungal infection (Larignon and Dubos, 2000; Serra *et al.*, 2008), although, it is difficult to relate these data with pathogen infection. Extended bleeding could explain the smaller lesions obtained in cv. Touriga Nacional in 2013, as this cultivar is characterised by late bud burst and delayed phenological development in the Dão wine region. Infections in 2013 could have been hampered by delayed sap flow in the inoculated vines.

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